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Protective effects of *Chlorella vulgaris* in lead-exposed mice infected with *Listeria monocytogenes*

Mary L.S. Queiroz[a,*], Ana P.O. Rodrigues[a], Claudia Bincoletto[a,b], Camila A.V. Figueirêdo[a], Solange Malacrida[a]

[a] *Departamento de Farmacologia/Hemocentro, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), C.P. 6111, CEP 13083-970, Campinas, SP, Brazil*

[b] *Centro Interdisciplinar de Investigações Bioquímicas (CIIB), Universidade de Mogi das Cruzes (UMC), CEP 08780-911, Mogi das Cruzes, SP, Brazil*

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Abstract

Chlorella vulgaris extract (CVE) was examined for its chelating effects on the myelosuppression induced by lead in *Listeria monocytogenes*-infected mice. The reduction in the number of bone marrow granulocyte-macrophage progenitors (CFU-GM) observed after the infection was more severe in the groups previously exposed to lead. Extramedullar hematopoiesis, which was drastically increased after the infection, was not altered by the presence of lead. Treatment with CVE, given simultaneously or following lead exposure, restored to control values the myelosuppression observed in infected/lead-exposed mice and produced a significant increase in serum colony-stimulating activity. The benefits of the CVE treatment were also evident in the recovery of thymus weight, since the reduction produced by the infection was further potentiated by lead exposure. The efficacy of CVE was evident when infected and infected/lead-exposed mice were challenged with a lethal dose of *L. monocytogenes* after a 10-day treatment with 50 mg/kg CVE/day, given simultaneously to the exposure to 1300 ppm lead acetate in drinking water. Survival rates of 30% for the infected group and of 20% for the infected/lead-exposed groups were observed. Evidence that these protective effects of CVE are partly due to its chelating effect was given by the changes observed in blood lead levels. We have observed in the group receiving the CVE/lead simultaneous exposure a dramatic reduction of 66.03% in blood lead levels, when compared to lead-exposed nontreated control. On the other hand, CVE treatment following lead exposure produced a much less effective chelating effect. CVE treatments for 3 or 10 days, starting 24 h following lead exposure, produced a reduction in blood lead levels of 13.5% and 17%, respectively, compared to lead-exposed nontreated controls. The significantly better response observed with the simultaneous CVE/lead administration indicates that the immunomodulation effect of CVE plays an important role in the ability of this algae to reduce blood lead levels. In this regard, additional experiments with gene knockout C57BL/6 mice lacking a functional IFN-γ gene demonstrated that this cytokine is of paramount importance in the protection afforded by CVE. The antibacterial evaluation measured by the rate of survival demonstrated that, in face of a 100% survival in the control group composed of normal C57BL/6 mice, which are resistant to *L. monocytogenes*, we observed no protection whatsoever in the IFN-γ knockout C57BL/6 mice treated with CVE and inoculated with *L. monocytogenes*.

Keywords: *Chlorella vulgaris*; Lead acetate; *Listeria monocytogenes*; Hematopoiesis; Interferon-γ; Knockout mice

* Corresponding author. Tel.: +55-19-3788-8751; fax: +55-19-3289-2968.
E-mail address: mlsq@fcm.unicamp.br (M.L.S. Queiroz).

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1. Introduction

Chlorella vulgaris, a unicellular green algae, has been studied in a variety of practical approaches. In the last years, several reports from our laboratory and others demonstrated the protective effects of this algae against bacterial and virus infections [1–8], tumours [9–12] and peptic ulcers [13]. Recently, the use of *C. vulgaris* for the removal of toxic metals from natural waters or waste waters has been reported by several authors [14–17]. This microalgae has been used for analytical purposes to quantitatively recover ions, such as lead, cadmium, chromium and nickel, from natural water samples. These experiments demonstrated the presence of very high affinity metal ion binding sites in these organisms [14,16–21]. This metal-binding property seems to be related to the presence of chloroplast in the cellular wall, an organelle rich in sulphur, potassium, calcium and phosphorus [16,17,22]. The ability of sulphydryl-containing compounds to chelate metals is well established in the literature and this could be the main factor involved in the in vitro removal by *C. vulgaris* of heavy metal contaminants [23].

Chelation therapy, first developed as a method for treating heavy metal poisoning, has recently been demonstrated to be effective against atherosclerosis, coronary heart disease and peripheral vascular disease. Its supposed benefits include increased collateral blood circulation, decreased blood viscosity, improved cell membrane function, improved intracellular organelle function, decreased arterial vasospasm, decreased free radical formation, inhibition of the aging process, reversal of atherosclerosis; decrease in angina, reversal of gangrene, improvement of skin color, and healing of diabetic ulcers [24–27].

Concerns about the safety of chelation have focused on experimental evidence in animals and on clinical experience. The adverse effects of chelators include hypersensitivity reactions, hyperpyrexia, tachycardia, hypertension, transient elevations of hepatic transaminases, conjunctivitis, lacrimation, salivation, renal failure, leukopenia, thrombocytopenia, hematuria, proteinuria, eosinophilia and anorexia [28–31]. However, the most serious potential adverse effect of chelation therapy is the nonspecific chelation, which causes the depletion of some essential elements such as calcium, iron, copper, manganese, molybdenum and zinc [31,32]. Moreover, although some chelating agents such as meso-2,3-dimercaptosuccinic acid (DMSA) do not directly bind magnesium, cysteine and gluthatione, its administration also produces some depletion of these nutrients [33].

Exposure to heavy metals, such as lead, has shown to downregulate various parameters of the immune response. In this regard, humoral and cellular immunosuppression appears to be a more subtle effect of exposure to low levels of lead [34–39]. In addition, a suppressive dose-dependent effect of lead on bone marrow granulocyte-macrophage progenitors was demonstrated by our group and others [40,41]. The accumulation of this metal in the bone marrow [42,43] could result in deleterious effects to the progenitor cells and consequently lower the resistance to a variety of pathogens. In this regard, it has been demonstrated that when lead-treated mice were challenged with *Listeria monocytogenes*, mortality increased significantly [36,40,44,45]. The potentiation by lead of the immunosuppression induced by *L. monocytogenes* infection is related to the diminished number of macrophages capable of responding to the bacterial insult [36,46,47] and to the reduced frequency of progenitor cells in the bone marrow that respond to hematopoietic growth factors [36,40,41].

In order to investigate if the in vitro chelating effects of CVE are also observed in vivo, we designed the present work to study the protective effect of this algae in mice exposed to lead and infected with *L. monocytogenes*. Our results demonstrated that, although the immunohematopoietic modulation produced by CVE was observed with all the treatment schedules used, the ability of CVE to reduce blood lead levels was much greater when the algae was administered simultaneously to lead exposure, and a much less expressive figure was found with the CVE treatment following lead exposure. These findings indicate that the effective chelating activity occurs in association to the immunoprotective effects of CVE. In this regard, the importance of IFN-γ was demonstrated with the use of IFN-γ gene knockout mice infected with *L. monocytogenes* and treated with CVE.

2. Material and methods

2.1. Mice

Male BALB/c (genetically susceptible to *L. monocytogenes*), C57BL/6 (genetically resistant to *L. monocytogenes*) and C57BL/6 interferon-γ gene knockout mice, 6–8 weeks old, were bred at the University Central Animal Facilities (Centro de Bioterismo, Universidade Estadual de Campinas, Campinas, SP), raised under specific pathogen-free conditions, and matched for body weight before use. Animal experiments were done in accordance with institutional protocols and the guidelines of the Institutional Animal Care and Use Committee.

2.2. L. monocytogenes infection

L. monocytogenes obtained at the Department of Clinical Pathology from the Universidade Estadual de Campinas was used to infect the animals. The intraperitonial (i.p.) lethal dose for BALB/c mice was established to be 4×10^6 bacteria/animal. Bacterial virulence was maintained by serial passages in BALB/c mice. Fresh isolates were obtained from infected spleens, grown in BHI medium and stored as 1-ml aliquots at −80 °C. Before use, each sample was thawed and diluted to an appropriated concentration in 0.9% NaCl. Mice were challenged i.p. with a sublethal dose of 4×10^4 viable *L. monocytogenes*/animal. To evaluate survival rate, mice were inoculated i.p. with a lethal dose of 4×10^6 viable *L. monocytogenes*/animal.

2.3. Treatment regimens

C. vulgaris extract was provided by Dr. T. Hasegawa (Research Laboratories, Chlorella Industry, Japan). Chemical analysis, performed by Hasegawa et al. [3], revealed that CVE contained 44.3 g protein, 39.5 g carbohydrates and 15.4 g nucleic acids in 100 g (dry weight) whole material. No lipids were detected.

CVE was dissolved in distilled water (37 °C) and diluted into appropriate concentrations immediately before use. Doses of 50 mg/kg were given orally by gavage in a 0.2-ml volume/mouse. Two time schedules were used for the CVE treatment. In one group, CVE was given simultaneously to 1300 ppm of lead acetate, for 10 consecutive days. In the other groups, CVE was given for periods of 3 or 10 days, starting 24 h following the administration of the same dose of lead. In all groups, the animals were challenged with a sublethal dose of *L. monocytogenes* 24 h after the last administration of CVE and femoral marrow was collected 24, 48 and 72 h after the inoculation of the bacteria. The selection of CVE and lead doses was based on previous study performed in our laboratory [8,40].

2.4. Progenitor cell assays

The bone marrow cells were aseptically removed by flushing the tibia with RPMI 1640 medium (Sigma, St. Louis, MO) using a syringe with a 25-gauge needle. The marrow plug was then converted to a dispersed cell suspension in 5 ml of RPMI 1640 by gently aspirating the suspension up and down using sterile 10-ml pipette. Spleens were removed aseptically and then converted to dispersed cell suspensions in RPMI 1640 medium by gently pressing through a stainless-steel mesh net.

Assays with suspensions from femoral marrow and spleen were performed in 1-ml agar cultures in 35-mm Petri dishes using 1×10^5 marrow cells or 2×10^5 spleen cells per culture. Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 20% fetal calf serum and 0.3% agar was used. Colony formation was stimulated by the addition of rmGM-CSF as described above. The cultures were incubated for 7 days in a fully humidified atmosphere of 5% CO_2 in air. Colony formation (clones >50 cells) was scored at 35× magnification using a dissection microscope and the whole cultures fixed using 2.5% glutaraldehyde followed by methanol. The intact cultures were floated onto glass slides, dried, stained with Luxol fast blue/hematoxylin, and visualized under microscope [48].

2.5. Hematopoietic stimulator

Recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF) was supplied by Sigma. The rmGM-CSF is an acid glycoprotein of 22 kDa expressed in *Escherichia coli*. Colony formation was stimulated by inclusion in the cultures of 0.025 ng/ml rmGM-CSF when 1×10^5 bone marrow cells were cultured in 1 ml of soft agar. This concentration

of rmGM-CSF was determined from the linear portion of the dose–response curve measured in our laboratory before the experiments started.

2.6. Assay for serum colony-stimulating activity (CSA)

Mice were bled from the ocular plexus under ether anesthesia. Within each experimental group, blood was pooled in periods of 24, 48 and 72 h after the *L. monocytogenes* challenge. Pooled blood was left at 37 °C for 30 min, and the clots were allowed to retract overnight at 4 °C. Following centrifugation, the serum was removed and stored at −20 °C. CSA was determined by ability of serum obtained from control and experimental groups to induce the growth and differentiation of normal bone marrow progenitor cells (CFU-GM).

2.7. Detection of blood lead levels

Lead concentration in blood was measured by atomic absorption spectrophotometry (Zeiss, FMD4) according to the direct chelation–extraction method [49] by Prevlab Laboratory, which is a reference in toxicological evaluation in the Region of Campinas. The accuracy and precision of this method were determined by analysing standardised bovine blood obtained from Koulson Laboratories. The results were 43 ± 7 μg/dl (cf. the control value 45 ± 7 μg/dl) for five samples. The lead concentration in the specimen was determined by comparing its absorbance with that of a standard curve prepared from reference samples. In the range from 20 to 100 μg/ml, the relative error was of the order of 10% with a standard deviation of approximately 8 μg/100 ml.

2.8. Antibacterial evaluation

The antibacterial activity of CVE was evaluated by measuring the survival time of BALB/c mice treated for 10 consecutive days with oral daily doses of 50 mg/kg of CVE given simultaneously to the administration of 1300 ppm of lead acetate in drinking water. Animals were infected with a lethal dose of *L. monocytogenes* 24 h after the end of the treatment. In addition, survival was also evaluated in interferon-γ gene knockout mice C57BL/6 after the treatment for 10 consecutive days with daily 50 mg/kg dose of CVE previously to the infection with *L. monocytogenes*. The study with these latter animals was performed in order to investigate the importance of endogenous IFN-γ production in the protection afforded by CVE. C57BL/6 normal mice are resistant to *L. monocytogenes* and the LD50 of the bacteria for this resistant strain is 100× greater than that for the susceptible BALB/c mice [58].

2.9. Statistical analysis

Analysis of data among all groups was done by one-way ANOVA. When the results were significant, the Tukey test was used to determine the extent of the differences. The probability of survival among the experimental groups was calculated by the Kaplan–Meier technique and the difference between the groups was compared using the log-rank test. Statistical significance was assigned when $P < 0.05$.

3. Results

3.1. Medular and peripheral hematopoiesis

The effects of the treatment with CVE, given simultaneously or following lead exposure, on the number of bone marrow granulocyte-macrophage colonies (CFU-GM), are presented in Figs. 1 and 2. As we can see in both figures, a dramatic reduction in the number of CFU-GM, although not observed at the 24-h evaluation, was present at 48 and 72 h after the infection. Exposure to lead, previously to infection, produced a more severe reduction in the number of CFU-GM at all stages studied, including the 24-h evaluation ($P < 0.05$). The administration of CVE, in addition to not producing any effect in the hematopoietic response of normal animals, led to a significant ($p < 0.05$) recovery in the number of these bone marrow progenitors in infected and infected/lead-exposed mice (Fig. 1). The same protection was afforded by the administration of CVE given simultaneously or following lead exposure. In addition, a full recovery of the hematopoietic response was already reached with CVE being given for 3 days following lead exposure (Fig. 2) and no differences were

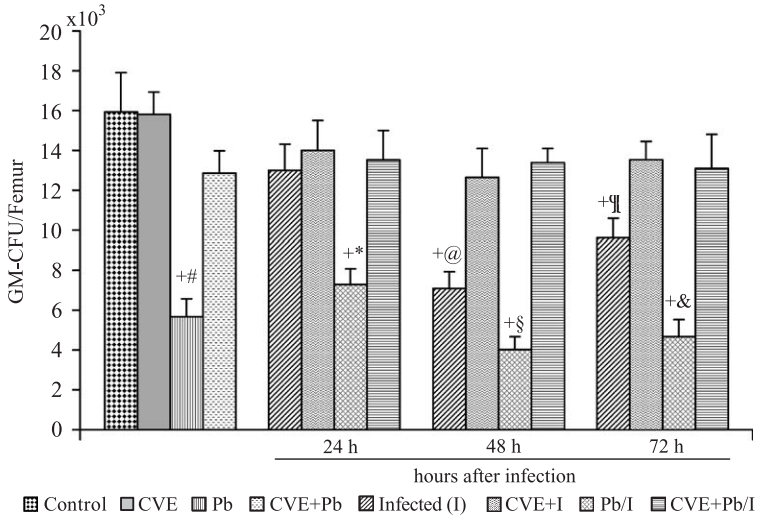

Fig. 1. The number of bone marrow granulocyte-macrophage colonies (GM-CFU) in mice infected with a sublethal dose of *L. monocytogenes* 24 h after the end of a 10-consecutive-day treatment with a daily 50 mg/kg oral dose of CVE, given simultaneously to the administration in the drinking water of 1300 ppm of lead acetate. GM-CFU number was determined at 24, 48 and 72 h after bacterial inoculation. Control mice received diluent only. Results represent the means ± S.D. of six mice per group. $^{+}P < 0.01$ in relation to control; $^{\#}P < 0.01$ in relation to CVE + Pb; $^{*}P < 0.01$ in relation to infected (24 h) and CVE + Pb/I (24 h); $^{@}P < 0.01$ in relation to infected (24 h), Pb/I and CVE + I (48 h); $^{\S}P < 0.05$ in relation to Pb/I (24 h) and CVE + Pb/I (48 h); $^{\P}P < 0.05$ in relation to infected (24 h), Pb/I and CVE + I (72 h); $^{\&}P < 0.05$ in relation to Pb/I (24 h) and CVE + Pb/I (72 h).

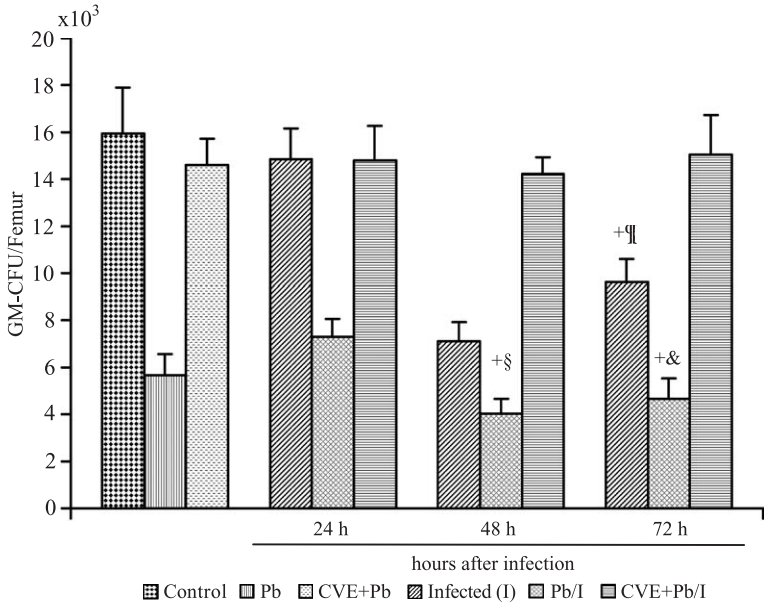

Fig. 2. The number of bone marrow granulocyte-macrophage colonies GM-CFU in mice infected with a sublethal dose of *L. monocytogenes* 24 h after the end of a 10-consecutive-day treatment with a daily 50 mg/kg oral dose of CVE. CVE treatment started 24 h following the administration for 10 days of 1300 ppm of lead acetate in the drinking water. GM-CFU number was determined at 24, 48 and 72 h after bacterial inoculation. Control mice received diluent only. Results represent the means ± S.D. of six mice per group. $^{+}P < 0.01$ in relation to control; $^{\#}P < 0.01$ in relation to Pb + CVE; $^{*}P < 0.05$ in relation to infected and CVE + Pb/I (24 h); $^{@}P < 0.05$ in relation to CVE + Pb/I (48 h); $^{\S}P < 0.05$ in relation to CVE + Pb/I (48 h); $^{\P}P < 0.05$ in relation to CVE + Pb/I (72 h); $^{\&}P < 0.05$ in relation to CVE + Pb/I (72 h).

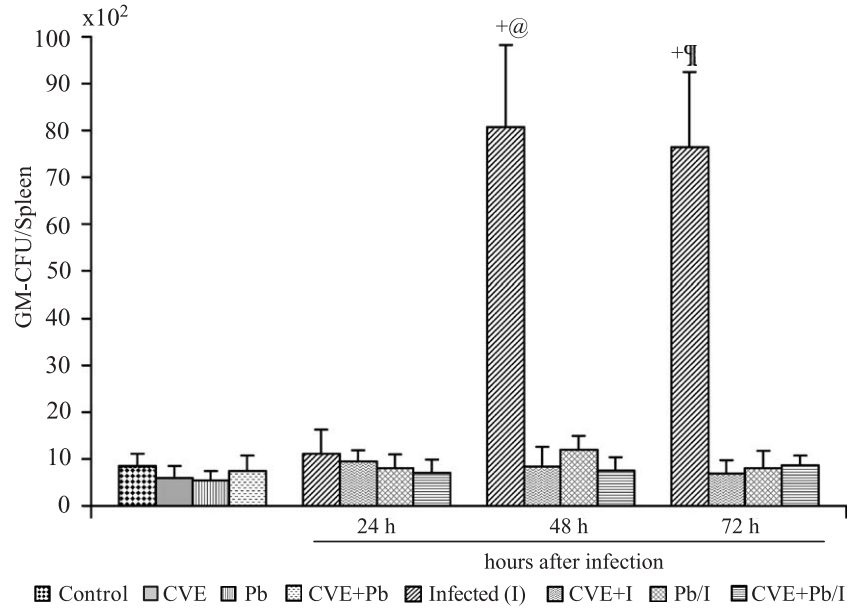

Fig. 3. The number of spleen granulocyte-macrophage colonies (GM-CFU) in mice infected with a sublethal dose of *L. monocytogenes* 24 h after the end of a 10-consecutive-day treatment with a daily 50 mg/kg oral dose of CVE given simultaneously to the administration in the drinking water of 1300 ppm of lead acetate. GM-CFU number was determined at 24, 48 and 72 h after bacterial inoculation. Control mice received diluent only. Results represent the means ± S.D. of six mice per group. $^{+}P < 0.001$ in relation to control; $^{@}P < 0.001$ in relation to infected (24 h), CVE + I, Pb/I, CVE + Pb/I (48 h); $^{¶}P < 001$ in relation to infected (24 h), CVE + I, Pb/I, CVE + Pb/I (72 h).

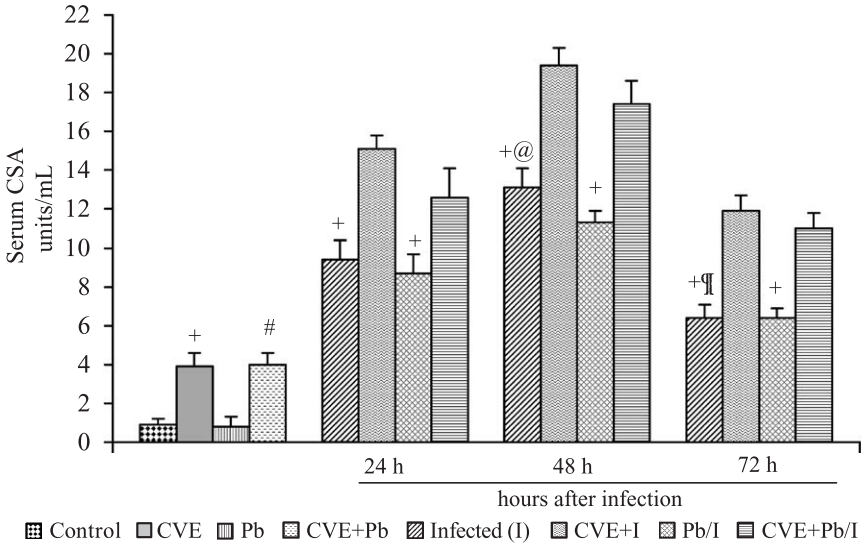

Fig. 4. Serum colony stimulating activity in mice infected with a sublethal dose of *L. monocytogenes* 24 h after the end of a 10-consecutive-day treatment with a daily 50 mg/kg oral dose of CVE given simultaneously to the administration in the drinking water of 1300 ppm of lead acetate. The serum was collected 24, 48 and 72 h after bacterial inoculation. Control mice received diluent only. Results represent the means ± S.D. of six mice per group. $^{+}P < 0.01$ in relation to control; $^{\#}P < 0.05$ in relation to Pb; $^{**}P < 0.05$ in relation to I (24 h); $^{*}P < 0.05$ in relation to Pb/I (24 h); $^{@}P < 0.05$ in relation to infected (24 h) and CVE + I (48 h); $^{\S}P < 0.05$ in relation to Pb/I (48 h); $^{¶}P < 0.05$ in relation to infected (24 h) and CVE + I (72 h); $^{\&}P < 0.05$ in relation to Pb/I (72 h).

Table 1
Changes in blood lead levels of mice exposed to lead acetate and treated with CVE

Blood lead levels (μg/%)					
Simultaneous Pb/CVE		Pb/CVE—10 days		Pb/CVE—3 days	
Pb	Pb/CVE	Pb	Pb/CVE	Pb	Pb/CVE
106	70	40	33	52	45
Reduction = 66.03%		Reduction = 17.05%		Reduction = 13.46%	

CVE treatment was given for 10 days simultaneously to the administration of lead acetate, and for 3 and 10 days following the 10-day Pb exposure.

observed between 3-day schedule results and those obtained after a longer (10 days) CVE treatment (data not shown).

Evaluation of peripheral hematopoiesis, as observed by the number of CFU-GM in the spleen, is presented in Fig. 3. The inoculation of bacteria produced a dramatic increase in the number of spleen colonies. This effect, reversed by the administration of CVE, was not affected by the presence of lead.

The effects of CVE in the serum colony-stimulating activity (CSA) are presented in Fig. 4. The results demonstrate that the increase in CSA, observed at 24, 48 and 72 h of infection, was significantly accentuated by the presence of CVE. It is worth pointing out that the CVE treatment was able to increase CSA activity not only in the infected and infected/lead-exposed groups, but also in the normal, nontreated mice. The presence of lead is shown not to produce by itself any effect in the CSA of the serum.

3.2. Blood lead levels

The changes produced in blood lead levels of mice exposed to lead acetate and treated with CVE are presented in Table 1. CVE was administered simultaneously or following Pb exposure. A dramatic reduction (66, 03%) was observed with the simultaneous CVE/lead exposure. CVE treatment following lead exposure produced reduction in blood lead levels of 17% and 13.5% when CVE was given for 10 or 3 days, respectively.

3.3. Thymus weight

The severe thymic atrophy observed in mice at 48 and 72 h after *L. monocytogenes* infection was further increased after lead exposure. In both cases, this effect was prevented by the oral treatment with 50 mg/kg CVE ($P < 0.05$) (Fig. 5).

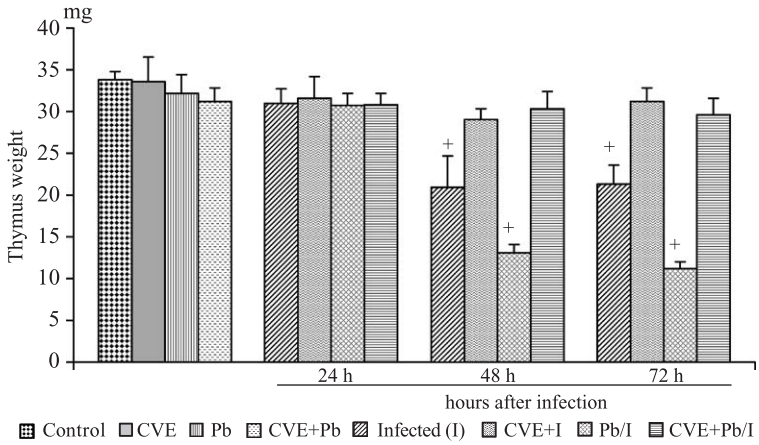

Fig. 5. Thymus weight of mice infected with a sublethal dose of *L. monocytogenes* 24 h after the end of a 10-consecutive-day treatment with a daily 50 mg/kg oral dose of CVE given simultaneously to the administration in the drinking water of 1300 ppm of lead acetate. Mice were scarified 24, 48 and 72 h after bacterial inoculation. Control mice received diluent only. Results represent the means ± S.D. of six mice per group. $^{+}P < 0.05$ in relation to control; $^{@}P < 0.05$ in relation to infected Pb/I (48 h); $^{\S}P < 0.05$ in relation to Pb/I 48 h; $^{¶}P < 0.05$ in relation to infected Pb/I (72 h); $^{\&}P < 0.05$ in relation to Pb/I (72 h).

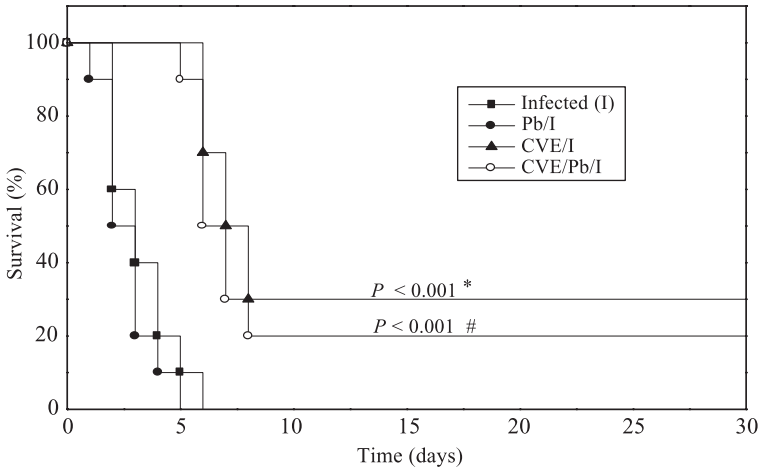

Fig. 6. Effects of a 10-consecutive-day treatment with oral daily dose of 50 mg/kg CVE, given simultaneously to the administration of 1300 ppm of lead acetate in the drinking water, on the survival of mice infected with a lethal dose of *L. monocytogenes* 24 h after the end of the treatment. Groups of 20 mice were checked daily for survival. $^{*}P<0.001$ in relation to infected mice; $^{\#}P<0.001$ in relation to Pb/I mice.

3.4. Antibacterial evaluation

As we can see from Fig. 6, all infected and infected/lead-exposed BALB/c mice presented a 100% mortality within 6 days. On the other hand, when these animals were treated with 50 mg/kg CVE for 10 days simultaneously to lead exposure, no deaths were observed up to the 6th day after infection, and the percentage of survivors in the infected and infected/lead-exposed groups was of 30% and 20%, respectively ($P<0.001$) (Fig. 6). However, these results were not significantly different, which suggests that lead does not hinder the immunoprotective effects of CVE. Our studies with

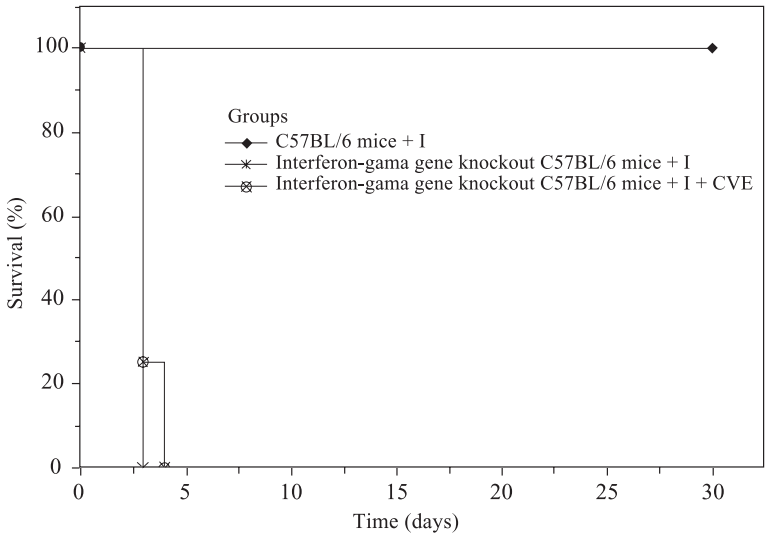

Fig. 7. The effects of a pretreatment with 50 mg/kg CVE given orally for 10 days to IFN-γ gene knockout mice infected with a lethal dose of *L. monocytogenes* 24 h after the end of the treatment. Groups of 20 mice were checked daily for survival.

the C57BL/6 mice resistant to *L. monocytogenes* demonstrated that, with the same dose of bacteria inoculated to BALB/c mice, there is a 100% survival of normal C57BL/6 mice. On the other hand, no protection was afforded by CVE in knockout C57BL/6 mice lacking a functional IFN-γ gene (Fig. 7).

4. Discussion

Chelating agents are considered as potential instruments for the treatment of heavy metal poisoning to prevent or reverse toxic effects and enhance the excretion of metals from the body [30]. However, some studies indicate that there is a lack of safety and efficacy when conventional chelating agents are used [31]. The search for new technologies involving the removal of toxic metals from waste waters has directed attention to biosorption, based on the metal-binding capabilities of algae and other aquatic plants. In this sense, the use of *C. vulgaris* for the removal of toxic metals from natural waters or waste waters due to its adsorption properties has been reported by several authors [14–17].

In the present study, we have demonstrated that CVE increases the levels of serum colony-stimulating factors, prevents the myelosuppression induced by lead exposure in normal and *L. monocytogenes*-infected mice, restores to normal the number of CFU-GM, and significantly enhances host survival. CVE was administered simultaneously or after Pb exposure. Two different time points, 3 and 10 days, were used for the study of the effects of CVE following lead exposure. Although, no differences were produced with the different treatment schedules on the ability of CVE to abrogate myelosuppression, the evaluation of blood lead levels demonstrated a more effective chelating effect when CVE was administered simultaneously to lead exposure. In this regard, reductions of 66%, 13.5% and 17.5% in blood lead levels were observed when CVE was given simultaneously, 3 and 10 days following lead exposure, respectively. These results indicate that the ability of CVE to restore the immunosuppression produced by lead contributes significantly to its ability to reduce blood lead levels. In this regard, we have also observed in this study that no protection as measured by the survival rate was afforded by CVE in IFN-γ knockout C57BL/6 mice infected with *L. monocytogenes*. The use of the C57BL/6 strain of mice in this experiment, which is 100× more resistant to *L. monocytogenes* than the BALB/c strain [58,60], reinforces the importance of IFN-γ for the manifestation of the protective CVE effects. In addition, CVE was able to restore the thymus involution produced by the infection and potentiated by lead exposure. It has been demonstrated that lead exposure produces a disruption in the balance between cell-mediated and humoral immunity by inducing the suppression of Th1-type responses, favouring the development of a Th2-type response and increasing tumour necrosis factor alpha (TNF-α) production [37,45,55,56]. In this regard, data recently obtained in our laboratory demonstrated that in *L. monocytogenes*-infected mice, CVE enhances the levels of Th1 cytokines (IFN-γ and IL-2) with no effects on IL-4 and IL-10 production [57]. Similarly, results reported by Hasegawa et al. [5] demonstrated that CVE treatment increases the levels of IFN-γ, IL-2, IL-1α and TNF-α in normal and murine syndrome of acquired immunodeficiency (MAIDS) mice after infection with *L. monocytogenes*. The same authors [59] showed that CVE oral administration may promote Th1 immune response via augmentation of IL-12 production. Enhanced levels of IL-12 may also be involved in the protective effects of CVE in lead-exposed mice, since this cytokine overcomes the lead-induced inhibition of IFN-γ, thus making the IL-12-driven Th1 development dominant over Pb-induced Th2 development [37]. Additional mechanism of action might contribute to the favorable effects of CVE in lead-exposed mice. It is known that lead can enhance the stress response which naturally occurs during infection and further compromises the innate and cell-mediated immunity by the inhibition of macrophage development and activation [45,52]. The effects of stress on the immune response have been attributed mainly to glucocorticoids, which induce a decrease in the number of immunocompetent cells, including thymocytes and peripheral T lymphocytes, thus contributing to thymic involution [52,53]. In this respect, Hasegawa et al. [54] have recently demonstrated that the oral administration of *C. vulgaris* extract significantly inhibited the elevation of serum glucocorticoid levels in mice after psychological stress and concurrently prevents the decrease in

the number of thymocytes and neutrophils. This effect was attributed to the ability of this algae to stimulate the production of pro-inflammatory cytokines, such as IL-1, TNF-α and IL-12, which are known to depress the glucocorticoid production by the adrenal cortex [45]. The chelating response demonstrated by CVE might also be related to the fact that this algae is a sulphydryl-containing substance which contains free radical content in its cellular wall [16,23]. It is well described in the literature the great affinity that heavy metals such as lead have for sulphydryl groups [50]. Pb modifies thiols on membranes indirectly by modulating oxidative products or altering the redox state of the cell. This effect is due to lipid peroxidation and/or modulation of the synthesis of immunoregulatory products, such as leukotrienes, which have a variety of effects on the immune system including the production of slow-reacting products that augment myeloid colony formation [51].

Our findings demonstrate that the ability of CVE to restore the immunosuppressive effects induced by lead might be the result of an interaction between its Th1-stimulating activity and its chelating in vivo ability. The dramatic chelating effect observed with the CVE treatment simultaneous to lead exposure reinforces its use as a food supplement. In view of the need for safer chelating agents, further studies are necessary to evaluate the detoxicating effects of CVE in humans.

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References

[1] Tanaka K, Koga T, Konishi F, Nakamura M, Mitsuyama K, Nomoto K. Augmentation of host defense by a unicellular green alga, *Chlorella vulgaris*, to *Escherichia coli* infection. Infect Immun 1986;53:267–71.

[2] Hasegawa T, Tanaka K, Ueno K, Okuda M, Yoshikai Y, Nomoto K. Augmentation of the resistance against *Escherichia coli* by oral administration of a hot water extract of *Chlorella vulgaris* in rats. Int J Immunopharmacol 1989;11:971–6.

[3] Hasegawa T, Yohikai Y, Okuda M, Nomoto K. Accelerated restoration of the leukocyte number and augmented resistance against *Escherichia coli* in cyclophosphamide-treated rats orally administered with a hot water extract of *Chlorella vulgaris*. Int J Immunopharmacol 1990;12:883–91.

[4] 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.

[5] Hasegawa T, Kimura Y, Hiromatsu K, Kobayashi N, Yamada A, Makino M, et al. Effect of hot water extract of *Chlorella vulgaris* on cytokine expression patterns in mice with murine acquired immunodeficiency syndrome after infection with *Listeria monocytogenes*. Immunopharmacology 1997;35:273–82.

[6] Ibusuki K, Minamishima Y. Effects of *Chlorella vulgaris* extract on murine cytomegalovírus infections. Nat Immun Cell Growth Regul 1990;9:121–8.

[7] Konishi F, Tanaka K, Kumamoto S, Hasegawa T, Okuda M, Yano I, et al. Enhanced resistance against *Escherichia coli* infection by subcutaneous administration of the hot-water extract of *Chlorella vulgaris* in cyclophosphamide-treated mice. Cancer Immunol Immunother 1990;32:1–7.

[8] Dantas DCM, Queiroz MLS. Effects of *Chlorella vulgaris* on bone marrow progenitor cells of mice infected with *Listeria monocytogenes*. Int J Immunopharmacol 1999;21:499–508.

[9] 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;19:73–8.

[10] Morimoto T, Nagatsu A, Murakami N, Sakakibara J, Tokuda H, Nishino H, et al. Anti-tumor-promoting glyceroglycolipids from the green alga, *Chlorella vulgaris*. Phytochemistry 1995;40:1433–7.

[11] Noda K, Ohno N, Tanaka K, Okuda M, Yadomae T, Nomoto K, et al. A new type of biological response modifier from *Chlorella vulgaris* which needs protein moiety to show an antitumour activity. Phytother Res 1998;12:309–19.

[12] Justo GZ, Silva MR, Queiroz MLS. Effects on the green algae *Chlorella vulgaris* on the response of the host hematopoietic system to intraperitoneal Ehrlich ascites tumor transplantation in mice. Immunopharmacol Immunotoxicol 2001;23:119–32.

[13] Tanaka K, Yamada A, Noda K, Shoyama Y, Kubo C, Nomoto K. Oral administration of a unicellular green algae, *Chlorella vulgaris*, prevents stress-induced ulcer. Planta Med 1997;63:465–6.

[14] Wilde EW, Benemann JR. Bioremoval of heavy metals by the use of microalgae. Biotechnol Adv 1993;11:781–812.

[15] Veglio F, Beolchini F. Removal of metals by biosorption: a review. Hydrometallurgy 1997;44:301–16.

[16] Wong SL, Nakamoto L, Wainwright JF. Detection of toxic

organometallic complexes in waste waters using algal assays. Arch Environ Contam Toxicol 1997;32:358–66.
[17] López CE, Castro JM, Gonzáles V, Pérez J, Seco HM, Fernández JM. Determination of metal ions in algal solution samples by capillary electrophoresis. J Chromatogr Sci 1998;36:352–6.
[18] Lustigman B, Lee LH, Khalil A. Effects of nickel and pH on the growth of *Chlorella vulgaris*. Bull Environ Contam Toxicol 1995;55:73–80.
[19] Carr HP, Cariño FA, Yang MS, Wong MH. Characterization of the cadmium-binding capacity of *Chlorella vulgaris*. Bull Environ Contam Toxicol 1998;60:433–40.
[20] Matsunaga T, Takeyama H, Nakao T, Yamazawa A. Screening of marine microalgae for bioremediation of cadmium-polluted seawater. J Biotechnol 1999;70:33–8.
[21] Slaveykova V, Wilkinson KJ. Physicochemical aspects of lead bioaccumulation by *Chlorella vulgaris*. Environ Sci Technol 2002;36:969–75.
[22] Traviesso L, Canizares RO, Borja R, Benitez F, Dominguez AR, Dupeyron R, et al. Heavy metal removal by microalgae. Bull Environ Contam Toxicol 1999;62:144–51.
[23] Apasheva LM, Gusareva EV, Kulinich AV, Shevchenko VA. Study of the role of thiols in determining the radiosensitivity of various strains of *Chlorella vulgaris*. Radiobiology 1972; 12:904–6.
[24] Chappell LT. EDTA chelation therapy should be more commonly used in the treatment of vascular disease. Altern Ther Health Med 1995;1:53–7.
[25] Kidd PM. Integrative cardiac revitalization: bypass surgery, angioplasty, and chelation. Benefits, risks, and limitations. Altern Med Rev 1998;3:4–17.
[26] Ernest E. Chelation therapy for coronary heart disease: an overview of all clinical investigations. Am Heart J 2000; 140:139–41.
[27] Quan H, Ghali WA, Verhoef MJ, Norris CM, Galbraith PD, Knudtson ML. Use of chelation therapy after coronary angiography. Am J Med 2001;111:729–30.
[28] Graziano JH, Lolacono NJ, Moulton T, Mitchell ME, Slavkovich V, Zarate C. Controlled study of meso-2,3-dimercaptosuccinic acid for the management of childhood lead intoxication. Pediatr Pharmacol 1992;120:133–9.
[29] Flora SJ, Bhattacharya R, Vijayaraghavan R. Combined therapeutic potential of meso-2,3-dimercaptosuccinic acid and calcium disodium edetate on the mobilization and distribution of lead in experimental lead intoxication in rats. Fundam Appl Toxicol 1995;25:233–40.
[30] Klaassen CD. Heavy metals and heavy-metal antagonists. In: Hardman JG, Gilman AG, Limbird LE, editors. Goodman & Gilman's the pharmacological basis of therapeutics. New York: McGraw-Hill; 1996. p. 1649–71.
[31] Gurer H, Ercal N. Can antioxidants be beneficial in the treatment of lead poisoning? Free Radic Biol Med 2000;29: 927–45.
[32] Tandon SK, Singh S, Jain VK. Efficacy of combined chelation in lead intoxication. Chem Res Toxicol 1994;7:585–9.
[33] Quig D. Cysteine metabolism and metal toxicity. Altern Med Rev 1998;3:262–70.
[34] Faith RE, Luster MI, Kimmel CA. Effect of chronic developmental lead exposure on cell-mediated immune functions. Clin Exp Immunol 1979;35:413–20.
[35] Blakley BK, Archer DL. Mitogen stimulation of lymphocytes exposed to lead. Toxicol Appl Pharmacol 1982;62:183–9.
[36] Kowolenko M, Tracy L, Lawrence D. Early effects of lead on bone marrow cell responsiveness in mice challenged with *Listeria monocytogenes*. Fundam Appl Toxicol 1991;17:75–82.
[37] Heo Y, Lee WT, Lawrence DA. Differential effects of lead and cAMP on development and activities of Th1- and Th2-lymphocytes. Toxicol Sci 1998;43:172–85.
[38] Chen S, Golemboski KA, Sanders FS, Dietert RR. Persistent effect of in utero meso-2,3-dimercaptosuccinic acid (DMSA) on immune function and lead-induced immunotoxicity. Toxicology 1999;132:67–79.
[39] McCabe MJ, Singh KP, Reiners JJ. Lead intoxication impairs the generation of a delayed type hypersensitivity response. Toxicology 1999;139:255–64.
[40] Bincoletto C, Queiroz MLS. The effect of Pb on the bone marrow stem cells of mice infected with *Listeria monocytogenes*. Vet Hum Toxicol 1996;38:186–90.
[41] Van Den Heuvel RL, Leppens H, Schoeters GE. Lead and catechol hematotoxicity in vitro using human and murine hematopoietic progenitor cells. Cell Biol Toxicol 1999; 15: 101–10.
[42] Westerman MP, Pfitzer E, Ellis LD, Jensen WN. Concentrations of lead in bone in plumbism. N Engl J Med 1965; 23: 1246–50.
[43] Barry PS. Letter: lead levels in blood. Nature 1975;258:775.
[44] Lawrence DA. In vivo and in vitro effects of lead on humoral and cell-mediated immunity. Infect Immun 1981; 31: 136–43.
[45] Kishikawa H, Song R, Lawrence A. Interleukin-12 promoters enhanced resistance to *Listeria monocytogenes* infection of Pb-exposed mice. Toxicol Appl Pharmacol 1997; 147:180–9.
[46] Kowolenko M, Tracy L, Muzinski S, Lawrence DA. Effects of lead on macrophage function. J Leukoc Biol 1988;43:357–64.
[47] Kim D, Lawrence DA. Immunotoxic effects of inorganic lead on host resistance of mice with different circling behavior preferences. Brain Behav Immun 2000;14:305–17.
[48] Metcalf D. The hemopoietic colony stimulating factors. New York: Elsevier; 1984.
[49] Hessel DW. A simple and rapid quantitative determination of lead in blood. At Absorpt Newsl, Norwalk 1968;7:55–6.
[50] Skoczynska A. Lipid peroxidation as a toxic mode of action for lead and cadmium. Med Pr 1997;48:197–203.
[51] Parker CW. Lipid mediators produced through the lipoxygenase pathway. Annu Rev Immunol 1987;5:65–84.
[52] Domínguez-Gerpe L, Rey-Méndez M. Modulation of stress-induced murine lymphoid tissue involution by age, sex and strain: role of bone marrow. Mech Ageing Dev 1998;104: 195–205.
[53] Dhabhar FS, Miller AH, McEwen BS. Effects of stress on immune cell distribution. J Immunol 1995;154:5511–27.
[54] Hasegawa T, Noda K, Kumamoto S, Ando Y, Yamada A, Yohikai Y. *Chlorella vulgaris* culture supernatant (CVS) re-

duces psychological stress-induced apoptosis in thymocytes of mice. Int J Immunopharmacol 2000;22:877–85.
[55] Tian L, Lawrence DA. Pb inhibits nitric oxide production in vitro by murine splenic macrophages. Toxicol Appl Pharmacol 1995;132:156–63.
[56] Heo Y, Parsons PJ, Lawrence DA. Lead differentially modifies cytokine production in vitro and in vivo. Toxicol Appl Pharmacol 1996;138:149–57.
[57] Queiroz MLS, Bincoletto C, Valadares MC, Dantas DCM, Santos LMB. Effects of *Chlorella vulgaris* extract on cytokines production in *Listeria monocytogenes* infected mice. Immunopharmacol Immunotoxicol 2002;24(3):483–96.
[58] Cheers C, McKenzie FC. Resistance and susceptibility of mice to bacterial infection: genetics of listeriosis. Infect Immun 1978;19:755–65.
[59] Kumamoto S, Ando Y, Yamada A, Nomoto K, Yasumobu Y. Oral administration of hot water extracts of *Chlorella vulgaris* reduces IgE production against milk casein in mice. Int J Immunopharmacol 1999;21:311–23.
[60] Skamene E, Kongshavn PAL. Phonotypic expression of genetically controlled host resistance to *Listeria monocytogenes*. Infect Immun 1979;25:345–51.