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Sodium Caseinate Induces Increased Survival in Leukaemic Mouse J774 Model

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Abstract. *Background: Acute myeloid leukaemia is a neoplastic disease of haematopoietic stem cells. Although there have been recent advances regarding its treatment, mortality remains high. Consequently, therapeutic alternatives continue to be explored. In the present report, we present evidence that sodium caseinate (CasNa), a salt of the principal protein in milk, may possess important anti-leukaemic properties. Materials and Methods: J774 leukaemia macrophage-like cells were cultured with CasNa and proliferation, viability and differentiation were evaluated. These cells were also inoculated into BALB/c mice as a model of leukemia. Results: We demonstrated that CasNa inhibits the in vitro proliferation and reduces viability of J774 cells, and leads to increased survival in vivo in a leukaemic mouse model. Conclusion: These data indicate that CasNa may be useful in leukaemia therapy.*

Acute myeloid leukaemia (AML) is a neoplastic disease of blood stem cells that is characterized by the continuous clonal proliferation of myeloid precursors that fail to differentiate or mature into terminal cells (1). The M5 subtype, or acute monocytic leukaemia, displays characteristic clinical features, and the disease is associated with a poor prognosis compared to other subtypes of AML, although this has not been clearly established (2-4). Most importantly, this disease may develop after chemotherapy, particularly following epipodophyllotoxin and anthracycline treatments (5). Thus, cases of M5 AML have been reported in patients with breast cancer and all of these AML cases were fatal. The increased crude incidence of AML is statistically significant (6). Despite the importance of the M5 subtype of leukaemia, very few studies on new drugs designed to treat this disease have been reported.

Casein, the principal protein in milk and an important component of the human diet, could be useful in leukaemia treatment because it acts as a proliferation and activation regulator and as an activator of blood cells. For example, beta-casein, a component of bovine casein, has been found to activate free radical production in granulocytes and to induce lymphocyte proliferation (7). Casein also functions as an inflammatory agent that can induce the migration of myeloid and lymphoid cells into the peritoneal cavity (8). We have recently shown that sodium caseinate (CasNa), a salt of casein, inhibits the proliferation of several leukaemia cell lines, including the J774 leukaemia macrophage-like cell line (9), a model of macrophage-like tumour M5 AML (10, 11).

The present study was performed to determine whether CasNa exerts anti-leukaemic effects *in vivo* as it does *in vitro*.

Materials and Methods

Experimental animals. Female BALB/c mice between 8 and 12 weeks of age were used and maintained in pathogen-free conditions. The experiments were carried out in the animal facility of Zaragoza School of Advanced Studies, National Autonomous University of Mexico in accordance with the institutional guidelines. The mice were provided with autoclaved water and fed a standard powdered rodent diet ad libitum. All experimental protocols were approved with the number FESZ/DEPI/CI/128/14 by the Ethics Committee of Zaragoza Faculty of Advanced Studies, in accordance with the national and international regulations for the care and use of experimental animals.

Cell culture. The J774 leukaemia macrophage-like cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were cultured in hydrophobic surface Petri dishes (Sarstedt AG & Co., Germany) with Iscove's modified Dulbecco's medium (IMDM) (Gibco-BRL, Carlsbad, CA,

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USA) supplemented with 10% foetal bovine serum (FBS) (Gibco-BRL, Carlsbad, CA, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The cells were maintained in a humidified atmosphere with 5% CO_2 at 37˚C, and the culture medium was changed every two days.

Sodium caseinate. CasNa (Spectrum, New Brunswick, NJ, USA) was dissolved in phosphate-buffered saline (PBS) at a concentration of 100 mg/ml. Autoclaved dilutions were prepared with PBS to yield concentrations of 0.5, 1.0 and 2.0 mg/ml.

Proliferation assay and cell viability. To evaluate cell proliferation, 1×10^3 J774 leukaemia macrophage-like cells were cultured for 72 h with a range of CasNa concentrations (0, 0.5, 1.0 or 2.0 mg/ml) in 96-well plates (Corning Costar, St. Louis, MO, USA). Proliferation was assessed by direct quantification using a Neubauer chamber, and Trypan blue (Sigma-Aldrich, St. Louis, MO, USA) exclusion assays were employed to determine the number of viable cells in each culture; unstained cells were counted as viable. The results are expressed as the mean percentage of cell viability±standard deviation (SD) of triplicate cultures. Additionally, an assessment of the J774 cells (in the presence or absence of 2 mg/ml of CasNa) at 24 h intervals for up to five days was carried out and representative photographs of cell morphology were taken.

Sulforhodamine B (SRB) assay. To evaluate cell viability, 1×10^3 J774 cells were cultured for 120 h with CasNa (2 mg/ml) in 96-well plates (Corning Costar, St. Louis, MO, USA) and subsequently assessed using the SRB colorimetric test (Sigma-Aldrich, St. Louis, MO, USA). Briefly, trichloroacetic acid was added to each well to fix the cells, which were then incubated at 4˚C for 1 h and washed several times. The plates were dried at room temperature, and SRB dye (0.4% acetic acid 1% dilution) was added and the cells incubated for a further 5 min at room temperature to stain cells. The plates were then washed with 1% acetic acid and dried at room temperature. Finally, the dye was solubilised with Tris base (pH 10.5), and the absorbance was determined using a plate reader (Tecan Spectra, Grödig, Austria) at a wavelength of 550 nm. The results are expressed as the mean percentage of cell viability±standard deviation (SD) of triplicate independent cultures.

Establishment of the leukaemia mouse model. J774 cells were washed twice with PBS, quantified with Trypan blue to confirm >95% viability, and adjusted to a density of 1.0×10^6 cells/0.5 ml. BALB/c mice (females and males) were divided into four groups of eight mice each, injected intraperitoneally (*i.p.*) with J774 cells, and monitored for survival. Upon death, the mice were weighed and spleens, livers and tumour samples were obtained and weighed individually; respective indices were determined as the ratio of either tumour or organ weight to body weight.

Antileukaemic activity in BALB/c mice. Four groups of eight BALB/c mice were used. One group of mice served as the negative control treated only with PBS (1 ml injected *i.p.*), and the three remaining groups were injected *i.p.* with 0.5 ml of J774 cells. These three groups were treated with PBS, CasNa (1 ml of CasNa 10% in PBS), or 0.5 mg/ml of arabinofuranosyl cytidine (Ara-C;) (Lamery, D.F. Mexico), an antineoplastic agent, starting 48 h after cell inoculum and every 48 h thereafter. The treated groups and control groups were observed for survival. At the end of treatment series, the mice were sacrificed and the tissues were weighed individually.

Presence of mononuclear cells in the bone marrow of BALB/c mice. Total bone marrow cells from mice that survived the inoculation of J774 cells and were treated with CasNa or Ara-C were extracted from the femur and flushed with IMDM supplemented with 10% FBS. Mononuclear cells (MNCs) were isolated from total cells *via* gradient separation with Ficoll-Paque (Amersham Biosciences AB, Uppsala, Sweden) at a density of 1.077 g/ml, and they were washed twice with PBS. MNCs were cultured for 120 h in IMDM supplemented with 15% (v/v) FBS, 5% (v/v) horse serum (Gibco-BRL, Carlsbad, CA, USA) and 5 ng/ml recombinant mouse interleukin-3 (rmIL3; R&D System, Minneapolis, MN, USA) or PBS. The cells were cultured in a humidified atmosphere with 5% CO_2 at 37˚C for a maximum duration of 120 h, cell proliferation was then assessed as previously described.

Statistical analyses. All individual experiments were carried out in triplicate. All experiments were repeated three times, and the values are expressed graphically as the average values±SD. One-way ANOVA was used for statistical analysis, and $p<0.05$ was considered statistically significant. Statistical software (SPSS, Inc., Chicago, IL, USA)) was used to perform the analyses.

Results

CasNa inhibits proliferation without affecting viability of J774 cells, but induces differentiation. CasNa was able to inhibit the proliferation of J774 leukaemia cells, as we have shown previously (9). Assessment *via* Neubauer quantification shows that CasNa at 1 and 2 mg/ml inhibits proliferation without affecting cell viability, while inducing an increase of macrophagic differentiation (Figure 1A-D).

CasNa inhibits proliferation of J774 cells in a time-dependent manner and reduces viability after 120 h of stimulation, as does Ara-C. We extended the culture time in the presence of 2 mg/ml CasNa and evaluated the proliferation and viability at 24-h intervals. The results show that after 48 h, CasNa inhibited the proliferation of J774 cells (Figure 2A). Interestingly, a Trypan blue exclusion assay revealed that J774 cell viability significantly decreased at 120 h (Figure 2B). Moreover, to compare this inhibition of viability with respect to the anti-neoplastic agent Ara-C, an SRB assay, revealed that CasNa induced a greater reduction in J774 cell viability (Figure 2C).

Establishment of a BALB/c-J774 leukaemia model. After determining that CasNa inhibits the proliferation of J774 cells, and reduces cell viability, as does anti-neoplastic Ara-C, we developed a murine leukaemia model with this cell line to evaluate the antileukaemic potential of CasNa. Our results show that animals (female or male BALB/c mice) challenged with J774 cells developed major splenomegaly,

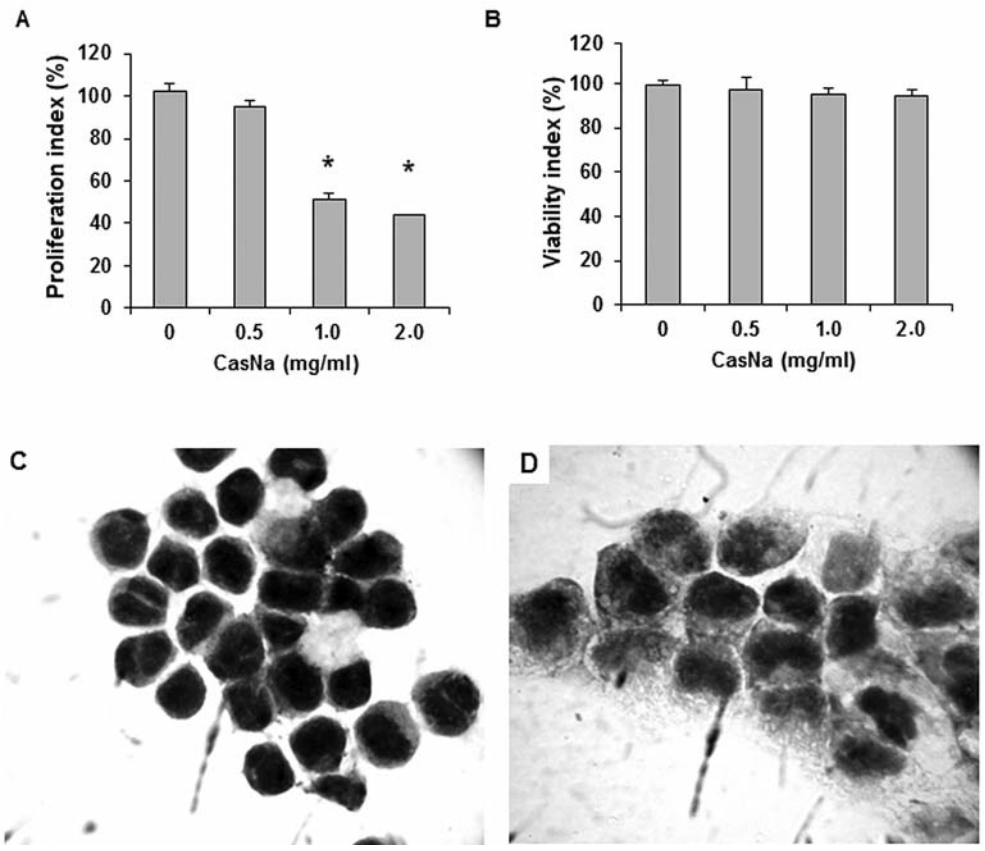

Figure 1. *Proliferation (A) and viability (B) of J774 cells in the presence or absence of CasNa. Each point represents the mean±standard deviation of at least three independent assays. ANOVA Tukey test, *p<0.05. C: Representative images showing differentiation of J774 cells in the absence and presence of 2 mg/ml of CasNa (J774+PBS and J774+CasNa, respectively). Wright stain, ×100.*

hepatomegaly and solid tumours (Figures 3A-C), thereby resulting in the death of both female and male mice, before one hundred days (Figure 4).

CasNa reduces splenomegaly, hepatomegaly and tumour burden, while it increases the survival of mice inoculated with J774 cells. Leukaemia was induced using J774 cells as described previously, and our results show that treatment with CasNa significantly reduced splenomegaly, hepatomegaly and the presence of solid tumours (Figures 3A-C). We observed that all animals that were not treated with CasNa died before 100 days, while those treated with CasNa survived for a longer period, with 60% survival after 160 days in females and 50% survival in males. A Kaplan-Meier (log-rank) test was run and we found that the three treatments were significantly different ($p<0.05$) between PBS *vs*, CasNa, PBS *vs* Ara-C or CasNa *vs* Ara-C. (Figures 4A and B). Overall, the increased survival of mice treated with CasNa, and the decreased splenic, liver and tumour index indicate that CasNa also inhibits the proliferation of J774 cells *in vivo*.

Mononuclear bone marrow cells isolated from mice that survived the inoculation of J774 cells treated with CasNa are unable to proliferate in vitro in the absence of rmIL3. We aimed to determine whether J774 cells persisted in BALB/c mice treated with CasNa that survived for 260 days. Therefore, we evaluated the proliferation of bone marrow MNCs derived from these mice compared with that of bone marrow cells from healthy mice. Our results show that the MNCs in the bone marrow of mice that survived the inoculation of J774 cells and were treated with CasNa are unable to proliferate *in vitro* in the absence of rmIL3 (Figure 3D). Therefore, our results suggest that CasNa induces cure of J774 leukaemia macrophage-like cell-induced leukaemia in surviving mice.

Discussion

One of the major problems concerning improved AML therapy is the long-term, low rate of disease-free survival (12); the scenario is worsened if the patient is over 65 years

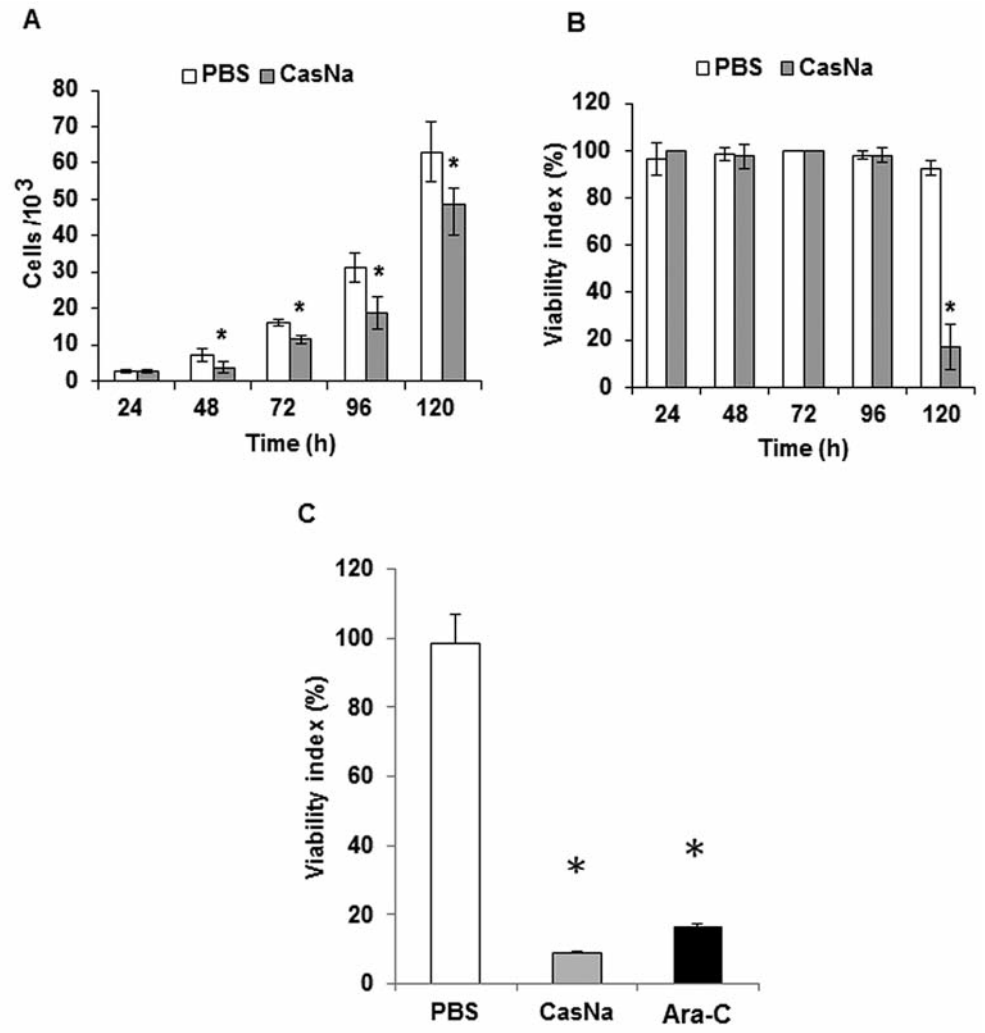

Figure 2. *Proliferation (A) and viability (B) of J774 cells at 24-h intervals in the absence or presence of 2 mg/ml CasNa (PBS or CasNa respectively). (C) Viability index of J774 cells in the presence and absence of 2 mg/ml CasNa or Ara-C in 120 h culture. Each point represents the mean±standard deviation of at least three independent assays. ANOVA Dunnet test, *p<0.05.*

of age, when intensive treatments are poorly tolerated or induce rapid death (13). Furthermore, after a course of chemotherapy for AML with epipodophyllotoxins and anthracyclines, it has been found that patients develop secondary leukaemias, particularly the M5 FAB subtype of AML or acute monocytic leukaemia (14).

Our recent studies have shown that CasNa, a casein salt, reduces the proliferation of various leukaemia cell lines, (but not normal hematopoietic cells) *in vitro* while promoting granulopoiesis in healthy mice *in vivo* (15). Although we found that CasNa reduces the proliferation of leukaemia cells, it was uncertain whether prolonging the exposure time also affected viability and differentiation, two key elements in the physiology of leukaemia cells closely associated with malignant growth (16).

Previously, we observed an increase in monocytic differentiation by 72 h which did not negatively affect viability. Notably, by increasing the treatment time to 120 h, cell viability dropped to 80%. However, we consider it important to clearly identify the transition of J774 monoblastic cells to fully-differentiated and functional J774 monocyte-macrophage, therefore, in the future, we aim to analyze whether differentiation is associated with a specific induction of the cell death path.

The establishment of murine leukaemia models using leukaemia cell lines is considered ideal for the study of possible therapeutic drugs (17). Unfortunately, although monocytic leukaemia is the most common subtype among secondary leukaemias (14) and is difficult to treat, and few monocytic leukaemia models have been reported (10, 11, 18).

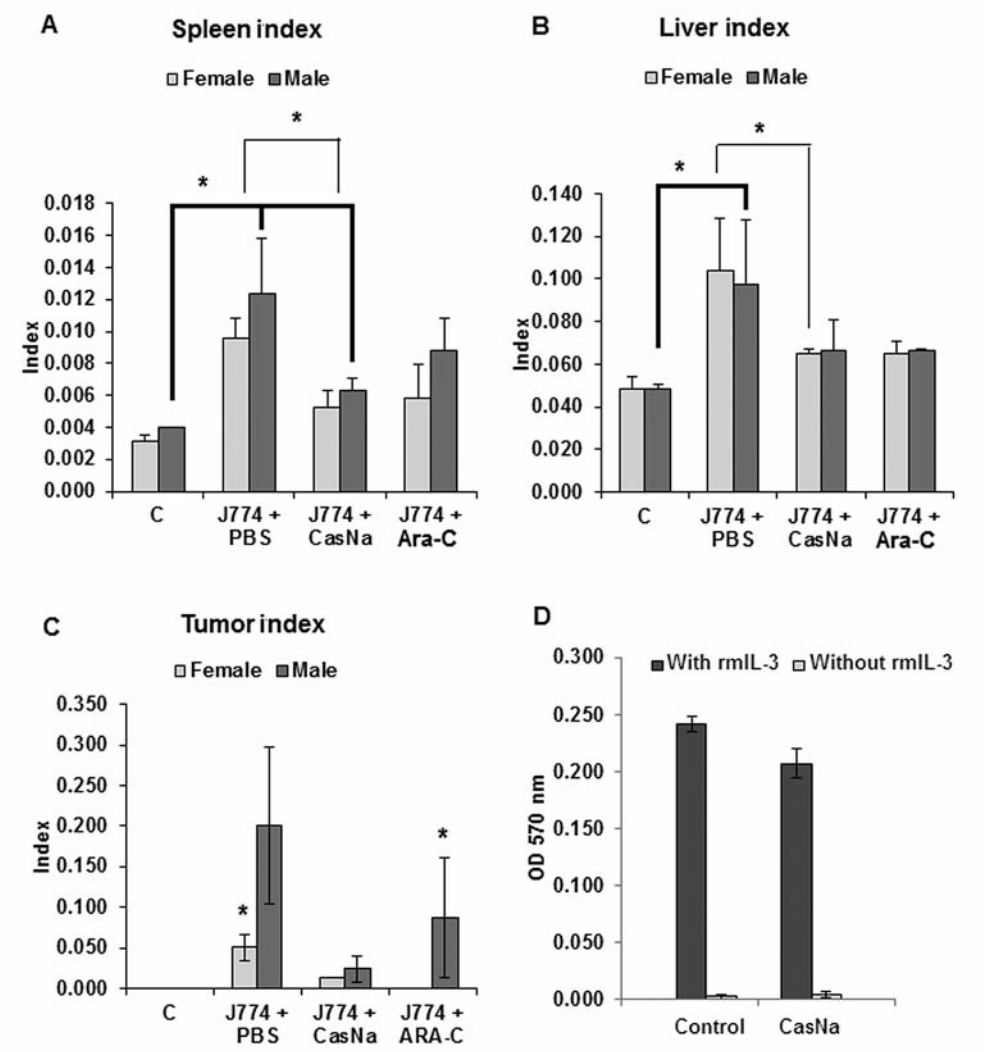

Figure 3. *Splenic (A) hepatic (B) and tumoural (C) index of female and male BALB/c mice intraperitoneally inoculated with 1×10^6 J774 cells. Each point represents the mean±standard deviation of at least three independent assays. (D) Proliferation of mononuclear cells of bone marrow of BALB/c mice, control or surviving BALB/c mice inoculated with J774 cells and treated with CasNa, with and without 5 ng/ml of rmIL3. Representative assay. ANOVA Dunnet test, *p<0.05.*

Therefore, we evaluated whether the inoculation of J774 monocytic leukaemia cells (19) generated from the murine BALB/c, induced macrophage tumours, as reported by others (10, 11).

Injection of this cell line *i.p.* into both male and female mice proved to be lethal, and all mice displayed similar symptoms, despite the gender of the animals. This appears to contrast with the incidence of monocytic leukaemia with little differentiation (M5a) in which there is a greater tendency for patients to be male (males: 57%, females: 43%) (14). However, considering the time point of the first symptoms, our results suggest that males are more likely to develop the disease earlier than females; in this sense, we wonder if CasNa could prolong survival in a scenario of worst disease. Males treated with CasNa had extended survival compared to those that were not treated or to animals that received Ara-C, which has been used as an anti-neoplastic control in *in vivo* models (11). Our data are consistent with the work of Castano *et al*., in which mice inoculated with J774 cells, either untreated or treated with anti-neoplastic cyclophosphamide, had similar survival curves, and in all cases the mice died (10). Interestingly, the prolonged survival of leukaemic mice *via* CasNa treatment is considered as evidence of growth inhibition of leukaemia cells *in vivo* (20).

A common problem with leukaemia treatment is the presence of minimum residual disease responsible for relapse (21); the problem is exacerbated with the M5 subtypes, which

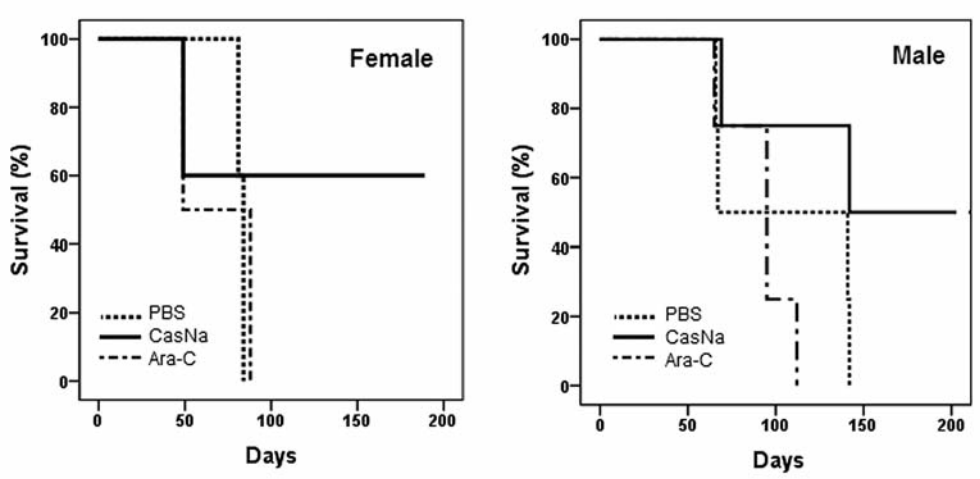

Figure 4. *Survival curve of BALB/c mice treated with PBS, CasNa (10% w/v) or Ara-C every 48 h. Kaplan–Meier (Log rank) test shows CasNa statistical difference with respect to PBS p<0.05.*

have high incidence in secondary leukaemias (22). No specific surface marker of J774 cells has yet been reported, however, the presence of residual malignant cells in the bone marrow can be established by assessing *in vitro* proliferation in the absence of growth factors (23). Interestingly, when we cultivated MNCs isolated from surviving mice inoculated with J774 and treated with CasNa, we found no evidence of MNCs that were capable of autonomous proliferation. Because cell proliferation in the presence of rmIL3 was similar in healthy mice and leukaemia survivors, these data suggest that CasNa treatment may be a curative alternative in this macrophage tumour model.

While it is clear that CasNa promotes survival, the mechanisms of *in vivo* antileukaemic action are unknown. In this regard, recently, we observed that *i.p.* administration of CasNa into healthy mice induces the production of cytokines in both the plasma and bone marrow (15); therefore, the antitumour properties described herein would seem to result from the massive induction of inflammatory cell migration into the peritoneal cavity (24) either *via* bioactive components of CasNa (25) or the secretion of growth factors, or differentiation or inflammatory systemic action (26). Additionally, we believe that CasNa must activate mechanisms beyond a simple inflammatory process because although other agents, such as zymosan or thioglycollate, increase the levels of proinflammatory cytokines (IL1β, TNF-α, MIP2 and MCP-1/CCL2) (27, 28), they have no inhibitory effect on proliferation of hematopoietic leukaemic cells. On the other hand, CasNa also promoted normal haematopoiesis in healthy mice, representing a suitable therapeutic framework in which leukaemic cells are reduced, and favouring the immune response, such as granulopoiesis, to counter the leukemia cells.

In conclusion, CasNa prolongs the survival of mice that were lethally-inoculated with J774 cells, which represents clear evidence of antileukaemic activity.

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