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Sodium Caseinate (CasNa) Induces Mobilization of Hematopoietic Stem Cells in a BALB/c Mouse Model

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Hematopoietic stem cells transplantation has high clinical potential against a wide variety of hematologic, metabolic, and autoimmune diseases and solid tumors. Clinically, hematopoietic stem cells derived from peripheral blood are currently used more than those obtained from sources such as bone marrow. However, mobilizing agents used in the clinic tend to fail in high rates, making the number of mobilized cells insufficient for transplantation. We investigated whether sodium caseinate induces functional mobilization of hematopoietic stem cells into peripheral blood of Balb/c mice.

Material/Methods: Using a mouse model, we administrated sodium caseinate or Plerixafor, a commercial mobilizing agent, and analyzed counts of hematopoietic stem cells in peripheral blood, and then cells were transplanted into lethally irradiated mice to restore hematopoiesis. All assays were performed at least twice.

Results: We found that sodium caseinate increases the number of mononuclear cells in peripheral blood with the immunophenotype of hematopoietic stem cells (0.2 to 0.5% LSK cells), allowing them to form colonies of various cell lineages in semisolid medium ($p<0.05$). This effect is similar to that of Plerixafor, and cells transplanted into lethally irradiated mice can restore hematopoiesis at higher percentages than mononuclear cells mobilized by Plerixafor (40% *vs.* 20%, respectively). Further, a secondary transplant rescued a separate group of irradiated mice from death, proving definitive evidence of hematopoietic reconstitution after hematopoietic stem cells transplantation. Data are presented as mean ± standard deviation. To determine significant differences between the data, one-way ANOVA and the Tukey test were used.

Conclusions: Collectively these results show the utility of sodium caseinate as a mobilizer of hematopoietic stem cells and its potential clinical application in transplantation settings.

MeSH Keywords: Caseins • Hematopoietic Stem Cells • Transplants

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Background

Almost 60 000 autologous and allogeneic stem cell and hematopoietic progenitor cell transplant (HSC/HPCT) procedures are performed annually to rebuild normal bone marrow (BM) in patients who had received a regimen of chemotherapy, radiation, or a combination of both to treat disease hematologic, metabolic, or autoimmune diseases or solid tumors [1]. Until recently, BM was the source of HSCs for transplantation, but it is now possible to obtain HSCs from other sources, such as mobilized peripheral blood (PB), so that almost all autologous transplants and 75% of allogeneic transplants use PB as a cell source for HSCs [2].

Under homeostasis, the number of HSCs in the PB is insufficient for transplantation [3–6], but it has been shown that HSC numbers can be pharmacologically increased by inducing mobilization of the HSC niche in the BM to PB [7,8]. Classical mobilization protocols include the use of cytokines, such as granulocyte colony-stimulating factor (G-CSF analogs, filgrastim or pegfilgrastim) and granulocyte macrophage colony-stimulating factor (GM-CSF analog, sargramostim) either alone or in combination with chemotherapy [9,10]. However, there are still many failures of mobilization, and the use of mobilizing agents may be associated with cytotoxicity and adverse effects such as low graft quality [9].

Recently, a selective antagonist molecule for CXCR4 receptor, known commercially as Plerixafor (AMD3100, Mozobil), has shown potential as a mobilizer of HSCs, reversibly regulating the output of HSCs to PB. Interestingly, this molecule quickly mobilizes HSC and promotes rapid recovery in patients who received a transplant. Unfortunately, even with Plerixafor mobilization, failures occur in 10–30% of cases, making it important to develop new mobilizing agents for clinical use, thereby improving outcomes and graft quality [9].

It is widely known that inoculation of sodium caseinate (CasNa), a salt of casein, the principal milk protein [11], induces the accumulation of granulocytes and macrophages in the peritoneal cavity of mice and increases the serum levels of cytokines related to HSC mobilization [3,12,13]. In this regard, we recently showed that granulocyte activation by CasNa, which resembles stimulation by G-CSF or GM-CSF [14], increases spleen size, weight, and cellularity [15–17]. This suggests extramedullary hematopoiesis activation, which may involve migration of HSC or HPC [18]. Therefore, in this paper we show that CasNa mobilizes MNCs with the HSC immunophenotype (LSK cells) and that these cells have the ability to form colonies in culture and rescue lethally irradiated mice from death through a secondary transplant, evidence of the presence of HSCs.

Material and Methods

Animal model

Syngeneic Balb/c mice, 8 to 12 weeks old, were maintained in pathogen-free conditions in the animal facility of Zaragoza Faculty of Advanced Studies (FES-Zaragoza), with *ad libitum* sterile standard powdered rodent diet. One week prior to transplantation, recipient mice were given water acidified to pH 2.5–3.0. All experimental protocols were approved with the EV number FESZ/DEPI/CI/128/14 by the Ethics Committee of Zaragoza Faculty of Advanced Studies, and were performed in accordance to the "Guide for the Care and Use of Laboratory Animals, Eighth Edition" published by the National Institutes of Health, and in accordance with the national regulation for the care and use of experimental animals, NOM-062-ZOO-1999.

Cell mobilization

All molecules used here were administered intraperitoneally (i.p.) in 1 mL of phosphate buffer solution (PBS) as vehicle. Mice in the donor groups received 0.1 g/mL of sodium caseinate (CasNa) (Spectrum, New Brunswick, NJ) or only 1 mL of PBS alone 4 times, once every 48 h. Plerixafor (Sigma-Aldrich, St Louis, MO) was administered in a single dose (5 mg/kg) 1 h before sacrifice. At 24 h after the last CasNa inoculation or 1 h after Plerixafor inoculation, mice were anesthetized with ether. Blood axillary plexus was obtained and then mononuclear cells (MNCs) of PB were isolated by density gradient using Ficoll (δ=1.077 g/mL) (Sigma-Aldrich, St Louis, MO). Once these MNCs were obtained, the cell number was assessed by performing a count in a Neubauer chamber on an inverted microscope at 10×.

Flow cytometric analysis

Cell preparation and analysis were performed as follows. Mouse HSCs were defined as Lin- Sca-1+ c-Kit+ (LSK). The immune subsets were gated as anti-CD34 antibody (clone RAM34) conjugated with FITC (fluorescein isothiocyanate), anti-c-Kit (clone 2B8) conjugated with PE (phycoerythrin) and anti-Sca-1 (D7 clone) conjugated to Cy-7 PE (phycoerythrin Cy-7). To purify cells committed to a hematopoietic lineage, a cocktail of antibodies was used (Lin), CD3 (clone 145-2C11), CD45R (B220) (clone RA3-6B2) Ly6C and Ly6G (Gr1) (clone was used RB6–8C5), CD11b (Mac1) (clone M1/70), TER-119 (clone TER-119) together with APC (allophycocyanin). All antibodies reactive with murine cell antigens were purchased from BD Biosciences San Diego, CA, USA.

Colony formation assay

Colony formation assays were performed using MethoCult GF M3434 (StemCell Technologies, Vancouver, BC, Canada). In accord to manufacturer's instructions, which suggest for

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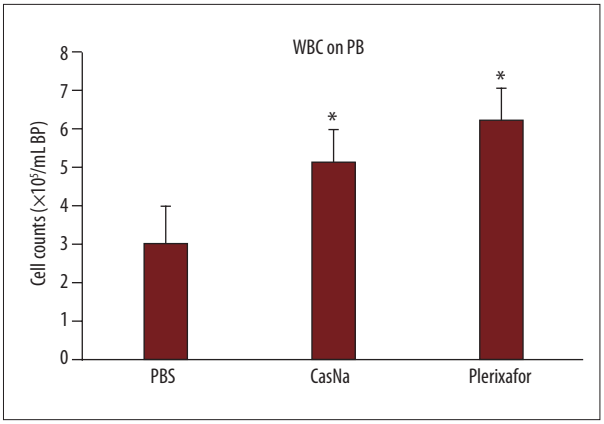

Figure 1. Count of mononuclear cells obtained from peripheral blood (WBC on PB) of Balb/c mice treated with vehicle (PBS), CasNa (0.1 g/mL), or Plerixafor (5 mg/kg).
* P<0.05, error bars represent standard deviation.

peripheral blood cells, seeding 1×10^5 MNCs cells, mouse CFU numbers evaluated *in situ* will be approximately 26 BFU-E progenitors. We seeded 1×10^5 of mobilized MNCs in petri dishes 35×10 mm (Corning, NY, USA) using MethoCult M3434 (Stem Cell Technologies, Vancouver, BC, Canada), which contains a cocktail of growth factors, including recombinant mouse stem cells factor (rmSCF), recombinant mouse IL-3 (rmIL-3), recombinant human IL-6 (rhIL-6), and recombinant human erythropoietin (rhEpo). Cultures were maintained at 37°C, 5% CO_2 and moisture dew point for 14 days. Colonies were counted with an inverted microscope (PrimoStar).

Transplantation and secondary transplant

Balb/c recipients were subjected to 8.5 Gy of irradiation using a Gammacell 1000 Nordion irradiator ^{137}Cs isotope. Four hours later, mice was transplanted via the tail vein with 2x10^6 MNC mobilized in 200 uL of PBS supplemented with 1% mouse serum. The lethally irradiated mice were housed in a sterile environment, and sterile food and acidified water was provided ad libitum. After transplantation, mice were monitored daily for at least 4 months (22 weeks).

Balb/c recipients that survived the first radiation were used for obtaining MNCs for transplanting a secondary group of irradiated mice, as detailed above. MNCs from BM mice aged 8–10 months, approximately the same age as the first transplant survivors, were used as a graft control. In both cases, 5×10^6 MNC-BM/mouse were transplanted, and mice were monitored daily for 6 months (26 weeks), as previously described.

Statistics

All assays were performed at least twice. Data are presented as mean ± standard deviation. To determine significant differences between the data, one-way ANOVA and the Tukey test ($p<0.05$) were used; survival is reported using Kaplan-Meier graphs. Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.

Results

CasNa increased MNC and mobilized LSK cells into peripheral blood

To determine the ability of CasNa to mobilize HSCs into PB, Balb/c mice were inoculated i.p. with CasNa, Plerixafor, or vehicle alone (PBS) and MNCs were obtained 24 h later. The results showed that CasNa induces a significant increase in counts of MNCs, similar to that of Plerixafor, in comparison to only PBS as vehicle treatment (Figure 1). Then, LSK cells mobilized by CasNa were compared with those mobilized by Plerixafor or PBS alone, and the data demonstrated that CasNa mobilized more LSK cells than Plerixafor (0.5% and 0.3%, respectively, compared to 0.2% of PBS) (Figure 2).

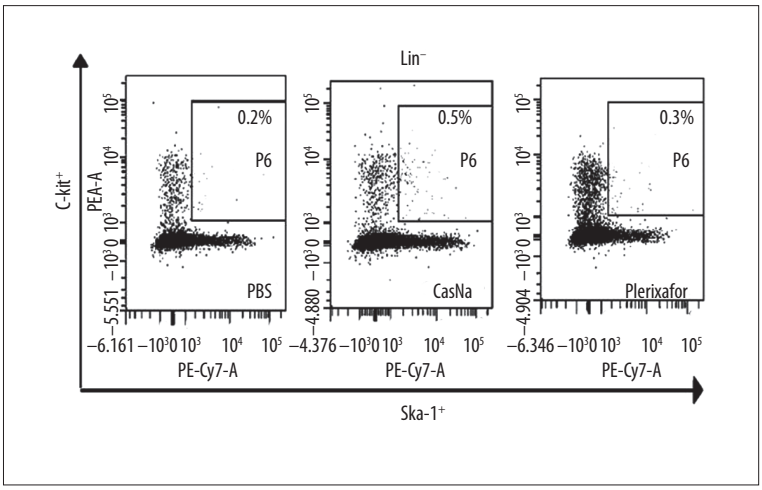

Figure 2. Mononuclear cells from peripheral blood stained for HSCs (lineage negative, Sca-1+ and C-kit+) obtained from mice treated with vehicle (PBS), CasNa (0.1 g/mL), or Plerixafor (5 mg/kg).

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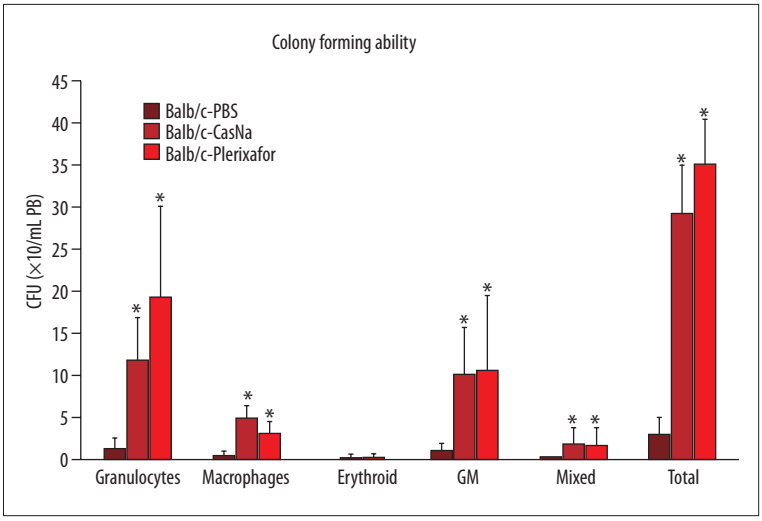

Figure 3. Colony-forming units (CFU) from mononuclear cells obtained from peripheral blood of mice treated with vehicle (BALB/c-PBS), 0.1 g/mL of CasNa (BALB/c-CasNa), or 5 mg/kg of Plerixafor (BALB/c-AMD3100). GM (granulocyte-macrophage). * P<0.05, and error bars represent standard deviation.

By contrast, colony formation assays revealed that cells mobilized by CasNa and AMD3100 contained significantly more colony-forming units than MNCs from mice treated with PBS alone, by approximately 15-fold. When the type of CFU was evaluated, the most common were granulocyte colonies, followed by granulocyte-macrophage, macrophages, and, interestingly, despite not finding erythroid colonies, we observed an increase in mixed colonies, which are characterized by containing cells from 3 lineages (macrophage, granulocyte, and erythroid) (Figure 3).

CasNa-mobilized grafts significantly enhanced survival of lethally irradiated Balb/c mice

The results show that CasNa and Plerixafor induced increase in the numbers of LSK cells and that they produce CFUs, which shows the presence of HSCs/HPCs. However, considering that the ultimate test of HSC functionality is their ability to rescue lethally irradiated individuals from death, MNCs mobilized to PB with CasNa, Plerixafor, or PBS alone were transplanted to lethally irradiated syngeneic mice (Figure 4). Of mice receiving grafts mobilized by CasNa, 40% survived to 20 weeks after transplantation. Interestingly, the engraftment of the cells mobilized by AMD3100 was significantly inferior to that of the cells mobilized by CasNa, with a survival rate of only 10% at 19 weeks, although the number of CFUs and LSK cells is comparable between treatments, even though Plerixafor mobilized more CFUs than CasNa. In contrast, mice receiving grafts mobilized by PBS alone died within 2 weeks, probably due to failure to reconstitute hematopoietic cells, as suggested by the much lower frequencies of CFUs and LSK cells then in the CasNa or AMD3100 treatments.

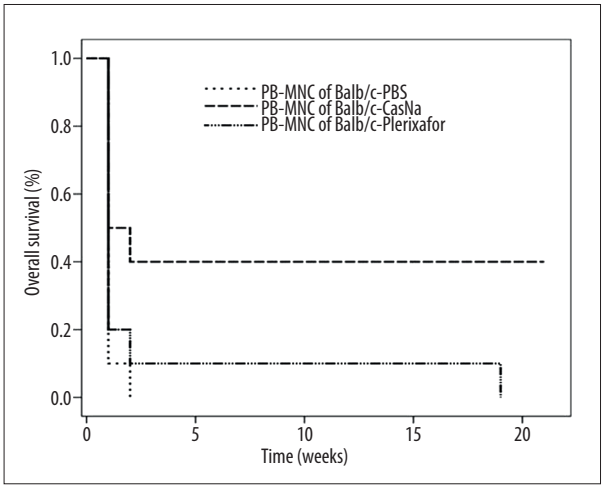

Figure 4. Survival analysis of lethally irradiated BALB/c mice receiving peripheral mononuclear cells mobilized by vehicle (PB-MNC of BALB/c-PBS), 0.1 g/mL of CasNa (PB-MNC of BALB/c-CasNa), or 5 mg/kg Plerixafor (PB-MNC of BALB/c-Plerixafor). Each group contains 5 mice. Kaplan-Meier analysis was performed over 22 weeks. * P<0.05 compared with BALB/c-PBS.

Secondary grafts of BM MNCs from mice previously grafted with MNCs from CasNa-treated mice significantly enhanced the survival of lethally irradiated Balb/c mice

The enhanced survival at 20 weeks suggested HSC engraftment and hematopoietic reconstitution. To verify the latter, we analyzed whether the BM of the survivors (22 weeks after being irradiated and transplanted with MNCs mobilized to PB with CasNa) contained HPCs, as a criterion for hematopoietic reconstitution following the transplant. Notably, MNCs of BM from survivors of first transplant have the ability to form colonies of all lineages (granulocytes, macrophages, erythroid, granulocyte-macrophage, and mixed), which indicates repopulation of

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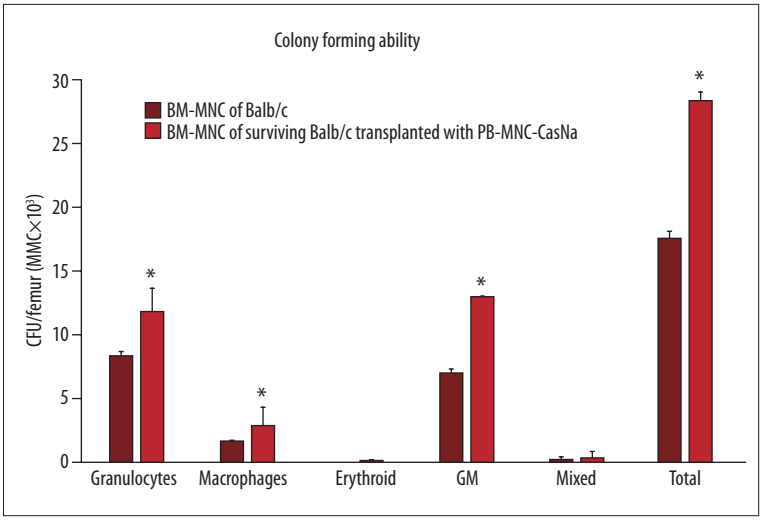

Figure 5. Colony-forming units (CFU) from mononuclear cells (MNC) obtained from bone marrow of surviving BALB/c mice transplanted with MNCs of peripheral blood from CasNa-treatment mice (Figure 4) or transplanted with MNCs obtained from bone marrow of healthy mice (BM-MNC of BALB/c). * P<0.05, error bars represent standard deviation. BM-MNC of surviving BALB/c transplants, contains 2 mice.

the bone marrow in irradiated mice. Indeed, we found a greater number of CFUs in surviving mice when compared with the number of CFUs from healthy mice of the same age (Figure 5). Next, we analyzed whether MNCs from BM of surviving mice had the ability to rescue a new group of lethally irradiated mice from death, and we found that 20% of mice receiving grafts of MNCs from surviving mice were still alive after 25 weeks, but mice transfused with MNCs from the BM of untreated mice of the same aged died at 2 weeks (Figure 6). This suggests that CasNa effectively induces functional HSC mobilization into PB.

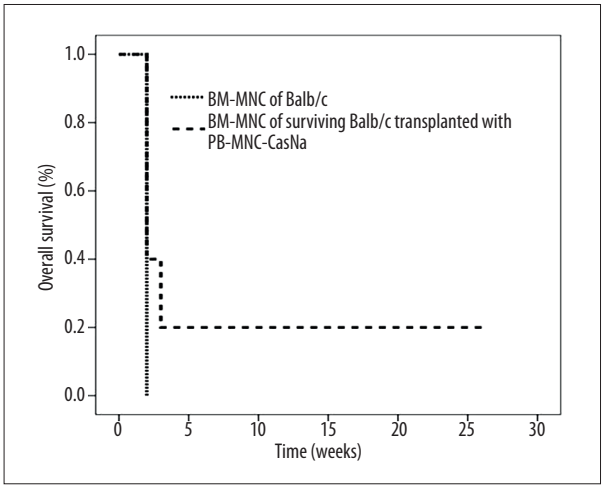

Figure 6. Survival analysis of lethally irradiated Balb/c mice receiving mononuclear cells of bone marrow of health BALB/c mice (BM-MNC of BALB/c) or bone marrow of surviving Balb/c mice transplanted with peripheral MNC mobilized by CasNa. (Figure 4). Each group contains 5 mice. Kaplan-Meier analysis.

Discussion

Although it is known that counts of HSCs/HPCs in PB are very low, they can be increased pharmacologically using various molecules, such as cytokines, chemokines, and certain regimens of chemotherapy [5,7]. The main obstacle to the use of mobilizing agents is the risk of insufficient mobilization of HSCs/HPCs, in addition to cytotoxicity and adverse effects, so there is a need for novel strategies to increase the performance of HSCs/HPCs mobilization, improving graft quality and transplant outcomes [9].

Historically, CasNa had been used in studies of inflammation to induce migration of granulocytes and monocytes [12] and increase the concentration of cytokines, such as G-CSF and GM-CSF [13], which are directly related to the generation of myeloid cells and the mobilization of HSC to the PB. Recently, our research group found that CasNa induces granulopoiesis on Balb/c mice [14] and increase the counts of MNC in PB, suggesting the induction of cell migration from the bone marrow to the PB [17]. However, it remained unknown whether CasNa induces mobilization of HSCs/HPCs.

In this work we confirmed that intraperitoneal inoculation of CasNa in healthy mice increased the counts of MNCs in PB compared to PBS-treated mice. Analysis of whether these cells were LSK cells, which is considered characteristic of HSCs/HPCs in the mouse [19], showed that CasNa, like the commercial mobilizing agent Plerixafor, increases the percentage of these cells in the PB. Interestingly, when analyzing whether these LSK cells had the ability to form colonies in culture, which is considered as evidence of HPCs [20], we noted that mice treated with either CasNa or Plerixafor showed 15 times more CFUs than PBS-treated mice and that analysis of the lineages of those colonies showed granulocytes, monocytes, GM, and mixed colonies, which suggests ample hematopoietic repopulation.

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Because the HSC functional test is a compelling demonstration of reconstitution of the hematopoietic system of a myelosuppressed individual [21–23], we performed a transplantation assay of mobilized MNCs into lethally irradiated mice. Our results showed that cells mobilized with CasNa had greater survival capacity than MNCs from Plerixafor-treated mice. This is interestingly because Plerixafor induced an increase in the percentage of LSK cells in MNCs of PB, but these cells had a limited capacity to induce survival in irradiated individuals, which is consistent with a report in the literature [24] indicating that immunophenotype, although useful for isolation and characterization of cells for transplantation, cannot be equated with functionality in terms of ability to rescue myelosuppressed individuals from death [25].

For many years the evidence for hematopoietic reconstitution has been counting lymphoid and myeloid cells in peripheral blood 4 months after transplantation [26]. However, Ema et al. [27] mentions that since the granulocytes have extremely short lifespans, their presence directly reflects HSC activity. Continuing the above analysis, we assessed the presence of granulocyte progenitors in the BM of mice surviving the first transplant and found that these mice have significantly more granulocyte progenitors, as well as macrophages and mixed GM, indicating a hematopoietic reconstitution. Finally, we conducted a secondary transplant to definitively demonstrate the presence of HSC in the graft [22,24,28–30] and found that the BM MNCs obtained from the surviving mice have the ability to rescue 20% of individuals myelosuppressed for at least 8 months. If we consider the survival time from the first transplant, this represents a total of 12 months of life after mobilization, which constitutes strong evidence that CasNa mobilizes HSCs to PB, and that this is capable of long-term reconstitution. However, success of our graft was only 20% in the secondary transplant, thus it is necessary to explore ways in which we improve graft efficiency to achieve survival in all cases, before we considering entering preclinical testing. In this sense, it has at present only been shown that the combination of mobilizers considerably increases the number of HSC/HPC in comparison to the administration of only 1 agent, for example, filgrastim [31].

Conclusions

Sodium caseinate, like Plerixafor, a commercial mobilizer of HSC, increases counts of MNCs and LSK cells in peripheral blood, with the ability to form colonies. However, only MNCs mobilized by CasNa enhanced the survival of lethally irradiated mice. Consequently, sodium caseinate induces functional mobilization of HSCs in PB of Balb/c mice.

Study limitations

The major limitation of this study is that we did not include controls on bone marrow in the first transplant; this is needed to compare the effectiveness of cells mobilized by grafting against a traditional bone marrow transplant.

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Conflict of interest statement

All authors declare no conflicts of interest.

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