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Article

A short oro-gastric imipramine administration in *Brucella abortus 2308* infected mice decreases CFU count at the spleen; improves the IFN-γ/IL-6 ratio; restores serotonin levels in the hippocampus, muscular strength, and mood.

Maldonado-Tapia Jesús Octavio [1,2,†], Maldonado-García José Luis [1,2,†], Pérez-Sánchez Gilberto [1], Becerril-Villanueva Enrique [1], Alvarez-Herrera Samantha [1], Pavón Lenin [1,*], Gutiérrez-Ospina Gabriel [3,4,*], López-Santiago Rubén [2] and Moreno-Lafont Martha C [2,*]

[1] Laboratorio de Psicoinmunología, Dirección de Investigaciones en Neurociencias del Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz, Ciudad de México 14370, Mexico; jmaldonadot1200@egresado.ipn.mx (M.-T.J.O.); joselmgarci@comunidad.unam.mx (M.-G.J.L.); gilberto.perez.sanchez@imp.edu.mx (P.-S.G.); lusenbeve@imp.edu.mx (B.V.E.); dra.alvarezherrera@gmail.com (A.-H.S.)

[2] Laboratorio de Inmunología Celular, Departamento de Inmunología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Ciudad de México 11340, Mexico; rlopezsantiago@hotmail.com (L.-S.R.).

[3] Laboratorio de Biología de Sistemas, Departamento de Biología Celular y Fisiología, Instituto de Investigaciones Biomédicas and Coordinación de Psicobiología y Neurociencias, Facultad de Psicología, Universidad Nacional Autónoma de México, Ciudad de México 04510, Mexico; gabo@iibiomedicas.unam.mx

[4] Department of Zoology and Physiology, University of Wyoming, Laramie, WY 82071, USA

* Correspondence: lkuriaki@imp.edu.mx (P.L.); gabo@iibiomedicas.unam.mx (G.-O.G.); mlafont@ipn.mx (M.-L.M.C.).
Tel.: +52-5541-605082 (P.L.); +52-56-2289-59(G.-O.G.).; +52-5729-6300 (ext. 62368) (M.-L.M.C.)

† These first authors contributed equally.

Abstract: Brucellosis infection induces fever, chills, sweats, headache, myalgia, arthralgia, anorexia, fatigue, and mood disorders. In mice, it causes a rise in IL-6, TNF-α, and IFN-γ; and reduces serotonin and dopamine levels in hippocampus. There is loss in muscle strength and equilibrium and increased anxiety and hopelessness. Imipramine (ImiP) is a tricyclic antidepressant that increases the capacity of macrophages to destroy intracellular microorganisms in vivo. The effect of ImiP was evaluated in Balb/c mice infected with *Brucella abortus 2308*. Serum levels were determined in IFN-γ, IL-6, TNF-α, IL-12, MCP-1, and IL-10 by FACS; the bacterial count in the spleen, by CFU; the serotonin concentration in the hippocampus, by HPLC; and strength, equilibrium, and mood by behavioral tests. Our results showed that infected vs. control mice had a significant rise in levels of IFN-γ, IL-6, TNF-α, and IL-12 with a low IFN-γ/IL-6 ratio, elevated bacterial-counts, alterations in serotonin concentration at hippocampus, and decreased muscular strength, equilibrium, and mood. Infected mice treated with ImiP vs. infected mice showed: 1) improved mood; 2) increased hippocampal serotonin availability, splenic dendritic cells, and macrophage phagocytic activity; and 3) upturn inflammation and reduced CFU ability. Our results support that ImiP favors positive outcomes in subjects handling Brucella infections likely by improving psychoimmune resilience.

Keywords: Depression, psychoimmune resilience, tricyclic antidepressants, serotonin, modulation of inflammatory response, infectious diseases

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1. Introduction

For at least a decade, it has been entertained the hypothesis that treatment-resistant mood disorders occur secondary to microbial infections either due to direct neural infection and/or mediated through chronic neuroinflammation [1–8]. Microbes may enter anew the body or may invade it from "inner" sources when mucosae integrity is compromised and commensal/symbiotic organisms that inhabit the body gain access to its internal milieu [3,9–11]. On the other hand, it is also believed that mood disorders, especially depression, provide an evolutionary advantage for hosts to deal with microbial disease by furnishing reciprocal modulatory interactions between the mood and the immune response (the pathogen-host defense theory of depression; [12,13]). Accordingly, depression may pre-condition the individual's risk to develop severe infections [14,15] and/or worsen disease progression and outcome [12,13,16]. Under this context, it might be fundamental to enhance psychoimmune resilience [17], by modulating the depression subjects already infected.

Brucellosis is a zoonosis present in Latin America, Africa, and the Mediterranean region. In humans, this infection induces non-specific symptoms such as fever, chills, sweats, headache, myalgia, arthralgia, anorexia, fatigue, weight loss, lymphadenopathy (10-20%), splenomegaly (20-30%) and diarrhea [18], as well as Dep/Anx and other neurological symptoms [19,20]. Brucellosis induced in experimental animal units displays a clinical picture similar to that observed in humans, including the presence of anxiety and depression-like symptoms, altered serotonin levels in the hippocampus, and increased peripheral inflammation [21]. Hence, this experimental mouse unit seems useful to explore whether down modulating depression could contribute to improve the clinical outcome in *Brucella* infected mice. Having this in mind, in this work, we evaluated this possibility by administering imipramine (ImiP), a tricyclic antidepressant drug with serotonin transporter blocker activity, which allows the restoration of serotonin levels in the hippocampus [21], and behavioral symptomatology.

2. Materials and Methods

2.1 Mice

Six-to-eight weeks old, female BALB/c mice (n=40; 18-21 grams of body weight), supplied by UPEAL, Mexico, were housed in groups of five per cage. Upon arrival, mice were allowed to habituate for 21 days before starting the experiments. Mice were kept in temperature (21°C) and light (7:00 on/19:00 off, regular cycle) controlled rooms at the animal facility of the Department of Immunology of the Escuela Nacional de Ciencias Biológicas (ENCB), Instituto Politécnico Nacional. Mice had free access to water and food (Lab Rodene Diet 500, USA) until their sacrifice. The study design considered four groups. Control (Ctrl; n=10), *B. abortus* 2308 infected mice (*Ba*; n=10), *B. abortus* 2308 infected and ImiP treated mice (Im*Ba*; n=10), and ImiP treated mice (ImiP; n=10). A single intraperitoneal inoculation was done inside a biosafety cabinet (Nuaire Class II type A/B3) at day 0 with either phosphate buffer-saline (PBS; 0.1M, pH 7.4; 100 µl; Ctrl and ImiP) or with $1x10^6$ Colony Forming Units (CFU) of *B. abortus* 2308 suspended in 100 µl PBS (*Ba* and Im*Ba* mice). Behavioral tests (see below) and blood samples were conducted withdrawn inside a biosafety cabinet 14 days later. Animals were then sacrificed by cervical dislocation and brain samples dissected (see below). Animal handling and experimentation protocols were revised and approved by the local Ethics Committee (Register No. ENCB/CEI/077/2020).

2.2 Imipramine administration

ImiP and Im*Ba* mice were treated daily (500µl), from day 8 to 14, with ImiP (15 mg/kg/day) through an orogastric tube (Cat: FTP-20-38; Instech, Plyouth Meeting, PA, USA). ImiP tablets of 25 mg (Psicofarma, CDMX, Mexico) were grounded and dissolved in sterile water until reaching the required concentration.

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2.3 Behavioral test

At day 14, each Ctrl, *Ba* or Im*Ba* mice was subjected to open field (OF) and forced swim (FST) tests precisely as described by us elsewhere [21]; OF and FST are tests designed to estimate motivation.

2.4 Collection of serum, spleen, and brain samples

At day 14, blood samples were withdrawn from the facial vein. These samples were centrifuged (2,000×g) at room temperature for 10 min. The serum was then collected aliquoted (100 µl) and stored at -80 °C until use. Mice were then sacrificed by cervical dislocation and decapitation. We proceeded to remove the brain from the skull and to dissect the hippocampus as previously described [21]. Tissue samples were weighed using an analytical balance, frozen in liquid nitrogen, and stored at -80 °C until further processing.

2.5 Determination of spleen *Brucella* colony forming units (CFU)

After decapitation, the spleen was removed, mechanically minced in a Petri's dish filled 5 mL of PBS (1M) and meshed using a cell straimer (Cat: 431750; Corning®, Corning, NY, USA). The cell suspension obtained was washed once in PBS (3 mL), centrifuged and re-suspended at room temperature. Twenty microliters of this cell suspension were then taken and sequentially diluted (1:10) in 1M PBS until achieving a final dilution of 10^{-4}. Then, 20µl samples of each dilution were plated in duplicates on tryptic soy agar (TSA) for 48 hours at 37 ºC. The number of *Brucella* CFUs was estimated by multiplying the number of colonies by the inverse of the dilution factor.

2.6 Flow cytometry analysis

Cells suspension from spleen were adjusted to 1 x10^6 splenocytes by mL. Then this suspension was incubated with anti CD16/CD32 anti-mouse antibodies (Ab) (Cat: 553141, BD Bioscience, San Jose, CA, USA) for 20 min to avoid non-specific binding through Fc. Ab anti CD11b PE/Cy5-coupled, anti MHC II (I-A/I-E) PE-coupled and a PE-Cy7-coupled anti F4/80 were used to stain cells. Following staining, two washes were performed with PBS solution and cells were fixed with 0.1% paraformaldehyde (PFA) in PBS for 15 min on ice. Cells were washed with 0.2% PBS-BSA and analyzed on a FACS-Aria III flow cytometer (BD Bioscience, San Jose, CA, USA), acquiring one million total events. Autofluorescence and compensation controls were used for each fluorochrome using uninfected mouse spleen cell suspension.

The analysis strategy followed was the elimination of detritus and double events by comparing area versus size forward scatter; then, in a plot of side scatter versus forward scatter, cells were selected. From this region, selection of CD11b+, I-A/I-E+ double-positive cells region was made, and subsequent to this, F4/80+ cells were selected in a histogram where macrophages were F4/80 positive cells (see supplementary S1 figure).

2.7 Determination of the percentage of macrophages and MHCII$^+$CD11b$^+$ cells in spleen

To determine the macrophage or dendritic cell phagocytic activity, we calculated the ratio between the number of *Brucella* CFU determined in the spleen by the number of events corresponding to macrophages or MHCII$^+$CD11b$^+$ in each group.

2.8 Cytokine quantification

Serum samples were used to estimate IL-6, IL-12, TNF-α, IFN-γ, MCP-1, and IL-10 concentrations by using the mouse inflammation kit (BD™ catalog # 552364), according to the manufacturer's instructions. Samples thus processed were analyzed on a FACSARIA III flow cytometer (BD Bioscience, San Jose, CA, USA).

2.9 Serotonin quantification by High Performance Liquid Chromatography (HPLC)

2.9.1. Hippocampal tissue processing

After cervical dislocation and decapitation, the brain of every mouse of different groups was removed and the cerebral cortex peeled off to expose hippocampal formation

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from the dorsal view. This structure was dissected bilaterally and homogenized by sonication (≈ 5 mg tissue) in 200 µL serotonin (5HT) extraction buffer containing 5% citric acid, 200 mM sodium phosphate, 2.5 mM L-cysteine, and 2.5 mM EDTA. Then, 50 µL 0.4 M perchloric acid was added to precipitate proteins after incubating the homogenate at 20 °C for 20 min. Lastly, homogenates were centrifuged at 12,000 × g during 10 min at 4 °C , and the supernatants collected, aliquoted (200 µL) and stored at -80 °C until used.

2.7.2. Solid phase extraction (SPE) of 5-HT

Serotonin concentration was enriched using a solid phase extraction (SPaE) chromatographic column (Octadecyl C_{18}, 1 mL, Cat. 702001; J.T. Baker). Briefly, we used 0.1% trifluoracetic acid (TFA) in water and 0.1% TFA in acetonitrile as mobile phase A (MP-A) and B (MP-B), respectively. The column was activated with 250 µL of MP-B and equilibrated with 250 µL of MP-A. Then, we added 250 µL of supernatant to the column, followed by a wash with 250 µL MP-A. Finally, 5-HT was eluted with 250 µL MP-B.

2.7.3. Chromatographic runs

Serotonin chromatograms were obtained by reversed-phase HPLC (RP-HPLC) in a JASCO HPLC system (PU-2089 plus pump, AS-2057 plus autosampler, and X-LC™3120FP fluorescence detector; Jasco, Inc. Easton, MD, USA). The system was controlled with ChromNav (Jasco, Inc. Easton, MD, USA). The column (CAPCELL PAK C_{18} column (300 Å, 5 µ, 4.6 × 250 mm, Shiseido®, Cat. 92533) was equilibrated with 100% MP-A; then we injected 90 µL of each sample in duplicate. All runs were performed with a column temperature of 30 °C. Chromatographic runs were set as follow: from 0 to 3 min 100% MP-A; from 3 to 33 min a linear gradient from 0-10 % MP-B; from 33 to 38 min a linear gradient from 10-20 % MP-B; from 38 to 39 min a linear gradient from 20-30 % MP-B; 30 % MP-B was maintained until minute 43 to clean the column; from 43 to 45 min we returned the column to 100 %MP-A with a linear gradient. Finally, 100 % MP-A was maintained until minute 60 to equilibrate the column for the next injection. The 5-HT peak was detected at minute 26 with the following detector parameters: gain:100; attenuation:32; response: 20 s; and 280 nm and 315 nm for excitation and emission, respectively.

2.8. Statical analysis

All statistical analyses were performed using GraphPad Prism, version 9.0.0 for Windows, GraphPad Software, San Diego, CA, USA. Data sets were subjected to normality tests. The results were analyzed using one-way ANOVA or Kruskal–Wallis multiple comparisons for parametric or non-parametric data, respectively, followed by Tukey's or Dunn's post hoc tests. The statistical difference of the IFN-γ/IL-6 ratio and CFU/Macrophages between groups *Ba* vs. Im*Ba* was evaluated using a Mann Whitney U-test. Significance was established when $P< 0.05$. All data sets passed the normality tests except for: ImIP group: IL-12 and FST; *Ba* group IL-6, MCP-1; whereas *ImBa* IL-6 and 5-HT. For all groups: OF, CFU in spleen and percentage of macrophages and MHC-II+CD11b+ cells.

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3. Results

3.1 Orogastric administration of imipramine restores mood and hippocampal serotonin levels in Im*Ba* mice.

Ba mice manifested decreased exploratory behavior (OF test; P<0.001) and increased immobility time (TST; P<0.0001), as compared with their Ctrl mates (Fig. 1). When comparing the Ctrl groups with the ImiP group, no differences were found in immobility time, These results combined indicate that *Ba* mice display lack of motivation (i.e., depression-like behavior) [21]. Low motivation in mice is commonly accompanied by reduced hippocampal serotonin levels. Accordingly, *Ba* mice had significantly decreased hippocampal serotonin levels (P< 0.001) when compared with Ctrl mice. Whereas ImiP group show no difference in comparison to Ctrl mice.

In sharp contrast to what was seen in *Ba* mice, Im*Ba* mice significantly improved their scores in both behavioral tests (OF: P<0.001); FST: P<0.01) and showed increased hippocampal 5-HT concentrations (P< 0.001) when compared to *Ba* mice. In fact, the values of all these parameters between Ctrl and Im*Ba* mice were fully comparable; both significantly departed from those obtained for *Ba* mice (Fig. 1; **OF:** H(3)= 31.37, P<0.0001; **FST:** H(3)= 18.85, P<0.001; **Serotonin:** H(2)=15.50, P< 0.01).

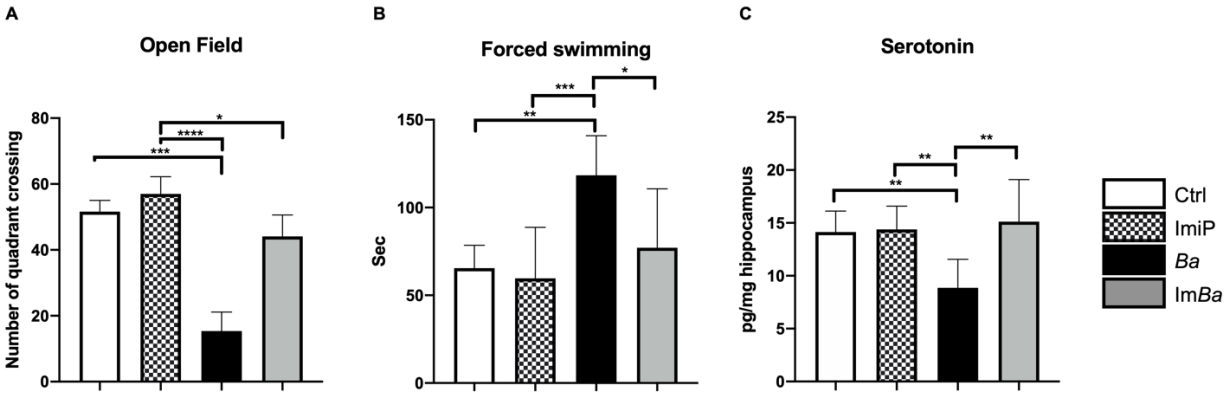

Figure 1. Behavioral tests and serotonin levels. **A)** Open field to evaluate the anxiety-like behavior; an increase in the number of quadrant crossing indicates an increase in anxiety-like behavior. **B)** Forced swim test to evaluate depressive-like behavior; an increase in immobility time indicates an increase in hopelessness. **C)** serotonin levels in hippocampus. Bar represent the mean SD of Control mice (white bars, Ctrl), Imipramine treated mice (grid bars; ImiP), *B. abortus 2308* infected mice (black bars, *Ba*), and imipramine-treated, *B. abortus 2308* infected mice (gray bars; Im*Ba*). In all cases Kruskal–Wallis test were utilized. Statistical significance is represented as follows: $p < 0.05$. **= $p < 0.01$; ***= $p < 0.001$; ****= $p < 0.0001$.

3.2 Orogastric administration of imipramine restores equilibrium and muscular strength in Im*Ba* mice.

Statistically differences were found in behavioral test among the analyzed groups MBCT: F= 19.19 df:(3, 36), P<0.0001; FGST: F= 12.13 df(3, 36), P<0.001.

As previously reported [21], our results showed that *Ba* group compared to Ctrl, increased latency in MBCT (P<0.0001), presence of muscle weakness in FGST (P<0.0001) (Figure 2). The ImiP group group showed no difference in balance and muscular strength when compared to the Ctrl group. Furthermore, the intragastric administration of ImiP between days 8-14 PI significantly improved the score of Im*Ba* mice in the applied behavioral tests MCTB (P<0.0001) and FGST (P<0.0001 in such a way that their performance was equivalent to that presented in the control group.

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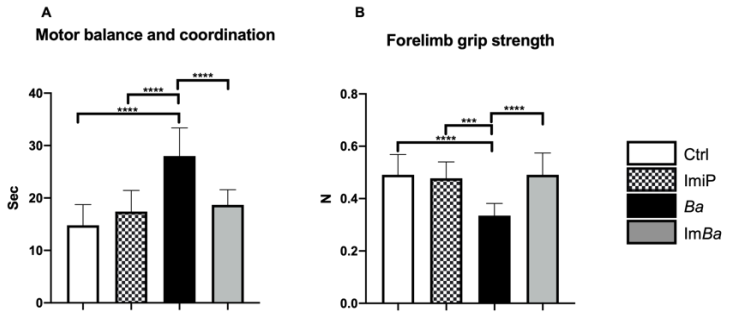

Figure 2. Evaluation of motor disabilities and muscular weakness. **A.** The motor balance and equilibrium test measures the time it takes the mouse takes to perform the test, a longer time to perform the test can be interpreted as a motor disability. **B.** The forelimb grip test quantifies the muscular endurance of a mouse in Newton, a lower value in the test indicates an increase in muscle weakness. Bar represent the mean SD of Control mice (white bars, Ctrl), Imipramine treated mice (grid bars; ImiP), *B. abortus 2308* infected mice (black bars, *Ba*), and imipramine-treated, *B. abortus 2308* infected mice (gray bars; Im*Ba*). In all cases ANOVA test were utilized. Statistical significance is represented as follows: ***= $p < 0.001$; ****= $p < 0.0001$.

3.3 Imipramine administration tended to decrease the inflammatory tone in Im*Ba* mice.

Ba mice's IL-6, TNF-α, IL-12, and IFN-γ serum levels, and thus inflammation, increased relative to base-line control levels at day 14 post-inoculation (Fig. 3; [21]). Im*Ba* mice, on the other hand, had serum levels of most of these cytokines at values that fell between those estimated for Ctrl and *Ba* mice (Fig. 2; IL-6: H(3)= 27.14, P<0.0001; IL-12: H(3)= 11.17, P<0.01; TNF-α: F= 9.32, df (3,36) P<0.001; IFN-γ: F= 25.66, df (3,36) P<0.0001). ImiP group group did not displayed difference in cytokines concentration when compared with Ctrl mice group. This observation, along with the one showing that Im*Ba* mice had a significant increase of the IFN-γ/Il-6 ratio (U=21; P<0.02) due to an increase in IFN-γ and a decrease in IL-6 (Fig. 4), it suggests that imipramine treatment moderates the intensity of *Brucella* induced, immune response. Because IFN-γ levels modulate macrophage phagocytic activity, we turned to evaluate and compare this parameter across experimental groups.

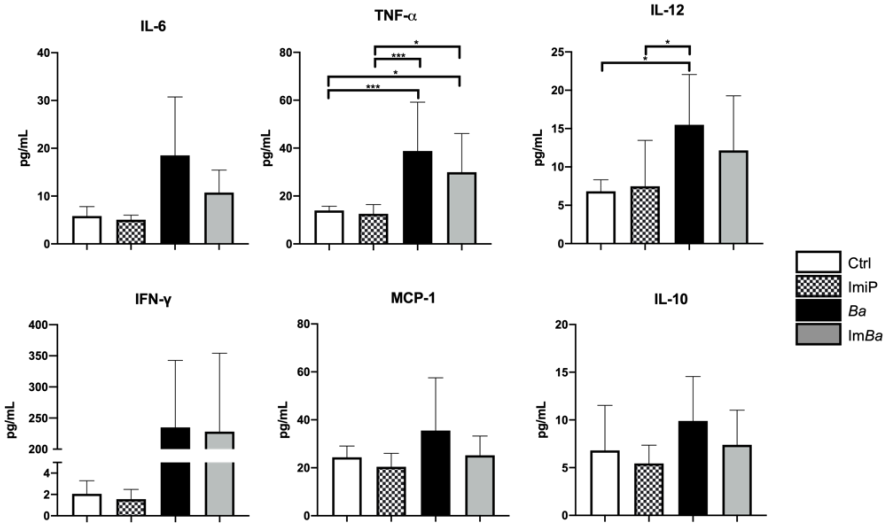

Figure 3. Inflammatory profile. Bar graphs that depict serum levels of pro-inflammatory cytokines. Orogastric administration of imipramine moderates the immune response in Im*Ba* mice. Bar represent the mean SD of Control mice (white bars, Ctrl), Imipramine treated mice (grid bars; ImiP), *B. abortus 2308* infected mice (black bars, *Ba*), and imipramine-treated, *B. abortus 2308* infected mice (gray bars; Im*Ba*). In all cases ANOVA test were utilized, except in IL-6, IL-12 and MCP-1, in which ANOVA test were used. Statistical significance is represented as follows: $p < 0.05$; ***= $p < 0.001$; ****= $p < 0.0001$.

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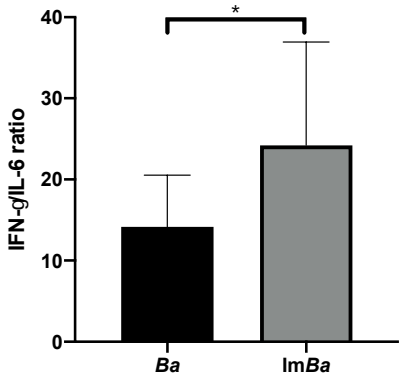

Figure 4. IFN-γ/IL-6 ratio. Bar graph that depicts IFN-γ/IL-6 ratio in *B. abortus 2308* infected mice (*Ba*) and imipramine-treated, *B. abortus 2308* infected mice (Im*Ba*). Im*Ba* mice showed a significant increase of this ratio at the expense of a reduction of IL-6, as compared with Ba mice. Mann Whitney U-test were utilized. Statistical significance is represented as follows: * $p < 0.05$.

3.4 Spleen macrophages increased their numbers and enhanced their *B. abortus 2308*-phagocytic activity following imipramine administration.

As expected during an active infection, macrophages increased their number in both *Ba* and Im*Ba* mice (H(3)= 14.30, P< 0.01). Interestingly, the percentage of the MHCII⁺CD11b⁺ cell population showed significant differences in the Ba and ImBa-mice groups in comparison to the control group animals (H(3)= 13.73, P<0.01), suggesting further analysis of dendritic cells in this model. In addition, ImiP group group did not show difference in the percentage of MHCII⁺CD11b⁺ cells and macrophages in comparison with Ctrl mice group. Significance was achieved for both cell types only in Im*Ba* mice when compared with Ctrl mice (P< 0.05; Fig. 5). So we thought that in addition to lessen depression, ImiP might favor phagocytosis thus improving psychoimmune resilience.

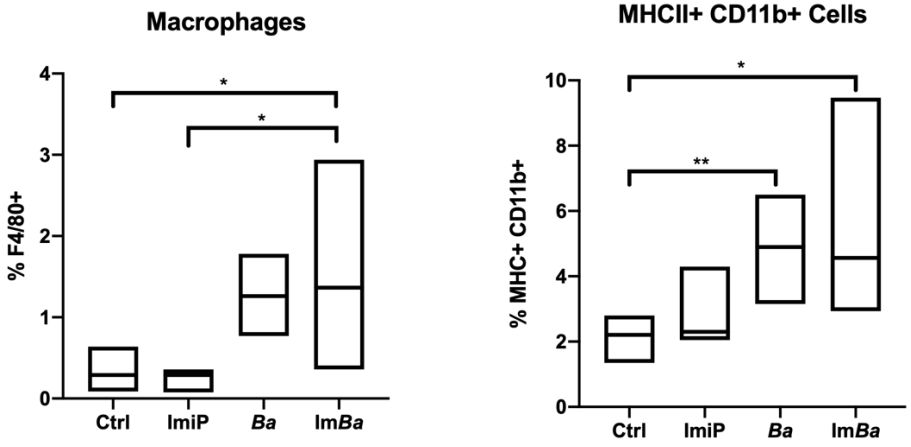

Figure 5. Phagocytic cell quantification. Box plots that depict the relative number of MHCII⁺CD11b⁺ and macrophage cells in control mice (Ctrl), *B. abortus 2308* infected mice (*Ba*), and imipramine-treated, *B. abortus 2308* infected mice (Im*Ba*). Both *Ba* and Im*Ba* mice increased the number of these cell types relative to Ctrl mice. In both cases Kruskal Wallis test were utilized. Statistical significance is represented as follows: *$p < 0.05$; **$p<0.01$

To estimate whether spleen professional phagocytes had increased ability to engulf *B. abortus 2308* in Im*Ba* mice, we first estimated the number of spleen *B. abortus 2308* CFU in *Ba* and Im*Ba* mice. We found a sixteen-fold decrease of this value in Im*Ba* as contrasted against *Ba* mice 14 days post-inoculation (U=0; P<0.0001; Fig. 6).

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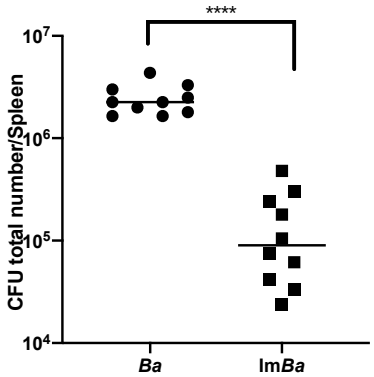

Figure 6. *Brucella abortus 2308* CFU in spleen. Scatter plot that shows *B. abortus* 2308 Colony Forming Units (CFU) derived from individual spleens in *B. abortus 2308* infected mice (*Ba*) and imipramine-treated, *B. abortus 2308* infected mice (Im*Ba*). Imipramine treatment led to a significant reduction of spleen *B. abortus* CFUs. Mann Whitney U-test were utilized. Statistical significance is represented as follows: ****$p < 0.0001$.

ImiP may have an indirect impact on *B. abortus 2308* CFU-forming abilities after improving mood, but it may also have a more direct effect on professional phagocytes function. A way to test this possibility is to estimate the CFU/macrophages ratios per spleen sampled in *Ba* and Im*Ba* mice. As shown in figure 7, ratio dropped significantly in Im*Ba* mice as compared to their *Ba* counterpart, thus suggesting that the efficiency of macrophages to get rid of *B. abortus 2308*, respectively, approximately 18 times higher after ImiP treatment. The reported effect is likely specific since ImiP directly does not compromise the viability of *B. abortus 2308* [22].

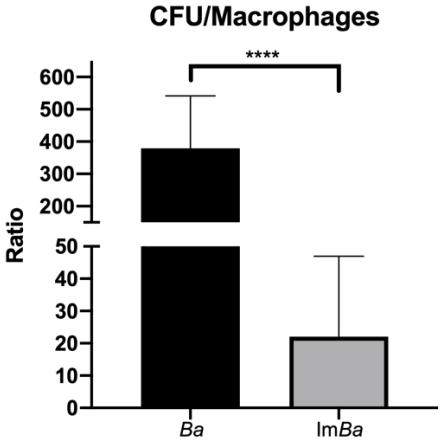

Figure 7. CFU/Macrophages ratio. Bar graphs that show the CFU/macrophages ratios in *B. abortus 2308* infected mice (*Ba*) and imipramine-treated, *B. abortus 2308* infected mice (Im*Ba*). Notice that ratio dropped significantly following imipramine treatment. Mann Whitney U-test were utilized. Statistical significance is represented as follows: ****$p < 0.0001$: ****$p < 0.0001$.

4. Discussion

Brucellosis is a debilitating and disabling illness. This bacterial disease causes severe chronic pain, anxiety, and depression that affect the quality of social and working lives [23]. The administration of antibiotics is not successful enough. This is one of the reasons why this zoonosis in humans evolves rapidly towards chronicity, generating economic effects in the countries where this disease is endemic [24,25].

Our results show that acute infection with Brucella abortus 2308 in mice induced a significant increase in circulating levels of IL-6, IL-12, TNF-α, and IFN-γ an increase in the number of macrophages and MHC-II+, CD11b+ in the spleen; a decrease in the 5-HT levels at hippocampus; a loss of physical strength and equilibrium and the appearance of behaviors associated with anxiety and hopelessness, as previously was reported by our group

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[21]. Alterations in serotonin levels in the hippocampus and behavioral changes are due to the effect of bacterial antigens, as lipopolysaccharides, and inflammatory molecules, such as IL-1β, and its synergistic effect with other inflammatory mediators [26]. So, the intensity of the inflammatory response and the relative abundance of bacterial antigens will be associated with neurochemical and behavioral changes [21].

Depression may be a chronic outcome of infectious diseases [1–8]. Depression, on the other hand, may also increase the individual's risk to develop severe infections [12–16]. Hence, in clinical setting, downsizing depression might help infected patients to successfully resolve microbial infection, even though a depressive tone seems to provide evolutionary advantages (e.g., resource shortage to microbial invaders) to hosts while combating infectious disease [12,13]. In agreement with this statement, in this work we showed that instrumenting a short-term course of ImiP treatment in *Brucella* infected mice reinstates near normal motivation and increased hippocampal serotonin availability, while upturn peripheral inflammation and improves body condition. The mechanisms by which ImiP improves motivation are fairly well established. Briefly, ImiP increases monoamines extracellular concentrations after inhibiting serotonin, norepinephrine, and dopamine reuptake [27–30], a circumstance that remedies mood. *Brucella abortus*, on the other hand, may induce depression after inducing inflammatory colitis [31] following the disruption of colorectal mucosae linings [32,33], colorectal serotonin availability [34,35] and dysbiosis [9,36]. Imipramine administration may reverse this condition by improving gut's visceral sensation, serotonin local availability and gut barrier [37]. Body condition, on the other hand, could have been improved in Im*Ba* mice following the drop on IL-6, since elevations of this cytokine sensitizes pain pathways and produces hyperalgesia through various mechanisms [38,39]. In addition, it is known that the ImiP consumption reduces circulating levels of IL-6 [40,41].

Imipramine, however, not only appears to improve the likelihood of recovery in Im*Ba* mice by acting counterbalancing depressive symptoms, it may also modulate more directly immune response. Accordingly, in our experimental series, MHC-II+, CD11b+ spleen-derived cells and macrophages seem to have increased their phagocytic activity against *B. abortus 2308* after ImiP treatment, thus reducing bacterial loads in the spleen of infected mice. How does this may come? Although we still lack evidence to explain our finding, given its molecular similitude with cholesterol (Fig. 8), ImiP might mimic cholesterol actions on signal transduction pathways that promote phagocytosis, immunological synapse formation, and antigen presentation in professional phagocytes [42] [43–45]. Imipramine might also reduce membrane fluidity, a circumstance known to enhance phagocytosis [43]. Previous work suggests that ImiP induces the production of superoxide and nitric oxide by macrophages *in vitro* [46] and *in vivo* too [43–45]. The results presented in figure 8 can be explained by the effect of imipramine on macrophages, it is important to note that there was no statical difference in the percentage of MHC-II+CD11b+ cells and macrophages obtained from the spleen of the Ba and ImBa group mice, nevertheless, there was a significant difference in the CFU obtained from the spleen of Ba and ImiP mice. This suggests that the ImBa mice despite having a similar number of macrophages as the Ba mice, had an increased clearance of the bacteria. Our results suggest a functional improvement in clearance of infection. Future studies will be required to understand how imipramine promotes Brucella clearance. Previously, it has been reported that imipramine has no a microbicidal effect on *B. abortus 2308* [22]. On the other hand, Mukherjee et al. have described the use of imipramine in murine models of leshmaniasis, achieving elimination of leshmania [43–45].

Another way in which ImiP may limit *B. abortus 2308* infections is acting directly upon mechanism used by the intracellular microorganisms to enter the target cell. Intracellular cholesterol trafficking is essential for *B. abortus* 2308 to consolidate the infecting condition in mice. *B. abortus* 2308 passes through the host cell membrane by using the VirB complex and lipid rafts [47]. The insertion of lipid rafts into the cell membranes depends, nonetheless, upon the transferring of cholesterol from the endosomal compartment to the cell membrane. Since ImiP may substitute cholesterol, such substitution could interfere

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with the integration of lipid rafts to cell membranes thus preventing *B. abortus* 2308 to enter the target cells [48], making it more susceptible to be cleared through phagocytosis.

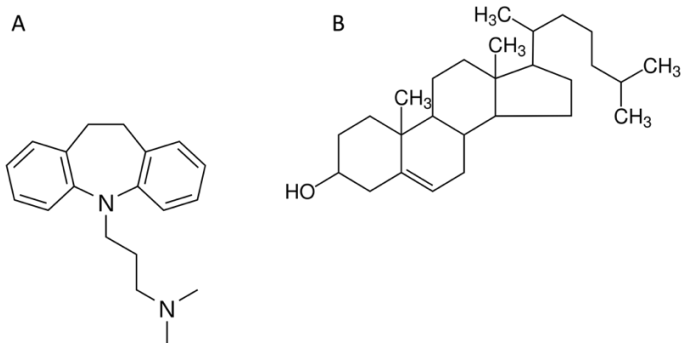

Figure 8. Imipramine and cholesterol structures. Schematic bidimensional representation of the structure of Imipramine (A), notice its similitude with cholesterol's structure (B).

Lastly, ImiP may have direct regulatory effects on pro-inflammatory cytokine synthesis and release. Indeed, as in the present study, it has been demonstrated that this drug decreases the secretion of pro-inflammatory cytokines from a variety of immune competent cells [49–51]. Such a decrease release may follow a rise of intracellular levels of cAMP [49–51]) and/or a decreased availability of intracellular serotonin in immune competent cells [52–56]. In addition, increments of extracellular serotonin concentrations after ImiP administration could feedback negatively pro-inflammatory cytokines' mRNA transcription [52–56]. The exception to this general principle is IFN-γ, a cytokine that remained elevated in Im*Ba* mice. This apparent dissonance might be explain by the fact that the elevated levels of extracellular serotonin associated with ImiP administration could have triggered IFN-γ release [57]. Such a hypothetical scenario may be feasible in the context of our experiments since IFN-γ released by NK cells [58] could have activated spleen macrophages, thus decreasing spleen *B. abortus 2308* CFUs counts.

IL-6 generates multiple biological effects, not all of them beneficial [39]. This inflammatory molecule may act as a trigger to initiate a neuropathic pain process throughout the ubiquitous glycoprotein gp130, which serves as its receptor or is associated with IL-6R and could be localized in nociceptor endings [59]. The increase of IL-6 levels favors the rise of expression of vanilloid receptors and ankyrin-1 in neuronal endings and the release of calcitonin gene-related peptide (CGRP) and substance P (SP), among others [38,39]. This peripheral sensitization mediated by IL-6 and gp130 promotes an increase of cytokines and proteases released by microglia and astrocytes at nerve ganglia due to the rise of neurotransmitters, adenosine prostaglandins, and nitric oxide [39]. All these mediators generate prolonged hyperalgesia, which could explain why *Ba* mice that presented high levels of IL-6 show a significant decrease in equilibrium and muscle strength. On the other hand, it has been described that the consumption of ImiP induces a reduction of circulating levels of IL-6 [40,41] . As observed in Im*Ba* mice, the decrement of IL-6 levels decreases their discomfort and recover their muscle strength and equilibrium capacity.

A numerical parameter that we consider helpful for the analysis of the results is the ratio of IFN-γ/ IL-6. Although we cannot determine the cellular source of these cytokines, this numerical value is associated with their circulating levels. It reflects the positive effects mediated by the administration of imipramine since the decrease of IL-6 improves physical performance in infected animals. This fact is relevant since it has been observed in humans that elevated circulating levels of IL-6 are associated with painful phenomena such as fibromyalgia and post-COVID [60,61]. In addition, the serum levels of IFN-γ have been associated with a more efficient performance of phagocytic cells.

In this work we present data that suggest that the use of ImiP may improve the clinical outcome of mice infected by *B. abortus 2308*. Although the mechanisms by which ImiP exerts its beneficial effect still are a matter of future studies (Fig. 9), it appears fair to say that when used at low doses (≈ two times lower the dose used in humans; [62]) and for

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short-term courses, it enhances the psychoimmune resilience of the individual while avoiding the unpleasant effects [41,63]. Pending is the development of clinical studies in humans affected by brucellosis to corroborate its clinical potential.

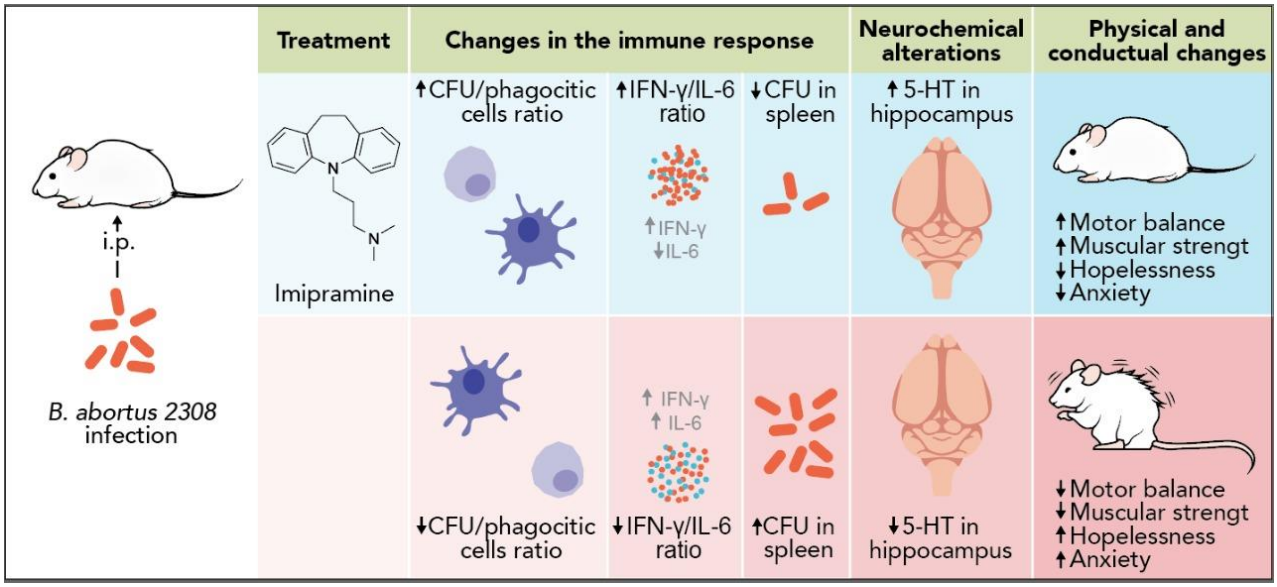

Figure 9. Imipramine effects on mice infected with *B. abortus 2308*. Our study showed that *Ba* vs.Ctrl mice had a significant rise in levels of IFN-γ, IL-6, TNF-α, and IL-12 with a low IFN-γ/IL-6 ratio, elevated bacterial counts, alterations in serotonin concentration at hippocampus, and decreased muscular strength, equilibrium, and mood. While infected Im*Ba* vs. *Ba* mice showed a significant increase IFN-γ/IL-6 ratio, an increase in phagocytic cell functionality, a significant reduction in bacterial loads, as well as significant improvement in the strength, equilibrium, and mood scores, and the restoration of serotonin concentrations at the hippocampus.

Limitations

As most experimental studies, the present work has limitations that need to be commented on. One of such limitations is the use of orogastric tubes to administer ImiP since it could have stressed the animals. We also think that a thorough dendritic cell typing must be conducted in upcoming studies with an extensive antibody panel to identify their phenotype. Further studies should also consider the effect of IL-1 on 5-HT levels in the hippocampus, which has been reported in other infectious models and in vivo assays as result of lipopolysaccharide or IL-1β administration. To include a more extensive battery of behavioral tests to deeply evaluate psychoimmune resilience is needed. Dose-effect curves are also necessary, as well conducting liver function tests.

5. Conclusions

Imipramine orogastric administration for six days improved the systemic inflammatory profile and the IFN-γ/IL-6 ratio, increased the capacity of macrophages, which is reflected in the decrease of the bacterial count in the spleen. It also restored hippocampal serotonin levels and increased motivation scores and improved the physical condition of infected mice. Therefore, ImiP may be a pharmacological agent worth to add to the arsenal to treat *B. abortus 2308* infections since improves psychoimmune resilience in a short time and in under a safe dose.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Analysis strategy for flow cytometry.

Author Contributions: Conceptualization, Lenin Pavón and Martha C Moreno-Lafont; Data curation, Jesús Octavio Maldonado-Tapia, José Luis Maldoando-García, Lenin Pavón, Gabriel Gutiérrez-Ospina and Rubén López-Santiago; Formal analysis, Jesús Octavio Maldonado-Tapia, José Luis Maldoando-García, Gilberto Pérez-Sánchez, Enrique Becerril-Villanueva, Lenin Pavón, Gabriel Gutiérrez-Ospina and Martha C Moreno-Lafont; Funding acquisition, Lenin Pavón and Martha C

Moreno-Lafont; Investigation, Jesús Octavio Maldonado-Tapia and José Luis Maldoando-García; Methodology, José Luis Maldoando-García, Gilberto Pérez-Sánchez, Enrique Becerril-Villanueva, Samantha Alvarez-Herrera and Rubén López-Santiago; Project administration, Lenin Pavón and Martha C Moreno-Lafont; Resources, Lenin Pavón; Supervision, Gilberto Pérez-Sánchez, Lenin Pavón and Martha C Moreno-Lafont; Validation, Lenin Pavón and Gabriel Gutiérrez-Ospina; Visualization, Lenin Pavón, Gabriel Gutiérrez-Ospina and Martha C Moreno-Lafont; Writing – original draft, José Luis Maldoando-García, Lenin Pavón, Gabriel Gutiérrez-Ospina and Rubén López-Santiago; Writing – review & editing, Jesús Octavio Maldonado-Tapia, José Luis Maldoando-García, Gilberto Pérez-Sánchez, Enrique Becerril-Villanueva, Samantha Alvarez-Herrera, Lenin Pavón, Gabriel Gutiérrez-Ospina, Rubén López-Santiago and Martha C Moreno-Lafont.

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Institutional Review Board Statement: The study was conducted according to the NIH Guide for the Care and Use of Laboratory Animals. Mouse handling, inoculation, and experimental protocols were revised and approved by the Research Ethics Committee from the Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, with Permit No. ENCB/CEI/077/2020.

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