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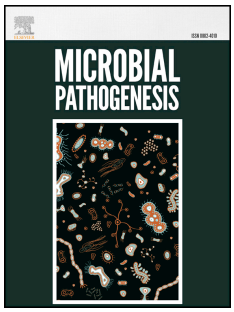


Increased of the hepatocytes and splenocytes apoptosis accompanies clinical improvement and higher survival in mice infected with *Trypanosoma cruzi* and treated with highly diluted *Lycopodium clavatum*

Gislaine Janaina Falkowski-Temporini, Carina Ribeiro Lopes, Paula Fernanda Massini, Camila Fernanda Brustolin, Fabiana Nabarro Ferraz, Patricia Flora Sandri, Luzmarina Hernandes, Denise Lessa Aleixo, Terezinha Fátima Barion, Luiz Gilson Esper, Silvana Marques de Araújo

PII: S0882-4010(16)30787-2

DOI: 10.1016/j.micpath.2017.06.027

Reference: YMPAT 2319

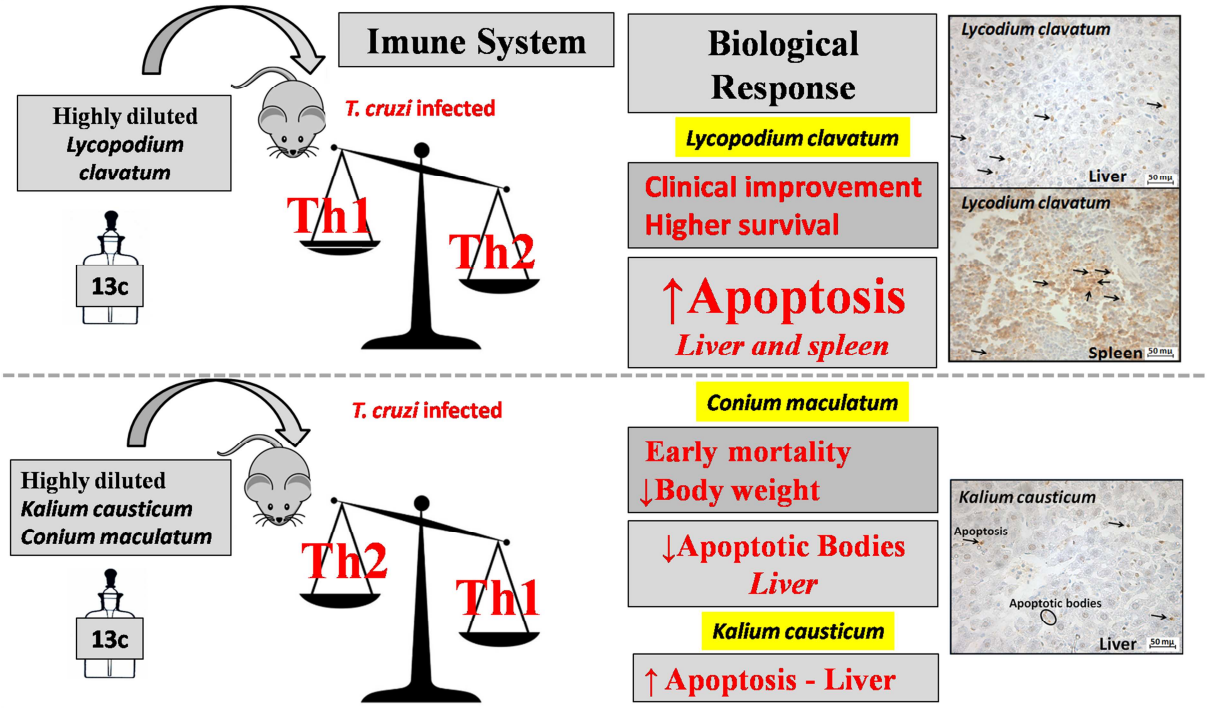
To appear in: *Microbial Pathogenesis*

Received Date: 14 November 2016

Revised Date: 6 June 2017

Accepted Date: 19 June 2017

Please cite this article as: Falkowski-Temporini GJ, Lopes CR, Massini PF, Brustolin CF, Ferraz FN, Sandri PF, Hernandes L, Aleixo DL, Barion TerezinhaFá, Esper LG, de Araújo SM, Increased of the hepatocytes and splenocytes apoptosis accompanies clinical improvement and higher survival in mice infected with *Trypanosoma cruzi* and treated with highly diluted *Lycopodium clavatum*, *Microbial Pathogenesis* (2017), doi: 10.1016/j.micpath.2017.06.027.



Title page

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Author names and affiliations

Gislaine Janaina Falkowski-Temporini[a*], Carina Ribeiro Lopes[a], Paula Fernanda Massini[a], Camila Fernanda Brustolin[a], Fabiana Nabarro Ferraz[a], Patricia Flora Sandri[b], Luzmarina Hernandes[c], Denise Lessa Aleixo[a], Terezinha Fátima Barion[d], Luiz Gilson Esper[d], Silvana Marques de Araújo[e].

[a]Post-Graduate Program in Health Sciences, Universidade Estadual de Maringá, Brazil.
[b]Post-Graduate Program in Biosciences and Physiopathology, Universidade Estadual de Maringá, Brazil.
[c] Department of Morphological Sciences, Universidade Estadual de Maringá, Brazil
[d]Homeopath Volunteer, Maringá, Brazil.
[e]Department of Health Sciences, Universidade Estadual de Maringá, Brazil.

E-mail adresses: gisajanaina@hotmail.com (G. J. Falkowski-Temporini); lopescarina@hotmail.com (C. R. Lopes); paulavet_massini@hotmail.com (P. F. Massini); brustolincamilaf@gmail.com (C. F. Brustolin); Fabiana_nabarro@hotmail.com (F. N. Ferraz); paty_sandri@hotmail.com (P. F. Sandri), luzhernandes@gmail.com (L. Hernades); deniseparasito@gmail.com (D. L. Aleixo); gsfatima@hotmail.com (Terezinha Fátima Barion); luizgilsonesper@gmail.com (Luiz Gilson Esper); smaraujo@uem.br (S. M. Araújo).

***Corresponding Author**: Gislaine Janaina Falkowski-Temporini. Departamento de Ciências da Saúde, Laboratório da Parasitologia, Bloco I90. Av. Colombo 5790, CEP: 87020-900 - Maringá, PR Brasil - **E-mail:** gisajanaina@hotmail.com; Tel.: +55 44 3011-4918

Authors' postal address

Gislaine Janaina Falkowski-Temporini: Post-Graduation Program in Health Sciences – UEM – Universidade Estadual de Maringá. UEM, Av Colombo 9727, Bloco I-90, sala 11, KM 130, 87070-000, Brazil. gisajanaina@hotmail.com

Carina Ribeiro Lopes: Post-Graduation Program in Health Sciences – UEM - Universidade Estadual de Maringá. UEM, Av Colombo 9727, Bloco I-90, sala 11, KM 130, 87070-000, Brazil. lopescarina@hotmail.com

Paula Fernanda Massini: Post-Graduation Program in Health Sciences – UEM - Universidade Estadual de Maringá. UEM, Av Colombo 9727, Bloco I-90, sala 11, KM 130, 87070-000, Brazil. paulavet_massini@hotmail.com

Camila Fernanda Brustolin: Post-Graduation Program in Health Sciences – UEM - Universidade Estadual de Maringá. UEM, Av Colombo 9727, Bloco I-90, sala 11, KM 130, 87070-000, Brazil. brustolincamilaf@gmail.com

Fabiana Nabarro Ferraz: Post-Graduation Program in Health Sciences – UEM - Universidade Estadual de Maringá. UEM, Av Colombo 9727, Bloco I-90, sala 11, KM 130, 87070-000, Brazil. fabaiana_nabarro@hotmail.com

Patricia Flora Sandri: Post-Graduation Program in Biosciences and Phisiopathology – UEM, Av Colombo 9727, Bloco I-90, sala 11, KM 130, 87070-000, Brazil. paty_sandri@hotmail.com

Luzmarina Hernandes: Department of Morphological Sciences – UEM - Universidade Estadual de Maringá. UEM, Av Colombo 9727, Bloco H-79, sala 108, KM 130, 87070-000, Brazil. luzhernandes@gmail.com

Denise Lessa Aleixo: Post-Graduation Programin Health Sciences – UEM - Universidade Estadual de Maringá. UEM, Av Colombo 9727, Bloco I-90, sala 11, KM 130, 87070-000, Brazil. deniseparasito@gmail.com

Terezinha Fátima Barion: Homeopath Volunteer – R. Martin Afonso, 1075, 87010 410, Maringá – Paraná, Brazil. gsfatima@hotmail.com

Luiz Gilson Esper: Homeopath Volunteer – Av. Centenário, 267, 87050-040, Maringá – Paraná, Brazil. luizgilsonesper@gmail.com

Silvana Marques de Araújo: Department of Basic Health Sciences – Laboratory of Parasitology, Universidade Estadual de Maringá. Av Colombo 5790, Bloco I-90, sala 11, KM 130, 87020-900 Maringá, PR, Brazil. smaraujo@uem.br

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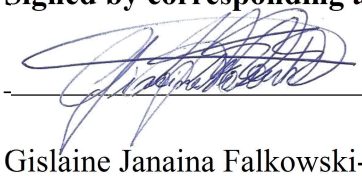

Gislaine Janaina Falkowski-Temporini

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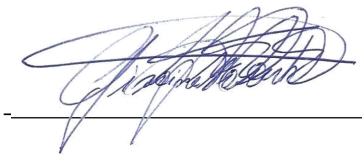

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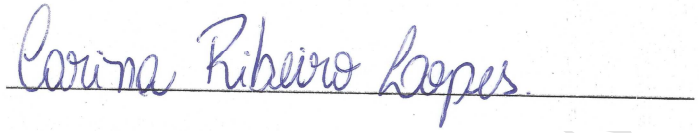

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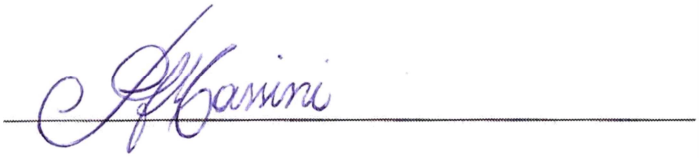
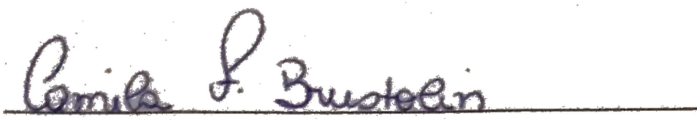


Paula Fernanda Massini



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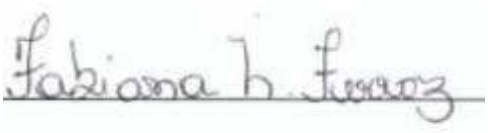
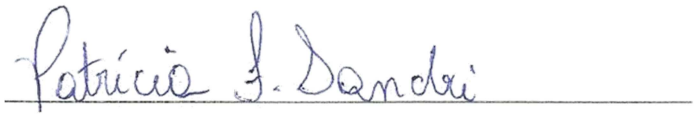


Fabiana Nabarro Ferraz



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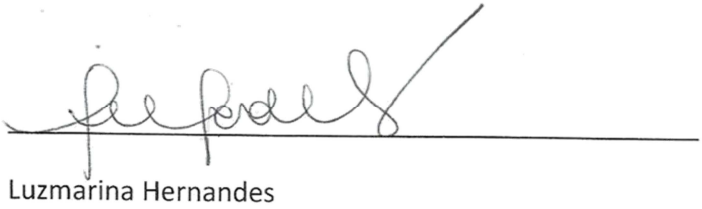
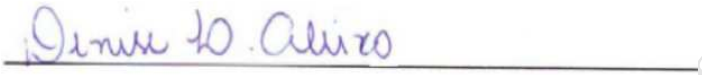


Luzmarina Hernandes



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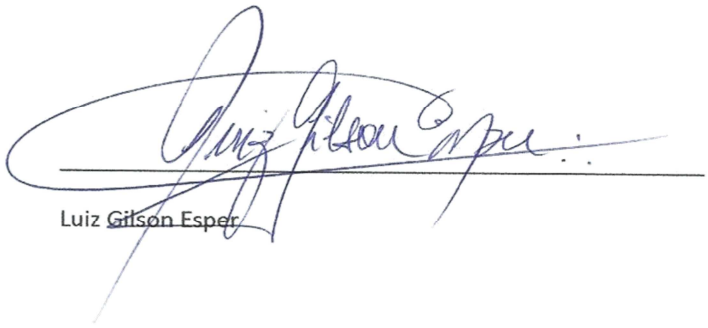
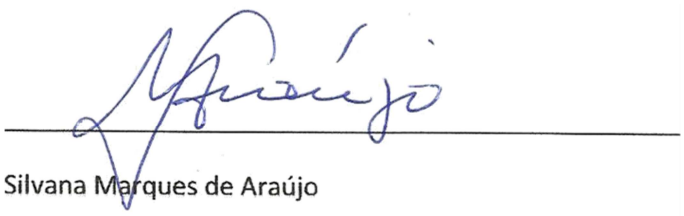


Luiz Gilson Esper



Silvana Marques de Araújo

ABSTRACT

Recent evidence includes apoptosis as a defense against *Trypanosoma cruzi* infection, which promotes an immune response in the host induced by T cells, type 1, 2 and 17. Currently, there is no medicine completely preventing the progression of this disease. We investigated the immunological and apoptotic effects, morbidity and survival of mice infected with *T. cruzi* and treated with dynamized homeopathic compounds 13c: *Kalium causticum* (GCaus), *Conium maculatum*, (GCon), *Lycopodium clavatum* (GLy) and 7% alcohol solution (control, vehicle compounds, GCI). There was significant difference in the increase of apoptosis in the treated groups, compared with GCI, which might indicate action of the compounds in these cells. Infected animals treated with *Lycopodium clavatum* presented better performance compared with other groups. GLy showed a higher amount of hepatocytes and splenocytes undergoing apoptosis, higher number of apoptotic bodies in the liver, predominance of Th1 response, increased TNF-α and decreased IL-6, higher survival, lower morbidity, higher water consumption, body temperature, tendency to higher feed intake and weight gain compared with GCI. *Conium maculatum* had worse results with increased Th2 response with increased IL-4, worsening of the infection with early mortality of the animals. Together, these data suggest that highly diluted medicines modulate the immune response and apoptosis, affecting the morbidity of animals infected with a highly virulent strain of *T. cruzi*, being able to minimize the course of infection, providing more alternative approaches in the treatment of Chagas disease.

Keywords

Apoptosis, cytokine, homeopathy, *Lycopodium clavatum, Trypanosoma cruzi.*

Abbreviations

Th1, T helper cell type 1; Th2, T helper cell type 2; TNF-α, tumor necrosis factor alpha; IFN-γ, IL-6, Interleukin 6; IL-4, Interleukin 4.

1. INTRODUCTION

Approximately 7-8 million people in the Americas are estimated to be carriers of Chagas disease, with approximately 12,500 deaths per year in the last two decades [1]. Recently, the largest multi-center clinical study ever conducted regarding Chagas disease, BENEFIT, demonstrad that the currently available drug is not able to protect

the progression of infection, stimulating new researches [2]. The search for a compound that stimulates the immune system has been the research subject for new therapies for this infection [3].

Trypanosoma cruzi infection releases numerous chemical mediators [4] with the participation of T-cells that, by expressing TNF-α, become highly susceptible to undergo apoptosis in order to control the morbidity of infection [5]. Expression of IL-4 signals increased susceptibility of the host against *T. cruzi* infection [6], and increased Th17 intensifies the infection [7]. Therefore, some biomarkers such as chemokines and cytokines are associated with progression of infection and lower survival [8,9,10], while the stimulus of defense cells, megakaryocytes and Kupffer cells, are related to higher survival of the host [3].

The host cell integrity allows the survival of the pathogen [11]. Activation of the programmed cell death seems to limit the progression of infection [12]. The involvement of the innate immune response and activation of caspase-8 as a mechanism of cellular defense against the spread of the parasite in infected organ were recently demonstrated [13,14], using the counterbalance of this pathway with less aggressive power when compared to other mechanisms of cell death, where there is release of several inflammatory mediators [15,16].

The use of dynamized, highly diluted compound, i.e., above the Avogadro constant ($6.02x10^{23}$), has been shown to stimulate the immune system in mice infected with *T. cruzi*, maintaining homeostasis, positively altering the course of infection [3,17]. Modulation of Th1, Th2 and Th17 responses and apoptotic profile has been the subject for therapeutic approaches [10,18]. However, some mechanisms still remain unanswered [19] and a broad understanding of the immune response is required for proposing strategic interventions, minimizing injuries in infected hosts [20].

The resistance to infection involves immunoregulatory mechanisms that can be induced to maximize response against the parasite, for example apoptosis. However, immunomodulation of host in activation of apoptotic signaling pathways remains a challenge in the search for the proposition of effective protection against *T. cruzi* infection [14,20]. Thus, in this study we evaluated the levels of cytokines Th1, Th2, Th17 and the biological response profile of apoptosis in survival of mice infected with *Trypanosoma cruzi* and treated with different highly diluted compounds, above the Avogadro constant.

2. MATERIALS AND METHODS

2.1. Experimental design

The experiment was performed twice as a blind, controlled, and randomized trial. Healthy, eight weeks-old, Swiss male mice, from the Central Vivarium at the Universidade Estadual de Maringá, were used. The experimental procedures were approved by the ethics committee, conducted in accordance with animal testing research and humane endpoint guidelines (protocol 54/2011- CEAE/UEM). The research adhered to the principles enunciated in the 8th Edition of the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011. The animals were kept in the sectorial experimentation laboratory for one week before the beginning of the experiment, for adaptation. Temperature and humidity conditions were controlled, with artificial lighting photoperiod of 12 h (light/dark), with water and food *ad libitum*. The groups were randomly divided, so that the average weight of each group was not statistically different from each other. The animals were infected intraperitoneally with 1,400 blood trypomastigotes of *T. cruzi* - Y strain [22]. Groups according to treatment with dynamized homeopathic compounds 13c (dilution $1:10^{26}$): GCaus - Group treated with *Kalium Causticum* 13c (n=10), GCon - Group treated with *Conium maculatum* 13c (n=11), GLy - Group treated with *Lycopodium clavatum* 13c (n=10), GCI - Group treated with the vehicle for medicine preparation (alcohol solution 7%) (n=11).

2.2. Dynamized compounds

Compounds selection

The compounds were selected by repertorization, in healthy animals [23,24], by observing the idiosyncratic characteristics of the group, considering general behavior, socialization and physiology. The observation of the animals was performed by three homeopaths for 30 minutes in a silent environment, from 19:00 to 19:30, considering the nocturnal activity for rodents. The characteristics were added to the Lynx Expert System Software (Albuquerque, USA), which recorded three compounds: (1) *Kalium Causticum*, (2) *Conium maculatum*, (3) *Lycopodium clavatum*. The compounds were selected considering the Law of Similitude in accordance with conventional homeopathic clinical practice described by Hahnemann [25]. The most notable collective characteristics in the animals were coexistence, fear, conscientiousness, shyness, high frequency in water intake in small quantity, diminished vision, hearing and keen sense of smell. The compounds *Kalium causticum, Conium maculatum* and

Lycopodium clavatum encompassed these characteristics to reestablish the balance of the biological system against considerable disturbances in the bioimmunophysiological system [25], caused, in this case, by *T. cruzi* infection. In Homeopathic Materia Medica [24], these compounds may be related to clinical conditions similar to pathological events observed in *T. cruzi* infection. *Kalium causticum* may be correlated to myasthenia; *Conium maculatum*, with anorexia. In addition, *Lycopodium clavatum* may be correlated with bowel disease [24] showing an effect in the treatment of rats infected with *Trypanosoma cruzi*, protecting intestinal neurons and modifying the immune response profile in treated animals [26]. The compounds were prepared in dynamization 13c (dilution $1:10^{26}$), by understanding the experimental murine infection by *T. cruzi* as an acute pathology and considering Swiss mice as small animals [27].

Preparation of compounds

The compounds *Kalium causticum, Conium maculatum* and *Lycopodium clavatum* were prepared from the mother tincture diluted in grain alcohol at 70% (Agro-Industrial Tarumã Ltda, São Pedro do Turvo, Brazil) until 12c dynamization [27]. The dynamization used for the compounds was prepared (13c) in 7% alcohol, using mechanical dynamizer (AUTIC® Mod. Denise). 7% Alcohol, the vehicle for compounds, was used as control.

Biological risk

Biological risk was determined by inoculating five healthy mice/group, male, 8 weeks old, with 0.1 mL of each compunds (*Kalium causticum, Conium maculatum* and *Lycopodium clavatum)* intraperitoneally, to assess the possible biological response to the medicine. For 30 days, mice were assessed for weight, temperature, fur appearance, appearance of feces and mortality [28,29]. No biological effects from the compounds studied were found in these healthy mice.

Treatment regimen

The treatment was administered orally, diluted in water (1mL/100mL), offered *ad libitum* in amber bottle, overnight, two days before infection and for three days after infection, every 48 hours, in order to stimulate the biological system and ensure the action of the compound on the immune system before and after the presence of the

parasite [30]. The GCI received only 7% hydroalcoholic solution, also diluted in water (1 mL/100 mL), in the same treatment regimen.

2.3. Parameters evaluated

Survival

Survival was evaluated up to 90 days after infection and the average lifespan for each group was calculated, considering the humane endpoint (minimal pain and suffering prior-to-death and well-being, in survival of the animals) for groups GCI (n=8) GCaus (n=7), GCon (n=8), GLy (n=7).

Clinical parameters

The animals were clinically assessed for five days before the infection, on the day of infection and on the 23 consecutive days after infection at a fixed time 09h00min [31]. **Quantitative analysis: Weight** - expressed in grams, it was individually evaluated on BEL engineering® - Class Mark II 500g digital balance; **Temperature** - expressed in degrees centigrade (ºC), it was individually measured in the anterior region of the left thigh (less fur) using Icel, infrared digital thermometer, model TD-920.0387; **Water and feed consumption** - expressed in milliliters (mL) and grams (g), respectively. These parameters were collectively evaluated, considering the initial value for each group subtracted from the measured value after a day of consumption. The value obtained was divided by the number of animals in order to estimate the individual values; **Amount of excreta** - expressed in grams (g), it was collectively evaluated and obtained by weighing the cage beds before use, subtracted from the weight obtained after one day of use. Feces and urine were considered together. All the sawdust to be used as bed in the experiment was dried in a kiln first. **Qualitative analysis:** it was performed using numerical category scaling, defined as: 0 for no, and 1 for yes, and we analyzed whether the animal was asleep at 8:00h, presence of diarrhea (by observing the anal region), scrotal swelling, presence of abdominal edema, fur appearance (bristly), agitation in the cage, naturally and with handling. Behavioral alterations of physiological signals were also evaluated through atypical, unpredictable alterations, without the addition of stimuli, as well as social inhibition and presence of convulsion.

Cytokines and Ratios Th1/Th2, Th1/Th17

The cytokines IL-2, IL-4, IL-6, IL-10, IL-17, IFNγ and TNFα were measured (pg/ml) in serum (aseptic cardiac puncture) on the 8th day of infection (peak of parasites), using

3 animals per group. The measurement performed using the commercial kit BD™ Cytometric Bead Array Mouse Th1/Th2/Th17 (CBA) (BD Biosciences Pharmingen, San Diego, USA), with analysis by flow cytometer (BD FCS CantoII) following the manufacturer's instructions. Quantitation of each cytokine was obtained by using specific software (FCAP Array – Becton & Dickinson).

The ratios of cytokines Th1/Th2 (IL-2/IL-4, IL-2/IL-10, IL-6/IL-4, IL-6/IL-10, TNFα-/IL-4, TNF-α-/IL-10, INF-γ/IL-4, INF-γ/IL-10) and Th1/Th17 (IL-2/IL-17, IL-6/IL-17, TNF-α-/IL-17, INF-γ/IL-17) were calculated by dividing the values obtained for cytokines Th1 by the values of cytokines Th2 or Th17 of each animal, calculating the mean after.

Apoptosis - In situ detection of DNA fragmentation

On the 8th day of infection, three animals from each group were euthanized and the spleen and larger lobe of the liver were collected and fixed in 10% formol for 24 hours. Subsequently, the organs were dehydrated, diaphanized and immersed in paraffin. Each organ was subjected to 4 semi-serial sections with 5μm thickness, with 20μm of separation between them (microtome SLEE MAINZ model CUT 5062 - Mainz, Germany), and fixed in slides pre-prepared with poly-L-lysine. Detection of apoptosis was performed by the TUNEL method [32] using a commercial kit for detecting apoptosis (ApopTag® Peroxidase-Chemicon). The number of apoptotic bodies (cells containing apoptotic material phagocytosed in the liver), and splenocytes and hepatocytes undergoing apoptosis were compared. Counterstaining with Harris hematoxylin was performed. For quantitative analysis of the sections, 20 and 10 random microscopic fields/section were counted, totaling 240 and 120 fields per group, from the liver and spleen, respectively, with 40X objective in Olympus CBA microscope (Tokyo, Japan).

2.4. Statistical analysis

Statistical analysis was performed by comparing the different parameters between the treated groups and control group, using Student "Z" test for qualitative variables (parametric data) and the Mann-Whitney test for quantitative variables (nonparametric data) using Software Statistica 8.0. Survival analysis was performed using Logrank test and Kaplan-Meier analysis - software R.3.0.2. The significance level used for the tests was 5%.

3. RESULTS

3.1. Survival

GLy presented the longest survival time (50.1 ± 37.3) compared with GCI (26.8 ± 25.6) (p=0.02), GCaus (17.4 ± 2.1) (p=0.00) and GCon (15.4 ± 1.9) (p=0.00). GCon group (15,4 ± 1,9) presented early mortality compared with GCI (26.8 ± 25.6) (p=0.03). There was no significant difference between GCI (26.8 ± 25.6) and GCaus (17.4±2.1) (p=0.63). By the Kaplan-Meier estimator, the probability of the treated animal in GLy group surviving up to 22 days is 42.9% (CI 95% = 18% to 100%). In the GCon group, in turn, the probability of surviving up to 17 days is 12.5% (CI 95% = 2% to 78.2%); in GCaus group, the probability of surviving up to 18 days is 33.3% (CI 95% = 10.8% to 100%) and the probability of surviving 21 days is 12.5% (CI 95% = 2% to 78.2%) in GCI (Table 1 and Figure 1).

3.2. Clinical parameters

In the overall average of the clinical parameters, all the compounds presented a significant increase in temperature compared with GCI ($p<0.05$) (Table 1). However, in average divided by periods of infection, only the GLy group presented a significant increase in temperature from the 6th-23rd day of infection, while GCaus presented a decrease in this parameter ($p<0.05$) (Figure 2). GCon presented decreased feed intake from the 6th-9th day of infection compared with GCI ($p<0.05$), and GCaus presented decrease in body weight compared with GCI from the 10th to the 13th day of infection ($p<0.05$) (Figure 2). GLy presented higher water consumption compared with GCI from the 2nd-5th day of infection and from the 10th-13th day ($p<0.05$) (Figure 2). In this comparison, it was observed that the animals treated with Ly showed no decrease in water or food consumption, presenting better performance than the other groups, which had significant decrease in these parameters.

The amount of excreta and presence of diarrhea had no significant difference between the groups. In the analysis of qualitative clinical parameters, GLy and GCaus presented greater drowsiness, greater genital edema and greater agitation, naturally and during handling. The bristliest fur was observed in GCon and GLy (Table 2). Social inhibition was not observed during the experiment. On the 4th day of infection, 14% of the animals (1/7) in GCaus had clonic convulsion, with observation of muscle spasms in the lower limbs.

3.3. Cytokines and Ratio Th1/Th2, Th1/Th17

Animals treated with *L. clavatum* presented predominance of Th1 response by increased TNF-α. This group also showed a significant increase in IL-10 and decrease in IL-6, compared with the control group ($p<0.05$). GCon had predominance of Th2 response by the increase of IL-4 and GCaus presented decreased Th1 response by the decrease in IL-2 and IFN-γ, both compared with the control group GCI ($p<0.05$) (Figure 3).

Figure 4 presents details of the Ratio Th1/Th2, Th1/Th17 for the proposed groups. The animals treated with *L. clavatum* presented a Th1 response predominantly due to an increase in TNF-α (TNF-α/IL-4 and TNF-α/IL-17), while GCaus and GCon presented a decrease in Th1/Th2 ratio and GCaus in Th1/Th17 ratio.

3.4. Apoptosis - In situ detection of DNA fragmentation

Detection of cells undergoing apoptosis on the eighth day of infection with *T. cruzi* in mice treated with different highly diluted compounds is described in Figure 5. GLy and GCcon groups had a higher number of splenocytes and hepatocytes undergoing apoptosis compared with GCI ($p<0.05$). However, GCon presented a decrease in apoptotic bodies in liver compared with GCI ($p<0.05$), while GLy had a significant increase in apoptotic bodies in the liver compared with GCI ($p<0.05$). GCaus only presented a higher number of hepatocytes in apoptosis when compared with GCI ($p<0.05$) (Figures 5, 6 and 7).

4. DISCUSSION

The murine model of infection with the Y strain of *T. cruzi* is a reference and is widely described in literature [33]. The use of this model may be useful for developing new treatments and understanding the mechanisms by which new tested compounds act [34,35]. In this study with highly diluted compounds, for the first time, it was possible showing the increased apoptosis, production of Th1 cytokines, clinical improvement and higher survival in mice infected with *Trypanosoma cruzi*. Our data complement studies regarding the compound *Lycopodium clavatum*, showing its positive effect on bio-immune-physiological responses with increased survival in animals susceptible to infection by highly pathogenic strain of *T. cruzi* [17, 36].

The clinical evaluation [31] of the animals infected with *T. cruzi* was an important factor to characterize and compare the morbidity in different experimental groups.

However, the assessment of clinical parameters divided into the period between the beginning of infection until the end of the patent period (23rd day of infection) was more efficient in evidencing the occurrence of clinical improvement. In GLy this improvement was significant throughout the infection, observed by the increase in temperature. A recent study, by adopting clinical evaluations during the course of infection, also observed the effect of highly diluted *Lycopodium clavatum* in mice experimentally infected with the parasite *Toxoplasma gondii* [37]. These findings help us to understand the importance of analyzing different periods of infection and not just the average of a given parameter as a whole, because the overall average of one parameter may mask the results obtained.

Although infection of Swiss mice by Y strain of *T. cruzi* is a good experimental model [38], the virulence of this strain for this lineage of mice is very high. This characteristic, in addition to the fact that Swiss mice do not constitute isogenic lineage and to the fact of having the number of experimental animals currently limited to the minimum for ethical reasons, prejudices the statistical demonstration of clinical benefits of the treatment on the experimental model used, which was definitively proved by the increased survival observed in GLy group.

In this study, aiming to statistically prove the beneficial effects of the highly diluted compounds, the evaluations of individual parameters (water and feed consumption, temperature and body weight), were performed at different periods of infection. The highest water consumption was presented by GLy (2nd-5th and 10th-13th day of infection); the lowest body weight (10th-13th day of infection) and lowest water consumption (18th-23rd day of infection) in GCaus; and lower feed intake in GCon (6º-9th day of infection), show the different clinical effects of highly diluted compounds in the biological system, since GLy represents the establishing of a greater physiological balance, promoting a more comfortable situation in physical and mental performance [39,40], resulting in increased motor coordination and faster cognitive reflexes [41]. The latter were also proven by the observation of increased motor activity in this group of animals, demonstrated by increased agitation which was detected, naturally and during handling, consistent with improved clinical condition.

The increased temperature indicates benefit to the host [42,43]. This increase will generate macrophages when necessary [44]. In a recent publication, it was demonstrated that the increase of macrophages (megakaryocytes and Kupffer cells), promoted greater survival in mice infected with *T. cruzi* [3]. Thus, these data can suggest that the

medication diluted beyond the Avogadro constant, indicated within the homeopathic precepts, might contribute to the activation of the immune response in mice.

The immune modulation between inflammatory and anti-inflammatory cytokines has a crucial role in controlling the response against the parasite and tissue damages [13], while a high level of TNF-α increased disproportionately can cause tissue damage [18], IL-10, on the other hand, acts as a factor of growth and differentiation of B cells, modulating the production of cytokines secreted by Th1 cells, i.e., promoting the cross-regulation of Th cells, modulating the intensity of response [44]. The diluted and dynamized compounds have the ability to modulate the immune system in mice infected with *T. cruzi*, controlling the Th1 response, not exacerbating the concentration of TNF-α, reducing the inflammatory state of the infection by this protozoan [6], causing an improvement in morbidity [3,17,44], as observed in our results through clinical improvement and increased survival. Thus, *Lycopodium clavatum* provided homeostasis information to the organism susceptible to infection, through the predominance of Th1 response, counterbalancing the Th2 response, since it was able to change information regarding the response against infection (i.e., "disease pattern"), improving the morbidity of the animals infected with a highly pathogenic strain. The balance between the cytokines with predominance of Th1 response in GLy can be clearly observed when we look at figure 3, and we found increased Th1 response (by the increase in TNF-α and decrease in Th17), and when comparing the ratio of cytokines in figure 4. We noticed that the Th1/Th17 ratio increased by increasing Th1, whereas the Th2 response was released to promote the immune balance due to the immunoregulatory action of IL-10 [3,44].

The action of cytokines on *Trypanosoma cruzi* infection is not completely elucidated [21,45]. In this work, we demonstrate predominance of Th1 response counterbalancing the Th2 response, while we observed increased apoptosis, clinical improvement with higher survival rate in animals treated with *Lycopodium clavatum*. These results are consistent with recent studies [6, 46].

IL-6, for being able to present both anti-inflammatory and proinflammatory activity [47], possibly signaled activation of the classical pathway in this model, acting as Th2 response (anti-inflammatory), demonstrating less tissue damage in the same experimental model [17]. This happens because in conditions in which less inflammation occurs, a lower amount of this interleukin is observed, i.e., its reduction, as observed in the GLy group, causes fewer cytotoxic effects [48,49].

In GCon, increase of Th2 response, by increased IL-4 and decrease of ratio Th1/Th2 (IL-6/IL-10, IL-2/IL-10, TNF-α/IL-4, TNF-α/IL-10, INF-α/IL4, IFN-γ/IL10), demonstrates modulation that contributes to the pathogenesis of infection [50]. When comparing the ratio TNF-α/IL4 for GCon and GLy, opposite results are observed, which is consistent with the early mortality in GCon and increased survival in GLy. Furthermore, the positive balance of Th1/Th2 response and higher concentration of TNF-α, proved to be a favorable condition to the increased survival of animals treated with *Lycopodium clavatum*, which may also be associated with less inflammation and lower cytotoxicity [17, 36].

It is suggested that the increase in IL-17 (Th17) might be related to the worsening of Chagas disease [51] and Th1 expression might be related to resistance to infection [52]. Although there are still contradictions regarding the role of cytokines as modulators of *T. cruzi* infection [21], emphasizing the role of dynamic Th1/Th17 is important to understand the mechanism of progression or improvement of the infection by this protozoan [53,54]. Our results demonstrate greater benefit in GLy, expressed by the increased ratio Th1/Th17 (TNF-α/IL-17) due to increase in TNF-α. On the other hand, the decrease in ratio Th1/Th17 observed in GCaus (IL-2/IL-17, IL-6/IL-17, IFN-γ/IL-17) by decreased IL-2 and INF-γ (Th1), and no significant reduction in IL-6, was not related to increased survival compared with the control group. These data indicate that there was activation of Th1 response for GLy, while no inhibition of this immune response occurred in GCaus.

Immunosuppressive drugs have the ability to reduce the concentration of circulating IL-2 [55]. It can be understood that *Kalium causticum* promoted immunosuppression, since in GCaus there was downregulation of IL-2. This result may be related to the unfavorable clinical course and unaltered survival observed in this group compared with GCI, contrasting with the positive results observed in GLy.

Although the mechanisms of action of highly diluted compounds are not known, evidence suggests that they are related to homeostasis [56,57] by increased apoptosis and reduced inflammation in animals infected with *T. cruzi* [58]. When this induction of apoptosis does not occur, the body may use more aggressive ways to cause cell death, such as autophagy and necrosis, which damage other tissues, mainly by increased inflammation and secretion of a number of mediators [15,16].

The mechanism of apoptosis is a general mechanism of programmed cell death, in response to different stimulus, depending on the balance of the host-parasite

relationship. In this experiment the results might indicate a more beneficial and balanced way to deal with the infection [16]. Thus, only GLy had significant, persistent increase in hepatocytes and splenocytes undergoing apoptosis compared with other treatments, in addition to hepatocytes with apoptotic bodies inside, which might indicate action of this compound on the cell. In future research it would be important to analyze whether apoptosis occurs in infected cells of animals not infected with *T. cruzi in vivo* as well *in vitro* experiments using culture of cells infected and not infected by the protozoan.

Highly diluted compounds are capable of regulating the immune system, preserving organs [59] and destroying cells with alterations [60]. Treatment with GLy activated converging pathways towards immune responses, mediated by expression and inhibition of cytokines with predominance of Th1 response and increased apoptosis, providing greater resistance to the animals, demonstrated by clinical improvement and increased survival in this group.

In summary, compounds diluted above the Avogadro constant and dynamized regulated the cytokines differently, increasing apoptosis and interfering with the morbidity with a difference in the survival of mice infected with *T. cruzi*. *Lycopodium clavatum* presented a better course of action with predominance of Th1 response by TNF-α increase counterbalancing the Th2 response and increased apoptosis, clinical improvement and higher survival, while *Kalium causticum* and *Conium maculatum* had decreased Th1 response. *Conium maculatum* promoted early mortality with increase of Th2 response by increased IL-4. Among the compounds studied, *L. clavatum* modified the functioning of the host's organism, promoting benefits, raising the possibility of using highly diluted compounds for the treatment of Chagas disease.

Conflict of Interest:

The Authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES/PROAP (no. 68/7-1), and Convênio Fundação Araucária-UEM (no. 251/12, protocol no. 22.390).

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TABLES AND FIGURES LEGENDS

Tab. 1. Quantitative clinical parameters and overall survival (probability probability) evaluated in Swiss male mice, 8 weeks-old, inoculated with 1400 trypomastigotes of the Y strain of *Trypanosoma cruzi*, in groups GCI, GCaus, GCon and GLy.

Data presented as mean ± standard deviation.
* Significative differences compared with GCI ($p<0.05$); GCaus: group treated with *Kalium Causticum* 13c; GCon: group treated with *Conium maculatum* 13c; GLy: group treated with *Lycopodium clavatum* 13c; GCI: group treated with 7% alcohol (vehicle medicine).
CI = confidence index of 95%.

Fig. 1. Kaplan-Meier curve of mice infected with 1,400 blood trypomastigotes of Y strain of *T. cruzi*, treated with 7% alcoholic solution of highly diluted compounds (GCI), *Kalium causticum* 13c (GCaus), *Conium maculatum* 13c (GCon) and *Lycopodium clavatum* 13c (GLy).

Tab. 2. Qualitative clinical parameters evaluated in Swiss male mice, 8 weeks-old, inoculated with 1400 trypomastigotes of the Y strain of *Trypanosoma cruzi*, for the groups GCI, GCaus, GCon and GLy.

GCI = control of infection group, treated with 7% hydroalcoholic solution of the highly diluted compounds; GCaus = treated with *Kalium Causticum* 13c, GCon = treated with *Conium maculatum* 13c; GLy = treated with *Lycopodium clavatum* 13c.
n/N: n= number of observations; N=overall number of elements in the group throughout infection.
*p significant by the Z test compared with GCI
Ref – Reference

Fig. 2. A: Average body weight (g) from the 1st to the 23rd days of infection; B: Average feed intake (g) from the 1st to the 23rd day of infection; C: Average water consumption (mL) from the 1st to the 23rd day of infection; D: Average body

temperature (ºC) from the 1st to the 23rd day of infection of Swiss male mice, 1,400 blood trypomastigotes of Y strain of *T. cruzi*, treated with 7% alcoholic solution (GCI) of highly diluted compounds, *Kalium causticum* 13c (GCaus), *Conium maculatum* 13c (GCon) and *Lycopodium clavatum* 13c (GLy). * $p<0.05$ compared with GCI.

Fig. 3. Serum concentration (pg/ml) of IL-2, IFN-γ, TNF-α, IL-6, IL-4, IL-10 and IL-17 on the 8th day after infection in Swiss male mice, 1400 blood trypomastigotes of the Y strain of *T. cruzi* treated with 7% hydroalcoholic vehicle of highly diluted compound (GCI), *Kalium Causticum* 13c (GCaus), *Conium maculatum* 13c (GCon) and *Lycopodium clavatum* 13c (GLy). n=3 animals per group; * $p<0.05$ compared with GCI between groups for the same cytokine.

Fig. 4. Ratios of IL-6/IL-4, IL-6/IL-10, IL-2/IL-4, IL-2/IL-10, TNFα/IL-4, TNFα/IL-10, IFNγ/IL-4, IFNγ/IL-10, IL-2/IL-17, IL-6/IL-17, TNFα/IL-17, IFNγ/IL-17 (balance Th1/Th2 and Th1/Th17) on the 8th day after infection in Swiss male mice, 1400 blood trypomastigotes of the Y strain of *T. cruzi* treated with 7% hydroalcoholic vehicle of highly diluted compound (GCI), *Kalium Causticum* 13c (GCaus), *Conium maculatum* 13c (GCon) and *Lycopodium clavatum* 13c (GLy). n=3 animals per group; *$p<0.05$ compared with GCI between groups for the same relationship between cytokines. Letters a, b, c, d, e, f = individualized citokines with better definition.

Fig. 5. Mean of the number of hepatocytes TUNEL+, splenocytes TUNEL + and apoptotic phagocytosed bodies (Liver) in histological sections of Swiss male mice, 8 weeks-old, inoculated with 1400 trypomastigotes of the Y strain of *Trypanosoma cruzi* on the 8th day after infection (40X magnification). Immunohistochemistry method: Terminal deoxynucleotidyl transferase-mediated UTP Nick end labeling (TUNEL) and APOPTAG (Millipore). n=3 animals per group; *$p<0.05$ compared with the GCI for each parameter.
Hepatocytes TUNEL +: liver cells undergoing apoptosis.
Splenocytes TUNEL +: Spleen cells undergoing apoptosis.
Apoptotic bodies: cells containing apoptotic material in the liver.
GCI = control of infection group, treated with 7% hydroalcoholic solution of the highly diluted compound; GCaus = treated with *Kalium Causticum* 13c, GCon = treated with *Conium maculatum* 13c; GLy = treated with *Lycopodium clavatum* 13c.

Fig. 6. Section of liver of Swiss male mice, 8th day of infection with 1400 blood trypomastigotes of the Y strain of *T. cruzi* treated with 7% hydroalcoholic vehicle of highly diluted compound (GCI), *Kalium causticum* 13c (GCaus) , *Conium maculatum* 13c (GCon) and *Lycopodium clavatum* 13c (GLy). n=3 animals per group; Technique used: Terminal deoxynucleotidyl transferase-mediated UTP Nick end labelling (TUNEL) - APOPTAG (Millipore). 40x objective. Arrow (→): hepatocytes undergoing apoptosis; Oval shape (o): apoptotic bodies - phagocytic cells with phagocytic vacuole with apoptotic material.

Fig. 7. Section of spleen of Swiss male mice, 8th day of infection with 1400 blood trypomastigotes of the Y strain of *T. cruzi* treated with 7% hydroalcoholic vehicle of highly diluted compound (GCI), *Kalium causticum* 13c (GCaus), *Conium maculatum* 13c (GCon) and *Lycopodium clavatum* 13c (GLy). n=3 animals per group; Technique used: Terminal deoxynucleotidyl transferase-mediated UTP Nick end labeling

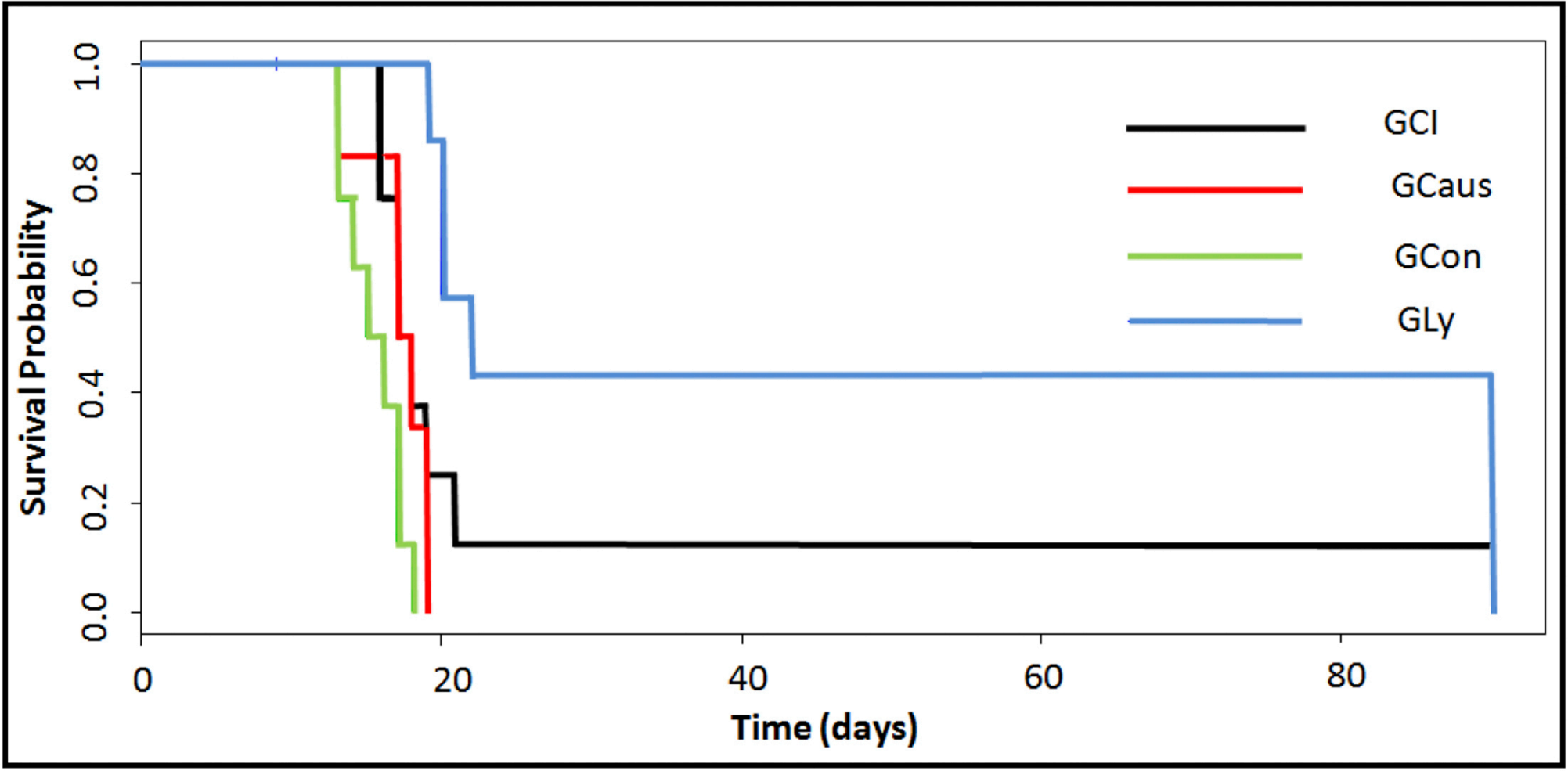
(TUNEL) - APOPTAG (Millipore). 40x objective. Arrow (→): splenocytes undergoing apoptosis.

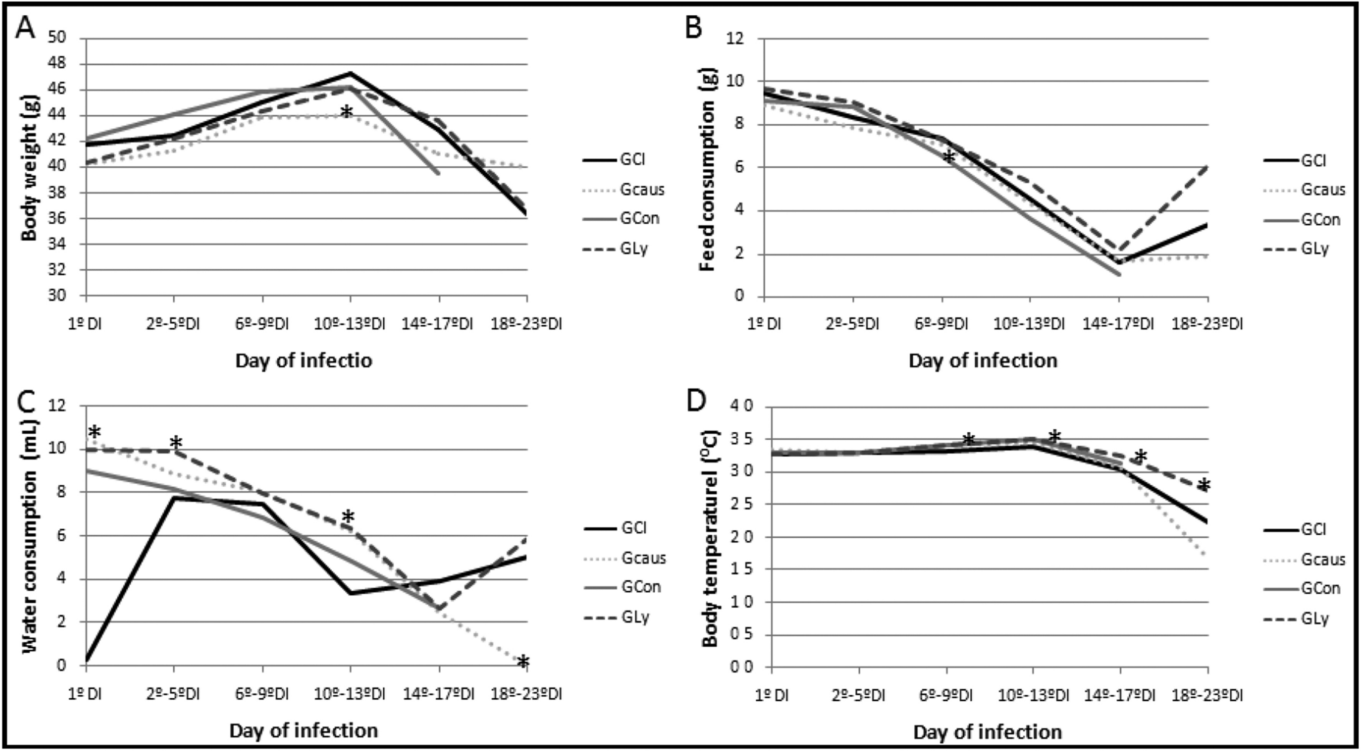
Tab. 1.

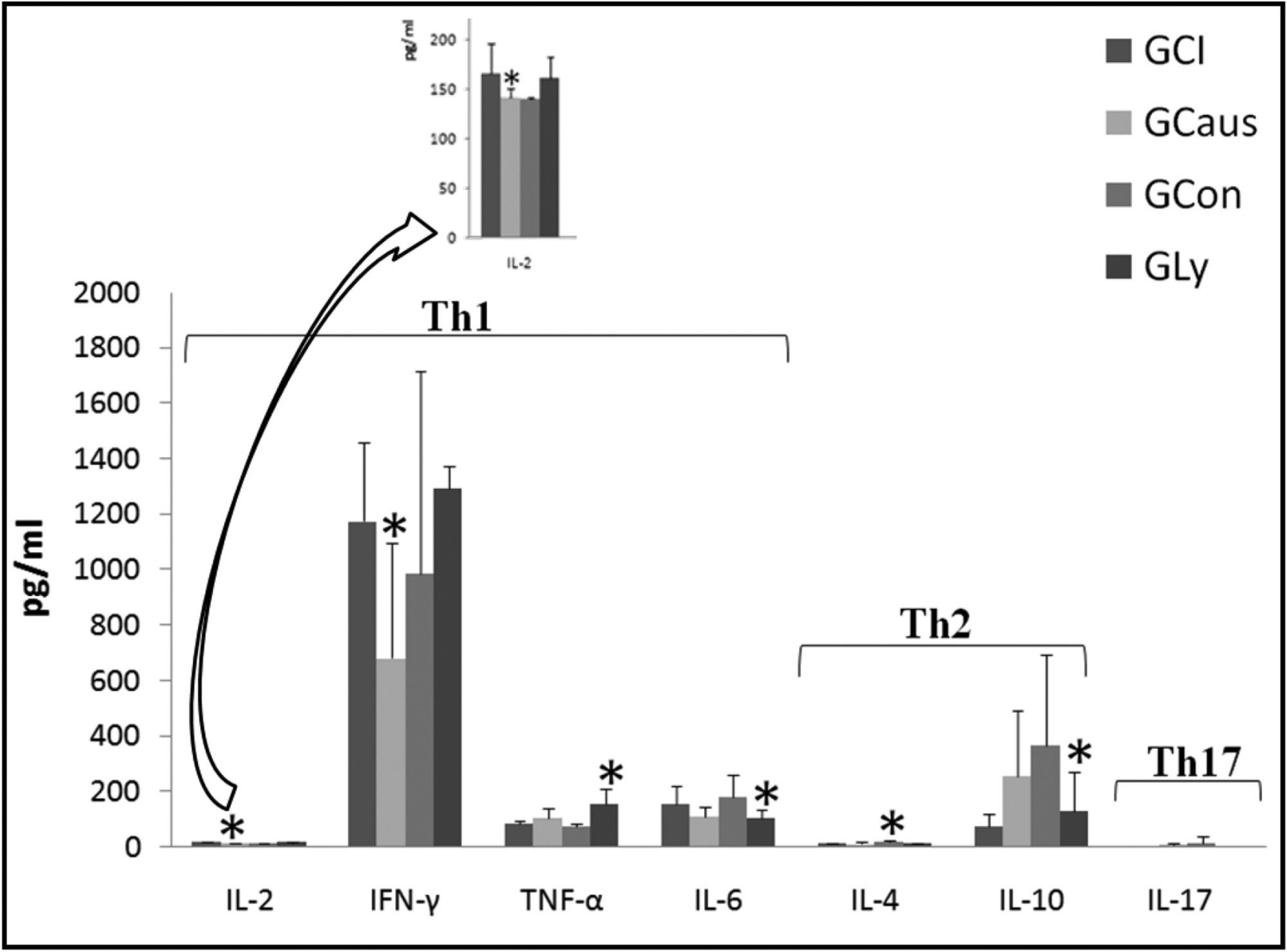
Groups	Temperature (ºC)	Body weight (g/day)	Feed Consumption (g/day)	Water Consumption (mL)	Overall survival, probability (95% CI)
GCI	32,61±2,2	42,7±4	6,8±2,7	6,8±2,8	12% (2%-78%)
GCaus	33,19±2,5*	41,2±4,5	6,5±2,3	7,6±2,4	33% (10%-100%)
GCon	33,74±1,2*	43,4±3,8*	7,3±2,7	7,5±2,2	12% (2%-78%)
GLy	33,04±3,2*	41,8±4,2	7,2±2,7	7,6±2,9	42% (18%-100%)

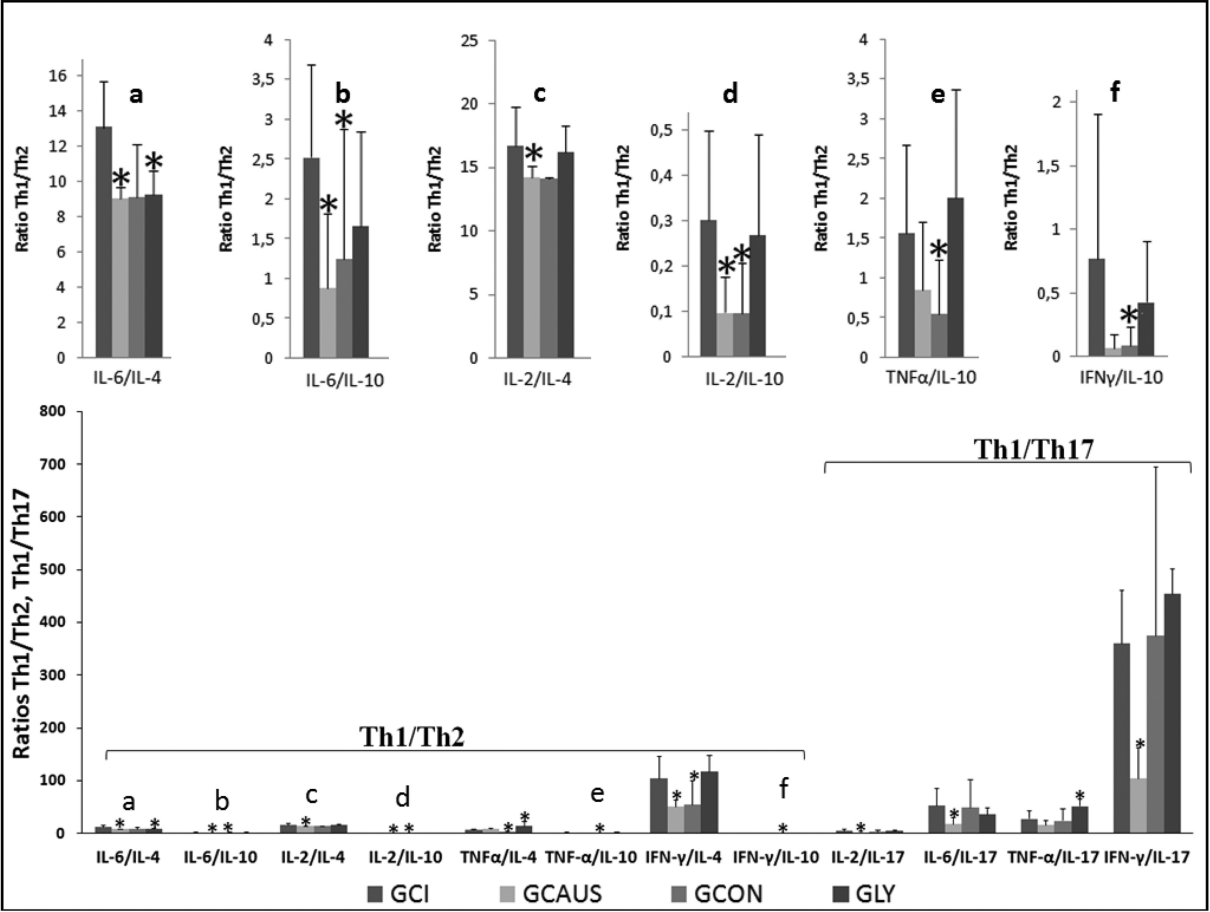
Tab. 2.

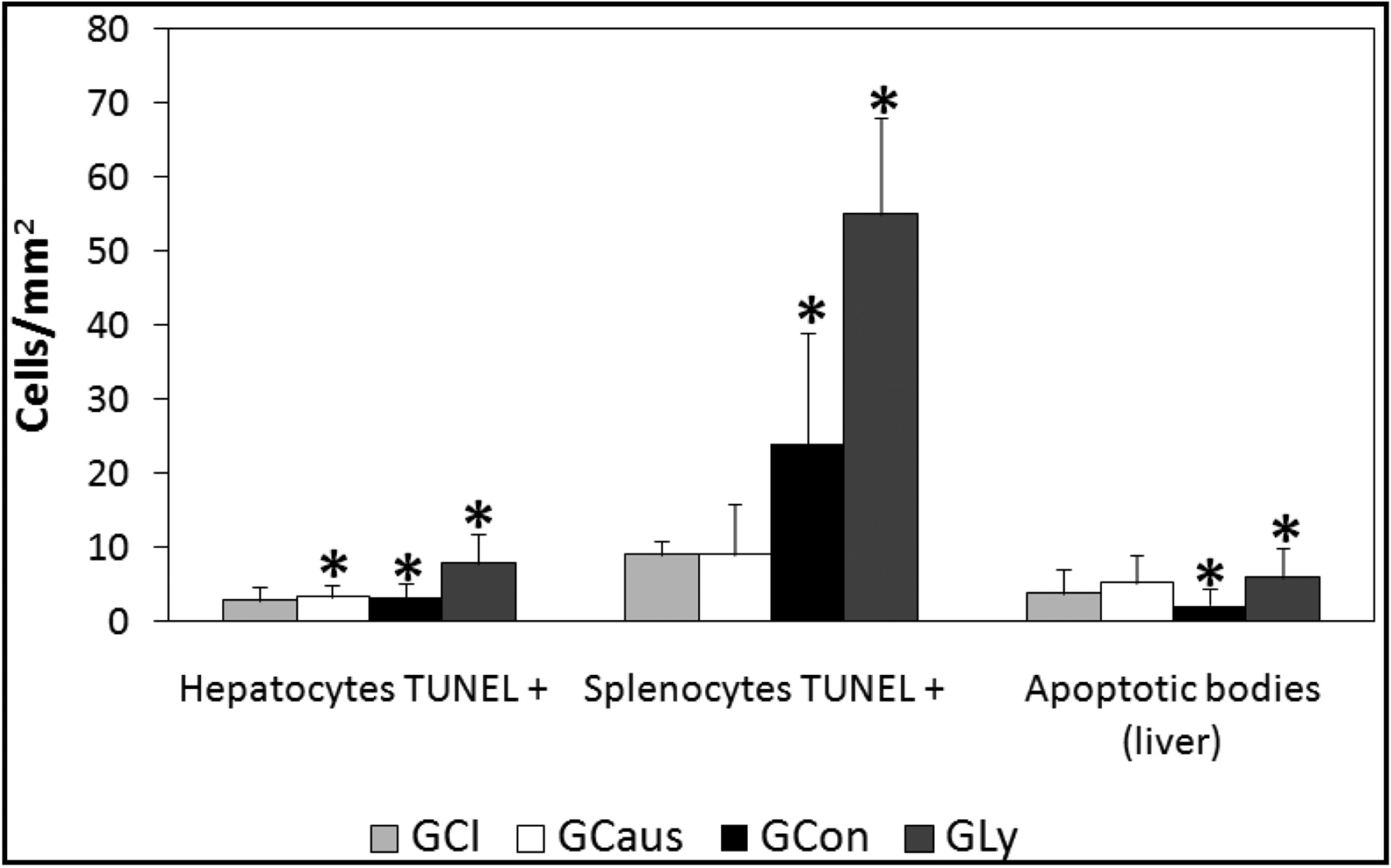
Groups	Sleepiness		Genital Edema		Abdominal Edema		bristly Fur		Natural Agitation		Agitation with Handling	
	n/N (%)	*P*	n/N (%)	*P*	n/N (%)	*P*	n/N (%)	*p*	n/N (%)	*P*	n/N (%)	*P*
GCI	166/226 (73,5)	Ref	74/226 (32,7)	Ref	55/226 (24,3)	Ref	78/226 (34,5)	Ref	6/226 (2,7)	Ref	67/226 (29,6)	Ref
GCaus	150/166 (90,4)	0,0000*	85/166 (51,2)	0,0000*	52/166 (31,3)	0,0230*	99/166 (59,6)	0,9999	12/166 (7,2)	0,0000*	71/166 (42,8)	0,0399*
GCon	136/205 (66,3)	0,9999	53/205 (25,9)	0,9999	34/205 (16,6)	0,9999	37/205 (18,0)	0,0174*	8/205 (3,9)	0,9999	61/205 (29,8)	0,9999
GLy	184/224 (82,1)	0,0000*	74/224 (36,1)	0,0000*	75/224 (36,6)	0,0000*	79/224 (38,5)	0,0266*	11/224 (5,4)	0,0000*	79/224 (38,5)	0,0266*

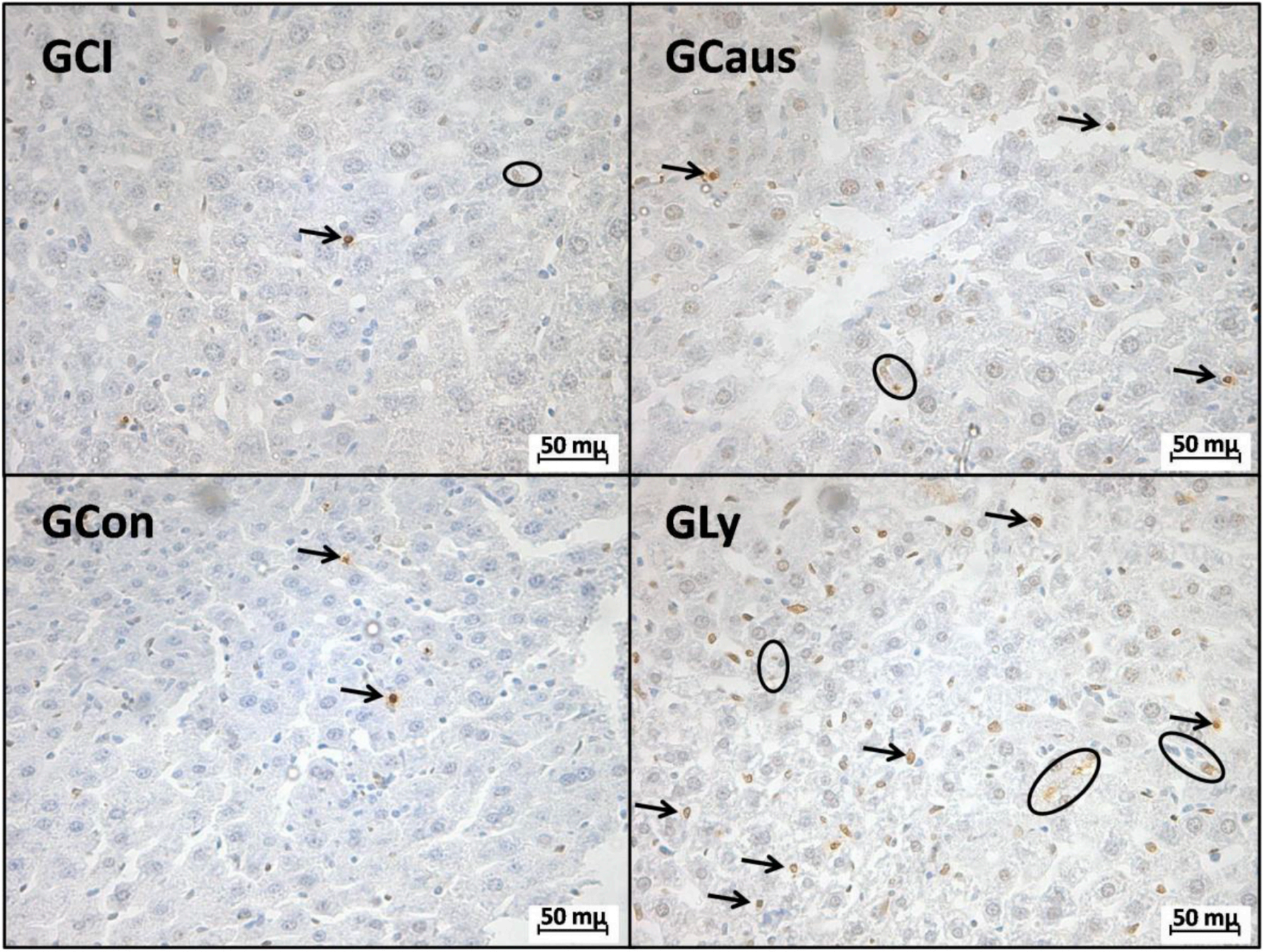

Survival Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Time (days)
GCl
GCaus
GCon
GLy

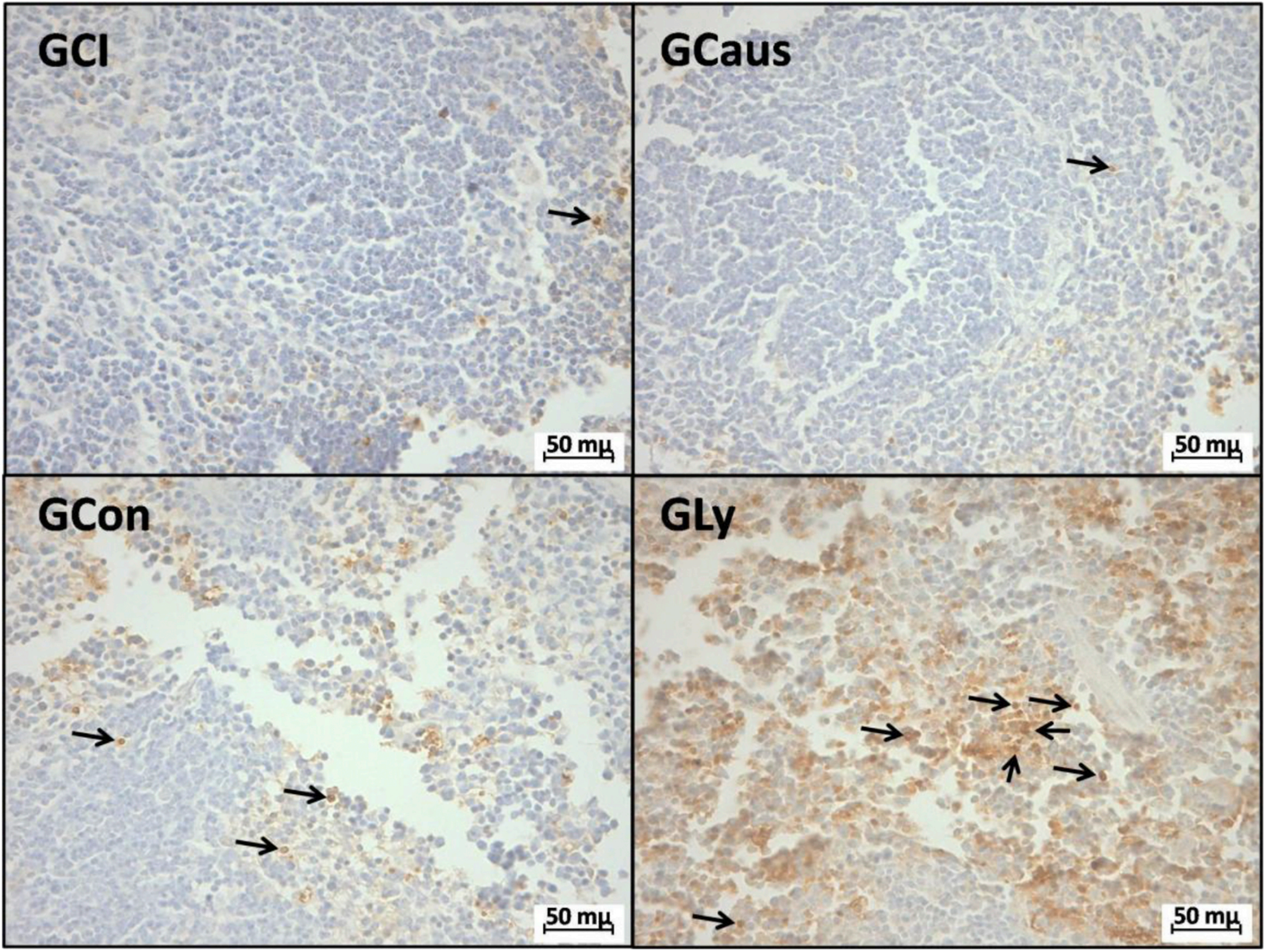

A
Body weight (g)
50
48
46
44
42
40
38
36
34
32
30
1º DI
2º-5ºDI
6º-9ºDI
10º-13ºDI
14º-17ºDI
18º-23ºDI
Day of infectio
GCl
Gcaus
GCon
GLy
B
Feed consumption (g)
12
10
8
6
4
2
0
1º DI
2º-5ºDI
6º-9ºDI
10º-13ºDI
14º-17ºDI
18º-23ºDI
Day of infection
GCl
Gcaus
GCon
GLy
C
Water consumption (mL)
12
10
8
6
4
2
0
1º DI
2º-5ºDI
6º-9ºDI
10º-13ºDI
14º-17ºDI
18º-23ºDI
Day of infection
GCl
Gcaus
GCon
GLy
D
Body temperaturel (ºC)
40
35
30
25
20
15
10
05
00
1º DI
2º-5ºDI
6º-9ºDI
10º-13ºDI
14º-17ºDI
18º-23ºDI
Day of infection
GCl
Gcaus
GCon
GLy


GCl
GCaus
GCon
GLy
pg/ml
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Th1
Th2
Th17
IL-2
IFN-γ
TNF-α
IL-6
IL-4
IL-10
IL-17
*


a
b
c
d
e
f
Ratio Th1/Th2
IL-6/IL-4
IL-6/IL-10
IL-2/IL-4
IL-2/IL-10
TNFα/IL-10
IFNγ/IL-10
Ratios Th1/Th2, Th1/Th17
Th1/Th2
Th1/Th17
IL-6/IL-4
IL-6/IL-10
IL-2/IL-4
IL-2/IL-10
TNFα/IL-4
TNF-α/IL-10
IFN-γ/IL-4
IFN-γ/IL-10
IL-2/IL-17
IL-6/IL-17
TNF-α/IL-17
IFN-γ/IL-17
GCI
GCAUS
GCON
GLY


80
70
60
50
40
30
20
10
0
Cells/mm2
*
*
*
*
*
*
Hepatocytes TUNEL +
Splenocytes TUNEL +
Apoptotic bodies (liver)
GCl
GCaus
GCon
GLy


GCl
50 mμ
GCaus
50 mμ
GCon
50 mμ
GLy
50 mμ


GCl
50 mμ
GCaus
50 mμ
GCon
50 mμ
GLy
50 mμ

Highlights

- *Lycopodium clavatum* increase apoptosis, clinical improvement and higher survival;
- *Lycopodium clavatum* causes predominance of Th1 response;
- *Kalium causticum* and *Conium maculatum* causes decrease Th1 response*;*
- *Conium maculatum* promoted early mortality with increase of Th2 response.