Original Article

High-diluted thymulin on Ehrlich tumor growth in mice and the importance of tumor microenvironment

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Abstract

Introduction: The aim of the present study was to describe different biological aspects of Ehrlich tumor in mice, such as body weight evolution, tumor growth rate, histological organization and systemic immune response after treatment with high-diluted thymulin (10^{-9} M, named 5CH).

Methods: Tumor assessment was focused on macro- and microscopic aspects; parameters included occurrence of necrosis, embolism and tumor development, in addition to quantitative analysis of apoptosis (caspase-3), cell proliferation (Ki-67) and angiogenesis (vascular endothelial growth factor - VEGF) by means of specific immunohistochemistry markers. Spleen cell populations were evaluated by flow cytometry analysis.

Results: Mice treated with thymulin 5CH exhibited changes in the tumor microenvironment, such as reduced micro-embolism incidence and cytokeratin expression, with increased caspase-3 expression in the tumor cells. These findings indicate some apoptotic activity by the tumor cells induced by the treatment, even though no reduction of the macroscopic tumor mass occurred. No changes in the systemic immune response were detected, as the balance among spleen cell populations remained unchanged.

Conclusions: The results indicate that treatment of mice bearing Ehrlich tumor with thymulin 5CH induces some specific changes in the tumor environment. However, it did not influence systemic immunity parameters. Adjuvant use of thymulin 5CH in oncological clinical practice is still a matter of discussion.

Keywords: Ehrlich ascites tumor; high dilutions; thymulin; tumor immunology; experimental oncology.

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Introduction

The current treatments for cancer are often associated with adverse effects and sequels that have negative effects on the patient’s life. For this reason, patients are increasingly seeking complementary treatments, including homeopathy and high-diluted substances, to avoid side effects (GHOSH et al., 2014; FRASS et al., 2015; GLEISS et al., 2016; ROSSI et al., 2018; SHALOM-SHARABI et al., 2018).

In vivo and in vitro experimental models to study the effects of drugs prepared as homeopathic preparations - i.e., agitated high dilutions - on the progression of cancer were developed in recent years, within the context of fundamental research (BISWAS, KHUDA-BUKHSH 2002; MCLAUGHLIN et al., 2006; TOLIOPOULOS et al., 2013; ARORA, TANDON, 2015; SAHA et al., 2015; MONDAL et al., 2016; WANI et al., 2016; SEKER et al. 2018). Some of these studies focus on immune modulation as the main mechanism for cancer control (SATO et al., 2005; BURBANO et al., 2009; GUIMARÃES et al., 2010; SAHA et al. 2013). The results thus achieved encourage the performance of further studies with high-diluted immune-modulatory substances in experimental oncology.

Thymulin is an endocrine peptide directly involved in T-cell differentiation. It was discovered in 1970 as a zinc-dependent nonapeptide produced by epithelial cells in the thymus and plays a relevant role in T-cell maturation. Thymulin purified peptide might be obtained from calf thymus acid lysate and acts on T, B and natural killer (NK) cells modulating their proliferation, differentiation and activity (BACH 1979; REGGIANI et al., 2009; 2014). Previous studies showed that high-diluted thymulin prepared according to the homeopathic pharmacopeia (ANVISA 2011) exerts immune-modulatory effects in different experimental situations, mainly in low potencies, whose concentration is near to the physiological level (BASTIDE et al., 1985; 1987; DAURAT et al., 1988; BONAMIN et al., 2013; RODRIGUES DE SANTANA et al., 2014; BONAMIN, BELLAVITE, 2015; POITEVIN, 2015; SIQUEIRA et al., 2016).

Ehrlich tumor has been previously used as model to elucidate the immune mechanisms of homeopathic medicines in tumors (SAHA et al., 2013). It is a transplantable experimental malignant neoplasm discovered in 1886 and described in 1905 by Paul Ehrlich as mammary carcinoma of female mice. This tumor develops an ascitic form when inoculated into the peritoneum and a solid form when inoculated into the subcutaneous tissue (EHRLICH, 1906). This tumor has been a common model in experimental oncology for studies about tumor-host interactions, since many autocrine factors influence its growth in mouse tissues, such as glutamine and tumor capillary web
vascular endothelial growth factor-dependent or VEGF-dependent (GHOSH et al, 2004), producing systemic dysfunctions like cardiomyopathy (MISHRA et al., 2018) and liver oxidative stress (MERAL et al., 2018).

Based on the considerations above, the aim of the present study was to investigate systemic and microscopic aspects of the Ehrlich tumor phenotype and of the immune response to it under treatment with homeopathically prepared high-diluted thymulin.

Methods

Animals

We used 13 randomized BALB/c adult male mice which were kept in micro-isolators (Techniplast®), with 6 to 7 animals per cage, at the vivarium of animal experimentation of Paulista University, with water and food ad libitum, under controlled temperature (22±2°C) and humidity (55-65%), continuous air flow changes (75/hour) and 12-hour light-dark cycle. Male mice were used to avoid hormonal variations on the outcomes due to the estral cycle.

Ethical issues

The present study is part of a larger research project approved by the institutional research ethics committee (CEUA) of Paulista University, on April 17th 2013, ruling number: 158/2013. The study design complied the current requirements for animal well-being and the 3Rs rule (reduction, replacement and refinement), as described in the Brazilian guidelines for ethics in animal experimentation (CONCEA), which is in accordance to the EU Directive 2010/63/EU for animal experiments. The procedures comply with the ARRIVE guidelines.

Experimental model

Preparation of cells and inoculation

To obtain the optimal number of tumor cells, a mouse carrying Ehrlich tumor ascites tumor for 10 days was subjected to abdominal puncture, the ascitic fluid was removed and cells were washed three times in phosphate buffer saline (PBS), after successive centrifugations. Cell counting was performed with 0.1% trypan blue (GIBCO®, USA) on a Neubauer chamber. The volume of 0.05 mL of suspension containing $10^5$ cells was inoculated into the subcutaneous tissue of the left footpad, to
induce the development of the tumor solid form. This method allows evaluating the macroscopic
development on a daily basis, with no need to kill the experimental animals and, at once, using an
optimal number of cells able to show the tumor development without cause pain and suffering. The
study duration was set to 10 days, i.e., before the occurrence of clinical worsening.

**Drugs**

Commercial thymulin 4CH (Boiron, France) was used as source, which was prepared from the zinc
free synthetic peptide or serum thymic factor (MW = 858.85), whose purity degree was 98.66%
(according to the supplier). The last dilution (5CH) was prepared at our laboratory anew every
week. For this purpose, 10 mL of ultra-pure water were used to dilute 100µL of thymulin 4CH in
amber glass vials. The vials were automatically agitated with a mechanical arm (Autic®, BRAZIL) in
compliance with the recommendations set in the Brazilian Homeopathic Pharmacopeia, 3rd edition
(ANVISA, 2011). Agitated pure water was used as control (vehicle). CH stands for serial centesimal
dilution of the starting substance in 70% hydroalcoholic solution. Thus, the number of dilutions
defines the so-called “homeopathic potencies” according to the specific technical nomenclature
used in this field. Thymulin 5CH dilution is theoretically equivalent to 10⁻⁹M.

**Blinding**

Medicines and controls were coded as A and B by a laboratory technician who did not participate in
the study; the code was broken only after the end of statistical analysis. Therefore, the investigators
were blinded as to the groups of allocation all throughout the study.

**Experimental design**

The animals were randomly divided in two groups: thymulin 5CH (n=6) and control, which was
treated with the vehicle (n=7). Twenty-four hours after tumor inoculation, the animals were
administered orally with 10µL of medicine (or vehicle)/10g body live weight/animal/day, every
afternoon, along 10 days. The order of administration was in accordance with the code A or B.
Clinical parameters were daily assessed, including body weight and tumor growth. If necessary, the
endpoint would be set wherever the step of the study. To evaluate the macroscopic tumor growth,
the footpad was measured before tumor inoculation and then every day, with a micrometer
(Mitutoyo® IP65, Japan). The experiment was performed once and the number of animals per
group was the minimum considered to a trustful statistical analysis, according to previous
experience of the laboratory with this model.
Sample collection for histopathological and flow cytometry analysis

All the animals were euthanized 10 days after tumor inoculation. For this purpose, the animals were deeply sedated with 20 mg/10g live weight of xylazine and 10mg/10g live weight of ketamine until the vital signs were no longer perceptible. Next, cervical dislocation was performed to ensure the animals were dead before the onset of necropsy.

Tumor samples were collected for histopathological analysis; in such a way that a latero-lateral metameric cut of the footpad was done. The standardization of the tumor sampling was important to perform histometry. The samples were fixated in 8% paraformaldehyde to ensure surface antigen preservation. Next, they were included in paraffin, sectioned and mounted on silanized (for immunohistochemistry) or conventional (for hematoxylin-eosin staining) slides. No tumor inoculated pads were used as histological control.

For flow cytometry, a central fragment of the spleen was used. The pool of spleen cell was prepared containing spleen fragment suspension from two animals of the same group.

Flow cytometry

Flow cytometry of spleen cells was performed to quantify the number of T (CD4+, CD8+), B1, B2, marginal zone (MZ) lymphocytes and macrophages, according to a panel of markers, as described in Table 1.

Table 1. Cell markers used for phenotype identification and quantification on flow cytometry

<table>
<thead>
<tr>
<th>CELLS</th>
<th>MARKERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1 lymphocytes</td>
<td>CD19+ CD23- CD11b+</td>
</tr>
<tr>
<td>B2 lymphocytes</td>
<td>CD19+ CD23+ CD11b-</td>
</tr>
<tr>
<td>Macrophages</td>
<td>CD19- CD23- CD11b+</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>CD19- CD4+ or CD19- CD8+</td>
</tr>
<tr>
<td>T reg or activated T lymphocytes</td>
<td>CD19- CD25+</td>
</tr>
<tr>
<td>Marginal zone B lymphocytes</td>
<td>CD19+ CD23- CD11b-</td>
</tr>
</tbody>
</table>

After treated with hemolytic buffer to remove red blood cells, spleen cells were washed two times with PBS 1% and, then, counted using Neubauer chamber. Samples contained $1 \times 10^6$ cells were incubated with anti CD16/CD32 diluted in PBS supplemented with 1% of bovine serum albumin (BSA, PBS-BSA1%), for 20 min at 4ºC. Then, cells were washed and divided in two aliquots re-
suspend in PBS-BSA 1%. Each aliquot was incubated at 4°C for 20 min with respectively monoclonal antibodies combination, as described in Table 2. All antibodies were supplied by INVITROGEN (Thermo Fisher Scientific, USA). Afterwards, cells were washed and re-suspended in PBS for flow cytometry acquisition, using a device FACS Canto II (BD Bioscences, Mountain View, CA, USA). The data analysis was performed using FlowJo software (FlowJo LLC, Data Analysis Software, Ashland, OR), according to gates strategy showed in Figures 1 and 2.

**Figure 1.** Gate strategy for analyzing spleen B cells population by flow cytometry. Images acquired with FlowJo 7.6.5 software shown the gate strategy to analyze spleen B leukocyte population by flow cytometry. (A) Total leukocyte population; (B) B1, B2 lymphocyte and phagocyte population; (C) MZB lymphocyte and B2 lymphocyte population; (D) B1a and B1b cell population.
Figure 2. Gate strategy for analyzing spleen T cells population by flow cytometry. Images acquired with FlowJo 7.6.5 software shown the gate strategy to analyze spleen T leukocyte population by flow cytometry. (A) Total leukocyte population; (B) CD19- cell population (T lymphocytes, phagocytes and NK cells) and CD19+ cell population (B lymphocytes); (C) T lymphocyte population (CD4+ and CD8+); (D) CD25+ cell population (T_{reg} lymphocytes or activated T lymphocytes); (E) CD4+CD25+ and CD8+CD25+ T lymphocyte population.

Table 2. Monoclonal antibodies combination used for Flow cytometry analysis of spleen cells

<table>
<thead>
<tr>
<th>Combo 1 B lymphocytes</th>
<th>Combo 2 T lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD 23 FITC</td>
<td>CD25 AF488</td>
</tr>
<tr>
<td>CD 5 PE</td>
<td>CD 4 PE</td>
</tr>
<tr>
<td>CD 19 PE Cy 5:5</td>
<td>CD 19 PE Cy 5:5</td>
</tr>
<tr>
<td>CD 11b PB</td>
<td>CD8 AF 405</td>
</tr>
</tbody>
</table>

Immunohistochemistry and histomorphometry

Indirect immunohistochemistry

Five-micrometer sections of footpad samples included in paraffin were mounted on silanized slides. The sections were deparaffinized, rehydrated and subjected to antigen unmasking using citrate...
buffer for 20 minutes at 100°C, in an electric cooker (PANASONIC®, Japan). The sections were left to cool and then subjected to endogenous peroxidase blocking with 5% H₂O₂ solution in methanol, for 5 minutes. The sections were rinsed 3 times and the unspecific adsorption sites were blocked with 2.5% normal horse serum (VECTOR®, USA) for 30 minutes. Next, the sections were incubated overnight with monoclonal antibodies in specific standard dilutions at 4ºC in a humidity chamber (Table 3) and incubated with peroxidase-polymer conjugated secondary antibody (Kit Impress Universal VECTOR®, USA) for 30 minutes at room temperature. Cell labeling was revealed by further 3,3′-diaminobenzidine - DAB (DAKO®, USA) staining. All the reactions included negative controls, involving the use of antibody solvent alone (DAKO®, USA).

Table 3. Antibodies used for immunohistochemistry: molecular targets, clones, dilutions and sources

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Function</th>
<th>Molecular target</th>
<th>Donor species</th>
<th>Clone</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>Angiogenesis</td>
<td>Growth factor</td>
<td>Rat</td>
<td>VG-1 monoclonal</td>
<td>1:200</td>
<td>Abcam®</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Cell proliferation</td>
<td>cytoplasmic protein</td>
<td>Rat</td>
<td>Polyclonal</td>
<td>1:1000</td>
<td>Abcam®</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>Apoptosis</td>
<td>Cytoplasmic protein</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>40µg/mL</td>
<td>Abcam®</td>
</tr>
<tr>
<td>CytoK</td>
<td>Epithelial cells</td>
<td>Cytoplasmic protein</td>
<td>Rat</td>
<td>Krt1-19</td>
<td>1:50</td>
<td>Serotec®</td>
</tr>
</tbody>
</table>

**Semi-quantitative histopathological assessment**

Slides with hematoxylin-eosin (HE)-stained footpad sections were analyzed under NIKON Eclipse 200 microscope and assessed as to the tumor size by means scores (Figure 3). The full section on each slide was screened and assessed with 4x magnification. A score system was applied as shown in Table 4.
### Table 4. Score for histological tumor size quantification

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No tumor</td>
</tr>
<tr>
<td>1</td>
<td>1 to 20% of the footpad section area with tumor</td>
</tr>
<tr>
<td>2</td>
<td>20 to 50% of the footpad section area with tumor</td>
</tr>
<tr>
<td>3</td>
<td>More than 50% of the footpad section area with tumor</td>
</tr>
</tbody>
</table>

**Figure 3.** Photomicrographs of footpad samples with solid form Ehrlich tumor stained by means of the hematoxylin-eosin method used to quantify the tumor size according to a preset score. Magnification: 4x.
Quantitative histopathological assessment

Four to 10 microphotographs were taken, ensuring the coverage of all the tumor-containing microscopic fields under 40x magnification using Nikon Eclipse 200 microscope coupled to a Coolpix digital camera with LCD screen (Nikon®, Japan). DAB-positive labeling for cytokeratin (cell differentiation), caspase-3 (apoptosis) and VEGF (angiogenesis) was automatically calculated for brown intensity, in pixels average per field, using software Metamorph® (Molecular Devices®, USA). The software was calibrated with digital color filters, with red, green blue bit regulation, so that only clearly labeled cells were included, in such a way that degenerative or necrotic areas were not considered in the quantification. For analysis, we considered the average intensity of brown staining in each analyzed field.

The number of Ki-67 labeled cells for was calculated by means on visual counting considering only the cells with nuclear staining, since Ki-67 cytoplasmic labeling has no biological significance for cell proliferation.

Quantitative assessment of the incidence of several tumor parameters was performed by means of visual counting on Nikon Eclipse 200 microscope, under different magnifications. The parameters assessed on each HE-stained footpad section were: presence of necrosis or not, evidences of local tumor invasion and of micro-embolism. Footpad swelling was quantified based on the HE-stained sections. The area with dissociated tissue (edema) was automatically calculated, in pixels, using the same software (Figure 4).

Statistical analysis

Student’s t-test and the Mann-Whitney test were used for analysis of histological parameters (in pixels or scores) as a function of the homoscedasticity of variables as assessed by means of Bartlett’s test. The results of flow cytometry were assessed by means of the Mann-Whitney test. Semi-quantitative data (incidence rates) were analyzed with Fisher’s exact test. Two-way ANOVA was performed to compare the progression of body weight over time. In all the analyses the significance level was set as p≤0.05.
Figure 4. Photomicrograph of footpad with HE-stained solid Ehrlich tumor (A-D) and control with no tumor (E-F). ED – edema; [] – areas with tumor invasion; arrows – inflammation cells; [ ] – necrosis; [Q] – keratin; [EP] – epithelium. Magnification: 4x, 10x and 40x.
Results

**Histology, immunohistochemistry and histomorphometry**

Animals treated with thymulin 5CH exhibited statistically significant lower incidence of microembolism (p=0.01), higher caspase-3 labeling (pixel intensity, p=0.05), reduced cytokeratin expression (p=0.03), but no statistically difference in tumor proliferation parameters (incidence of invasion, angiogenesis and Ki67 labeled cells per field) compared to the controls (Table 5).

**Table 5.** Assessment of histopathological parameters on HE-stained sections and tumor labeling on immunohistochemistry. Values are expressed as number of positive cases over the total (percentage in parentheses) and as mean ± standard deviation. *p=0.01 Fisher’s exact test. **p=0.05 Student’s t-test. #p=0.03 Mann-Whitney test, U=6.000, compared to control. CytoK= cytokeratin.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle</th>
<th>Thymulin 5CH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Necrosis (incidence)</td>
<td>3/6 (50%)</td>
<td>1/6 (16%)</td>
</tr>
<tr>
<td>Micro-embolism (incidence)</td>
<td>5/6 (83%)</td>
<td>0/6 (0%) *</td>
</tr>
<tr>
<td>Tumor invasion (incidence)</td>
<td>6/6 (100%)</td>
<td>5/6 (83%)</td>
</tr>
<tr>
<td>Edema (area in pixels × 10⁴)</td>
<td>25.31 ± 12.76</td>
<td>23.43 ± 11.04</td>
</tr>
<tr>
<td>Caspase-3 (intensity, in pixels)</td>
<td>96.58 ± 2.91</td>
<td><strong>108.78 ± 4.83</strong> **</td>
</tr>
<tr>
<td>CytoK (intensity, in pixels)</td>
<td>113.08 ± 18.07</td>
<td>95.60 ± 9.02 #</td>
</tr>
<tr>
<td>VEGF (intensity, in pixels)</td>
<td>117.97 ± 3.10</td>
<td>111.19 ± 25.29</td>
</tr>
<tr>
<td>Ki-67 (positive cells per field)</td>
<td>09/16</td>
<td>13/14</td>
</tr>
</tbody>
</table>

**Clinical parameters**

There was no significant difference for body weight and tumor growth over time and no adverse effect in treated mice (Figure 5).
Figure 5. Temporal progression of body weight (g) and paw thickness (mm) – representing tumor growth - after inoculation of Ehrlich tumor cells into the footpad of mice daily treated with vehicle or thymulin 5CH. N=6. Values are expressed as mean ± standard deviation. Two-way ANOVA.
**Flow cytometry**

Significant difference was not found between the groups in regard to any cell subtype considering their concentration in the spleen cell suspension, percentage and mutual ratios, although a mild increase of CD8+/CD4+ ratio was seen (Table 6).

**Table 6.** Number of cells x 10^2 in 10,000 events analyzed by flow cytometry. Each marker was identified on the selected gates of spleen cells suspension from mice bearing solid Ehrlich tumor along 10 days. Values are expressed as mean ± standard deviation. Proportions were calculated as simple average ratios. Mann-Whitney test and Fisher’s exact test. MZB= marginal zone B lymphocytes.

<table>
<thead>
<tr>
<th>Cell subtypes</th>
<th>Vehicle</th>
<th>Thymulin 5CH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total B lymphocytes (CD19+)</strong></td>
<td>53.43 ± 5.59</td>
<td>50.00 ± 5.93</td>
</tr>
<tr>
<td><strong>Total T lymphocytes (CD19-)</strong></td>
<td>46.46 ± 5.598</td>
<td>54.40 ± 0.424</td>
</tr>
<tr>
<td><strong>B2 lymphocytes (CD19+ CD23+)</strong></td>
<td>50.50 ± 7.98</td>
<td>56.95 ± 14.35</td>
</tr>
<tr>
<td><strong>B1 lymphocytes (C19+ CD23-)</strong></td>
<td>6.01 ± 2.12</td>
<td>5.39 ± 0.12</td>
</tr>
<tr>
<td><strong>MZB</strong></td>
<td>51.43 ± 2.30</td>
<td>41.70 ± 13.71</td>
</tr>
<tr>
<td><strong>Macrophages</strong></td>
<td>43.10 ± 5.48</td>
<td>44.45 ± 6.43</td>
</tr>
<tr>
<td><strong>CD4+</strong></td>
<td>63.93 ± 4.74</td>
<td>60.05 ± 3.04</td>
</tr>
<tr>
<td><strong>CD8+</strong></td>
<td>18.66 ± 2.65</td>
<td>21.40 ± 0.14</td>
</tr>
<tr>
<td><strong>CD25+</strong></td>
<td>8.93 ± 0.84</td>
<td>6.89 ± 0.66</td>
</tr>
<tr>
<td><strong>CD 25+/CD19- (Treg/Ttotal) ratio</strong></td>
<td>0.19</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Taking the data all together, treatment with thymulin 5CH was not able to revert several parameters of the systemic immune response in the spleen cell populations, nor clinical aspects, such as body weight and tumor growth progression over time. However, histopathological assessment of the tumor microenvironment evidenced statistically significant reduction of necrosis and micro-embolism incidence, with increase of caspase-3 labeling associated to reduction in cytokeratin positivity.

**Discussion**

T lymphocytes are responsible for the cell-mediated immune response, which involves cell-to-cell contact. Under these circumstances, helper (CD4), suppressor (CD8), cytotoxic (CD8), natural killer (NKT) and natural killer (NK) cells are activated, together with macrophages and monocytes. The T-cell maturation is controlled by thymic hormones, cytokines and chemokines, including thymulin, thymosin, thymopentin, interleukins, interferons, and also by hormones secreted by other endocrine glands, such as melatonin, neuropeptides and insulin (DARDENNE et al., 2009; REGGIANI et al., 2009; 2014; CSABA 2016).

Thymulin has a broad scope of physiological actions involving endocrine and paracrine mechanisms, including intra- or extra-thymic T-cell maturation. Thymulin modulates the activity of NK, polymorphonuclear cells and monocytes, as well as the production of interleukins (IL) such as IL-1 and IL-6, tumor necrosis factor (TNF) and prostaglandin E2 (PGE2) (BACH 1979; LIN, 1989; REGGIANI et al., 2014; CSABA 2016). Increase of the serum thymulin levels shifts the immune response to the Th1 pattern at the expense of the Th2 response. In turn, lack of zinc induces shift to the humoral response, because this metal is an important cofactor of thymulin. Secretion of thymulin is controlled by immune and extra-immune factors, including thyroid and growth hormones, prolactin, melatonin and zinc, among others (REGGIANI et al., 2014; CSABA 2016).
T-cell balance recovery has been described in cancer patients treated with thymulin or thymic extract since the 1970s without any mutagenic or toxic effect (TUROWSKI et al., 1976; CAVDAR et al., 1978; BENA, MORDOH, 1980). The first studies on the use of very low doses of thymulin as immunomodulator factor in the literature date from the 1980s (BASTIDE et al., 1985; 1987; DAURAT et al., 1988). More recently, the immunomodulatory effects of high dilutions of thymulin were described in viral, bacterial and protozoan experimental infections in vivo (SATO et al., 2012; BONAMIN et al., 2013; RODRIGUES de SANTANA et al., 2014; SIQUEIRA et al., 2016).

RODRIGUES de SANTANA, et al., 2014, studied the effect of thymulin 5CH on the modulation of the inflammatory response in murine cutaneous leishmaniasis. Following a transient increase of the peritoneal regulatory T cells, the percentage of peritoneal B1 cells in the spleen and peritoneum of the treated mice increased, leading to extensive macrophage infiltration in the primary site of infection and reduction of the parasite population. The interaction between macrophages and B1 cells in chronic inflammation is already described (RUSSO; MARIANO, 2010), as well as the modulation of this interaction by thymulin 5CH (BONAMIN et al., 2013). Besides the modulation of protozoan and bacterial infections, thymulin 5CH also increased B2 lymphocyte population in the spleen of mice inoculated with soluble viral antigens (SIQUEIRA et al., 2016) and improved the quality of life of broiler chickens with viral arthritis (SATO et al., 2012).

In the present study, treatment of mice bearing Ehrlich solid tumor with thymulin 5CH induced some mild changes in the tumor microenvironment, including reduction of cytokeratin expression and incidence of microembolism. Moreover, it increased Caspase-3 labeling by tumor cells. These findings denote a trend for pro-apoptotic activity and reduced tumor spreading. Some reduction of the incidence of necrotic areas within the tumor was also found in the thymulin 5CH treated mice. Within the context of the Ehrlich tumor dynamics, it is known that the expression of caspase related genes is also involved in the mechanisms of necrosis (REUNOV et al., 2018). Also, previous studies described significant changes in Ehrlich tumor microenvironment following treatment of mice with thymulin 5CH combined with cyclophosphamid. In this case, treatment induced bone metaplasia at the tumor site together with the reduction of tumor mass (TOLEDO, 2005).

Changes in the systemic immune response do not seem to be involved in this case, since there are not significant differences in spleen cell populations between the treated and control groups. Probably, higher doses of thymulin are necessary to change such parameters. Nevertheless, direct
effects of high-diluted thymulin on the tumor microenvironment and on the tumor cells themselves should be also considered, since many studies reported pro-apoptotic effects of high diluted/homeopathic prepared substances on tumor cells \textit{in vitro} (SAHA \textit{et al}., 2013; SAHA \textit{et al}., 2015; ARORA; TANDON, 2015). Most such effects were related to epigenetic changes, as well as in the expression of critical genes (MONDAL \textit{et al}., 2016; SEKER \textit{et al}., 2018). Thus, further specific studies about tumor microenvironment changes and local tumor – immune cells interaction are needed to confirm this hypothesis. Unfortunately, \textit{in vivo} models are required to study tumor evolution in a host microenvironment context. Herein, the use of standard and controlled environmental conditions, the daily observation of animal welfare and the reduced number of mice per group to a satisfactory limit to perform a trustful statistical analysis were the methodological refinement used, to be in accordance with the 3Rs rules for an ethical use of experimental animals.

The results of the present \textit{in vivo} study represent a contribution to the knowledge about the action of homeopathic medicines in tumor biology, considering the microenvironment in which it is inserted, since different histopathological features were described, which would not be possible with cell monocultures \textit{in vitro}, as most shown by most of the papers found in the literature about this topic. High-diluted homeopathically prepared medicines are already used in the oncological practice in some countries, with interesting results (GHOSH \textit{et al}., 2014; FRASS \textit{et al}., 2015; GLEISS \textit{et al}., 2016; ROSSI \textit{et al}., 2018). Since homeopathic medicines have a universal applicability, in which different species can be treated with (MATHIE \textit{et al}., 2012; SATO \textit{et al}., 2012; BONAMIN; BELLAVITE, 2015), these experimental results may have a large translational character, in order to give cellular-level explanations to the observed effects. However, the understanding of how the application of these medicines in oncological clinical practice could be optimized, even if used together with conventional therapies, is still a matter of discussion.

\textbf{Conclusion}

Treatment of mice bearing solid Ehrlich tumor with thymulin 5CH induced some mild but specific changes in the tumor environment, such as reduced incidence of microembolism and cytokeratin expression with increased Caspase-3 labeling. However, treatment with thymulin alone did not seem to influence systemic immunity parameters or the tumor clinical progression, as expected according to previous results obtained in other experimental models. Thus, the use of thymulin 5CH a complementary resource in oncological clinical practice is still a matter of discussion.
Conflict of interest

The authors declare there is no conflict of interest.

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