Author's response to reviews

Title: Immunological response in mice bearing LM3 breast tumor undergoing Pulchellin treatment

Authors:

Djamile C de Matos (djamatos@terra.com.br)
Lívia C.A. Ribeiro (liviacarol@gmail.com)
Aline Tansini (alinetansini@yahoo.com.br)
Lucas S. Ferreira (gigabreath@hotmail.com)
Marisa C.P. Placeres (marisapolesi@yahoo.com.br)
Lucas L. Colombo (lucascol2003@yahoo.com.ar)
Iracilda Z. Carlos (carlosiz@fifar.unesp.br)

Version: 2 Date: 30 May 2012

Author's response to reviews: see over
BMC Complementary and Alternative Medicine

May 29, 2012

Dear Editor,

We are returning the manuscript originally entitled “Immunological response in mice bearing pulchellin-treated breast tumor” modified, when possible, according to the referees’ suggestions. One of the referees suggested that the manuscript’s title should be adjusted, therefore this revised version is now entitled “Immunological response in mice bearing pulchellin-treated breast tumor”.

All the incorporated revisions are highlighted in the manuscript and explained here.

With kind regards,

Prof. Iracilda Zeppone Carlos
Laboratory of Clinical Immunology
School of Pharmaceutical Sciences
Araraquara, SP
Brazil.

Suggestions made by referee #1:

Title:
The referee suggested that the title needed to be adjusted.
- Authors' answer: The previous title was "Immunological response in mice bearing pulchellin-treated breast tumor", which was changed to "Immunological response in mice bearing LM3 breast tumor undergoing pulchellin treatment".

Introduction:
The referee suggested that on “lines 3 and 4... the authors wrote ‘Cancer immunotherapy aims to stimulate the immune system to destroy tumors by enhancing the production of cytokines and immune mediators...’ However, this is one strategy and thus it should be mentioned in the statement above.”
- Authors' answer: In order to address this, we changed the text from “...to destroy tumors by...” to “...to destroy tumors, i.e. by...”. 
Methods:
The referee asked for the average body weights of mice.
- Authors’ answer: We then added the following section to the manuscript: “2.3 Animals: Fifteen female BALB/c mice (2-3-month-old) weighing around 20g were taken from the animal facility of UNICAMP, São Paulo, Brazil. Sterilized water and food were administered *ad libitum*. All animal procedures were performed in accordance with the regulations of the Research Ethics Committee (# 28/2008), Faculty of Pharmaceutical Sciences, UNESP, São Paulo, Brazil.”.

The referee asked "why the mice were sacrificed after 7 days post pulchellin injection?"
- Authors' answer: The reason for this is that the time-frame for animal sacrifice after pulchellin treatment was determined based on previous research like Xue, H., Field, C.J., Sawyer, M.B., Dieleman, L.A., Baracos, V.E., 2009: Prophylatic ciprofloxacin treatment prevented high mortality, and modified systemic and intestinal immune function in tumour-bearing rats receiving dose-intensive CPT-11 chemotherap. Br J Cancer, 100 (10): 1581-8.”.

Results
The referee suggested that "the type of cells with their adhesion molecules that pulchellin enhanced should be mentioned, not just ‘adhesion and costimulatory molecules expression on macrophages...’.”
- Authors' answer: We addressed this by adding the following text on page 6, line 3: “Pulchellin treatment (group P) promoted an enhancement of adhesion (CD54) and costimulatory (CD80) molecules expression in peritoneal macrophages (p<0.01)...”.

The referee noted that “since a non-significant reduction in the number of CD8 cells and dendritic cells, p value should be >0.5 and not <0.5!”.
- Authors' answer: This mistake was corrected and the corresponding text on page 6, line 5 was changed from "... dendritic cells, p<0.05..." to "... dendritic cells, p>0.05 ..."
The referee noted that "Figure 3 needs to include IL-10 and IFN-γ instead of TNF-α".
- Authors' answer: This mistake was corrected and Figure 3 now shows the correct graphs for IL-10 and IFN-γ.

**Discussion**

The referee stated that "since the authors did not work on IL-12, it would be difficult to say that IFN-γ-induced pulchellin activates macrophages and it is not IL-12-IFN-γ activation pathway i.e. it could that pulchellin activates macrophages and the activated macrophages activates Th1 cells. So both possibilities could take place".
- Authors' answer: The reason we did not consider that both possibilities could take place is that, in fact, we determined IL-12 production by macrophages from tumor-bearing mice treated with pulchellin, but we found no detectable levels of this cytokine. In order to clarify the text in the revised manuscript, we reformulated the entire paragraph, as follow: "In this study, we evaluated the production of some immune mediators and the expression of surface markers in different immune cells obtained from tumor-bearing mice treated or not with pulchellin. We observed greater production of IFN-γ by spleen cells from group P than in those from group T. Since we couldn't detect IL-12 production by PECs from group P (data not shown), it can be suggested that the increased IFN-γ production found in this group is most likely the cause of the enhanced expression of adhesion (CD54) and costimulatory (CD80) molecules and the production of TNF-α and NO by macrophages [11]. In some cases, production of IFN-γ can be triggered in the absence of IL-12 stimulation, when there is enough antigen levels to keep a sustained TCR activation with subsequent fosforilation of the mitogen-activated Erk kinase protein, resulting in IFN-γ-producing Th1 cells [12]."

The referee suggested that “since also pulchellin induces proinflammatory (IFN-γ, TNF-α) and reduce some anti-inflammatory cytokines (TGF-β, IL-6) but not IL-10 or IL-4, it is worth noting inflammation role in cancer development and cancer suppression in the discussion. Also, it is preferable to mention the reduction of TGF-β and IL-6 on Treg and Th17 development in cancer-bearing animals.”
- Authors' answer: We agreed with this suggestion and therefore added the following text on page 7, lines 9 to 13: “besides that, IL-6 is known to promote, together with TGF-β, naive T cells differentiation into Th17 cells [16], which are found in greater quantities in mice as breast cancer progresses, reaching their highest levels at the later stages of the disease [17].”

**Figures**

Regarding table 1, the referee suggested that "the authors should differentiate between the source of cells (i.e. peritoneal vs. spleen)".

- Authors' answer: We accordingly modified the table title to “Percentage of macrophages (peritoneum), dendritic cells and lymphocytes (both from spleen)...”.

As suggested by the referee, the English language was revised and proof read.

**Suggestions made by referee #2:**

This referee did not make any suggestions.

**Suggestions made by referee #3:**

The referee commented that “this study aimed to study the potential effect of pulchellin on treating tumor, however, it did not have data regarding the change of tumor in mice by treating with pulchellin.”

- Authors' answer: In this regard, pulchellin-treatment did not reduce significantly the tumor size. Thus, we proposed pulchellin as a coadjuvant treatment to improve immune response to tumor, but it should be used together with other chemotherapy in order to increase its effectiveness.”

The referee stated that “the present manuscript showed only some preliminary observations which were difficult to reach conclusion.”

- Authors' answer: We think that this study will be important to the flourishment of further research aiming to investigate pulchellin’s functions within the immune system.
The referee also stated that “It is better that these data should also be studied in normal mice with pulchellin treatment.”
- Authors' answer: The reason we did not treat healthy mice with pulchellin is that we choose the intratumoral administration route as a way to limit pulchellin’s toxic properties to the tumor microenvironment. So, if we tried to treat healthy mice with pulchellin, we would have to necessarily use another administration route, which would then result in the obtainment of data not directly comparable with that of pulchellin-treated tumor-bearing mice.

The referee commented that "authors should pay more attention on preparing their figures. In Figure 3, data for IL-10 and IFN-γ were not found. Instead, same figure from Figure 2 was shown in Figure 3 twice?"
- Authors' answer: This mistake was corrected and Figure 3 now shows the correct graphs for IL-10 and IFN-γ.

The referee also stated that the quality of written English was “not suitable for publication unless extensively edited.”
- Authors' answer: We sought to address this concern by extensively reviewing and editing the entire manuscript.