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## **Immunomodulatory Effects of Swainsonine from *Ipomoea carnea* in Healthy Mice**

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**Abstract:** The objective of this study was to more clearly characterize the immunomodulatory effects of swainsonine and an *Ipomoea carnea* aqueous fraction using two different mouse strains: Swiss outbred mice and C57BL/6 inbred mice. The swainsonine is the main toxic principle found in the *Ipomoea carnea* a poisonous plant native from Brazil and other tropical countries. Many studies have shown that swainsonine promotes biological response modifications in different cell lines, such as increased murine splenic NK lymphocyte activity, improvement of peritoneal macrophage activity and macrophage cytotoxicity against tumor cells. In addition, it is suggested that swainsonine stimulates bone marrow cell proliferation in inbred mice. Therefore, we evaluated in this study the immunomodulatory effects of swainsonine and *I. carnea* aqueous fraction using for this analyses of macrophages activities and histology evaluation of lymphoid organ. Thereby, analyses of peritoneal macrophage activities showed decreased phagocytosis of aqueous fraction-treated Swiss mice and enhancement of both the spreading activity and PMA-induced H<sub>2</sub>O<sub>2</sub> production of swainsonine-treated Swiss mice; however, no alterations in these parameters were observed in C57BL/6 mice. In addition, swainsonine and aqueous fraction treatment showed no differences for both Swiss and C57BL/6 mice in the thymus, spleen and bone marrow evaluations and histological analyses of liver and kidney. In conclusion, a clear difference in swainsonine immunostimulant effect was observed when considering mouse strain, while the use of swainsonine alone did not induce bone marrow cellularity in healthy mice.

**Key words:** *Ipomoea carnea*, swainsonine, immunomodulation, bone marrow, macrophage

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### **INTRODUCTION**

The use of herbal extracts and preparations for health and well-being has extended for millennia and still comprise the principal source of primary health care for much of the rural population worldwide (Fowler, 2006). Despite the wide-scale manufacture of synthetic chemistry of new molecules for drug production, the plant kingdom still is the major source of chemical scaffold for the development of a wide range of high-value therapeutic agents. Among these plants, several have received attention regarding their main active principle

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because of their potential immunomodulatory properties, including the indolizidine alkaloid, swainsonine (SW). Plants containing SW are distributed throughout the world, such as *Swainsona canescens* in Australia (Jolly, 1997), certain species known as locoweed, belonging to the genera *Astragalus* and *Oxytropis*, are found in North America (Ralphs *et al.*, 2008) and *Ipomoea carnea* is found in Brazil and other tropical countries (Agra *et al.*, 2007).

The SW has a dual biological effect, since it inhibits two distinct intracellular enzymes, one is lysosomal  $\alpha$ -mannosidase, which, depending on the species and dose administered, can cause toxic accumulation of incompletely processed oligosaccharides into lysosomes, resulting in loss of cellular function and, ultimately, in cell death (Tulsiani *et al.*, 1988). Histologically, cellular vacuolation is observed in cells of different tissues, such as thyroid, liver, pancreas, kidney, CNS and other cells, like alveolar macrophages and Kupffer cells (Stegelmeier *et al.*, 1995; De Balogh *et al.*, 1999). The second enzyme inhibited by swainsonine is Golgi  $\alpha$ -mannosidase II, which is involved in N-linked glycoprotein processing (Elbein, 1989) resulting in the production of hybrid types of oligosaccharides. This alteration in glycoprotein synthesis can cause impairment and dysfunction of cell adhesion molecules and various membrane receptors (Stegelmeier *et al.*, 1998) and promote biological response modifications in different cell lines, such as increased murine splenic NK lymphocyte activity (Humphries *et al.*, 1988), enhanced human large granular lymphocyte cytotoxicity against NK-resistant colon carcinoma cells (Yagita and Saksela, 1990) and improvement of peritoneal macrophage activity (Das *et al.*, 1995; Hueza *et al.*, 2003) and macrophage cytotoxicity against tumor cells in different mouse strains (Grzegorzewski *et al.*, 1989).

It is also been suggested that SW stimulates bone marrow cell proliferation in inbred mice and Oredipe *et al.* (2003) attributed these effects to alterations in carbohydrate composition of cell surface glycoprotein, leading to mitogenic events mediated through the release and action of cytokines and growth factors. However, all of these studies evaluating immunomodulatory processes and bone marrow cell proliferation were conducted on inbred mice. Thus, the objective of this study was to more clearly characterize the immunomodulatory effects of SW and an *I. carnea* Aqueous Fraction (AF) using two different mouse strains: Swiss outbred mice and C57BL/6 inbred mice.

## MATERIALS AND METHODS

### Animals and Experimental Design

Male Swiss (outbred) and C57BL/6 (inbred) mice, aged 60 days-old, bred in the Department of Pathology, School of Veterinary Medicine and Animal Sciences, were used. The mice were maintained under controlled conditions of temperature (22-25°C), humidity (50-65%) and lighting (12/12 light/dark cycle). Drinking water and standard laboratory food pellets (Nuvilab-CR1®, Nuvital Nutrientes LTDA) were provided ad libitum. All procedures using animals were performed following Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, text available at <http://www.nap.edu/readingroom/books/labrats/>) and were reviewed and approved by the Bioethics Committee of the FMVZ-USP.

### *Ipomoea carnea* and Swainsonine

*Ipomoea carnea* leaves were collected from the Research Centre for Veterinary Toxicology (CEPTOX), Department of Pathology, University of Sao Paulo (USP), Pirassununga, Brazil, in May 2002. A voucher herbarium specimen was deposited in the Botanical Institute of São Paulo (SP), Brazil, under number SP-360911. Taxonomic

identification was performed by Dr. Rosângela Simão Bianchini, Botanical Institute of São Paulo. An aqueous fraction (AF) from *I. carnea* dry leaves was obtained as previously described by Hueza *et al.* (2003) and submitted to SW quantification by the method described by Haraguchi *et al.* (2003), resulting in 0.013% for *I. carnea* dry leaves. In addition, SW was extracted and isolated from *I. carnea* leaves as previously described by Haraguchi *et al.* (2003) and the purity level obtained was 98%.

### **Chemicals and Reagents**

Brewer thioglycollate, Phorbol myristate acetate (PMA), RPMI-1640 medium and Zymosan-A were purchased from Sigma Chemical Co (Saint Louis, Missouri, USA). Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was obtained from Merck and Co (Whitehouse Station, New Jersey, USA). *Bacillus Calmette Guerin* (BCG) was produced by the Butantan Institute, Sao Paulo, Brazil.

### **Aqueous Fraction Administration**

Both experimental mice strains, Swiss and C57BL/6, were treated by gavage for 14 days using AF at 1 and 3 g k<sup>-1</sup> b.wt. of *I. carnea* dry leaves (0.13 and 0.39 mg of swainsonine, respectively) using the same protocol proposed by Hueza *et al.* (2003). Control (Co) mice received only water and were treated at the same time as the experimental mice. The volume used for the gavage exposures ranged from 0.3 to 0.4 mL according mouse weight. The body weights of the mice were measured every three days for dose adjustment throughout the experimental period.

### **Swainsonine Administration**

Experimental C57BL/6 and Swiss mice were treated twice-daily by intraperitoneal (ip) via with 200 µL of SW solution (100 µg mL<sup>-1</sup>) for 8 consecutive days using the same protocol proposed in the Oredipe *et al.* (2003) studies. The daily injections of SW were spaced 8 h apart. The control mice were treated with i.p. injections of plain vehicle, Ca<sup>2+</sup> and Mg<sup>2+</sup> free Phosphate-Buffered Saline (PBS) and were treated at the same time as the experimental mice.

### **Evaluation of Spreading and Phagocytosis of Peritoneal Macrophages**

A modification of the method described by Rabinovitch and De Stefano (1973) was used to study macrophage spreading and phagocytosis. Briefly, a total of 200 cells per slide were counted for each mouse and the macrophage Spreading Index (SI) and Phagocytosis Index (PI) were calculated as follows: SI or PI = Number of spreading macrophages or phagocytic activity x 100 ÷ 200 adherent cells counted, i.e., SI = Percentage of spreading macrophages and PI = Percentage of macrophages with phagocytized zymosan particles. The mean of four counts obtained from two slides for each mouse was used to express the SI or PI. In order to promote the activation of peritoneal macrophages, seven days before the end of the AF treatment period, Swiss mice were treated by i.p. via with (8 mg kg<sup>-1</sup>) of BCG suspension. To elicit peritoneal macrophages, five days before the end of SW treatment period both Swiss and C57BL/6 mice were treated by i.p. via with 3 mL of 3% Brewer thioglycollate.

### **Hydrogen Peroxide Release**

Spontaneous and phorbol myristate-acetate solution (PMA)-induced H<sub>2</sub>O<sub>2</sub> release by macrophages were measured by the method described by Russo *et al.* (1989). H<sub>2</sub>O<sub>2</sub> concentration was calculated using absorbance measurements. Spontaneous and PMA-induced H<sub>2</sub>O<sub>2</sub> production experiments were repeated four times in each mouse in each group and the mean value of the four counts was used to determine H<sub>2</sub>O<sub>2</sub> concentration.

### Thymus, Spleen and Bone Marrow Evaluation

Lymphoid organ evaluation was performed with the same protocol proposed by Descotes (2006). Mouse thymus and spleen were harvested and weighed. A single cell suspension was prepared by crushing the spleen between two pieces of ground glass. Bone marrow cell suspensions were obtained by flushing the femur cavity with ice-cold RPMI-1640 medium using a sterile syringe with a 26-gauge needle. Cell viability and cell number were determined by the trypan blue assay.

### Histological Evaluation

Histological analyses were performed in sections (5  $\mu\text{m}$ ) of liver and kidney stained with hematoxylin and eosin (HE).

### Statistical Analysis

Data were analyzed using GraphPad Prism 5.00® software (GraphPad Software, Inc., San Diego, CA). The data from three or more groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons and data from two groups were compared by the Unpaired t-test. Percentage data from three or more groups were compared by the Kruskal-Wallis test followed by Dunn's test and percentage data from two groups were analyzed by the Mann-Whitney test. All data were expressed as Mean $\pm$ SD and differences were considered to be statistically significant at  $p < 0.05$ .

## RESULTS

### Spreading, Phagocytosis and Hydrogen Peroxide Release by Peritoneal Macrophages

The peritoneal macrophages of Swiss mice treated with the lowest dose of AF showed decreased phagocytosis and no difference in the other parameter, as shown in Table 1.

In contrast, when these mice were treated with SW alone, phagocytosis remained unaltered; however, enhancement of both the spreading activity and PMA-induced  $\text{H}_2\text{O}_2$  production of peritoneal macrophages was observed later, as shown in Table 2. Although,

Table 1: Spreading, phagocytosis, spontaneous and induced  $\text{H}_2\text{O}_2$  release by peritoneal macrophages from Swiss mice treated by gavage for 14 days with *Ipomoea carnea* aqueous fraction (1 and 3 g/kg/day) and single-dose by i.p. via with BCG (8 mg  $\text{kg}^{-1}$ ) seven days before the end of treatment period

Macrophage's activities	Groups		
	Co	1	3
Spreading (%)	42.68 $\pm$ 13.4	37.28 $\pm$ 9.34	33.08 $\pm$ 7.23
Phagocytosis (%)	46.37 $\pm$ 7.53	34.42 $\pm$ 7.29*	46.33 $\pm$ 11.35
Spontaneous $\text{H}_2\text{O}_2$ (nMols)	2.41 $\pm$ 1.37	1.88 $\pm$ 1.46	2.08 $\pm$ 1.51
Induced $\text{H}_2\text{O}_2$ (nMols)	4.10 $\pm$ 0.88	3.99 $\pm$ 1.24	4.34 $\pm$ 1.37

The data are expressed as Mean $\pm$ SD. \* $p < 0.05$  Kruskal-wallis test followed by Dunn's test. Co (n = 8), 1 (n = 7) and 3 (n = 6)

Table 2: Spreading, phagocytosis and induced  $\text{H}_2\text{O}_2$  release by peritoneal macrophages from Swiss (SSw) and C57BL/6 (CSw) mice treated twice-daily by i.p. via with 200  $\mu\text{L}$  of swainsonine solution (100  $\mu\text{g mL}^{-1}$ ) for 8 days and single-dose by i.p. via with thioglycollate 3% (3 mL/mouse) five days before the end of treatment period

Macrophage's activities	Swiss <sup>1</sup>		C57BL/6 <sup>2</sup>	
	SCo	SSw	CCo	CSw
Spreading (%)	47.94 $\pm$ 12.07	61.55 $\pm$ 9.19*	34.25 $\pm$ 16.02	38.40 $\pm$ 8.92
Phagocytosis (%)	76.72 $\pm$ 7.36	79.22 $\pm$ 10.39	68.75 $\pm$ 9.64	72.20 $\pm$ 18.06
Induced $\text{H}_2\text{O}_2$ (nMols)	0.12 $\pm$ 0.35	0.46 $\pm$ 0.26 <sup>c</sup>	3.15 $\pm$ 1.08	2.43 $\pm$ 1.03

The data are expressed as Mean $\pm$ SD. \* $p < 0.05$  Mann-whitney test. <sup>c</sup> $p < 0.05$  Unpaired t-test. <sup>1</sup> SCo (n = 9), SSw (n = 9). <sup>2</sup> CCo (n = 8) and CSw (n = 10)

Table 3: Bone marrow, spleen and thymus evaluation from Swiss mice treated by gavage for 14 days with aqueous fraction of *Ipomoea carnea* (1 and 3 g/kg/day)

Parameters	Groups <sup>1</sup>		
	Co	1	3
Bone marrow cellularity x10 <sup>7</sup>	1.490±0.24	1.750±0.38	1.700±0.24
Spleen cellularity x10 <sup>7</sup>	11.420±2.90	12.510±4.58	12.050±3.60
Relative spleen weight (g/100 g b.wt.)	0.280±0.11	0.300±0.14	0.270±0.05
Relative thymus weight (g/100 g b.wt.)	0.071±0.013	0.069±0.020	0.054±0.007

The data are expressed as Mean±SD. <sup>1</sup> Eight mice per group (n = 8)

Table 4: Bone marrow, spleen and thymus evaluation from Swiss (SSw) and C57BL/6 (CSw) mice treated twice-daily by i.p. via with 200 µL of swainsonine solution (1.00 µg mL<sup>-1</sup>)

Parameters	Swiss <sup>1</sup>		C57BL/6 <sup>2</sup>	
	SCo	SSw	CCo	CSw
Bone marrow cellularity x10 <sup>7</sup>	2.71±0.17	2.83±0.30	2.19±0.39	2.47±0.65
Spleen cellularity x10 <sup>7</sup>	15.34±2.19	15.78±3.94	13.41±3.11	13.57±1.42
Relative spleen weight (g/100 g b.wt.)	0.31±0.04	0.35±0.09	0.32±0.06	0.35±0.03
Relative thymus weight (g/100 g b.wt.)	0.08±0.01	0.08±0.01	0.15±0.03	0.18±0.02*

The data are expressed as Mean±SD. \*p<0.05 Mann-whitney test. <sup>1</sup>SCo (n = 10), SSw (n = 10). <sup>2</sup>CCo (n = 9) and CSw (n = 10)

these results were observed in peritoneal macrophages of Swiss mice, no differences were observed in activity of these cells in C57BL/6 mice (Table 2).

### Thymus, Spleen and Bone Marrow Evaluation

The AF and SW treatments in Swiss mice did not stimulate bone marrow cell proliferation and did not alter spleen cellularity or the relative weight of spleen and thymus, as shown in Table 3 and 4.

Similarly, this treatment in C57BL/6 mice did not enhance bone marrow or spleen cellularity. However, increased relative thymus weight was observed (Table 4).

### Histological Evaluation

Histological evaluations of mouse liver and kidney submitted to AF treatment showed no differences from the control group (data not shown).

## DISCUSSION

There are several in vitro investigations showing that SW stimulates the immune system in different effector cells, such as murine NK cells (Humphries *et al.*, 1988), lymphokine-activated killer (LAK) cells (Bowlin *et al.*, 1989) and peritoneal macrophages (Das *et al.*, 1995). In addition, a study performed in rats subjected to AF of *I. carnea* revealed that rat macrophages showed enhanced phagocytosis and H<sub>2</sub>O<sub>2</sub> production (Hueza *et al.*, 2003). In contrast, in the present study, while SW treatment in Swiss mice presented a stimulatory effect on macrophage spreading and PMA-induced H<sub>2</sub>O<sub>2</sub> production by peritoneal cells, no alterations were detected in the cells of C57BL/6 mice, revealing differences in the response to macrophage activity between these breeds; however, at present, it is not possible to hypothesize why this distinction between isogenic and nonisogenic mice occurs.

Interestingly, contrary to expectation, when Swiss mice were treated with lower dose of AF of *I. carnea*, a decrease in macrophage phagocytosis was verified. Since, SW inhibits lysosomal α-mannosidase, this suggested that the impairment of this enzyme may have led to vacuolization of different tissue cells, including those of the myeloid system (macrophages); however, in the present study, histopathological exams revealed no

vacuolization in kidney and liver cells, which are known to be highly sensitive to SW effects on  $\alpha$ -mannosidase inhibition (Novikoff *et al.*, 1985) thus, this hypothesis was ruled out.

On the other hand, inhibition of Golgi mannosidase II can cause impairment of various membrane receptors (Stegelmeier *et al.*, 1998), such as macrophage endocytosis receptors (Arunugham and Tanzer, 1983). In this manner, the reduction in macrophage phagocytosis verified here, with lower dose of AF of *I. carnea*, can be a consequence of alteration in macrophage endocytosis receptors. Nevertheless, the normal response observed in the present study with highest dose of AF of *I. carnea* can be an adaptive response (hormesis effect) (Calabrese, 2008) due to stimulation of an alternative mannosidase (Moremen, 2002). The stimulation of an alternative enzyme happens in rats with lysosomal  $\alpha$ -D-mannosidase that is not inhibited by swainsonine (Tulsiani and Touster, 1983).

In addition, considering that *Ipomoea carnea* contains not only SW, but also nortropane alkaloids, calystegines B<sub>2</sub> and C<sub>1</sub> (De Balogh *et al.*, 1999), that both calystegines are potent inhibitors of  $\beta$ -glucosidase and that calystegine B<sub>2</sub> is also an inhibitor of  $\alpha$ -galactosidase enzymes (Asano *et al.*, 1995), it is feasible to suggest that the lack of stimulatory effects found in mice treated with this plant may be linked to the effects of nortropane alkaloids.

Oredipe *et al.* (1991) showed unequivocally that SW protects bone marrow cells and the hematopoietic system against the toxic effects of chemotherapeutic agents and radiation. In other study, the authors demonstrated that SW promoted an increase in bone marrow cellularity in different lineages of inbred mice: C57BL/6; C3H-HeN; BALB/c and DBA-2 (Oredipe *et al.*, 2003). In the present study, using the same protocol proposed in the Oredipe *et al.* (2003) study no differences between inbred and outbred mice strains were found in relation to bone marrow cellularity.

In a similar study, Klein *et al.* (1999) verified that SW did not stimulate the number of murine bone marrow cells of isogenic mice, C57BL/6 and B6C3F1, when administered alone, which corroborate with the present study; however, when SW was administered in mice treated with doses of 3'-azido-3'-deoxythymidine (AZT) that typically leads to severe myelosuppression, the authors verified increased total bone marrow cellularity.

It is known that the chemotherapeutic agents methotrexate, 5-fluorouracil, cyclophosphamide and doxorubicin used to evaluate the protective effect of SW on bone marrow cellularity (Oredipe *et al.*, 1991) promote myelosuppression by inhibiting DNA synthesis, preventing the DNA replication process or by DNA adduction (Lind, 2008). The depletion of cells from the bone marrow or blood stream induces different cell lineages to release cytokines and growth factors in order to repopulate these organs. In relation to AZT, it has been suggested that this drug causes myelosuppression due to down regulation of various cytokine receptors (Chitnis *et al.*, 2002).

Considering that, SW an inhibitor of mannosidase II involved in N-linked glycoprotein processing, modifies the expression of cell surface receptors and it is correlated with increased activity in different immune cell lineages (Galustian *et al.*, 1994), in which an increase in IL-2 receptor expression was observed, it is possible to hypothesized that SW alone could not promote enhanced bone marrow cellularity. Nonetheless, in the presence of chemical or physical injury which leads to diminished bone marrow cellularity and, consequently, the release of growth factors, SW acts synergically to recolonize this organ. Possibly this is the reason why SW acts only when an injury produced by these drugs occurs in the bone marrow and not when administered in healthy animals. Further studies in order to confirm this hypothesis by which SW stimulates hematopoietic cells are clearly required.

In conclusion, the data presented here, combined with the studies using SW to protect bone marrow suppression during cytotoxic chemotherapy, suggest that this indolizidine

alkaloid may be a potential adjuvant in treatment protocols that use high-dose chemotherapy treatment in which myelosuppression is a major preoccupation. On the other hand, it seems that the use of SW alone does not induce bone marrow cellularity in healthy mice. A clear difference in SW immunostimulant effect was also observed when considering the strain of mice; thus although studies of SW administration in mice are important in elaborating the potential of SW as an immunostimulant, the extrapolation of such data to clinical application in humans or any other animal species should be carefully evaluated.

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