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Alleviation of cyclophosphamide-induced immunosuppression in Wistar rats by onion lectin (*Allium cepa* agglutinin)

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Cyclophosphamide (PubChem CID: 2907)
N-(1-naphthyl)-ethylenediamine dihydrochloride (PubChem CID: 15106)
Nitric oxide (PubChem CID: 145068)
Sulfanilamide (PubChem CID: 5333)
Zinc sulfate (PubChem CID: 24424)

ABSTRACT

Ethnopharmacological relevance: In various traditional medicines, onion has been classified as an immune-boosting food. Recent studies have claimed this property due to the presence of bioactive organosulfur compounds, prebiotic fructo-oligosaccharides and an immunomodulatory protein, lectin (*Allium cepa* agglutinin; ACA) (Prasanna and Venkatesh, 2015. Characterization of onion lectin (*Allium cepa* agglutinin) as an immunomodulatory protein inducing Th1-type immune response *in vitro*. Int. Immunopharmacol. vol. 26, pp. 304–313).
Aim of the study: The aim of this study was to evaluate the immunoprotective properties of ACA in normal and cyclophosphamide (CP; 100 μg/kg)-induced immunosuppressed Wistar rats.
Materials and methods: Wistar rats were administrated different doses of ACA (1, 10, and 100 μg) to respective groups in normal as well as immunosuppressed animals. The effect of ACA on the status of immune organs was assessed by examining the splenic and thymic indices, and histopathological changes. The biomarkers for humoral immunity (serum IgG and IgA levels) and serum pro-inflammatory markers (COX-2, TNF-α and IL-10) were measured by ELISA.
Results: ACA showed immunoprotective properties by significantly promoting the restoration of lymphoid cell count by ~6 fold vs. model control (immunosuppressed animals) and promotes the immune response significantly (~1.5-fold) in CP-induced immunosuppressed animals compared to model control; production of pro-inflammatory molecules (COX-2 and nitric oxide) and expression levels of immune regulatory molecule (TNF-α) were elevated in a dose-dependent manner.
Conclusions: The observed *in vivo* results suggest that ACA has the potential to be used as a nutritional therapeutic to boost the immune status of immunosuppressed subjects brought about by CP administration.

1. Introduction

Various pathophysiological and severe clinical conditions, including extensive chemotherapy and radiation therapy for cancer and long-term immunosuppressive therapy to organ transplant subjects are reported to be associated with unwanted, clinically significant direct immunosuppression. Several cytotoxic drugs are being used as chemotherapy treatment to cancer subjects as well as for long-term immunosuppressive therapy, viz., azathioprine, cyclophosphamide (CP), fludarabine monophosphate, methotrexate and mycophenolic acid (Nijkamp and Parnham, 2011). The molecular mechanism of CP inducing immunosuppression has been described by Huang and Li (2013); CP is an effective alkylating agent and is able to bind to the DNA through covalent linkages (alkylation) particularly to the N7 of guanine residue and forms DNA double strand adducts (Huang and Li, 2013). At cellular level, this leads to triggering of apoptosis and induces the pronounced cytotoxic effect on murine lymphocytes. The drug also causes senescence by inducing generation of reactive oxygen radicals. In addition, acrolein, a secondary metabolite of CP has been reported to inhibit the cell proliferation, and also modulates the expression levels of genes and transcription factors, by lowering the activation of nuclear factor-kappa B (NF–κB) and activator protein-1 (AP-1) (Sulkowska et al., 1998).

Myelosuppression which occurs in subjects undergoing chemotherapy for various diseases is a common side-effect observed during immunosuppression (Inoue and Tani, 2014). Impairment of

Abbreviations: ACA, *Allium cepa* agglutinin; ASA, *Allium sativum* agglutinin; COX-2, cyclooxygenase-2; CP, cyclophosphamide; Hb, hemoglobin; LPS, lipopolysaccharide; LYM, lymphocytes; NO, nitric oxide; PBS, phosphate-buffered saline; PECs, peritoneal exudates cells; SRBC, sheep red blood cells; TNF-α, tumor necrosis factor alpha; WBC, white blood cells

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the host defense system leads to various opportunistic diseases/infections (Elad et al., 2010). Immune suppression can be brought back to nearly healthy/normal immune status using various modes of therapy that have come into use in clinical practice, although many have failed to produce impressive results and are not cost-effective. In recent years, research in clinical nutrition has focused more on the role of food and its bioactive components in improving the immunological response and also their role in promoting the protective efficiency of vaccines (Marcos et al., 2003; Chandra, 1997; Słotwiński and Słotwińska, 2012).

Among dietary proteins, lectins have received additional focus in immunological research for gaining insights into the interactions between lymphoid cell receptors and proteins. It has been clearly shown that mucosal epithelium has affinity towards various plant lectins (Souza et al., 2013). Plant-derived mitogenic lectins such as concanavalin A (Con A), phytohemagglutinin (PHA), *Pisum sativum* agglutinin (PSA), *Ulex europaeus* agglutinin (UEA) and ArtinM were found to induce pro-inflammatory cytokines in vitro (Muraille et al., 1999; Souza et al., 2013). The immunomodulatory dietary proteins have to withstand the digestive processes to interact with the gut-associated lymphoid cells. Most of the dietary proteins are digested within the gut lumen by secretory enzymes and absorbed by microvillus of enterocytes' membranes by fluid-phase transcytosis. However, a small amount of resistant intact antigens escape the enzymatic degradation and some are available to interact with the gut-associated lymphoid cells, thus regulating the immune mechanism (Heyman, 2001).

Several Indian spices and condiments have been described as natural healers (Bakhru, 2001). In Ayurveda (traditional Indian system of medicine), 'rasayana' plants are described as specific anti-aging, improving quality-of-life with enhanced intelligence and memory, and hence increases resistance toward various diseases suggesting that such plants possess immunostimulant effect (Tripathi and Singh, 1999; Doshi et al., 2013; Kumar et al., 2012). Both garlic (*Allium sativum*) and onion (*Allium cepa* L. belonging to Amaryllidaceae; known as pyaz in Hindi and eerulli in Kannada) have been considered as 'rasayana' plants.

Various in vivo studies have been conducted to evaluate the mucosal immunity of some plant lectins such as *Lycopersicon esculentum* agglutinin (LEA), wheat germ agglutinin (WGA), PHA and UEA-I lectins by inducing the lectin-specific IgG and IgA responses (Lavelle et al., 2001). Among the dietary lectins, ArtinM and garlic lectins (ASA I and ASA II) are well studied in the field of nutritional immunomodulation (Chandrashekar and Venkatesh, 2009; Clement and Venkatesh, 2010; Clement et al., 2010; Silva et al., 2014; Souza et al., 2013). Onion has been widely described for its various biological activities such as antimicrobial, antiviral, anticancer and immunomodulatory activities (Block, 2010; Corzo-Martínez et al., 2007; Kumar et al., 2015; Mirabeau and Samson, 2012; Upadhyay, 2016). Onion lectin (*Allium cepa* agglutinin; ACA) is a strictly mannose-specific 50 kDa protein devoid of glycans and is a non-covalent tetramer of four 12.5 kDa subunits (Prasanna and Venkatesh, 2015). It has been demonstrated recently that ACA enhances immunostimulation by inducing the proinflammatory molecules in murine lymphocytes and macrophages in vitro (Prasanna and Venkatesh, 2015). It appeared interesting to investigate the immunomodulatory aspects of ACA in vivo using a suitable animal model. For this purpose, an immunosuppressed animal model was used wherein immunosuppression was induced in Wistar rats using CP (Hou et al., 2007). The results of intraperitoneal administration of ACA on the immunological/hematological parameters in immunosuppressed rats are presented in this article.

2. Material and methods

2.1. Materials

All cytokine-specific antibodies (antibodies to rat IL-10, TNF-α and COX-2 raised in mouse) were obtained from BioLegend, San Diego, CA. Mouse anti-rat IgG and IgA antibodies were procured from Santa Cruz Biotechnology, Dallas, TX. The secondary antibody-enzyme conjugate was rabbit anti-mouse IgG-horseradish peroxidase conjugate. TMB/H_2O_2 was a product of GeNei, Bengaluru, India. Cyclophosphamide, skimmed milk powder, sulfanilamide, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, lipopolysaccharide (LPS) from *Escherichia coli* and other chemicals were purchased from HiMedia Laboratories Ltd., Mumbai, India. Endotoxin quantification kit (catalog # N183) was obtained from Lonza, Basel, Switzerland. All other reagents and chemicals used in this study were of analytical grade.

The onion plant (*Allium cepa* L.) has been listed in 'The Plant List' website (http://www.theplantlist.org/tpl1.1/record/kew-295261; The Plant List, 2013). ACA was purified from onion bulbs (cv. Arka Kalyan) as described in detail recently by Prasanna and Venkatesh (2015); this cultivar was developed and maintained at the Indian Institute of Horticultural Research (Division of Vegetable Crops), Hesaraghatta Lake Post, Bengaluru, India. A voucher specimen of this cultivar is available at ICAR – National Bureau of Plant Genetic Resources (NBPGR), Pusa Campus, New Delhi, India [IC (indigenous collection) No. IC393740].

The endotoxin level in purified ACA preparation was quantified by *Limulus* amebocyte lysate (LAL) assay using the endotoxin quantification kit following the manufacturer's protocol. The presence of endotoxin in purified ACA (50 μg/mL solution) could not be detected by the LAL assay; however, since the assay detected limit was 0.125 endotoxin units (EU)/mL, the amount of endotoxin in purified ACA is taken as < 0.125 EU/mL. This corresponds to < 0.05 ng LPS/mL in a 50 μg/mL solution of purified ACA, and hence, is essentially considered as endotoxin-free. Hemagglutination (HA) assay and lymphocyte proliferation assay (by MTT reagent) were performed essentially as described previously (Prasanna and Venkatesh, 2015).

2.2. Animals and grouping

Male CFT Wistar rats (4–5-weeks-old) were obtained from the Central Animal House facility of CSIR–Central Food Technological Research Institute, Mysuru, India following approval from the Institutional Animal Ethics Committee (IAEC). All experimental procedures involving the handling and caring of animals have been carried out in accordance with the ethical guidelines. All animals were housed and maintained on a standard commercial diet at ambient temperature in a clean environment as mentioned in the ethical guidelines. The temperature was maintained at 22 ± 3 °C and relative humidity at 55 ± 10% with a 12 h light/dark cycle.

CFT Wistar rats were divided into eight groups ($n = 6$) for this study. The random distribution of animals was done to ensure the same response from animals in each group receiving the therapy; the average weight of the animals in each group was almost similar. The eight groups of animals and their experimental protocol are shown in Table 1.

All animals were allowed to acclimatize for one week before starting the administration. The administration protocol is shown in detail in Fig. 1. The dosages of ACA used in the present study were determined based on previous studies from our laboratory on the immunogenicity and adjuvanticity of garlic lectins *in vivo* (Clement and Venkatesh, 2010), and of several *in vivo* immunomodulatory studies of plant lectins (Lavelle et al., 2001;