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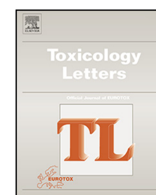

Immunomodulatory potential of Rhein, an anthraquinone moiety of *Cassia occidentalis* seeds

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HIGHLIGHTS

- Rhein (10 μM) suppresses the functional responses of the T- and B-lymphocytes.
- Rhein suppresses Con A and LPS induced lymphoproliferation in splenocytes.
- B-cells and T-cells surface receptors are modulated by Rhein treatment.
- Rhein treatment modulates both B-cell and T-cell regulatory cytokines.

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ABSTRACT

Rhein, the most toxic anthraquinone moiety in *Cassia occidentalis* seeds, has been associated with hepatomyoencephalopathy (HME) in children. Structural and functional alterations in the lymphoid organs have been reported both in HME patients and experimental animals indicating a possibility of the dysfunction of immune system following exposure to CO seeds or its toxic anthraquinones (Panigrahi et al., 2014a). In the present study the mechanism of immune response of Rhein in splenocytes has been investigated by measuring functional assays of lymphocyte, cell surface receptor expression and analysis of cytokine levels. Results indicate that Rhein at a maximum dose of 10 μM is non cytotoxic up to 72 h in splenocytes. In addition to its potential to decrease the allogenic response of T-cells, Rhein significantly suppresses the proliferation of the concavalin A (Con A) and lipopolysaccharide (LPS) stimulated splenocytes. Lymphocyte receptor expression analysis revealed that Rhein exposure significantly down regulate the expression of CD3e, CD4, CD8, CD28, CD69 molecules in T-cells. The expression of CD19, CD28, CD40 in B-cells were also found to be significantly decreased following Rhein exposure. In accordance with the functional responses, Rhein treatment significantly lowered the expression of IL2 and IL6 cytokines in Con A stimulated splenocytes, and IL6, IL10, IFNγ and TNFα in LPS stimulated splenocytes. Over all, the study suggests the immunomodulatory activity of Rhein and that it would be useful in understanding the immune response of CO seeds in human subjects.

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1. Introduction

Accidental poisoning of *Cassia occidentalis* (CO) seeds is known to be the contributing agent for hepatomyoencephalopathy (HME) in several parts of India (Vashishtha et al., 2007a,b; WHO, 2008; Panigrahi et al., 2014a). Our recent studies have identified the toxic anthraquinones (AQs) including Aloe-emodin, Chrysophanol, Emodin, Physcion and Rhein in CO seeds (Panigrahi et al., 2015a). These AQs were detected in the serum of HME patients as well as in the CO seeds exposed experimental rats, connecting their role in the CO seeds toxicity (Panigrahi et al., 2015a). Among the above five AQs, Rhein was found to be the most toxic anthraquinone in CO seeds. Further, the maximum concentration of Rhein in serum, the highest toxicity and the highest protein binding affinity as

Abbreviations: APC, allophycocyanin; APC-Cy7, allophycocyanin-cyanine 7; CD, clusters of differentiation; Con A, concanvalin A; DMEM, Dulbecco's Modified Eagles Medium; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MLR, mixed lymphocyte reaction; PE, phycoerythrin; PerCP, peridinin chlorphyll protein; TNF, tumor necrosis factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffer saline; FBS, fetal bovine serum; HEPES, hydroxyethyl piperazineethanesulfonic acid; EDTA, ethylenediamine tetraacetic acid; DMSO, dimethyl sulfoxide.

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compared to other AQs clearly suggest that Rhein is one of the main etiological agents in CO poisoning (Panigrahi et al., 2015a,c). It has been observed that Rhein, at a dose of 50 μM, causes apoptosis in primary hepatocytes by generating reactive oxygen species, increasing intracellular Ca^{2+}, decreasing the mitochondrial membrane potential and depleting intracellular glutathione content (Panigrahi et al., 2015b). At the molecular level, Rhein-induced DNA damage results in over expression of γ-H2AX protein, thereby causing enhancement of p53 and p21, which leads to intrinsic pathway mediated apoptosis involving bax, bcl2, cytochrome c, caspases 3 and 9 and poly-ADP ribose polymerase (Panigrahi et al., 2015b).

Earlier studies have shown that CO seeds are toxic to hepatocytes, neurons, glial cells and muscle cells (Barbosa-Ferreira et al., 2005; Panigrahi et al., 2014b). Our systemic toxicity study on CO seeds in rats showed alterations of parameters related to immune system (Panigrahi et al., 2014a). In this study, the relative organ weight of the spleen was found to be increased by 31% in 2% CO seeds treated rats. Furthermore, CO seeds treated rats showed 54% decrease in white blood cells. Additionally, CO seeds exposure treatment also reduced the number of red blood cells, which is accompanied by lowering of hemoglobin content and packed cell volume (Panigrahi et al., 2014a). Interestingly, similar alterations of the above parameters were also documented in HME patients (Vashishtha et al., 2007a,b, 2009).

Studies related to immunotoxicological aspect of CO seeds or its toxic anthraquinones are scarce in the literature. Some studies described the preliminary immunotoxicity of CO seeds in chicken and rats, however, mechanism of immunotoxicity has not been established (Mariano-Souza et al., 2010; Hueza et al., 2007). Further, a recent study has indicated a protective role of Rhein in the chronic renal injury (Guan et al., 2015). To understand the mechanism of immune response following CO exposure, it is imperative to study the effect of Rhein on splenocytes, which represents both innate and adaptive immune system of the body.

2. Materials and methods

2.1. Chemicals and reagents

Rhein, Concanvalin A (Con A), lipopolysaccharide (LPS), mitomycin C, N-acetyl cysteine (NAC), non essential amino acids, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium pyruvate, L-glutamine, β-mercaptoethanol were purchased from Sigma Chemical Co (St. Louis, MO). Dulbecco's Modified Eagles Medium (DMEM) was purchased from Invitrogen Co (Carlsbad, CA). Cytometric Bead Assay Kit for $T_H1/T_H2/T_H17$ cytokines and Antibodies (Anti-CD3e-APC-Cy7/Alexa Fluor 488, Anti-CD4-FITC, Anti-CD8-PE, Anti-CD19-Alexafluor 700, Anti-CD25-APC, Anti-CD28-APC, Anti-CD40-APC, Anti-CD86-FITC and Anti-CD95-PE) for immunophenotyping were purchased from BD Biosciences (San Diego, CA). Tritiated thymidine was purchased from Amersham Life science (Uppsala, Sweden). All the other chemicals and solvents used were of the highest purity available from commercial sources.

2.2. Animals

Inbred strains of female Balb/c and Swiss mice (8–10 weeks old, 18-20 grams) were procured from the animal breeding colony of CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Lucknow, India. All animal handling procedures were performed following the rules laid by of Institutional Animal Ethics Committee with prior approval for using the animals (Ref. no. ITRC/IAEC/16/2009). Animals were acclimatized under standard laboratory condition for one week prior to the start of experiment and were housed in polycarbonate cage maintained at 22 ± 2 °C under standard laboratory conditions of light and dark cycles (12–12 h). Animals were kept on normal diet (Provini Animal Nutrition India Pvt Ltd, Bangalore, India) and water ad libitum.

2.3. Mouse splenocyte culture

In the present study, splenocyte preparations were obtained following the method described earlier (Yadav et al., 2012, 2013, 2016). In brief, Balb/c or Swiss mice were sacrificed by cervical dislocation according to the institutional animal ethics guidelines of CSIR-IITR, Lucknow, India. Initially, spleens were dissected out and washed with cold PBS. Cell suspension was prepared by mincing the tissue in incomplete DMEM. Erythrocytes present in the splenocyte preparations were lysed by the lysis buffer (1 mM $NaHCO_3$, 150 mM NH_4Cl, 0.1 mM EDTA, pH 7.4). Subsequently, cells were washed with incomplete media and centrifuged at $300 \times g$. The pellet was then resuspended in DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 25 mM HEPES, 25 mM dextrose, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol. Finally, the cells were maintained at a concentration of 2×10^6 cells/ml and incubated overnight for acclimatization.

2.4. Cell viability assay following Rhein exposure to splenocytes

Cytotoxicity of Rhein in splenocyte was determined by MTT assay following the method described earlier (Mosmann, 1983). In brief, splenocytes were cultured in 96 well plates (U bottom) at a density of 2×10^5 per well and treated with different concentrations of Rhein (10–200 μM) for 72 h. MTT (at a concentration of 0.5 mg/ml) was added to cell culture four hours prior to the termination of experiment. Subsequently, the culture plates were centrifuged ($300 \times g$) and the pellets were dissolved in DMSO (100 μl/well). Finally, the plates were read at 550 nm and 660 nm in a plate reader (Synergy HT, BIO-TEK International, Winooski, VT).

2.5. Lymphoproliferation assay following Rhein exposure to splenocytes

Lymphoproliferation assay was carried out as described earlier (Yadav et al., 2012, 2013). Briefly, Con A and LPS were used to induce proliferation of T cell and B cell populations present in the splenocyte culture, respectively. Splenocytes (2×10^5/100 μl/well) in a 96 well plate were treated with Con A (5 μg/ml) or LPS (10 μg/ml) in the presence of Rhein and incubated at 37 °C for 72 h. The proliferation of cells was determined by the uptake of tritiated thymidine in the cells. Tritiated thymidine (2 μ Ci/ml) was added to cells, 18 h prior to the termination of experiment. The cells were then harvested with cell harvester (PerkinElmer, Waltham, MA) in glass fiber filters. Later, these filters were transferred to 3 ml of scintillation cocktail-W (Sisco Research Laboratories Pvt. Ltd. Mumbai, India) and β-counts were recorded on β-counter (Hewlett-Packard, Palo Alto, CA).

2.6. Effect of Rhein on mixed lymphocyte reaction (MLR) in splenocytes

MLR was carried out following the method described earlier (Yadav et al., 2012, 2013). In brief, stimulator cells (Swiss albino splenocyte) (2×10^7 cells/ml) were treated with mitomycin C (25 μg/ml) for 30 min to prevent mitogenic activity. Responder cells (Balb/c splenocyte without mitomycin C treatment) were co-cultured with stimulator cells in a ratio of 1:4 (0.5×10^5:2×10^5 cells per 200 μl). Additionally, the splenocyte culture media was supplemented with 1% non essential amino acids and 10 mM NAC. The co-culture was incubated at 37 °C in humidified CO_2 incubator in the presence or absence of Rhein for five days. Proliferation of the cells in term of relative fold change was determined by tritiated thymidine uptake as described earlier in responder cell.

2.7. Immunophenotyping studies following Rhein exposure to splenocytes

Splenocytes were labeled with surface marker specific antibodies for identification of individual populations of B-cells and T-cells in control and Rhein (10 μM) treated groups for 72 h. Cells were suspended in staining buffer (2% FBS, 1% sodium azide in PBS) and stained with Anti-CD3e-APC-Cy7, Anti-CD8-APC, Anti-CD4-FITC, Anti-CD19-Alexafluor700, Anti-CD25-APC, Anti-CD28-APC, Anti-CD40-APC, Anti-CD69-FITC, Anti-CD80-FITC, Anti-CD86-FITC and Anti-CD95-PE antibodies for 20 min on ice. Stained cells were washed twice with wash buffer (0.01% Sodium azide in PBS) and finally suspended in 500 μl PI solution (1 μg/ml). The samples were kept on ice and analyzed within 1 h by flow cytometer (FACS Canto II, BD Biosciences, San Jose, CA). Analysis was carried out by gating CD19+ and CD3+ population in dot plot showing the live cells only. CD3+ population was further resolved into CD4+ and CD8+ sub-populations. Both, CD19+ and CD3e+ populations were also analyzed for the expression of activation markers, CD40, CD80, CD86, CD95 in B-cells and CD25, CD28, CD69, CD95 in T-cells. Mean fluorescence intensity was measured and represented as levels of receptor expression in cells. In each sample, a total of 10000 events were collected and the data was analyzed by Flowing Software 2.5.1.

2.8. Cytokines analysis following Rhein exposure to splenocytes

$T_H1/T_H2/T_H17$ cytokines were measured in Con A or LPS stimulated splenocytes in the absence and presence of Rhein (10 μM) after 72 h using Cytometric Bead Array Flow Cytometry Kit (BD Biosciences, San Jose, CA). Samples were prepared for cytokines analysis as per the manufacturer's instructions and analyzed on the same day. CBA FCAP array software (Version 3.0) evaluated the level of cytokines present in the samples on the basis of standard curve obtained for each cytokine.

2.9. Cell Cycle phase distribution following Rhein exposure to splenocytes

Cell cycle analysis was carried out by the method described earlier (Darzynkiewicz and Huang, 2004). In brief, Rhein (10 μM) treated splenocytes (1×10^6 cells) were washed and fixed in 70% ethanol for 2 h at −20 °C. The fixed cells were washed with PBS and stained with PI solution (2 mg DNase free-RNase A

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