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Zearalenone induces immunotoxicity in mice: possible protective effects of radish extract (*Raphanus sativus*)

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Abstract

Radish (*Raphanus sativus*) has been extensively studied for its preventive effects against different degenerative diseases. Zearalenone (ZEN) is a mycotoxin produced by *Fusarium* spp and is frequently implicated in immunological disorders and occasionally in hyperoestrogenic syndromes contributing to the increased risk of cancer and other diseases. The aims of this study were, firstly, to quantitatively evaluate the Tunisian radish extract (TRE) for its total flavonoids, isothiocyanates and antioxidant activity and, secondly, to investigate the protective role of TRE against immune system disorders in Balb/c mice treated with ZEN for two weeks. The results indicated that mice treated with ZEN (40 mg kg^{-1}) alone showed a significant decrease in lymphocytes of the total white blood cells, immunoglobulin profile (IgG and IgM), B cells, T-cell sub-types ($CD3^+$, $CD4^+$ and $CD8^+$) and natural killer and pro-inflammatory cytokines. Mice treated with TRE (5, 10 or 15 mg kg^{-1}) for 7 days before, during or after ZEN treatment, however, showed a significant improvement in lymphocyte, immunoglobulin profile, T-cell sub-types, B cells and pro-inflammatory cytokines. Moreover, treatment with the highest dose of TRE (15 mg kg^{-1}) enhanced the release of tumour necrosis factor-α and interleukin-1β but the other parameters were comparable with those of the control. It could be concluded that TRE was effective in protecting against ZEN-induced immunological disorders. These results supported our hypothesis that TRE contains several compounds that are able to prevent or inhibit ZEN toxicity.

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Introduction

Zearalenone (ZEN) is a non-steroidal estrogenic mycotoxin biosynthesized through a polyketide pathway by certain *Fusarium* fungi, which are regular contaminants of cereal crops worldwide (Bennett & Klich 2003). It is a resorcyclic acid lactone reduced in mammals to two sterioisomeric metabolites that are more toxic than the paternal compound (α- and β-isomers). These metabolites are also produced by the fungi, but at much lower concentrations than ZEN (FAO/WHO 2000). ZEN is rapidly absorbed after oral administration. It appears to be extensively absorbed in mice, rats, rabbits and man (Kuiper-Goodman et al 1987) and has been implicated in several human and animal outbreaks of mycotoxicosis (Hussein & Brasel 2001). ZEN has been known to be hepatotoxic, haematotoxic, genotoxic and immunotoxic. Recent studies have reported that several alterations of immunological parameters were found in-vitro associated with ZEN concentrations in mice (Marin et al 1996) and man (Berek et al 2001). According to Eriksen & Alexander (1998), alterations in immunological parameters, such as the inhibition of mitogen stimulated, lymphocyte proliferation and an increase in interleukin (IL)-2 and IL-5 production, were found at high ZEN concentrations in-vitro.

Recently, Abbès et al (2006b) demonstrated that oral administration of a single dose of ZEN (40 mg kg^{-1}) to mice resulted in several immunotoxicological effects within 24 h of oral ingestion. Indeed, a significant difference was found between ZEN-treated mice and the control group regarding the serum concentration of IgG and IgA, total white blood cell count and the differential counts of white blood cells (WBCs). Moreover, ZEN stimulates the growth of human breast cancer cells through its effect on oestrogen receptor response (Ahamed et al 2001). Oestrogenic and immunotoxic effects of ZEN, in combination with the temporal concordance of its high concentrations in foodstuffs worldwide with

increasing age-adjusted incidence rates of breast cancer, supported the hypothesis that exposure to ZEN may contribute to increasing the occurrence of breast cancer (Yu et al 2005). Consequently the regulation of ZEN-induced immunotoxicity and its importance in carcinogenesis have recently gained special interest in the development of new therapeutic agents.

Radish (*Raphanus sativus*) is among the cruciferous vegetables with considerable anti-carcinogenic properties; it is grown worldwide and has been in use for medicinal purposes (Nadkarni 1976). It has been ethnically used as a laxative, a stimulant, a digestive aid, an appetizer and in other stomach disorders (Kapoor 1990). Several reports indicated that the main therapeutic constituents of radish are flavonoids 4-(methylthio)-3-butenyl isothiocyanate, allyl isothiocayanate, benzyl isothiocyanate and phenethyl isothiocyanate, as well as kaempherol glycosides, peroxidases and antioxidants (Wang et al 2004; Katsuzaki et al 2004; Hashimoto et al 2006; Suh et al 2006). It was found to be antimicrobial (Esaki & Onozaki 1982), anti-mutagenic (Hashem & Saleh 1999) and anti-carcinogenic (Hecht 1999). Recently, Suh et al (2006) demonstrated that radish extracts inhibit the abnormal growth of vascular smooth muscle cells as a prominent feature of vascular disease, including atherosclerosis and restenosis.

Lugasi et al (2001) reported that radish root extract has an antioxidant activity in-vitro, inhibits the membrane changes caused by a fat-rich diet, beneficially influences the natural scavenging activity of rat colon mucosa and protects the cell membranes against lipid peroxidation (Sipos et al 2002). Takaya et al (2003) suggested that the crude extract of radish contains antioxidant enzyme activity and the antioxidant L-tryptophan was isolated from radish extract (Katsuzaki et al 2004). In recent work, we demonstrated that the Tunisian radish extract (TRE) protects against ZEN toxicity regarding liver and kidney functions and the antioxidant enzyme status (Ben Salah-Abbès et al 2008). Despite the clinical importance of radish for it therapeutic potential, there have been no reports on elucidating other immunopharmacological activity. The objectives of the current work were to evaluate TRE for its total flavonoids, isothiocyanates and antioxidant activity and to investigate the protective role of TRE against immune system disorders in Balb/c mice treated with ZEN with regards to the total and differential count of leucocytes, peripheral lymphocytes, cytokines and immunoglobulin profile.

Materials and Methods

Chemicals and kits

ZEN was purchased from Sigma Chemical Co (St Louis, MO). Antibodies anti-$CD3^+$, anti-$CD4^+$, anti-$CD8^+$, anti-$CD56^+$ and anti-B cells were obtained from Serotec (Kidlington, Oxford, UK). TNF-α and IL-1β ELISA kits were purchased from Biosource International (Camarillo, CA). The lower detection limits of these kits are 4 pg mL^{-1}. The optical density was measured by using an automatic plate reader (Spectra Image; Tecan) at 450 and at 620 nm for reference. All chemicals used were at the highest purity available.

Plant material

Radish plants (*Raphanus sativus*, Brassicaceae) were harvested from Eletha region (Central Tunisia) in October, 2005 and were botanically identified by Prof. El Ouni (Department of Botany, Faculty of Sciences, Bizerte, Tunisia), according to the flora of Tunisia (Cuénod 1954).

Preparation of radish extract

Freshly harvested roots and aerial parts of radish were crushed, and the juice was extracted through a fine mesh. The extracts were purified and characterised according to the method described previously (Keilin & Hartee 1951; Wang et al 2004; Ben Salah-Abbès et al 2008). The filtrate was concentrated with a vacuum concentration apparatus and stored at −20°C until used.

Identification and quantification of flavonoids

Radish extract was screened for the presence of flavonoids using the methods described by Kilani et al (2005). The identification was carried out by thin-layer chromatography (TLC) on silica gel 60 F254 Merck (layer thickness 0.25 mm). The TLC was developed in n-butanol–acetic acid–water (4:1:5) and the spots were visualized with 1% $AlCl_3$ in methanol under UV (366 nm). The flavonoid quantification was made according to Zhishen et al (1999). Two milligrams of the lyophilised radish extract were dissolved in 3 mL of distilled water, 0.3 mL $NaNO_2$ (1:20 w/v) and 3 mL of $AlCl_3$ (1:10 w/v). After 6 min, 2 mL of 1 N NaOH were added and the total absorbance was measured at 510 nm. Quercetin was used as a standard for constructing a calibration curve.

Measurement of total isothiocyanate content

The level of total isothiocyanate was measured using the method of Zhang et al (1992) with a slight modification. In brief, 50 μL of n-hexane extract from radish extract was diluted with a mixture of 0.45 mL of methanol and 0.45 mL of 50 mM $Na_2B_4O_7$ HCl buffer (pH 8.5), 50 μL of 8 mM 1,2-benzenedithiol were added and mixed well in a 1.5 mL plastic tube. The tube was heated at 65°C for 1 h and the isothiocyanate content was calculated by reading the sample absorption at 365 nm from a linear standard equation derived from the absorption readings of a serial dilution of known phenethyl isothiocyanate concentrations.

Antioxidant activity of radish extract

The methanol extract of radish was subjected to the antioxidant assay using the modified BLM-Fe (III) method reported by Ekimoto et al (1985). Tris-HCl buffer (0.2 M; pH 7.4), 8 mM arachidonic acid, sample solution, 1 mM BLM, and 1.08 mM $FeSO_4$ (each 100 μL) were successively added to a test tube. The solution was incubated at 37°C for 5 min, then 0.2 M HCl (10 μL) was added followed by 0.5% 2-thiobarbituric acid (TBA) (0.2 mL). This mixture was incubated at 37°C for 30 min then 0.4 mL of water and 1 mL of 1-butanol were added, the mixture was vigorously shaken and centrifuged for

10 min at 300 *g*. Out of the resultant 1-butanol layer, 0.7 mL of the solution was diluted with 5.0 mL of 1-butanol in another tube. The absorbance of the solution at 532 nm (AS) was measured by a spectrophotometer. Absorbance using water instead of a sample solution in the above protocol (AC) served as the control. The antioxidant activity of the sample (AAS) was expressed as the inhibition rate of the sample using the following formula:

$$AAS = (1 - AS/AC) \times 100\ (\%) \quad (1)$$

The concentration of sample solutions as well as the positive control, L-ascorbic acid, was fixed as 3 mg mL^{-1} for crude samples, including the methanol extract, and 1 mM for isolated compounds. At these concentrations, the antioxidant activity is in proportion to the concentrations of L-ascorbic acid, so as to get reliable data. The index of activity (IA) and total activity (AAT) were defined as follows:

$$IA = AS/AVC\ (VC) \quad (2)$$

$$AAT = IA \times \text{content}\ (\%)\ \text{in radish extract}/100 \quad (3)$$

where AVC is the antioxidant activity of L-ascorbic acid at the same concentration of the samples, and the index of activity was expressed with a unit, VC, which is the vitamin C equivalent antioxidant activity.

Animals

Balb/c male mice, 21 ± 1 g (6–7 weeks old), were purchased from Pasteur Institute of Tunis. The mice were housed at 23 ± 1°C, with relative humidity of 55 ± 10%, a 12-h light–dark cycle and had free access to standard pellet diet (Vegetable fibre, 7.5%; protein, 14.5%; fat, 3%; and metabolic energy, 0.017 MJ) free from mycotoxins and free access to water.

Experimental design

After an acclimatization period mice were randomly divided into thirteen groups (10 mice/group) as presented in Table 1 and treated orally with the respective treatment in accordance with procedures adhering to the European Community regulations controlling experiments in live animals (86/609). The selected doses of ZEN and TRE were based on our previous work (Abbès et al 2007; Ben Salah-Abbès et al 2008). At the end of the treatment period, blood samples were collected from the retro-orbital venous plexus.

Measurement of lymphocyte T sub-types and total white blood cells

Mononuclear cells were isolated from blood samples as described by Boyum (1968). T-cell subsets were evaluated by an indirect immunofluorescence technique using CD3$^+$, CD4$^+$, CD8$^+$ and CD56$^+$ antibodies. CD3$^+$ antibodies recognized antimouse CD3 T-lymphocytes, CD4$^+$ antibodies recognized helper T-lymphocytes, CD8$^+$ antibodies recognized suppressor T-lymphocytes, and CD56$^+$ recognized natural killer cells. At least 200 cells per sample were counted using a fluorescence microscope (Nikon Eclipse E 400). Total white blood cells (WBCs), lymphocytes (LYs), eosinophils (EOs), neutrophils (NEs) and monocytes (MOs) were determined using a blood counter (Coulter STKS model; Coulter electronics Ltd, Luton, UK) with adapted dilutions.

Table 1 Experimental design

Group	Treatment period		
	Week 1	Week 2	Week 3
Group 1	Control	Control	
Group 2	Olive oil	Olive oil	
Group 3	TRE 3	TRE 3	
Group 4	ZEN	ZEN	
Group 5	TRE 1	ZEN	ZEN
Group 6	TRE 2	ZEN	ZEN
Group 7	TRE 3	ZEN	ZEN
Group 8	TRE 1 + ZEN	TRE 1 + ZEN	
Group 9	TRE 2 + ZEN	TRE 2 + ZEN	
Group 10	TRE 3 + ZEN	TRE 3 + ZEN	
Group 11	ZEN	ZEN	TRE 1
Group 12	ZEN	ZEN	TRE 2
Group 13	ZEN	ZEN	TRE 3

TRE 1, 5 mg kg^{-1}; TRE 2, 10 mg kg^{-1}; TRE 3, 15 mg kg^{-1}; ZEN, 40 mg kg^{-1}.

Immunoglobulin profile

Serum total IgG and IgM levels were measured by kinetic nephelometry (Roche Diagnostics, Barcelona, Spain).

Cytokines assay

Plasma TNF-α and IL-1β were measured by ELISA method in accordance with the manufacturer's instructions and were expressed in pg mL^{-1}.

Statistical analysis

All data were statistically analysed using the General Linear Models Procedure of the Statistical Analysis System (SAS, 1982). The significance of the differences among treatment groups was determined by Waller–Duncan k-ratio (Waller & Duncan 1969). All statements of significance were based on a probability of $P \leq 0.05$.

Results

Phytochemical study

The phytochemical study of radish extracts revealed the presence of various quantities of total isothiocyanate and total flavonoids, and an antioxidant activity. These results indicated

that TRE had a higher content of flavonoids (quercetin equivalents) and isothiocyanate, which reached 344 ± 3.8 and 38.98 ± 4.2 μmol/100 mg, respectively. Moreover, the index of antioxidant activity reached 2.4/100 mg extract and the total antioxidant activity reached 33.8/100 mg extract.

Body and lymphoid organ weights

After two weeks of ZEN treatment, general toxicity was observed in the mice; the body and lymphoid organs weights were decreased ($P < 0.05$) in treated mice (data not represented).

Total and differential white blood cell counts

Total WBCs were found to be increased in the ZEN alone-treated group (Table 2). The differential counts of WBCs revealed a decrease in lymphocytes and an increase in the other cell counts in mice treated with ZEN alone ($P < 0.05$). On the other hand, total and differential counts of WBCs in mice treated with TRE alone at the highest level (15 mg kg^{-1}) were comparable with the controls ($P < 0.05$) except eosinophils, which were found to be decreased ($P < 0.05$). Pre-, co- and post-treatment of the ZEN-treated group with TRE resulted in an improvement ($P < 0.05$) in both total and differential counts of WBCs, although these values still differed from those of the control group ($P < 0.05$) especially in mice treated with ZEN then TRE.

Lymphocyte T sub-types

The effects of TRE on lymphocyte T sub-types and B lymphocytes on ZEN-treated mice are presented in Table 3. Oral administration of TRE at the higher dose (15 mg kg^{-1}) had no

Table 2 Effect of TRE on total white blood cell and differential counts of leucocytes in Balb/c mice treated with ZEN

Group	WBC ($10^3/\mu L$)	NE ($10^3/\mu L$)	LY ($10^3/\mu L$)	MO ($10^3/\mu L$)	EO ($10^3/\mu L$)
Control	8.03 ± 0.20[a]	6.11 ± 0.09[a]	1.38 ± 0.09[a]	0.41 ± 0.05[a]	0.11 ± 0.01[a]
Olive oil	7.98 ± 0.26[a]	6.01 ± 0.11[a]	1.40 ± 0.06[a]	0.45 ± 0.06[a]	0.10 ± 0.02[a]
TRE (15 mg kg^{-1})	8.09 ± 0.21[a]	6.07 ± 0.07[a]	1.41 ± 0.05[a]	0.43 ± 0.07[a]	0.06 ± 0.01[b]
ZEN	12.44 ± 0.42[b]	9.84 ± 0.21[b]	0.82 ± 0.10[b]	1.69 ± 0.11[b]	0.61 ± 0.03[b]
TRE 1 then ZEN	9.91 ± 0.13[b]	7.01 ± 0.11[b]	0.91 ± 0.03[b]	1.13 ± 0.22[b]	0.59 ± 0.03[b]
TRE 2 then ZEN	8.60 ± 0.21[b]	6.73 ± 0.03[a]	0.98 ± 0.02[b]	1.01 ± 0.18[b]	0.51 ± 0.02[b]
TRE 3 then ZEN	8.12 ± 0.14[a]	6.01 ± 0.03[a]	1.27 ± 0.06[a]	0.62 ± 0.12[a]	0.21 ± 0.03[a]
ZEN + TRE 1	9.53 ± 0.14[b]	7.01 ± 0.03[b]	0.81 ± 0.03[b]	1.29 ± 0.05[b]	0.31 ± 0.02[a]
ZEN + TRE 2	8.29 ± 0.21[a]	6.31 ± 0.04[a]	1.07 ± 0.05[a]	0.82 ± 0.03[b]	0.21 ± 0.03[a]
ZEN + TRE 3	8.11 ± 0.16[a]	6.21 ± 0.07[a]	1.36 ± 0.03[a]	0.57 ± 0.02[a]	0.12 ± 0.01[a]
ZEN then TRE 1	11.97 ± 0.23[b]	8.91 ± 0.03[b]	0.95 ± 0.03[b]	1.53 ± 0.11[b]	0.59 ± 0.02[b]
ZEN then TRE 2	9.94 ± 0.21[b]	8.13 ± 0.05[b]	0.97 ± 0.02[b]	1.01 ± 0.12[b]	0.42 ± 0.01[b]
ZEN then TRE 3	9.18 ± 0.12[b]	7.53 ± 0.04[b]	1.09 ± 0.04[a]	0.78 ± 0.18[b]	0.22 ± 0.01[a]

Data are means ± s.e.m. TRE 1, 5 mg kg^{-1}; TRE 2, 10 mg kg^{-1}; TRE 3, 15 mg kg^{-1}; ZEN, 40 mg kg^{-1}. Within each column, means superscript with different letters are significantly different ($P < 0.05$). [a]Not significantly different to the control; [b]significantly different to the control.

Table 3 Effect of TRE on lymphocyte B and T sub-types in ZEN-treated mice

Group	Lymphocyte T sub-types (10^6/mL)			NK(10^6/mL)	Lymphocyte B (1×10^6/mL)
	CD_3	CD_4	CD_8	CD_{56}	
Control olive oil	4.50 ± 0.11[a]	3.34 ± 0.19[a]	1.39 ± 0.20[a]	1.11 ± 0.45[a]	4.34 ± 1.23[a]
Control distilled water	4.54 ± 0.12[a]	3.30 ± 0.31[a]	1.41 ± 0.22[a]	1.13 ± 0.42[a]	4.27 ± 1.54[a]
TRE (15 mg kg^{-1})	4.47 ± 0.11[a]	3.23 ± 0.15[a]	1.47 ± 0.27[a]	1.17 ± 0.14[a]	4.45 ± 1.33[a]
ZEN	2.72 ± 0.12[b]	2.18 ± 0.12[b]	0.92 ± 0.23[b]	0.73 ± 0.07[b]	2.03 ± 0.11[b]
TRE 1 then ZEN	3.11 ± 0.09[b]	2.71 ± 0.11[b]	0.96 ± 0.12[b]	0.83 ± 0.12[b]	2.34 ± 0.32[b]
TRE 2 then ZEN	4.40 ± 0.14[a]	3.23 ± 0.12[a]	1.27 ± 0.11[a]	1.11 ± 0.18[a]	3.56 ± 0.45[b]
TRE 3 then ZEN	4.52 ± 0.12[a]	3.31 ± 17.2[a]	1.35 ± 0.09[a]	1.12 ± 0.13[a]	4.09 ± 0.56[a]
ZEN + TRE 1	4.23 ± 0.14[a]	2.85 ± 0.12[b]	0.91 ± 0.05[b]	0.97 ± 0.19[a]	4.06 ± 0.65[a]
ZEN + TRE 2	3.67 ± 0.12[b]	2.97 ± 0.14[a]	1.27 ± 0.04[a]	1.07 ± 0.14[a]	4.11 ± 0.83[a]
ZEN + TRE 3	4.51 ± 0.18[a]	3.24 ± 0.16[a]	1.36 ± 0.03[a]	1.12 ± 0.18[a]	4.33 ± 0.67[a]
ZEN then TRE 1	2.97 ± 0.16[b]	2.51 ± 0.14[b]	0.81 ± 0.13[b]	0.73 ± 0.21[b]	3.73 ± 0.45[b]
ZEN then TRE 2	3.74 ± 0.13[b]	2.73 ± 0.09[b]	0.97 ± 0.02[b]	0.90 ± 0.19[b]	3.95 ± 0.76[b]
ZEN then TRE 3	4.38 ± 0.12[a]	3.13 ± 0.06[a]	1.21 ± 0.04[a]	1.03 ± 0.18[a]	4.26 ± 0.36[a]

TRE 1, 5 mg kg^{-1}; TRE 2, 10 mg kg^{-1}; TRE 3, 15 mg kg^{-1}; ZEN, 40 mg kg^{-1}. Within each column, means superscript with different letters are significantly different ($P < 0.05$). [a]Not significantly different to the control; [b]significantly different to the control.

effect on lymphocyte T phenotype ($P > 0.05$), reinforcing its non-immunotoxic properties. Mice treated with ZEN alone showed a remarkable reduction in $CD3^+$, $CD4^+$, $CD8^+$ and NK counts of lymphocyte T and B cells. This reduction was restored in the mice treated with TRE. The numbers of different phenotypes of T and B lymphocyte cells was recovered or greatly improved in ZEN-treated mice following the administration of TRE. Treatment with TRE pre- or simultaneous with ZEN showed that all the numbers of $CD3^+$, $CD4^+$, $CD8^+$ and NK, and B lymphocytes were at the normal level of the control. Nevertheless the counts and of T sub-type and B cells showed an obvious dose-dependent enhancement in the mice treated with TRE after ZEN (Table 3).

Immunoglobulin profile

Data presented in Figures 1 and 2 represent the humoral immune response (IgM and IgG) in mice. These results clearly indicate that treatment with ZEN alone induced a decrease ($P < 0.05$) in both IgM and IgG concentrations. However, treatment with TRE alone at the three tested doses did not show any humoral immune response. The combined treatment of ZEN plus TRE (5, 10, 15 mg kg^{-1}) succeeded in normalizing the humoral immune profile. On the other hand, pre-treatment with TRE at the three tested doses and post-treatment with the highest dose (15 mg kg^{-1}) overcame the immunoglobulin toxicity, respectively (Figures 1 and 2).

TNF-α and IL-1β plasma levels

TNF-α was found to be increased in an abrupt manner in TRE-treated mice (Figure 3). In ZEN-treated mice, the TNF-α level was obviously lower than was found in the control. Oral administration of TRE resulted in a remarkable increment in peripheral release of TNF-α in a dose-dependent manner, approaching the level of normal mice in the pre- and simultaneous TRE treatment modality. No difference ($P > 0.05$) was observed in the TRE-evoked release of TNF-α in ZEN-treated mice between the doses used in pre- and simultaneous treatment (Figure 3). In post-treatment with TRE, TNF-α level showed a slight enhancement at the higher dose (15 mg kg^{-1}). Treatment of mice with ZEN for two weeks increased the levels of circulating IL-1β (Figure 4). Meanwhile, mice treated with TRE alone at the three tested levels showed fluctuation compared to the control. Pre- and co-treatment with TRE of ZEN-treated mice resulted in a decrease ($P < 0.05$) in the elevated levels of IL-1β.

Discussion

The toxicity of ZEN on human and experimental animals is well documented in a series of reports. It is associated with a high incidence of liver and breast cancer worldwide (FAO/WHO 2002; Labuda et al 2005; Yu et al 2005). There is a great demand to find a method for detoxification, since people may consume ZEN-contaminated food daily in some areas. Nutrition is likely to be important in this respect. This study deals with the preventive effects of TRE (*Raphanus sativus*) against ZEN-induced immunotoxicity in Balb/c mice. The results of this study indicated that ZEN induces a reduction in peripheral lymphocytes similar to that reported previously (Berek et al 2001; Abbès et al 2006b) and indicated that ZEN induces its immunosuppressive effect via

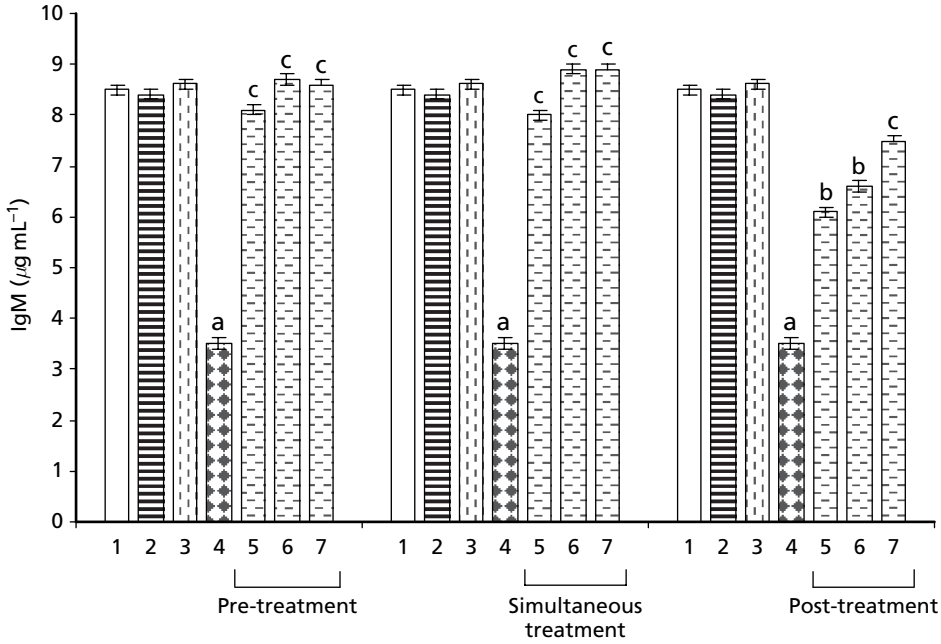

Figure 1 Effect of TRE on the plasma IgM level in ZEN-treated mice (means ± s.e.m.). 1, Control; 2, TRE 15 mg kg^{-1}; 3, olive oil; 4, ZEN 40 mg kg^{-1}; 5, ZEN + TRE 5 mg kg^{-1}; 6, ZEN + TRE 10 mg kg^{-1}; 7, ZEN + TRE 15 mg kg^{-1}. Data are means ± s.e.m. Each column with different letter differs significantly to the control ($P < 0.05$).

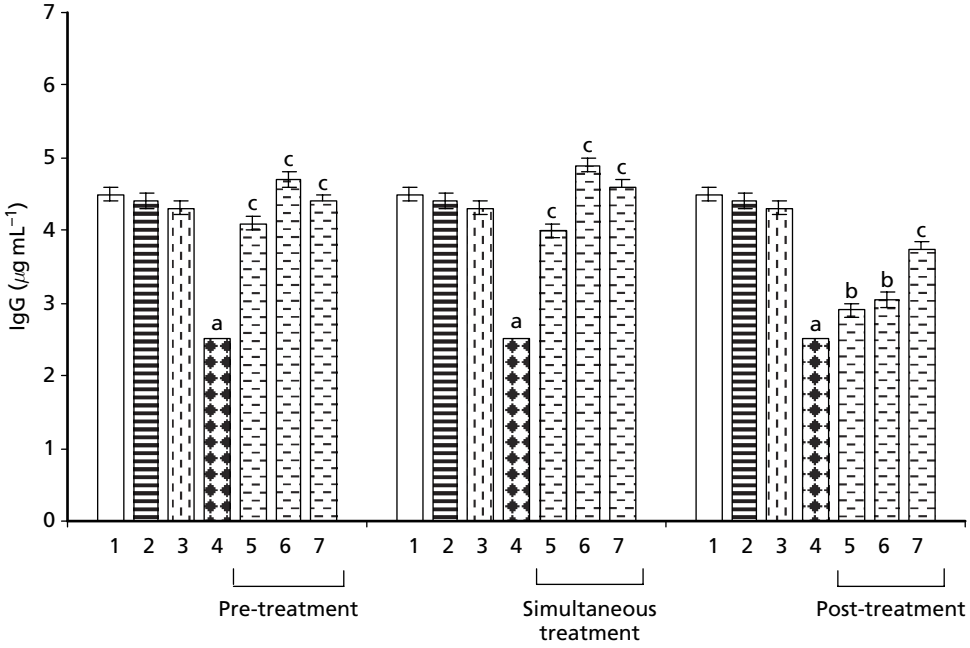

Figure 2 Effect of TRE on the plasma IgG level in ZEN-treated mice. 1, Control; 2, TRE 15 mg kg^{-1}; 3, olive oil; 4, ZEN 40 mg kg^{-1}; 5, ZEN + TRE 5 mg kg^{-1}; 6, ZEN + TRE 10 mg kg^{-1}; 7, ZEN + TRE 15 mg kg^{-1}. Data are means ± s.e.m. Each column with different letter differs significantly to the control ($P < 0.05$).

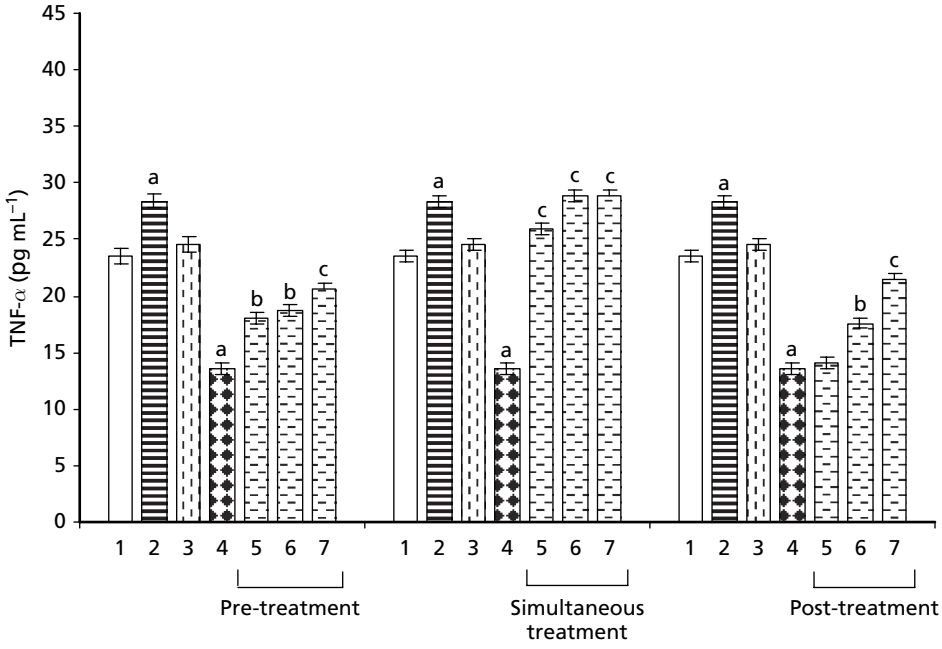

Figure 3 Effect of TRE on the plasma TNF-α level in ZEN-treated mice. 1, Control; 2, TRE 15 mg kg^{-1}; 3, olive oil; 4, ZEN 40 mg kg^{-1}; 5, ZEN + TRE 5 mg kg^{-1}; 6, ZEN + TRE 10 mg kg^{-1}; 7, ZEN + TRE 15 mg kg^{-1}. Data are means ± s.e.m. Each column with different letter differs significantly to the control ($P < 0.05$).

the depression of activity and number of T and B lymphocytes. The search for an underlying mechanism revealed apoptosis since ZEN induced immune cell death in the spleen (Abbès et al 2006b). Because the spleen is one of the most important organs for maturing lymphocytes, the death of lymphocytes in spleen definitely reduced peripheral lymphocytes. It directly participates in immune system toxicity by monitoring monogenesis or tumorigenesis and releasing pro-inflammatory or anti-tumour cytokines (Zhang et al 2004).

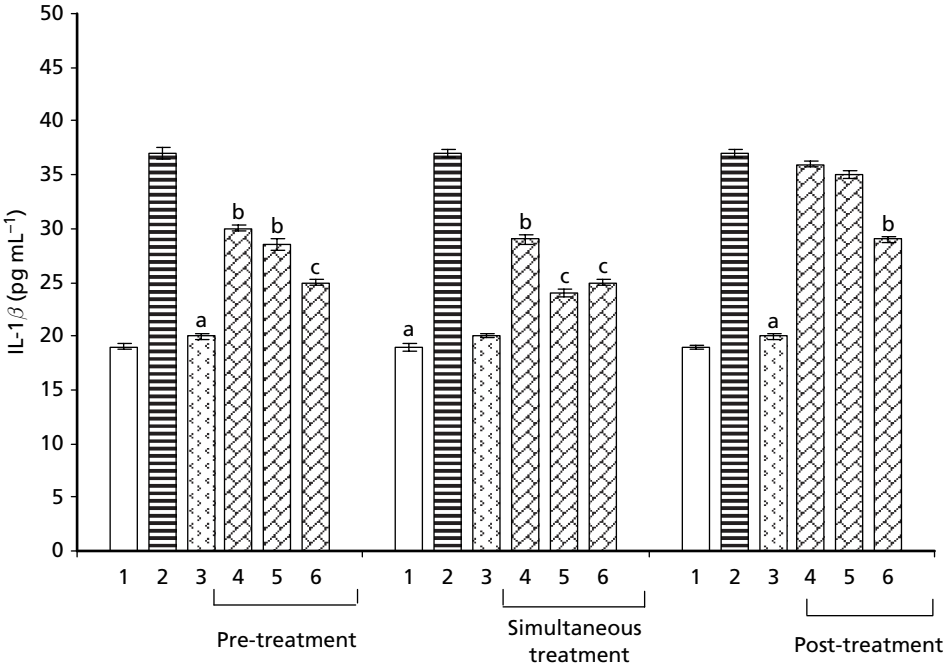

Figure 4 Effect of TRE on the plasma IL-1β level in ZEN-treated mice. 1, Control; 2, TRE 15 mg kg^{-1}; 3, olive oil; 4, ZEN 40 mg kg^{-1}; 5, ZEN + TRE 5 mg kg^{-1}; 6, ZEN + TRE 10 mg kg^{-1}; 7, ZEN + TRE 15 mg kg^{-1}. Data are means ± s.e.m. Each column with different letter differs significantly to the control ($P < 0.05$).

In this study, it was observed that the lymphocyte phenotype count was greatly reduced following ZEN treatment. The decreased expression of T cells, CD3$^+$, CD4$^+$, CD8$^+$ and NK, and B lymphocytes on peripheral blood observed in this study provides evidence for the immunosuppressive role of ZEN. The effect of ZEN on the mouse immune system was also demonstrated by Pestka et al (1987). In this regard, Berek et al (2001) stated that ZEN induced its immunosuppressive effect via the depression of T and B lymphocyte activity. Moreover, Swamy et al (2004) demonstrated that a ZEN-contaminated diet linearly reduced the B-cell count in broiler chickens. The progressive loss of CD3$^+$, CD4$^+$, CD8$^+$ and NK cell numbers has been related to many factors, such as reduced interferon-γ levels and IL-2 expression, and the presence of immunosuppressive factors, mainly prostaglandin E2, produced by natural suppressor cells resident in the spleen after ZEN damage (Segura et al 2000). These results were also supported by the findings of Karagezian (2000) who stated that a single intravenous administration of ZEN (15 mg kg^{-1}) to rats led to the formation of pronounced abnormalities in lymphocyte membrane phospholipid metabolism.

In this study, the increased number of total and differential leucocyte counts after ZEN treatment is supported by our previous work (Abbès et al 2006a) and the pro-inflammatory cytokines released by activated macrophages, NK cells and T lymphocytes (Mocellin et al 2005). In this study ZEN-treated mice showed a reduction in peripheral lymphocytes and decrease in the release of TNF, hence its content in the periphery. Moreover, the decrease in both IgM and IgG concentrations observed in ZEN-treated mice corresponds with the development of active humoral immunity and thus infers that ZEN affected both the cellular and humoral immune system. Indeed, the B cell count and immunoglobulin production were decreased after two weeks of ZEN treatment. This may be due to the decreased number and the disordered functions of regulatory T cells caused by ZEN treatment (Lim et al 2005). Moreover, it is well established that certain T-regulatory cytokines are involved in regulating the immunoglobulin response via enhancement of activation, switching and differentiation of B cells into immunoglobulin-secreting plasma cells (Beagley & Elson 1992). These findings were supported by the histological changes in the spleen tissue, which is considered the target organ for infectious agents such as ZEN mycotoxin (Abbès et al 2006b).

Our data revealed that TRE showed a protective effect in most cases against ZEN-induced immunotoxicity. We also showed that oral treatment with TRE resulted in an increase in TNF-α release, which indicates that the inhibition of carcinogenesis by TRE is also attributed to its effective enhancement of TNF-α release in peripheral ZEN-treated mice and that it is a potential candidate for an anti-tumour drug. Previous reports suggested that the mycotoxins are metabolised and detoxified by the cellular cytochrome P450 enzyme system; when the latter was insufficient, ZEN metabolites formed a reactive intermediate, which in turn reacted with macromolecules, such as lipids, nucleic acids and proteins, leading to lipid peroxidation and cellular injury (Abid-Essefi et al 2004; Abdel-Wahhab et al 2006). In a crossover study, the impact of a mixed vegetable diet on cytochrome P450 1A2 in man was investigated by Lampe et al (2000). The participants received first a vegetable-free diet followed by a diet enriched in cruciferous vegetables including radish. After a

consumption period of 6 consecutive days, a pronounced increase in cytochrome P450 1A2 activity was seen, enhancing potential detoxification. Moreover, our previous work demonstrated the efficacy of TRE as an antioxidant, radical scavenging and normalizing enzyme in ZEN-treated Balb/c mice (Ben Salah-Abbès et al 2008). In this regard, treatment with ZEN plus TRE leads to the restoration of peripheral and phenotype lymphocyte count by diminution of cytolytic activity of ZEN.

Isothiocyanates are highly reactive compounds in which the central carbon atom is strongly electrophilic and is attacked by nucleophiles such as amino groups (Nakamura et al 2001). Isothiocyanates are, therefore, capable of reacting with various cellular targets and inducing several biological responses: antimicrobial (Hashem & Saleh, 1999), anti-mutagenic (Hamilton & Teel 1996) and anti-carcinogenic activities (Hecht 1999). Talalay & Fahey (2001) have demonstrated that high intake of phytochemicals known as glucosinolates and isothiocyanates protects against cancer diseases. Isothiocyanates are well-known protectors against carcinogenesis and modulators of the activity of enzymes involved in the metabolism of carcinogens, especially by the induction of phase 2 detoxification enzymes. The flavonoid compounds were found to reduce the production of reactive oxygen species (ROS), the apoptosis caused by carcinogenic chemicals (i.e. aflatoxin) and showed good scavenging power, in accordance with the observed inhibition of NO production (Guerra et al 2006; Abdel-Wahhab et al 2007). The presence of flavonoids as a major constituent in TRE suggests it to be an effective agent against carcinogenic mycotoxins. The treatment with TRE effectively protected the spleen from the effects of ZEN and thereby maintained the number of peripheral lymphocyte cells at the level of that in normal mice.

The protective role of radish extract against the decrease in the number of lymphocytes by inhibiting lipid peroxidation resulting from ZEN treatment supported the antioxidant properties, which may be due to its higher content of isothiocyanate, kaempherol glycosides and L-tryptophan compounds and the ability to scavenge free radical intermediates of lipid peroxidation. In this concern, Sipos et al (2002) demonstrated that granule radish root extract protected cell membrane against lipid peroxidation in rats fed on a fat-rich diet. According to Rastogi et al (2001) and Abdel-Wahhab et al (2007), lipid peroxidation is one of the most prominent factors in mycotoxin damage and carcinogenicity. Hecht (1999) demonstrated that isothiocyanates represent the main compounds in radish extract, providing good chemoprevention and modification of carcinogen metabolism. Similarly, Nakamura et al (2001) have shown that 4-(methylthio)-3-butenyl isothiocyanate is a principal anti-mutagen in radish extract. Phenethyl isothiocyanate and benzyl isothiocyanate isolated from radish extract prevent lung tumorigenesis in A/J mice induced by benzo [a] pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Hecht et al 2000). The cruciferous compounds in the radish extract activate the antioxidant defence system in liver, which is mediated by several enzymes functioning in a concerted manner by removing peroxide and superoxide anions generated within the cell after ZEN damage (Shklar 1998). Besides, the peroxidase enzymes in radish (Wang et al 2004), such as glutathione peroxidase (GSH-PX) and catalase, are able to react with hypochlorous acid and hydrogen peroxide generated by superoxide dismutase in cytosol and mitochondria (Chance et al 1979) by oxidizing the GSH to GSSG (Halliwell 1994).

Conclusion

In conclusion, these results clearly indicated that ZEN induces immunological alterations in mice. TRE through its higher content of flavonoids, isothiocyanates and antioxidants exhibited significant inhibition of immunotoxicity of ZEN and can be considered as an active extract against mycotoxin-induced toxicity.

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