Original article

The potential protective role of Akropower against Atrazine-induced humoral immunotoxicity in rabbits

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\begin{abstract}

Introduction to the herbicide Atrazine (ATR) can bring about immunotoxicity, aside from other unfavorable results for the creature and human wellbeing. We went for clarifying the genotoxic mechanisms required in humoral immunotoxicity of Gesaprim\textsuperscript{®} (ATR) and their constriction by Akropower. Forty rabbits (1.5 kg \pm 20\%) were utilized and appointed into 4 equal groups. group 1: control; group 2: Received Atrazine at 1/10 LD\textsubscript{50} via food; group 3: Received Akropower at 1 ml/1 l/day by means of drinking water; group 4: Received both Atrazine and Akropower associatively by the same said dosage and course. Atrazine and Akropower exposure were accomplished for 60 days. The genotoxic mechanisms of Atrazine-induced humoral immunotoxicity were explained by increased serum total protein and albumin levels, decreased RHDV antibody titer only after four weeks of vaccination and increased level of spleen Fas and Caspase-III genes expression in Atrazine-exposed rabbits. Marked splenocytes apoptosis were detected in the immunohistochemical examination by caspase-III technique and TUNEL assay. Akropower attenuated ATR-induced apoptosis through down-regulation of Fas and Caspase-III genes expression and suppression of their signaling pathway. In conclusion, induction of apoptosis by overexpression of Fas and Caspase-III genes gives a new insight into the mechanism of ATR immunotoxicity. The protective part of Akropower, on the other hand, was characterized by attenuation of Fas and Caspase-III genes mediated apoptosis.

\end{abstract}

1. Introduction

Atrazine (6-chloro-N-ethyl-N'-(1-methylethyl)-triazine-2,4-diamine-ATR) is a selective post-emergence chlorotriazine herbicide worldwide used in many countries for the control of broadleaf and grassy weeds in agricultural crops [7]). It is applied to crops such as corn, sugarcane, sorghum, pineapple and in conifer reforestation plantings [9]). Environmental or occupational exposure to ATR herbicide can produce toxic consequences in animals and humans [12]). The potential immunotoxicity of ATR was reported through suppression of the innate immune response [28]), and the cell-mediated; humoral and non-specific immune function [31]). Recently, [33] recorded ATR-induced apoptosis. Fas-interceded apoptosis might be included as a system for ATR immunotoxicity in splenocytes of mice however extra components for immunotoxicity may likewise exist [34]).

Akropower\textsuperscript{®} is a nutritional adjuvant, consists of licorice fermented with Aspergillus oryzae, malic acid, vitamins (ascorbic acid, B1, B2, B6, B12, pantothenic acid and biotin), choline chloride, inositol, sodium propionate and BHT. It’s formulation is based on biotechnological processes in which the formula including water with the violet method that potentiates the kinetic charge of the various components and so increases the pharmacological potential of the individual compounds at the tissue and cellular levels (Akron@akronbio.com). Previous studies confirmed the immune stimulant and/or antioxidant actions of the individual Akropower\textsuperscript{®} components including Glycyrrhizic acid [19]); Glycyrrhetinic acid [20] and [1] ascorbic acid [2] and malic acid [13].

The present investigation was intended to investigate the genotoxic mechanisms of Gesaprim\textsuperscript{®} (Atrazine) initiated humoral immunotoxicity in the spleen of rabbits and the protective part of Akropower\textsuperscript{®} against such adverse impacts.

2. Materials and methods

2.1. Animals and experimental design

The Local Research Ethical Committee of the Faculty of Veterinary Medicine, Cairo University, Egypt approved the design of the experiment (approval No CU II S 19 16) and the protocol conforms to the
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2.1. Chemicals

Gesaprim® (Atrazine) was obtained from Syngenta Co., Switzerland while Akropower was obtained from Akron Srl Co., Italy. All other chemicals and kits were analytically pure and purchased from Thermo Scientific and Bioscience companies.

Antigens used in HA and HI tests:

- The hemagglutinating antigen of RHDV: Commercial vaccine used as antigen for HI-test.
- Sheep red blood cells (RBCs): Blood of susceptible sheep was collected on 4% sodium citrate and used for the preparation of washed sheep RBCs in PBS at a concentration of 1% for rapid HA and 0.5% in HI test.

2.2. Blood protein levels

Serum total protein and serum albumin were measured using test kits from Biodiagnostic Co. (Cat. No. 310 002). Serum globulin and albumin/globulin (A/G) ratio were then calculated.

2.3. Determination of RHDV antibody titer using (HAI)

Estimation of the antibody titers against RHDV was done in the serum samples, taken at zero, two and four weeks of vaccination, using the standard HAI method [21] after application of haemagglutination (HA) tests [8].

2.4. Quantification of spleen fas and caspase-III genes mRNAs (apoptosis markers) levels of expressions using real-time RT-PCR

2.4.1. Total RNA extraction

RNA isolation was done by Total RNA Purification Kit (Jena Bioscience, Cat. No. PP-2105) according to the manufacturer instructions. Both the concentration and purity of RNA were determined by Nanodrop ND1000

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) RT-PCR was done by using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Cat. No. #K1622).

2.5. Real-time PCR (qPCR)

Real-time PCR (qPCR) was done by Luminaries Color HiGreen Low ROX qPCR Master kit (Thermo Scientific, Cat. No. #K0371).

Each assay included triplicate samples for each tested cDNAs and no-template negative control. Each reaction contained 30 pg/ml of each of the following primers: Caspase-III 5-CACGCTGATGAGGAGTC-3; GCAAGCCTGAATAATGAA-3; 5-Fas-CAACCGAGCAGAAGAAATTGC-3; 5-CCGAAGAGCCAAAGATTGC-3; GAPDH-5-TCACCATCTTCCAGGACAGG-3; 5-CACAACTGCAGAGTGGCTGCT-3.

Data were normalized by using GAPDH gene as a housekeeping gene [16]. Two-steps cycling protocol was adjusted as follows; UDG pretreatment at 50 °C for 2 min, Initial denaturation at 95 °C for 10 min then 45 cycles of denaturation at 95 °C for 20 s and annealing/extension at 60 °C for 45 s. Fluorescent data were acquired during each extension phase. The fold change compared to control samples was calculated using CT, ΔCT, ΔΔCT by Mxpro software Stratagene.

2.6. Methods used for immunohistochemical investigation

It was done on paraffin sections prepared from spleen of the control and treated groups using Caspase-III technique for immunohistochemical detection of apoptosis Ibrahim et al., 2015)

2.7. Methods used for histopathological investigation

Histopathological examination of spleen taken from the control and treated groups at the end of exposure duration (60 days) was done according to [5].

2.8. Detection of the splenocyte apoptosis using TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed according to the method described by [6] using fluorometric TUNEL kit. Apoptotic cells displaying green fluorescence (due to the catalytic incorporation of fluorescein-12-dUTP at 390H DNA ends) were determined as TUNEL positive. The positive apoptotic splenocytes were counted in five high microscopic fields (X, 400)/group. The percentage of positive apoptotic splenocytes was then calculated.

2.9. Methods used for statistical analysis

Data were expressed as a mean ± standard error (SEM). Statistical analysis was performed using SPSS software program version 16 (SPSS Inc., USA). One way analysis of variance (ANOVA) was used to assess significant differences among different groups at P < 0.05.

3. Results

3.1. Effects on the blood proteins level

The effects of Gesaprim (ATR) (2475 ppm via food) and/or Akropower (1 ml/L D.W.) daily exposure for 60 days on blood proteins level of treated rabbits were presented in Table 1. The results revealed that oral exposure to Atrazine induced a significant decrease in serum total protein and albumin levels compared to the control. However, oral treatment with Akropower induced an insignificant increase in the

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>Control</th>
<th>Atrazine</th>
<th>Akropower</th>
<th>Akropower plus</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/G ratio</td>
<td></td>
<td>2.06 ± 0.22a</td>
<td>2.09 ± 0.23a</td>
<td>5.91 ± 0.24a</td>
<td>8.68 ± 0.29a</td>
</tr>
<tr>
<td>Globulin</td>
<td></td>
<td>1.76 ± 0.63a</td>
<td>2.34 ± 0.28b</td>
<td>4.12 ± 0.56b</td>
<td>6.46 ± 0.42b</td>
</tr>
<tr>
<td>Albumin</td>
<td></td>
<td>1.78 ± 0.19a</td>
<td>3.11 ± 0.11c</td>
<td>5.55 ± 0.52a</td>
<td>8.39 ± 0.47b</td>
</tr>
<tr>
<td>Total protein</td>
<td></td>
<td>1.87 ± 0.15a</td>
<td>2.64 ± 0.19bc</td>
<td>5.31 ± 0.33a</td>
<td>8.1 ± 0.41a</td>
</tr>
</tbody>
</table>

All values are presented as mean ± SE (n = 10 rabbits/group). Values with different letters within the same column are significantly different at P ≤ 0.05 with One-way ANOVA and Turkey’s HSD Post hoc test.)
serum total protein, albumin, globulin levels and A/G ratio compared to the control. Oral Atrazine plus Akropower co-treatment induced a significant increase in serum total protein and albumin levels compared to Atrazine-exposed animals.

3.2. Effects on RHDV serum antibody titers

The effects of Atrazine and/or Akropower daily exposure for 60 days on RHDV antibody titers determined by HI after the application of HA tests were presented in Table 2. The results revealed that oral exposure to Atrazine induced a significant decrease in the RHDV antibody titer only after four weeks of vaccination compared to the control. However, Akropower and Atrazine plus Akropower treatment induced an insignificant increase in its titer after zero, two and four weeks of vaccination compared to the control and Atrazine-exposed groups, respectively.

3.3. The effects of atrazine and/or akropower on the level of spleen fas and caspase-III genes expression

Oral exposure to Atrazine resulted in a significant increase in the level of spleen Fas (11.6 folds) and Caspase-III (23.3 folds) genes expression compared to control. However, Akropower treatment induced an insignificant increase in the expression level of Fas (1.83 folds) gene expression and an insignificant decrease in the level of Caspase-III (0.95 folds) gene expression compared to control. On the other hand, oral exposure to Atrazine plus Akropower induced a significant decrease in the level of spleen Fas (5.36 folds) and Caspase-III (10.56 folds) genes expression compared to the Atrazine alone-exposed group (Table 3).

Table 2
RHDV antibody titer* at zero, two and four weeks of vaccination of control; Atrazine (2475 ppm in food) and/or Akropower (1 ml/L in D.W.)-exposed rabbits for 60 days.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>4th week</th>
<th>2nd week</th>
<th>Zero</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.6 ± 0.4a</td>
<td>4.40 ± 0.4a</td>
<td>0.4 ± 0.2a</td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>5 ± 0.7b</td>
<td>4 ± 0.7a</td>
<td>0.2 ± 0.2a</td>
<td></td>
</tr>
<tr>
<td>Akropower</td>
<td>6.8 ± 0.8b</td>
<td>4.8 ± 0.8a</td>
<td>0.4 ± 0.2a</td>
<td></td>
</tr>
<tr>
<td>Atrazine plus Akropower</td>
<td>5.8 ± 0.4ab</td>
<td>4.2 ± 0.5a</td>
<td>0.2 ± 0.2a</td>
<td></td>
</tr>
</tbody>
</table>

Values are presented mean ± SE (n = 10 rabbits/group).

*HI antibody titre is the reciprocal of the highest dilution of serum at which no HA was observed.

Values with different letters within the same column are significantly different at P ≤ 0.05 with One-way ANOVA and Turkey’s HSD Post hoc test.

Table 3
The level of spleen Fas and Caspase-III genes expression of Atrazine (2475 ppm in food) and/or Akropower (1 ml/L in D.W.)-exposed rabbits for 60 days.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Groups</th>
<th>Relative Quantitation (RQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fas</td>
<td>Atrazine</td>
<td>11.6 ± 1.4a</td>
</tr>
<tr>
<td></td>
<td>Akropower</td>
<td>1.83 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>Atrazine plus Akropower</td>
<td>5.36 ± 1.1a</td>
</tr>
<tr>
<td>Caspase-III</td>
<td>Atrazine</td>
<td>23.3 ± 1.3a</td>
</tr>
<tr>
<td></td>
<td>Akropower</td>
<td>0.95 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>Atrazine plus Akropower</td>
<td>10.56 ± 1.3a</td>
</tr>
</tbody>
</table>

All values are presented mean ± SE (n = 3 rabbits/group).

A: Significantly different from control group at P < 0.05.

Fig. 1. Spleen's photomicrographs (By immune-histo-chemistry, caspase-III technique) of: (a) A control rabbit showing no apoptosis (X 400); (b) An atrazine-exposed rabbit showing apoptosis (X 400); (c) An akropower alone-treated rabbit showing no apoptosis (X 400); (d) An atrazine plus Akropower-treated rabbit showing no apoptosis (X 1000).
3.4. Immunohistochemical results

Immunohistochemical examination by caspase-III technique revealed no apoptosis in the spleen of control; Akropower and Akropower plus Atrazine treated rabbit groups Fig. 1a, c, d, while the spleen of Atrazine exposed rabbits showed a marked level of apoptosis Fig. 1b.

3.5. Histopathological results

The observed splenic histopathological changes in rabbits exposed to Atrazine; Akropower alone and Akropower plus Atrazine compared to control are shown in Fig. 2. No pathological changes could be seen in the spleen of control rabbits (Fig. 2a). The spleen of Atrazine- exposed rabbits showed severe depletion of lymphoid follicles (In the white pulp) (Fig. 2b). On the other hand, a hyperplasic activity of lymphoid follicles (Fig. 2c) was seen in the spleen of Akropower alone- treated rabbits. The spleen of Atrazine plus Akropower treated group showed slight depletion of lymphoid follicles (Fig. 2d).

3.6. TUNEL assay results

The effects of Atrazine and/or Akropower on the incidence of positive apoptotic splenocytes were shown in Table 4 and Fig 3.

The results revealed that the number of positive apoptotic splenocytes was significantly increased (13 ± 0.09) in Atrazine- exposed animals compared to control. However, the number of these positive apoptotic splenocytes was significantly reduced (5.2 ± 0.06) in Atrazine plus Akropower — treated rabbits compared to Atrazine alone-exposed ones.

4. Discussion

In the current work, we aimed at elucidating the genotoxic mechanisms required in humoral immunotoxicity of ATR in rabbits and exploring the potential ameliorating role of Akropower against such toxic effects.

Atrazine exposure induced a significant decrease in serum total protein and albumin levels compared to the control. This may be attributed to the hepatotoxic effects of Atrazine which were reflected on protein synthesis by the liver. Similarly, [15] reported Atrazine- induced significant decrease in serum total proteins and serum albumin in mature male Japanese quail. The recorded data indicated that oral Atrazine plus Akropower co-treatment induced a significant increase in serum total protein and albumin levels compared to the Atrazine-exposed animals. In agreement with these results, [32] approved the...
ameliorative effect of glycyrrhetinic acid (one of Akropower components) against the Acetaminophen-induced hepatic injury in rats.

Atrazine induced a significant decrease in the RHDV antibody four weeks after vaccination compared to the control. A significantly decreased IgM antibody response in Atrazine exposed rats was detected by [24]. On the other hand, Atrazine plus Akropower treatment increased the RHDV antibody titers after zero, two and four weeks of vaccination compared to the control and Atrazine exposed groups, respectively. Similarly, [4] reported that ascorbic acid (one of Akropower components) supplementation at 1000 ppm in chickens diet for 30 days improved the antibody response to IBD vaccine.

Atrazine exposure, in our investigation, induced apoptosis in splenocytes of exposed rabbits as shown in the immunohistochemical results by caspase-III technique. Several apoptotic pathways have been defined, including the extrinsic (death receptor) pathway, the intrinsic (mitochondrial) pathway, and the perforin/granzyme pathway. The extrinsic pathway mainly induces apoptosis via Fas/FasL-mediated caspase-8 activation [10]). The level of Fas expression was increased by ATR treatment, indicating that ATR may mediate the extrinsic pathway. [29] reported an increased expression level of Bax and Caspase-9, and down-regulated levels of tyrosine hydroxylase (TH), Bcl-xl and Bcl-2. These findings indicated that ATR may induce apoptosis-related changes. Atrazine elicited an immunotoxic effect by inducing endoplasmic reticulum stress causing apoptosis in T-cells. This provided an evidence for the molecular mechanism by which ATR induces dysregulation of the immune system [18]). ATR caused a significant decrease in spleen and thymus weight, degenerative micromorphology which is indicative for apoptosis in splenocytes. The percentage of apoptotic lymphocytes increased in a dose-dependent manner after ATR treatment [34]). We found that the spleen of Atrazine- exposed rabbits showed severe depletion of lymphoid follicles (white pulp). Similarly, ATR exposure showed depletion in the total number of spleen cells and the spleen cellular composition [11]). Moreover, the spleens appeared atrophic and were characterized by effacement of germinal centers, diminution of white pulp, and congestion of red pulp following ATR administration [35]). Atrazine exposure of rats at 200 mg/kg ATR for 48 days resulted in increased DNA disintegrity, chromatin de-condensation with increased apoptosis using TUNEL assay [3]).

Oral treatment with Atrazine for 60 days resulted in splenocytes apoptosis with concomitant over expressions of spleen Fas and Caspase-III genes mRNAs (apoptosis markers). The up-regulation of Fas and Caspase-III genes in these cells suggested activation of the Fas-mediated pathway. The activation of Fas led to the recruitment of FADD (Fas-associated protein with death domain) and procaspase-8. Caspase-8 activation is a complex process occurring via autocatalysis. The active caspase-8 cleaves caspase-III, that Atrazine significantly upregulated its gene [17]. However, Akropower co-treatment with Atrazine resulted in down-regulation of Fas and caspase-III genes. This indicated that Akropower provided an effective protection for the splenocytes against apoptosis by an extrinsic pathway which initiated by activation of Fas/
FasL pathway. In coincidence with these results, [1] studied the protective role of 18β-Glycyrrhetinic acid (50 and 100 mg/kg) (one of Akpower components) on methotrexate-induced injury in rats through attenuation of oxidative and nitrosative stresses mitigated inflammation as well as enhancement of the antioxidant defenses and prevention of apoptosis. The Fas-mediated, Caspase-8 dependent pathway led to activation of effector caspase-III followed by cleavage of its downstream target poly [ADP-ribose] polymerase 1 (PARP1) which execute DNA fragmentation. Consistently, [14] recorded Atrazine induced DNA double-strand breaks and triggering the DNA damage response in normal human breast epithelial cells (MCF-10A). ATR treatment resulted in activation of caspase-III, releasing its 17 kDa active fragment which further cleaved full-length PARP1 to its inactive 89 kDa fragment thereby, reducing the DNA repair function of PARP1 [27]. Akpower inhibited the anti-Fas antibody-induced apoptosis [22] through the down-regulation of caspase-III and inhibition of cytochrome C release from mitochondria into the cytoplasm [30].

Apoptosis is the real type of immune cell death and is required for homeostasis of lymphocyte numbers taking after the development of an immune response to a xenobiotic [25]). However reduction in lymphoid tissues has been associated with increased apoptosis indicating dysfunctional cell-mediated immunity [23]).

5. Conclusion

It is concluded that the recorded Atrazine-induced immune toxicity in rabbits may be related to its genotoxic activity where it enhanced Fas and Caspase-III genes expression levels. This is considered a fundamental part of the outward pathway of cell apoptosis, prompting apoptosis in splenocytes and lymphocytes. Thus, the upregulation of these genes has an essential role in the extrinsic pathway of cell apoptosis and Caspase-III genes expression levels. This is considered a fundamental part of the outward pathway of cell apoptosis, prompting apoptosis in splenocytes and lymphocytes. Thus, the upregulation of these genes has an essential role in the extrinsic pathway of cell apoptosis and lymphocytes depletion that was reflected as a humoral immunosuppression. On the other hand, Akpower elicited anti-apoptotic activity by downregulating these two genes, thereby protected splenocytes from ATR-induced apoptosis.

References


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