Original Article

Immunotoxicity of Acrylamide in Female BALB/c Mice*

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Abstract

Objective To investigate the immunotoxicity of acrylamide (ACR) in female BALB/c mice.

Methods A total of 200 female mice weighing 18-22 g were randomly divided into four clusters based on body weight, and each weight-based cluster included five groups (10 mice per group): negative control, positive control (cyclophosphamide), low, intermediate, and high dose ACR groups, and all the groups were administered ACR by gavage for 30 days. At the end of the study, the immunotoxicological effects of the ACR were evaluated through immunopathology, humoral immunity, cellular immunity, and non-specific immunity.

Results The terminal body weight, spleen and thymus weights, lymphocyte counts in the ACR-H group were decreased, pathological changes were observed in lymph glands, thymus and spleen. %T cells in blood lymphocytes were significantly increased in all ACR-treated groups, and a significant reduction of % natural killer(NK) cells and increase of %Th cells were observed in the ACR-H group. interleukin-6(IL-6), Concanavalin A(ConA)-induced splenocyte proliferation and serum half hemolysis value (HC_{50}) were also significantly suppressed in the ACR-H group.

Conclusion ACR elicited an inhibitory effect on cellular and humoral immunity of mice after 30 day feeding.

Key words: Acrylamide(ACR); Immunotoxicity; Female BALB/c mice

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INTRODUCTION

Neo-formed contaminants (NFCs) are compounds produced during heating or preservation processes, exhibiting potential harmful effects to humans[1]. Acrylamide (ACR) has been added to the list of NFCs since 2002 when the Swedish National Food Administration identified relevant amount of acrylamide in several heat processing, carbohydrate-rich foods, such as potato chips and crisps, coffee and bread[2]. The identification of ACR in food immediately raised public health concern and attracted great interest in ACR among researchers. Many studies indicated that acrylamide was neurotoxic to animals and humans and it was a reproductive toxicant, germ-cell mutagen and carcinogen in rodents[3-5]. The neurotoxicity of acrylamide in humans has also been well established following occupational or accidental exposures, while experimental studies have shown its adverse reproductive effects in animals[6].

Exposure to ACR and its toxic effects have been

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evaluated by the International Agency for Research on Cancer (IARC) and by WHO/JECFA[7]. IARC has classified acrylamide as 'probably carcinogenic to humans'. According to Dybing et al., six people in every ten thousand people will develop cancer as a result of ingesting acrylamide in foodstuffs[8]. In rats, increased cases of thyroid and mammary gland tumors and scrotal mesothelioma have been noted. In humans, increased risks of renal, ovarian, endometrial, and breast cancers and oral cavity cancer in non-smoking women have been reported[9]. As a harmful food component, ACR causes structural damage to various systems and organs and also leads to functional failure due to its direct toxic tissue effects. However, intensive studies have focused on the carcinogenicity of ACR, and its immunotoxic potential has rarely been elucidated.

In fact, the immunotoxic potential of ACR has been reported in a number of previous studies. For instance, Zaidi et al. demonstrated that ACR induced immunotoxicity via producing a significant decrease in the weight of spleen, thymus, and mesenteric lymph nodes in rats[10]. Y. Yener et al. demonstrated that toxicity of orally administrated ACR appeared to decrease alpha-naphthyl acetate esterase(ANAE)-positive lymphocytes counts and caused histopathological lesions in a dose-dependent manner in ileal Peyer's patches(IPPs); moreover ACR was shown to have toxic effect on immune system[11]. To elucidate the immunotoxicity of ACR, we conducted a comprehensive investigation of its immunomodulatory effect on female BALB/c mice by performing a panel of immune assays for a longer duration of 30 d. Meanwhile, cyclophosphamide (CP), a known immunosuppressant, served as a positive control as recommended by the immunotoxicity guideline of the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency (OPPTS 880.3550, U.S.EPA)[12]. Cell-mediated responses, humoral immune system, and non-specific immune capability after a 30-Day sub-acute exposure were evaluated. In the meanwhile, phenotyping of peripheral blood lymphocytes and serum cytokine levels were determined.

MATERIALS AND METHODS

Animals and Reagents

Female BALB/c Mice (age 6 to 8 weeks; weight 18 to 22 g) of Specific Pathogen Free (SPF) grade were purchased from Beijing HFK Bioscience Co., Ltd. [Certificate number: SCNK (CHUAN) 2008-29]. The animals were housed with five mice per cage with free access to standard laboratory diet and water under the following conditions: room temperature, 20 °C to 25 °C; relative humidity, 40% to 70%; and light/dark cycle, 12 h intervals.

Acrylamide (CAS No: 79-06-1, ≥98% purity) was purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA). Cyclophosphamide (CP) was purchased from Hengrui Medicine Co., Ltd. (Jiangsu, China). Sheep red blood cells (SRBC) were obtained from Beijing Laboratory Biology Technology Co., Ltd. (Beijing, China). FITC hamster anti-mouse CD3e, APC rat anti-mouse CD19, PE rat anti-mouse CD49b, APC rat anti-mouse CD4, PE rat anti-mouse CD8a, lysing buffer and Mouse CBA flex sets(IL-2, IL-4, IL-6, IFN-γ, and TNF) were purchased from Becton, Dicknson and Company (Franklin Lakes, NJ, USA). Mouse IgG ELISA, Mouse IgA ELISA and Mouse IgM ELISA kits were purchased from GenWay Biotech, Inc. (San Diego, CA, USA). Concanavalin A (ConA) and Lipopolysaccharide (LPS) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Thiazoyl blue tetrazolium bromide (MTT) was from Amresco LLC (Solon, OH, USA). RPMI 1640 medium, Hanks' balanced salt solution (HBSS), fetal bovine serum, phosphate buffered saline (PBS) and penicillin-streptomycin solution were from Thermo Fisher Scientific Inc (Logan, UT, USA). All of the other chemicals used were of analytical grade or above.

Dosage and Treatment Procedures

The acute oral toxicity experiment of acrylamide on female BALB/c mice, was carried out to determine the LD_{50} of acrylamide. According to the experiment result, the LD_{50}=147 mg/kg bw of female BALB/c mice was determined. One fourth of the LD_{50}e was determined as the high dose of acrylamide (36 g/kg bw). Then one third of the high dose was determined as the medium dose of acrylamide (12 mg/kg bw), and one third of the medium dose was determined as the low dose of acrylamide (4 mg/kg bw).

After acclimation for 7 d, 200 mice were randomly divided into four clusters based on body weight, and each weight cluster included five groups (10 mice per group): (1) negative control group [0.2 mL/kg body weight (bw) distilled water]; (2) positive control group [0.2 mL/kg body weight (bw) distilled water, administered with 200 mg/kg of CP by intraperitoneal injection 24 h before the

termination of the study][13-15]; (3) low-dose ACR group (ACR-L; 4 mg/kg bw of ACR dissolved in distilled water); (4) medium dose ACR group (ACR-M; 12 mg/kg bw of ACR dissolved in distilled water); and (5) high-dose ACR group (ACR-H; 36 mg/kg bw of ACR dissolved in distilled water). The total exposure duration was 30 consecutive days. The mice of five groups were weighted once every week to adjust the intragastric volume.

The study was approved by the Animal Ethics Committee of National Institute for Nutrition and Food Safety, Chinese Center for Disease Control and Prevention. Animals used were cared for in accordance with the guidelines established by the Association for Assessment and Accreditation of Laboratory Animal Care (ACRALAC). The study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Chinese Center for Disease Control and Prevention. All of the animals received humane care according to the criteria outlined in the Guide for the Care and Use of the Animal Management Rules of the Ministry of Health, the People's Republic of China (documentation Number 55, 217 2001, China). At termination, all animals were anaesthetized by carbon dioxide inhalation and killed by exsanguinations.

Organ/body Weight and Hematological Parameters

The mice were sacrificed after the total exposure duration of the treatment. Body and organ weights of the spleen and the thymus were determined for each animal. The organ index was calculated as percentage of terminal body weight. For haematological studies, whole blood was collected from the retro-orbital plexus of each mouse in the presence of anticoagulant, then white blood cell count (WBC) was measured with a COULTER Ac·Tdiff2 Hematology Analyser (Beckman Coulter Inc., USA).

Each animal was observed twice daily for abnormalities, physical appearance and mortality. Body weight of mice was measured immediately prior to dosing, weekly thereafter, and at the end of the experiment.

Phenotyping of Peripheral blood lymphocytes

Whole blood was collected from the retro-orbital plexus of each animal in the presence of anticoagulant. 50 μL blood cell suspensions were stained with three-color combinations of antibodies of FITC hamster anti-mouse CD_3e, APC rat anti-mouse CD_{19} and PE rat anti-mouse $CD_{49}b$ or antibodies of FITC hamster anti-mouse CD_3e, APC rat anti-mouse CD_4 and PE rat anti-mouse CD_8 for 20 min at room temperature in the dark, then the blood samples were lysed with 2 mL of ammonium chloride-based lysing buffer for 20 min at room temperature in the dark and washed with 2 mL of PBS. Subsequently, the samples were resuspended in 0.5 mL of PBS and analyzed on FACSCalibur flow cytometer using CellQuest software (Becton, Dicknson and Company, USA)[16-18]. Appropriate isotype controls were used for compensation controls.

Serum Cytokine Quantification

Whole blood was collected from retro-orbital plexus of each mouse without the addition of anticoagulant. Following centrifugation, the sera were obtained and assayed for levels of cytokine including IL-2, IL-4, IL-6, IFN-γ, and TNF by Mouse CBA Flex sets. The assay was performed as described in the manufacture's instruction. Briefly, 50 μL mixed capture beads were added into each assay tube, and 50 μL standards, blank or serum samples were added into the appropriate tubes, then PE detection reagent were added into each tube and incubated for 2 h at room temperature. The samples were washed with 1 mL wash buffer, then resuspended in 300 μL wash buffer and analyzed on FACS Calibur flow cytometer using CellQuest software.

Serum Immunoglobulin Quantification

The sera were obtained as described in the part of Serum Cytokine Quantification and assayed for concentrations of immunoglobulin including IgG, IgM, and IgA by ELISA kits. The assay procedures were in accordance with descriptions in the manufacturer's instructions. Briefly, 100 μL standards, blank or diluted serum samples were added into predesignated wells of 96-well plates in duplicate, the microtiter plates were covered and incubated at room temperature for 1 h, then washed 4 times with wash solution. 100 μL of enzyme-antibody conjugate was added into each well, and the plates were covered and incubated at room temperature for 30 min, then washed 4 times with wash solution. Subsequently, 100 μL of TMB substrate solution was added into each well, and incubated at room temperature for 10 min, finally, 100 μL of stop solution was added into each well, and the absorbance (450 nm) of the contents of each well was determined using an ELISA Reader (BioTek,

USA).

Hemolysis Test

Mice were immunized on day 25 with 0.2 mL of 2% (v/v) SRBC suspension in sterile saline via intraperitoneal injection. Five days post-immunization, whole blood was collected from retro-orbital plexus of each mouse without the addition of anticoagulant. Following centrifugation, the sera were obtained and assayed for HC_{50}. 1 mL of SA buffer solution, 0.5 mL of 10% (v/v) SRBC, 1 mL of diluted guinea pig complement (1:8 diluted with SA buffer solution) and 2 μL of mouse serum were added into sample tubes. Control tube without mouse serum was set at the same time. All tubes were kept in water bath for 15-30 min at 37 °C, and then the tubes were kept in ice bath to terminate the reaction. After centrifugation for 10 min at 2000 rpm, 1 mL of supernatant was collected and added into 3 mL of drabkin solution (1.0 g of $NaHCO_3$, 0.05 g of KCN and 0.2 g of $K_3Fe(CN)_6$ solved in 1000 mL stilled water), at the same time, 0.25 mL of 10% (v/v) SRBC and 3.75 mL of drabkin solution were mixed and standing for 10 min as positive control. The absorbance at 540 nm was detected, the HC_{50} value was got by the equation: $HC_{50}= (OD_1/OD_2)\times500$ (OD_1= the OD value of sample well subtract that of control well, OD_2=the OD value of positive control well subtract that of control well).

Preparation of Spleen Cell Suspensions

Single cell splenocyte suspensions were prepared as described in Technical Standards for Testing and Assessment of Health Food (2003) published by the National Ministry of Health. The spleens were aseptically removed and transferred to culture dishes containing 2 mL cold Hank's balanced salt solution (HBSS). Forceps and carbasus were used to finely triturate the spleen, and then the cell suspensions were transferred to tubes and washed twice in HBSS and centrifuged for 10 min at 1 000 rpm at 4 °C. The cell pellet was resuspended in appropriate culture medium for the assay to be performed. Spleen cell numbers were determined using a hemocytometer.

Plaque-forming Cell (PFC) Assay

The PFC assay was performed by a modified Jerne's method[19]. Mice were immunized on day 25 with 0.2 mL of 2% (v/v) SRBC suspension in sterile saline via intraperitoneal injection. Five days post-immunization, the mice were sacrificed and spleen cell suspensions were prepared as stated in the part of Preparation of Spleen Cell Suspensions. 25 μL of spleen cell suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution (5×10^6 cells/mL), 50 μL of 10% (v/v) SRBC in SA buffer solution (0.46 g of $C_4H_4N_2O_3$, 0.1 g of $MgCl_2\cdot6H_2O$, 0.2 g of $CaCl_2\cdot2H_2O$, 8.38 g of NaCl, 0.252 g of $NaHCO_3$ and 0.3 g of $C_8H_{11}N_2NaO_3$ solved in 1 000 mL stilled water) and 500 μL of agar solution (0.5 g/mL in HBSS, pH 7.2-7.4) were mixed in a glass tube, then poured onto slides. The slides were covered on a special frame after the mixtures were solidified, and incubated at 37 °C for 1-1.5 h, then guinea pig complement was added to the slot between the slides and bottom of the frame. The slides were incubated at 37 °C for another 1-1.5 h, then plaque production was enumerated and the results were expressed as the number of PFC per 10^6 splenocytes.

***Mitogen-induced Splenocyte Proliferation*[19]**

The mitogens used in this assay were ConA and LPS, which were dissolved in distilled water to reach a concentration of 100 μg/mL and 400 μg/mL. The mice were sacrificed on day 30 and spleen cell suspensions were prepared as stated in part of Preparation of Spleen Cell Suspension. 1 mL of spleen cell suspension (3×10^6 cells/mL) was added into 24-well plate and cultured with 75 μL of the mitogen solutions or distilled water for control cultures. The plates were incubated at 37 °C with 5% CO_2 for 68 h, then 0.7 mL supernatant in each well was discarded and 0.7 mL RPMI 1640 medium was added, in the meanwhile, 50 μL fresh prepared MTT solution (5 mg/mL, dissolved in PBS, pH 7.2) was added into each well, the plates were then incubated under the same conditions for an additional 4 h. Finally, 1 mL acid-isopropanol (4 mL of 1 mol/L HCl added to 96 mL of isopropanol) was added into each well, the contents in each well were mixed thoroughly to make the purple crystallize fully dissolved and transferred to 96-well plates in triplicate, the absorbance at 570 nm was determined, and the results were calculated by subtracting the OD value of control well from OD value of sample well.

Splenic Natural Killer (NK) Cell Activity

NK cell activity test was performed by lactate dehydrogenase-release assay[19-20]. 100 μL of spleen cell suspension (effector cells, 2×10^7 cells/mL) and 100 μL of YAC-1 cell suspension (target cells, 4×10^5 cells/mL) were added into 96-well round-bottom

plate and cultured. Spontaneous LDH release and total LDH release of target cells were determined by addition of culture solution and 2.5%Triton solution, respectively. All tests were performed in triplicate. The plates were incubated at 37 °C with 5% CO_2 for 4 h. The plates were centrifuged at 1 500 r/min for 5 min, then 100 μL of supernatant in each well was added into another 96-well flat-bottom plate, in the meanwhile, 100 μL of LDH freshly prepared LDH substrate solution (5×10^{-2} mol/L lithium lactate, 6.6×10^{-4} mol/L 2p-iodophenyl-3p-nitrophenyl tetrazolium chloride, 2.8×10^{-4} mol/L phenazine metosulphate, and 1.3×10^{-3} mol/L nicotineamide nucleotide NAD in 0.2 mol/L Tris-HCl buffer, pH 8.2) was added to each well. The plates were incubated light-protected at room temperature for 10 min, and the reaction was stopped by addition of 30 μL of 1 mol/L HCl/well. The absorbance at 490 nm was determined, and the results were calculated by the formula: %NK cell activity=[(E-S)/(M-S)]×100 (where E=experimental release of effector/target co-culture; S=spontaneous target cell LDH release; and M=total target cell LDH release).

Histopathology

All mice were humanely sacrificed at the end of the test, and a complete necropsy was performed. The liver, kidneys, spleen, thymus, femur, lymph glands including cervical lymph node, axillary lymph node, and mesenteric lymph nodes from all animals were fixed with 10% formal saline for 24 h, embedded in paraffin, sectioned, stained with hematoxylin and eosin for microscopic observation.

Statistical Analysis

All the results were presented as means±SD, the data were analyzed using the SPSS software (v11.5, Chicago, IL, USA). Comparisons between multiple groups were carried out using one-way ANOVA followed by Bonferroni post hoc test while equal variances assumed or Dunnett's T3 post hoc test while equal variances not assumed. Statistically significant differences between groups were defined as *P*-value <0.05.

RESULTS

Organ/Body Weight and Haematological Parameters

As shown in Table 1, CP in the positive control group induced a significant reduction in terminal body weight, spleen weight and thymus weight of mice when compared with animals in the negative control group. The terminal body weight, spleen weight and thymus weight of mice in the ACR-H group were significantly lower than those in the negative control group, Table 2 shows that the mean WBC value and LYM count of mice in the CP group or the positive control group was lower than that in the negative control group, and the LYM count of mice in the ACR-H group were significantly lower than that in the negative control group.

Phenotypic Analysis of Peripheral Blood Lymphocytes

CP in the positive control group induced a significant increase in percentage of T lymphocytes ($CD_3^+CD_{19}^-$), CD_4^+ T lymphocytes (CD_3^+ CD_4^+, Th cells), and CD_4^+/CD_8^+ (Th/Ts) ratio when compared with the negative control group, and a reduction in percentage of B lymphocytes (CD_3^- CD_{19}^+) was observed compared with negative control group. All ACR-treated groups revealed a significant increase in

Table 1. Effect of Acrylamide on Terminal Body Weight, Absolute, and Relative Organ Weights of Mice (mean±SD) (*n*=10)

Group	Initial Body Weight (g)	Terminal BodyWeight (g)	Spleen Weight		Thymus Weight	
			Absolute (g)	Relative (%)	Absolute (g)	Relative (%)
Negative Control	20.2±0.7	20.1±0.8	0.092±0.008	0.458±0.038	0.049±0.006	0.245±0.032
ACR-L	21.4±1.2	20.7±0.7	0.103±0.022	0.496±0.094	0.052±0.007	0.252±0.033
ACR-M	20.0±1.4	20.3±1.3	0.102±0.014	0.505±0.055	0.049±0.010	0.244±0.048
ACR-H	20.3±0.9	16.3±1.8*	0.052±0.017*	0.316±0.086*	0.036±0.011*	0.199±0.086
Positive Control or CP	20.3±1.1	18.9±1.3*	0.044±0.005*	0.220±0.028*	0.030±0.007*	0.170±0.058*

Note. *P<0.05 as compared with negative control group. Relative weight(%)=organ weight/terminal body weight×100%.

percentage of T lymphocytes ($CD_3^+CD_{19}^-$) when compared with the negative control group, and the percentage of CD_4^+ T lymphocytes (CD_3^+ CD_4^+, Th cells) in the ACR-H group were significantly increased compared with that in the negative control group. The percentage of NK cells ($CD_3^-CD_{49}^+$) in all ACR-treated groups were significant decreased compared with that in the negative control group (Table 3).

Serum Cytokine Levels and Serum Immunoglobulin

A significant reduction in serum IL-6 level was observed in the CP and ACR-H groups when compared with that in the negative control group, while no significant differences were found in serum IL-2, IL-4, IFN-γ, or TNF level in all ACR-treated groups when compared with those in the negative control group. Table 5 shows that the IgM and IgG level in the CP or positive control group was significantly decreased compared with that in the negative control group. There were no significant changes in IgM, IgG, or IgA level in all ACR-treated groups when compared with those in the negative control group (Table 4).

Table 2. Effect of Acrylamide on Hematology Values of Mice (mean±SD) (*n*=10)

Group	WBC ($\times10^9$/L)	LYM ($\times10^9$/L)	MON ($\times10^9$/L)	GRN ($\times10^9$/L)
Negative Control	6.13±0.84	5.11±0.68	0.21±0.07	0.80±0.48
ACR-L	7.10±2.91	4.66±2.05	0.27±0.52	2.13±1.18
ACR-M	7.20±2.06	5.49±2.11	0.27±0.49	1.44±0.39
ACR-H	4.63±1.39	3.05±1.19*	0.25±0.53	1.32±0.58
Positive Control or CP	2.86±1.23*	0.31±0.07*	0.21±0.09	1.98±1.15

Note. *P<0.05 as compared with negative control group.

Table 3. Effect of Acrylamide on Phenotypic Analysis of Peripheral Blood Lymphocytes of Mice (mean±SD) (*n*=10)

Group	$CD_3^+CD_{19}^-$ (%)	$CD_3^-CD_{19}^+$ (%)	$CD_3^-CD_{49}^+$ (%)	$CD_3^+CD_4^+$ (%)	$CD_3^+CD_8^+$ (%)	CD_4^+/CD_8^+
Negative Control	48.62±10.62	31.06±10.70	17.13±6.86	40.48±8.85	14.08±3.10	2.91±0.28
ACR-L	63.30±6.40*	26.55±6.77	7.60±1.39*	45.68±6.44	13.55±1.10	3.39±0.53
ACR-M	63.65±5.39*	25.40±4.50	8.04±2.18*	42.40±2.90	15.43±2.29	2.78±0.32
ACR-H	70.98±3.88*	21.24±4.12	6.07±1.29*	50.06±9.77*	15.22±3.01	3.30±0.32
Positive Control or CP	68.86±13.79*	4.19±1.97*	20.28±15.26	60.27±6.83*	15.64±2.97	3.94±0.64*

Note. *P<0.05 as compared with the negative control group.

Table 4. Effect of Acrylamide on Serum Cytokine Levels of Mice (mean±SD) (*n*=10)

Group	IL-2 (pg/mL)	IL-4 (pg/mL)	IL-6 (pg/mL)	TNF (pg/mL)	IFN-γ (pg/mL)
Negative Control	8.01±4.17	4.79±0.79	47.89±20.33	28.16±18.31	3.70±3.59
ACR-L	13.35±10.10	4.68±1.51	40.83±17.52	44.10±34.04	15.40±7.36
ACR-M	15.03±8.50	5.56±1.78	48.55±12.14	52.33±18.16	12.07±5.29
ACR-H	9.27±5.21	5.40±3.07	18.63±12.14*	18.48±18.30	8.48±4.44
Positive Control or CP	7.32±4.41	6.70±4.82	24.54±13.66*	14.93±9.97	3.18±1.57

Note. *P<0.05 as compared with the negative control group.

Immune Function Responses

The PFC assay and hemolysis test were commonly used to detect humoral immunity, the mitogen-induced splenocyte proliferation were commonly used to detect cellular immunity, and NK cell activity test was commonly used to detect non-specific immunity. A single dose of CP (200 mg/kg) induced a significant reduction in PFC/10^6 splenocytes, HC_{50}, ConA-induced splenocyte proliferation and LPS-induced splenocyte proliferation when compared with the negative control group. In the ACR-H group, HC_{50} and the ConA-induced splenocyte proliferation were significantly decreased compared with the negative control group (Table 6).

Histopathology

Histological examinations were performed on all the preserved organs, and pathological changes were observed in the CP and ACR-H group, which included mild atrophy of thymus (10/10 in the CP group, and 5/10 in the ACR-H group) and decrease in the number of bone marrow (hematopoietic) stem cells (10/10 in the CP group and 0/10 in the ACR-H group). Several lymphoid nodules in germinal center shrank or disappeared in spleen, and there were white pulp atrophy of spleen (10/10 in the CP group, 4/10 in the ACR-H group), lymphopenia and proliferation of fibrous tissue in lymph glands, unclearness or disappearance of follicle structure and atrophy of lymph glands (10/10 in the CP group, 3/10 in the ACR-H group). No obvious pathological changes of liver and kidney were found in CP the group or ACR-H group. Neither pathological changes of liver, kidney, spleen, thymus, mucous membrane of small intestine and lymph glands including cervical lymph nodes, axillary lymph nodes, mesenteric lymph nodes, and Peyer's patches were observed in the negative control group, ACR-M group or ACR- L group (Figure 1).

DISCUSSION

The immune system is highly sensitive to a variety of chemical and physical stressors and as such can be used as a tool to examine the subclinical effects of chemical exposure[21]. The purpose of the present study was to evaluate the immunotoxicological effect of ACR in female BALB/c mice in a span of 30 d. In this study, immunopathological parameters were examined, while the assays reflecting the function of humoral immunity, cellular immunity and non-specific immunity were conducted.

Table 5. Effect of Acrylamide on Serum Immunoglobulin of Mice (mean±SD) (*n*=10)

Group	IgG (μg/mL)	IgM (μg/mL)	IgA (μg/mL)
Negative Control	634.66±92.89	245.79±30.81	23.12±5.80
ACR-L	718.59±124.95	281.14±39.68	28.45±2.55
ACR-M	697.69±96.11	271.25±54.03	27.99±3.53
ACR-H	694.59±110.33	265.38±36.54	29.84±4.29
Positive Control or CP	447.10±85.59*	187.72±24.72*	20.76±1.35

Note. *$P<0.05$ as compared with the negative control group.

Table 6. Effects of Acrylamide on Immune Function Assays of Mice (mean±SD) (*n*=10)

Group	PFC (/10^6 cells)	HC_{50}	ConA-induced Splenocyte Proliferation	LPS-induced Splenocyte Proliferation	NK Cell Activity
Negative Control	32.6±11.2	26.77±1.08	0.83±0.29	0.42±0.13	8.51±4.75
ACR-L	21.3±8.8	24.98±4.66	1.72±0.55	0.36±0.14	7.11±3.77
ACR-M	40.4±24.6	26.79±2.23	0.82±0.37	0.46±0.34	11.82±5.81
ACR-H	24.8±13.0	23.08±1.34*	0.27±0.05*	0.40±0.14	8.38±3.87
Positive Control or CP	14.0±4.0*	23.23±1.01*	0.25±0.05*	0.17±0.06*	6.22±1.63

Note. *$P<0.05$ as compared with negative control group.

The treatment doses used in the present study were induced from our previous acute toxicity test (LD_{50} of ACR 147 mg/kg bw) for the female BALB/c mice. As a whole, a single dose of CP (200 mg/kg) induced a significant reduction of spleen weight and thymus weight, WBC value, LYM count, %B cells, serum IL-6 level, serum IgM and IgG, PFC/10^6 splenocytes, HC_{50} and mitogen-induced splenocyte proliferation, while a significant increase of %T cells, %Th cells and Th/Ts ratio was detected, and some histopathological changes in thymus, spleen, femur and lymph nodes were also observed. These findings indicated that the testing procedures and the experimental techniques presented here for immunotoxicity testing in female BALB/c mice were reliable and feasible.

The present study demonstrated that there were significant decreases in terminal body weight, spleen and thymus weights, LYM count in the ACR-H group when compared with mice in the negative control group. The histological examination revealed an adverse effect to immune organs in the ACR H group, indicating that high-dose ACR had a systemic toxicity to the female BALB/c mice and might compromise the immune function of BALB/c mice at a high dosage, which is in agreement with the studies of Zaidi and Y. Yenera[10-11].

Stability of lymphocyte subsets is necessary for maintaining normal immune function; it is also an important indicator to evaluate the cellular immunity. As regards the blood phenotyping test, the method is known as a streamlined procedure utilizing commercially available three-color 'cocktails' of immune cell markers[22]. Our study identified a significant increase of %T cells and a significant reduction of %NK cells in all ACR treated groups, and however, the percentage of %Th cells was significantly increased in the ACR H group, suggesting that ACR might possibly disturb the stability of lymphocyte subsets, and T cells and NK cells were more sensitive to the ACR treatment than B cells and that ACR might be toxic to cellular immunity of the mice. According to Y. Yenera, toxicity of orally administrated ACR appeared to decrease alpha-naphthyl acetate esterase(ANAE)-positive peripheral blood lymphocyte counts and caused histopathological lesions in a dose-dependent manner in ileal Peyer's patches in rat. Alpha-naphthyl acetate esterase (ANAE) is a lymphocyte lysosomal enzyme which is assumed to be responsible for the cytotoxic effects of T lymphocytes and the phagocytic activity of monocytes. The present study also revealed a toxicity of acrylamide to the T lymphocytes.

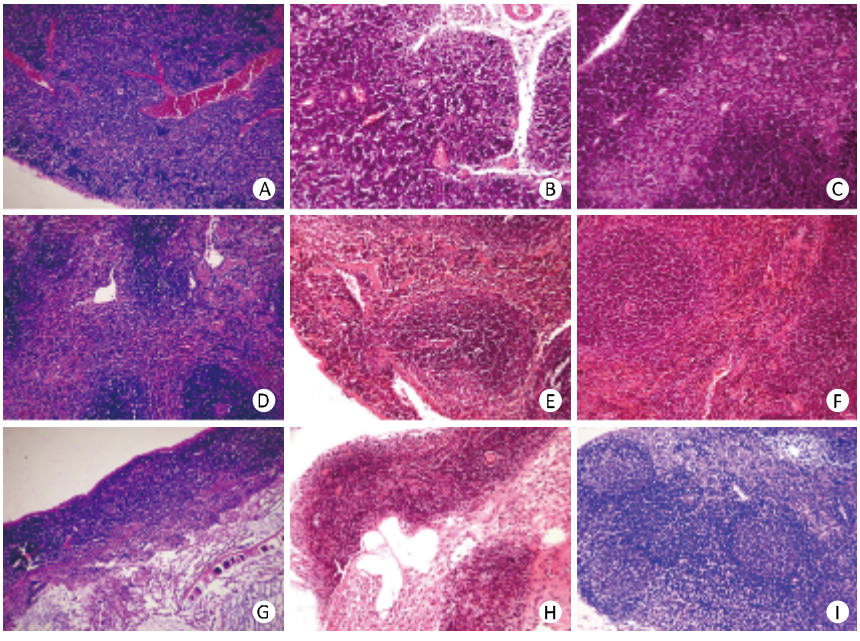

Figure 1. Photomicrographs of thymus (A-C, the CP group, the ACR-H group and the negative control group), spleen (D-F, the CP group, the ACR-H group and the negative control group) and lymph nodes (G-I, the CP group, the ACR-H group and the negative control group) tissues of mice stained with H&E ×100.

T and B lymphocyte is the most important marks of the specific immune function, and the serum cytokines and immunoglobulin secreted by immune cells are vital in modulating the immune response. It has been known that Th cells are further subdivided into Th1 cells and Th2 cells; the former is involved in cellular immunity, and the latter promotes humoral immune responses[23]. Immune responses are regulated by the secretion of Th1 and Th2-specific cytokines, including IL-2, IFN-γ, and IL-12 (Th1), as well as IL-4, IL-5, IL-6, IL-9, and IL-10 (Th2). IL-6 mainly mediates the humoral immunity, B cells activation and the production of antibodies. As shown in the present stufy, serum IL-6 level in the ACR-H group was significantly decreased compared with that in the negative control group, which indicates that ACR may have humroal immunity toxicity to the mice. For immune function assays, a significant reduction of ConA-induced splenocyte proliferation and HC_{50} was observed in the ACR-H group when compared with the negative control group, in combination with the result of blood phenotyping test and serum cytockines test, which demonstrated an obvious toxic effect of ACR to the cellular and humoral immune function.

In conclusion, ACR exerts immunotoxicological effects at certain dose range when feeding mice for 30 d. It can extensively compromise the immune system of female BALB/C mice, including cellular immunity and humoral immunity. ACR is a common occurrence in the environment, and consequently, exposure to ACR should be carefully monitored, and further studies are necessary to investigate the detailed mechanisms behind its immunotoxicity .

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