Original Article

DOI: 10.5582/bst.2013.v7.4.193

Sub-acute exposure to the herbicide atrazine suppresses cell immune functions in adolescent mice

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Summary **Atrazine (ATR), one of the most widely used herbicides worldwide, has caused a series of toxicological and environmental problems. This study sought to investigate the effects of ATR on the immune system of mice. Four-week-old female C57B l/6 mice were treated with 5, 25, and 125 mg/kg ATR for 28 days. On day 29, blood was collected and the spleen was harvested to detect lymphocyte transformation, natural killer (NK) cell activity, cellular phenotypes, and cytokines. Results indicated that the thymus and spleen weights decreased after ATR treatment, and the spleen was found to be more sensitive to ATR than the thymus. Decreases in lymphocyte transformation and NK cell activity were also observed in mice treated with 25 mg/kg ATR and 125 mg/kg ATR compared to the control group. In addition, there were also alterations of lymphocyte phenotypes in the spleen, and the percentages of CD3+ and CD4+ cells decreased in mice treated with 25 mg/kg ATR and 125 mg/kg ATR compared to the control group. Moreover, serum interleukin-4 level decreased significantly after treatment with 25 mg/kg and 125 mg/kg ATR, but ATR did not affect the expression of interleukin-2, interferon-γ, and tumor necrosis factor-α. These results suggest that ATR may have induced damage in spleen cells. As ATR is an environmental contaminant, its immunosuppressive action raises concerns that it may potentiate clinical conditions such as tumors, inflammation, and infections. Thus, it needs to be carefully monitored and studied.**

Keywords: Atrazine, sub-acute exposure, immunotoxic potential, adolescent mice

1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine, or ATR), is registered for the control of broadleaf weeds and certain grassy weeds. It has been used mostly to prevent weeds from growing in corn, sorghum, coffee, wheat, and sugarcane fields, conifer forests, Christmas tree farms, golf courses, and residential lawns (*1*). Since it first came on the market in 1958 in Switzerland, ATR has been one of the most commonly used herbicides worldwide (*2*). As a result, ATR has been found regularly in soil and surface moisture, where it tends to persist for months and migrate great distances from where it is used. ATR has even been found above the Arctic Circle, albeit at low concentrations (*3-5*). An estimated 2-3 million people who use groundwater as their primary drinking water are exposed to 0.2 ppb ATR.

The extensive use of ATR and its persistence underline the importance of understanding its general impacts on the environment. Many epidemiological studies have been performed, and some have indicated a possible correlation between atrazine exposure and an increased incidence of neoplastic diseases (*6*). Other studies have found that atrazine may act as an endocrine-disrupting compound (EDC) with effects on the central nervous system (*7*), endocrine system (*8*), and immune system (*9*).

Laboratory studies have revealed that exposure to environmental contaminants may suppress the amphibian immune system, rendering animals more susceptible to infection (*10-13*). Fatima found that herbicides at concentrations present in water in Europe caused

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immune suppression in goldfish (*11*). A meta-analysis reveals that ATR exposure consistently compromises the immune function of fish and amphibians (*5*). Brodkin *et al*. reported that in frogs ATR exposure suppressed the thioglycollate-stimulated recruitment of white blood cells to the peritoneal cavity compared to the background levels and also decreased the phagocytic activity of these cells (*10*). Langerveld *et al*. reported that ATR altered the expression of genes associated with growth and metabolism, proteolysis, fibrinogen complex formation, and immune regulation (*14*). A significant decrease in the number of intracelomatic cells and a significant decrease in the phagocytic index are observed after ATR exposure. In addition, silver catfish fingerlings exposed to ATR are more susceptible to intracelomatic challenge with the pathogen *Aeromonas hydrophila* (*15*).

Some studies have examined the immunotoxic potential of acute ATR exposure in adult animals. Exposure to ATR for 14 days can induce a significant decrease in the number of hematopoietic progenitors in murine bone marrow (*16*), cause a transient suppression of IgM production and T cell proliferation (*17*), decrease the CD4+ lymphocytes and MHC-II+ cells (*18*), and increase the mixed lymphocyte reaction (MLR) and cytotoxic T lymphocyte (CTL) response (*19*). Rajkovic *et al*. reported that ATR increased the number of degranulated mast cells of rats (*20*). Đikic *et al*. detected apoptotic cells in the thymus and lymph nodes of rats exposed to ATR (*21*).

Prenatal ATR exposure often causes substantially different effects in adult offspring from those noted in adult animals that are exposed (*22,23*). In one study, Rooney *et al*. orally administered ATR to Sprague-Dawley rats from day 10 of gestation to day 23 postnatally when pups were weaned. Two weeks after weaning, male pups that were inoculated with sheep red blood cells (SRBC) had a delayed hypersensitivity response that decreased in magnitude and a decreased IgM antibody response (*24*). Rowe *et al*. used time-release pellets to subcutaneously expose pregnant Balb/c dams to 700 μg/day of ATR for 21 days from day 10 and day 12 of pregnancy. At 3 months of age, male, but not female, mouse pups had increases in T cell proliferation, cytolytic activity, and the number of IgM-secreting B cells in the spleen, but there were no marked changes in the body weight, organ-to-body weight ratio of the spleen, and the numbers of CD8+ T cells, CD4+ T cells, and B220+ B cells in the spleen (*25*).

Previous studies described the toxic effects of atrazine on the adult and prenatal immune systems of animals. However, the immune system is still developing in the early postnatal period. The developing immune system is more sensitive to xenobiotic exposure than the adult immune system (*26-28*). A continuous remodeling of the primary and secondary immune organs occurs in mice throughout their lives, *i.e.*, a decline in the relative weight of the thymus, spleen, and lymph nodes occurs before puberty, while only the thymus continues to involute after puberty (*29*). One of the proposed windows of vulnerability for the developing rodent immune system occurs postnatally, which is from day 30 to sexual maturity (*30*). Combined with the limited immunotoxicity data in terms of age and with the possible impact and relevance of such data for immunotoxicological risk assessment, the current study sub-acutely exposed 4-week-old C57B l/6 mice to ATR for 28 days in order to assess the immunotoxic potential of ATR.

2. Materials and Methods

2.1. *Chemicals and reagents*

ATR (99% purity) and concanavalin A (ConA) were obtained from Sigma Chemical Company (St Louis, Missouri, USA). RPMI-1640 medium and fetal calf serum (FBS) were purchased from Gibco Laboratory (Gaithersburg, Maryland, USA). Interleukin-2 (IL-2), interleukin-4 (IL-4), interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α) enzyme-linked immunosorbent assay (ELISA) kits were from R&D Systems (Minneapolis, Minnesota, USA). CD3, CD4, and CD8 fluorescence-conjugated antibody were from Ebioscience (San Diego, California, USA). ATR solutions (0.5 mg/mL, 2.5 mg/mL, and 12.5 mg/mL) were prepared by dissolving ATR in corn oil. These solutions were kept at 4ºC for a maximum of 1 week.

2.2. *Animals and treatment*

Male mice housed three or more per cage often fight, and this may cause unpredictable stress responses that affect the immunological parameters being studied. Thus, 4-week-old pathogen-free female C57B l/6 mice were used in this study. Animals were purchased from the Experimental Animal Center of Norman Bethune Medical College, Jilin University (Changchun, China). This study was conducted in accordance with internationally recognized guidelines and approved by the Animal Research Committee of Norman Bethune College of Medicine, Jilin University. Animals were housed in groups of five in a temperature- and humidity-controlled environment and given access to food and water *ad libitum*. These animals were randomly divided into 4 groups by body weight (ten/group) and were orally given 0, 5, 25, or 125 mg/kg ATR for 28 consecutive days. Blood and the spleen and thymus were collected 24 h after the final round of ATR treatment.

2.3. *Body weight and organ weight*

Mice were weighed once every 7 days starting on day 1 after ATR treatment. The organs were collected aseptically and weighed on day 29. The relative weights

of organs were calculated as organ weight (mg)/body weight (g).

2.4. *Pathological examination*

The spleen was collected, fixed in 10% buffered formaldehyde, embedded in paraffin, and then sectioned. The sections were stained with hematoxylin-eosin for histological assessment.

2.5. *Preparation of splenocytes*

Suspensions of single cells from the spleen were prepared by gently passing the spleen through a nylon mesh filter. Cellular debris was removed with a 400-μm stainless steel mesh. Red blood cells were lysed with a hypotonic buffered solution and lymphocytes were washed with PBS and re-suspended in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Viable cells were counted using the trypan blue exclusion method with a hemocytometer.

2.6. *Lymphocyte transformation test (LTT)*

Freshly isolated splenocytes were suspended in complete RPMI-1640 medium supplemented with 10% FBS. The splenocytes were seeded into 6 wells in a 96-well plate (each well containing 2×10^4 cells). Cells in three wells were treated with PBS and served as controls, while cells in the remaining three wells were treated with 20 μg/mL ConA. After culturing in an incubator containing 5% CO_2 at 37ºC for 48 h, cell counting was done using a 3-(4,5-dimethylthiazol-2-yl) -2,5-diphe-nyltetrazolium bromide (MTT) assay. The optical density (OD) was quantified using a microplate reader. The following formula was used to calculate the stimulation index (SI): SI = OD of ConA-treated cells/OD of control cells.

2.7. *Natural killer (NK) cell cytotoxicity assay*

NK cells from mouse spleens were prepared as described above and used as effector cells. Mouse lymphoma YAC-1 cells, which are sensitive to NK cells, were used as target cells. Briefly, 1×10^4 YAC-1 cells and effector cells were separately added to a 96-well plate. After incubation for 48 h, NK cytotoxicity was assessed using the MTT assay described above. Cytotoxic activity was calculated using the following formula: NK cytotoxicity (%) = $[1 - (OD_{E+T} - OD_E)/OD_T \times 100\%]$. (E + T = a mixture of effector cells and target cells, E = effector cells, T = target cells).

2.8. *Flow cytometry*

For cell phenotype analysis, fluorescence-conjugated antibody was used in accordance with the manufacturer's instructions. Briefly, splenocytes were re-suspended in 100 μL of binding buffer followed by addition of 10 μL of anti-CD3-PE-Cy5, anti-CD4-FITC, or anti-CD8-PE. After incubation for 15 min at 37ºC, cells were washed thrice and then subjected to flow cytometry on the FAC Scan (Becton Dickinson Immuno-cytometry, San Jose, CA), in which 1×10^5 cells were counted. Analysis was performed using the CELL Quest software package (BDIS) in list mode and the lymphocyte gate as defined by forward/side scatter characteristics.

2.9. *Enzyme-linked immunosorbent assay (ELISA) of IL-2, IL-4, IFN-γ, and TNF-α*

After treatment with ATR for 28 days, animals were sacrificed and blood was collected. The serum was obtained by centrifugation at 3,000 g × for 10 min. The serum levels of murine IL-2, IL-4, IFN-γ, and TNF-α were detected with corresponding ELISA kits in accordance with the manufacturer's instructions.

2.10. *Statistical analysis*

Data were analyzed using the Statistical Package for Social Sciences (SPSS) version 10. Data were expressed as means ± standard deviations (S.D.). Comparisons among multiple groups were done using two-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. *General state and body weight*

All animals survived to the end of study. There were no overt changes in appearance and/or behaviors and no significant differences in body weight (Figure 1A) after ATR exposure.

3.2. *Toxic effects of ATR on the spleen*

The mean weights of the spleen and thymus decreased in mice treated with 25 mg/kg ATR and 125 mg/kg ATR ($p < 0.05$) compared to the control group (Figure 1B). Histopathological examination of the spleen revealed degenerative changes. The spleens appeared atrophic in mice treated with 25 mg/kg ATR and 125 mg/kg ATR and were characterized by effacement of germinal centers, diminution of white pulp, and congestion of red pulp (Figure 1C). There were no significant changes in mice treated with 5 mg/kg ATR in comparison to the control group.

3.3. *Lymphocyte transformation*

An MTT assay was performed to evaluate splenic lymphocyte proliferation in mice treated with different

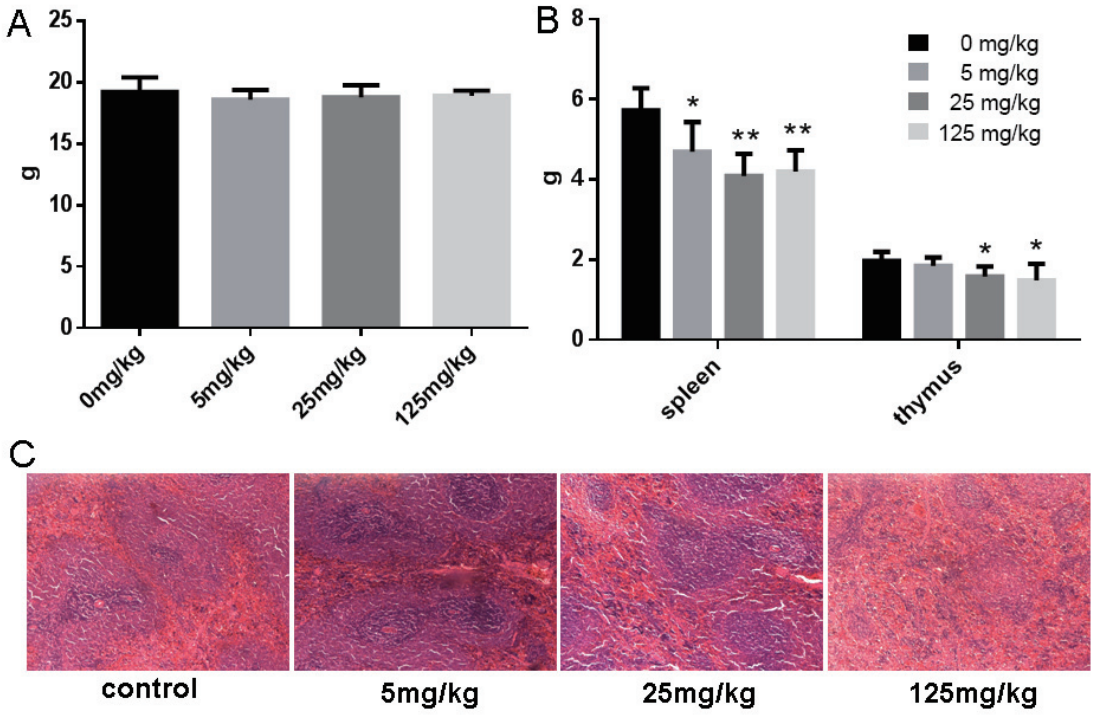

Figure 1. Toxic effects of ATR on the spleen. (A) Body weight was comparable among the 4 groups treated with ATR. **(B)** The mean weight of the spleen and thymus decreased in mice treated with 25 mg/kg ATR and mice treated with 125 mg/kg ATR (** $p < 0.01$, * $p < 0.05$). **(C)** Histopathological examinations of the spleen revealed degenerative changes in mice treated with 25 mg/kg ATR and mice treated with 125 mg/kg ATR.

concentrations of ATR. The SI was 2.69 ± 0.21 in mice treated with 25 mg/kg ATR and 1.64 ± 0.67 in mice treated with 125 mg/kg ATR, reflecting a significant decrease compared to the SI in the control group (3.19 ± 0.10) ($p < 0.05$, Figures 2A). The SI did not differ significantly in mice treated with 5 mg/kg ATR (2.66 ± 0.72) and the control group. These results suggest that ATR exposure reduced lymphocyte proliferation in mice.

3.4. *NK cell cytotoxicity assay*

To determine the impact of ATR on NK cell function, the activity of NK cells was measured *via* a cytotoxicity assay. Results indicated that NK cell cytotoxic activity was 71.16 ± 2.29% in mice treated with 125 mg/kg ATR, reflecting a significant decrease compared to the control group (85.08 ± 6.43%) ($p < 0.05$) (Figures 2B). Compared to the control group, there were no significant differences in the NK cell cytotoxic activity in mice treated with 25 mg/kg ATR (90.54 ± 3.23%) and 5 mg/kg ATR (84.04 ± 12.53).

3.5. *Lymphocyte phenotypes in the spleen*

Flow cytometry was used to detect the surface markers of lymphocytes. Results revealed that the percentage of CD3+ lymphocytes in the spleen decreased in mice treated with 25 mg/kg ATR (36.22 ± 2.62%) and 125 mg/kg ATR (34.80 ± 4.22%) compared to the control group (45.22 ± 0.55%) ($p < 0.05$) (Figure 3A). The percentage of CD4+ lymphocytes in the spleen decreased

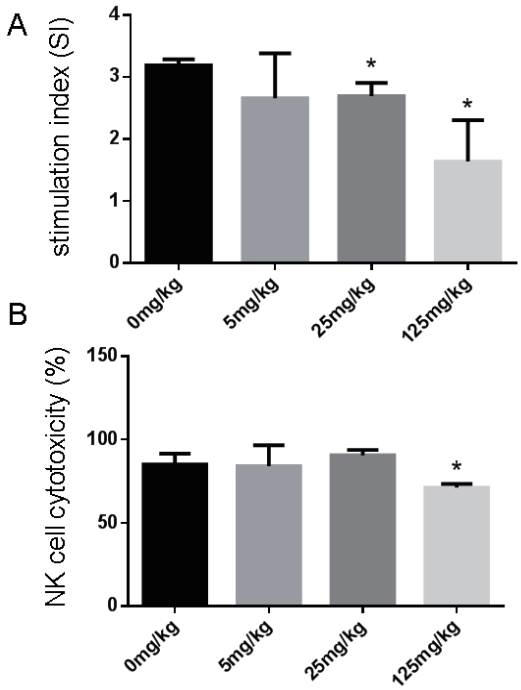

Figure 2. Toxic effects of ATR on splenocyte function. (A) Splenic lymphocyte proliferation decreased significantly ($p < 0.05$) in mice treated with 25 mg/kg ATR and mice treated with 125 mg/kg ATR compared to the control group. **(B)** NK cell cytotoxic activity decreased significantly ($p < 0.05$) in mice treated with 125 mg/kg ATR compared to the control group ($p < 0.05$).

significantly in mice treated with 25 mg/kg ATR (8.39 ± 0.56%) and 125 mg/kg ATR (7.14 ± 0.88%) compared to the control group (11.08 ± 0.49%) ($p < 0.05$) (Figure 3B).

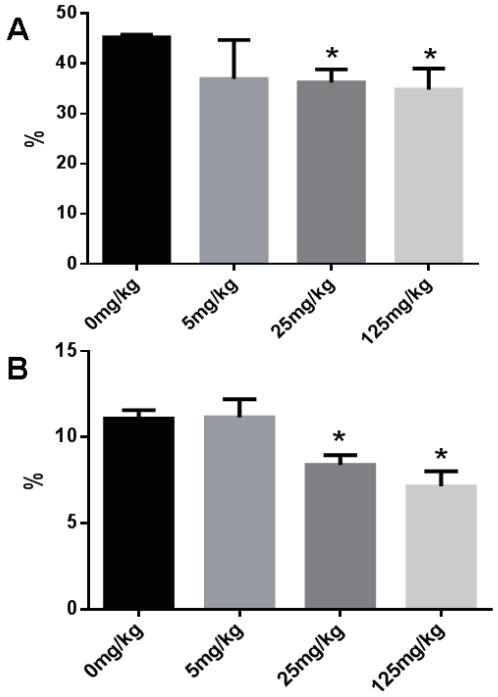

Figure 3. Effect of ATR on percentage of splenic lymphocyte phenotypes. (A) The percentage of CD3+ splenic lymphocytes decreased in mice treated with 25 mg/kg ATR and mice treated with 125 mg/kg ATR compared to the control group ($p < 0.05$). **(B)** The percentage of CD4+ splenic lymphocytes decreased significantly in mice treated with 25 mg/kg ATR and mice treated with 125 mg/kg ATR compared to the control group ($p < 0.05$). There were no significant changes in the percentage of CD8+ splenic lymphocytes in the four groups treated with ATR ($p > 0.05$).

Neither CD3+ (36.94 ± 7.79%) nor CD4+ (11.14 ± 1.16%) lymphocytes changed significantly in mice treated with 5 mg/kg ATR. There were no significant changes in the percentage of CD8+ lymphocytes in the groups treated with ATR and the control group. In fact, ATR treatment reduced the splenic CD4+/CD8+ ratio.

3.6. *IL-2, IL-4, IFN-γ, and TNF-α*

To further investigate the mechanisms by which ATR inhibits NK activation and decreases T lymphocytes, the serum levels of pro-inflammatory and immunosuppressive cytokines such as IL-2, IL-4, IFN-γ, and TNF-α were detected. As shown in Figure 4A, IL-2 levels in mice treated with ATR were comparable to those in the control group ($p > 0.05$), and the serum level of IL-2 was 330.88 ± 50.40 in mice treated with 0 mg/kg ATR, 307.24 ± 58.35 in mice treated with 5 mg/kg ATR, 322.70 ± 53.38 in mice treated with 25 mg/kg ATR, and 330.42 ± 53.05 in mice treated with 125 mg/kg ATR. As shown in Figure 4B, the IL-4 levels in mice treated with 25 mg/kg ATR (16.93 ± 1.14 ng/mL) and 125 mg/kg ATR (17.25 ± 1.98 ng/mL) had decreased about 15% from levels in the control group (20.45 ± 2.56 ng/mL) ($p < 0.05$), while there were no significant differences in those levels in mice treated with 5 mg/kg ATR (18.03 ± 2.50 ng/mL) and the control group. Figure 4C shows that IFN-γ levels in mice treated with ATR were similar to those in the control group ($p > 0.05$). The serum level of IFN-γ was 131.58 ± 40.95

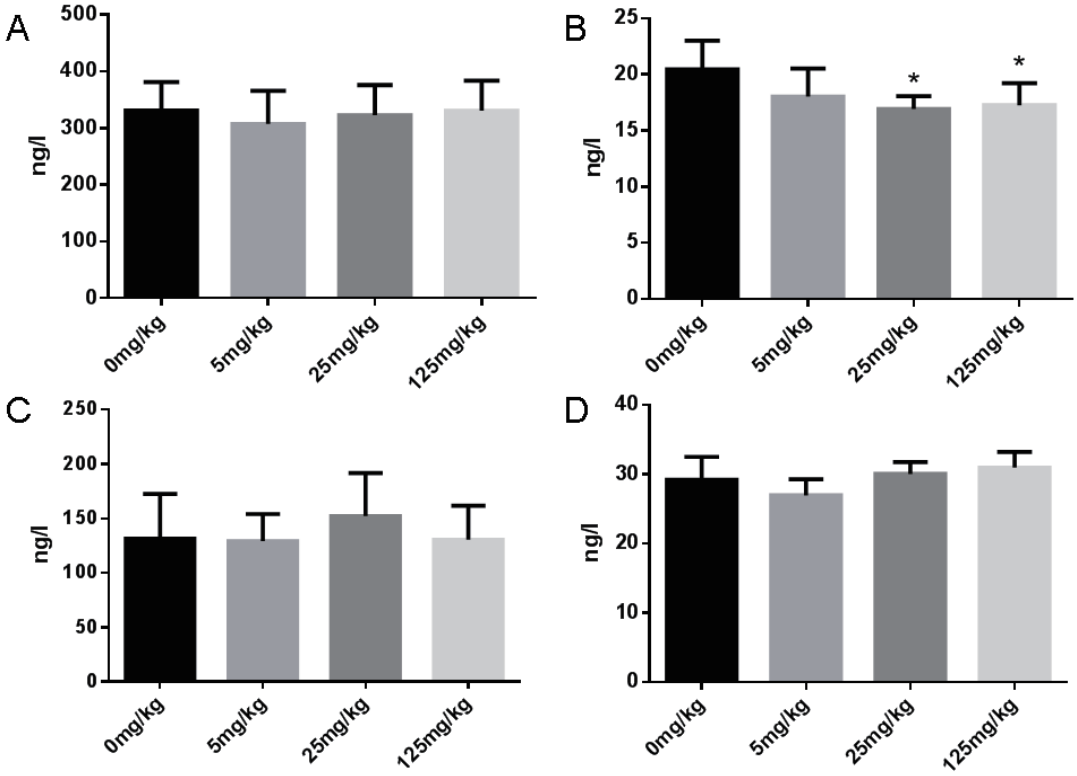

Figure 4. Effects of ATR on serum cytokine levels. (A) Serum IL-2 levels in groups of mice treated with ATR were comparable to those in the control group ($p > 0.05$). **(B)** Serum IL-4 levels decreased significantly ($p < 0.05$) in mice treated with 25 mg/kg ATR and mice treated with 125 mg/kg ATR. **(C)** The serum IFN-γ level in groups treated with ATR was similar to that in the control group ($p > 0.05$). **(D)** There were no significant changes in TNF-α in the four groups treated with ATR ($p > 0.05$).

in mice treated with 0 mg/kg ATR, 129.29 ± 24.77 in mice treated with 5 mg/kg ATR, 152.21 ± 39.59, in mice treated with 25 mg/kg ATR, and 130.54 ± 31.26 in mice treated with 125 mg/kg ATR. As shown in Figure 4D, there were no significant differences in TNF-α among the four groups treated with ATR ($p > 0.05$). The serum level of TNF-α was 29.45 ± 3.30 in mice treated with 0 mg/kg ATR, 26.99 ± 2.33 in mice treated with 5 mg/kg ATR, 30.10 ± 1.70 in mice treated with 25 mg/kg ATR, and 30.97 ± 2.28 in mice treated with 125 mg/kg ATR.

4. Discussion

The immune system is a complex network of interacting regulatory genes, hormones, and cells that has evolved in multicellular organisms for the purpose of maintaining homeostasis against a dynamic battery of foreign environmental agents and/or pathogens. Environmental pollutants can interfere with the normal operation of the immune system, leading to a broad range of disorders, including endocrine dysfunction, tumorigenesis, increased rates of inflammatory infections, and autoimmune diseases. Widespread use of the herbicide ATR increases the chance for and rate of exposure to other foreign agents that the immune system must contend with, and yet the immunotoxicological potential of ATR has not been studied extensively. The present study sought to examine the effects of ATR on selected immune parameters in juvenile mice. ATR (up to 125 mg/kg per day) was orally administered to 4-week-old female C57BL/6 mice for 28 days. One day after the final round of ATR treatment, mice were sacrificed and their serum and spleens were collected.

During the experiment, overt changes in the appearance and behaviors were not observed in mice treated with ATR, and no significant difference in body weight was noted after ATR exposure. However, relative spleen and thymus weights decreased significantly in mice treated with 25 mg/kg ATR and 125 mg/kg ATR compared to the control group, but there were no significant changes in those weights in mice treated with 5 mg/kg ATR. Since abnormal weights of the spleen and thymus are important indicators used to investigate immunotoxic potential (*9*), the current results indicate that ATR was likely to be immunotoxic to juvenile mice. The decrease in immune organ weight may be associated with the inhibition of lymphocyte proliferation and/or the increase in lymphocyte death in the spleen and thymus (*9*). In the current study, the histopathological features of the spleen were detected by light microscopy, and results revealed degenerative changes, compared to the control group, in the spleens of mice treated with 25 mg/kg ATR and 125 mg/kg ATR but not in mice treated with 5 mg/kg ATR. These results suggested that ATR exposure had an adverse impact on the immunity of the spleen in mice. This conclusion is supported by other studies reporting the adverse effects of ATR on the immune system (*18*).

T cells play a central role in organizing the immune defense and are practically involved in all types of immune reactions either by orchestrating the type of immune response or as effectors themselves (*31*). ConA is one of the biologically relevant stimuli applied to activate lymphocytes (*32*). In order to understand the effects of ATR induced T-cell response, the ability of ConA to stimulate the proliferation of T lymphocytes was investigated 1 day after the final round of ATR treatment. Results indicated that 5 mg/kg ATR induced similar T-cell proliferation in the control group while the SI of lymphocytes decreased significantly in mice treated with 25 mg/kg ATR and 125 mg/kg ATR in comparison to the control group. These findings suggest that ATR exposure for 28 days could lead to the inhibition of T-cell proliferation. NK cells play a central role in the immune defense against tumor development and viral infections. Thus, any agent that interferes with the ability of NK cells to lyse their targets may increase the risk for tumorigenesis and/or viral infections (*1*). To determine the impact of ATR on NK cell function, the antitumor activity of NK cells was detected *via* a cytotoxicity assay. Results showed the NK cell cytotoxic activity in mice treated with 125 mg/kg ATR was significantly lower than that in the control group, but 5 mg/kg ATR and 25 mg/kg ATR did not significantly affect NK cell cytotoxicity. These results suggest that treatment with 125 mg/kg ATR for 28 days may induce NK cell dysfunction.

A lymphoproliferative assay provides information only about overall proliferative responses without detailing the specific cell subset involved in these responses. Expression of activation molecules prior to proliferation, on the other hand, offers a useful method to predict lymphocyte proliferative activity (*33*). Exposure to foreign substances induces or stimulates a specific immune system. Some immune responses are directed to specific cells or cytokines that contribute to the immune response (*34*). To better understand the immune environment after ATR treatment, whether the cell phenotypes obtained in flow cytometry are modulated or whether they provide a true reflection of these cells must be determined.

Lymphocyte subsets are major cellular components of the adaptive immune response (*35*). CD3 is a signaling component of the TCR (T-cell receptor) complex, almost all T-lymphocytes express CD3, and therefore the CD3+ antibody is used as a genetic marker of T-lymphocytes. In addition, cytotoxic T lymphocytes express CD8 and recognize endogenous antigens by binding to MHC class I molecules; helper T-lymphocytes express CD4 and require the processing and presentation of antigens in association with MHC class II molecules by antigen-presenting cells and helper T-lymphocytes coordinate and assist other immune cells (*36*). In order to determine if ATR decreased the

yields of CD3+, CD4+, and CD8+ T lymphocytes, flow cytometry was used to detect surface markers of T lymphocyte subsets in this study. Interestingly, results indicated a significant decrease in the number of CD3+ T lymphocytes and a reduction in the ratio of CD4+/CD8+ T lymphocytes in the spleens of both mice treated with 25 mg/kg ATR and mice treated with 125 mg/kg ATR. A possible explanation for this is that ATR may affect T lymphocytes by decreasing CD4+ T lymphocytes mainly by affecting macrophage activation and production of cytokines (*37*).

CD4+ T lymphocytes can be further subdivided by the patterns of cytokine expression. Th1 CD4+ T lymphocytes, involved in cellular immunity, are known to produce IL-2, IFN-γ, and TNF-α and enhance macrophage function, cellular immunity, and synthesis of opsonizing and complement-fixing antibodies. Th2 CD4+ T lymphocytes involved in humoral responses are known to enhance the immunoglobulin G-mediated response and activation of eosinophils through the actions of IL-4, IL-5, and IL-13 (*36*). Numerous studies have found that the immune responses in mice and humans are critically influenced by the balance between Th1 and Th2 cytokines (*38*). Any absence in the cytokine network would cause an imbalance between Th1 and Th2 cytokines and potentially cause inflammation-related diseases. Inflammation-related cytokines have been regarded as either pro-inflammatory or anti-inflammatory according to their contribution to the inflammatory response. Th1 cells express mainly pro-inflammatory cytokines, and Th2 cells primarily secrete anti-inflammatory cytokines. The current results indicated that 25 mg/kg ATR and 125 mg/kg ATR reduced serum IL-4 levels while the serum levels of IFN-γ, IL-2, and TNF-α remained unchanged. IL-4, produced particularly during allergic, cellular, and humoral responses to selected pathogen infections, may modulate other lymphoid cell activities such as regulation of the differentiation of antigen-stimulated T lymphocytes and control of immunoglobulin class switching in B lymphocytes (*39*). Turnbull *et al*. found that IL-4 could inhibit macrophage cytokine production and restrain macrophage activation (40). The current results demonstrated that ATR at a concentration higher than 25 mg/kg could alter the production of inflammation-related cytokines in serum. As a result, the balance between Th1 and Th2 immune responses might be disrupted.

The immunotoxicity of ATR manifests as a decrease in immune response capacity, including the suppression of immune cell function and atrophy of immune organs (*10,18*), which were also observed in the present study. The ATR concentrations in this study ranged from 5 mg/kg to 125 mg/kg, which were determined based on findings in previous studies (*21,41*) and higher than what is expected in nature. However, these amounts ensured that the effects of ATR were noticeable in this study. In California, potential occupational exposure to ATR was assessed during mixing, loading, and application of ATR on field corn, and the absorbed daily dosage (ADD) of ATR for a mixer/loader/applicator was estimated to be 1.8-6.1 μg/kg/day in the event of short-term exposure (15-21 days) (*9, 21*). However, the ADD of ATR is expected to be higher for commercial applicators and farmers in developing countries due to inappropriate personal protective equipment and unintentional overspraying. In the surface water in the East Liaohe River Basin of Jinlin Province, the average ATR content was 9.7 μg/L in regions with agricultural plots and 8.854 μg/L in regions without agricultural plots; ATR content was as high as 18.93 μg/L in July (*42*). These data indicate that the daily ATR exposure for ordinary residents in the watershed is about 0.37-0.79 μg/kg/day, and this value is higher for farmers. ATR is not removed from the body within 24 h and its metabolites can still be detected in the urine 48 h after exposure (*9*). Thus, some effects may occur after repeated exposures and result in accumulation of substances above a critical threshold.

5. Conclusions

This study found that ATR interferes with immune function by inducing degenerative changes in the spleen, inhibiting the proliferation of T lymphocytes and NK cell cytotoxic activity, decreasing the percentage of CD3+ and CD4+ T lymphocytes, and reducing the serum levels of IL-4. Results indicated that ATR may be associated with inflammation, infections, and other diseases. However, the many molecular mechanisms involved in this process are still unclear. Therefore, further studies are needed to elucidate the exact mechanisms underlying the deleterious effects of ATR on the immune system and to investigate the effects of prolonged exposure to ATR under natural conditions.

Acknowledgements

The authors wish to thank Prof. Xuejian Zhao and Dr. Hejun Ren for their helpful suggestions. The authors also wish to thank Kun Li for his help critically reading and revising this manuscript. This work was supported by grants from the Jilin Provincial Science & Technology Department (grant No. 201215081) and the Jilin Provincial Department of Finance Projects (grant no. 2012845).

References

1. Whalen MM, Loganathan BG, Yamashita N, Saito T. Immunomodulation of human natural killer cell cytotoxic function by triazine and carbamate pesticides. Chem Biol Interact. 2003; 145:311-319.
2. Hayes TB, Khoury V, Narayan A, Nazir M, Park A,

Brown T, Adame L, Chan E, Buchholz D, Stueve T, Gallipeau S. Atrazine induces complete feminization and chemical castration in male African clawed frogs (Xenopus laevis). Proc Natl Acad Sci U S A. 2010; 107:4612-4617.
3. Dorfler U, Feicht EA, Scheunert I. S-triazine residues in groundwater. Chemosphere. 1997; 35:99-106.
4. Koskinen WC, Clay SA. Factors affecting atrazine fate in north central U.S. soils. Rev Environ Contam Toxicol. 1997; 151:117-165.
5. Rohr JR, McCoy KA. A qualitative meta-analysis reveals consistent effects of atrazine on freshwater fish and amphibians. Environ Health Perspect. 2010; 118:20-32.
6. Simpkins JW, Swenberg JA, Weiss N, Brusick D, Eldridge JC, Stevens JT, Handa RJ, Hovey RC, Plant TM, Pastoor TP, Breckenridge CB. Atrazine and breast cancer: A framework assessment of the toxicological and epidemiological evidence. Toxicol Sci. 2011; 123:441-459.
7. Weselak M, Arbuckle TE, Wigle DT, Krewski D. In utero pesticide exposure and childhood morbidity. Environ Res. 2007; 103:79-86.
8. Lasserre JP, Fack F, Revets D, Planchon S, Renaut J, Hoffmann L, Gutleb AC, Muller CP, Bohn T. Effects of the endocrine disruptors atrazine and PCB 153 on the protein expression of MCF-7 human cells. J Proteome Res. 2009; 8:5485-5496.
9. Zhang X, Wang M, Gao S, Ren R, Zheng J, Zhang Y. Atrazine-induced apoptosis of splenocytes in BALB/C mice. BMC Med. 2011; 9:117.
10. Brodkin MA, Madhoun H, Rameswaran M, Vatnick I. Atrazine is an immune disruptor in adult northern leopard frogs (Rana pipiens). Environ Toxicol Chem. 2007; 26:80-84.
11. Fatima M, Mandiki SN, Douxfils J, Silvestre F, Coppe P, Kestemont P. Combined effects of herbicides on biomarkers reflecting immune-endocrine interactions in goldfish. Immune and antioxidant effects. Aquat Toxicol. 2007; 81:159-167.
12. Kiesecker JM. Synergism between trematode infection and pesticide exposure: A link to amphibian limb deformities in nature? Proc Natl Acad Sci U S A. 2002; 99:9900-9904.
13. Koprivnikar J, Forbes MR, Baker RL. Contaminant effects on host-parasite interactions: Atrazine, frogs, and trematodes. Environ Toxicol Chem. 2007; 26:2166-2170.
14. Langerveld AJ, Celestine R, Zaya R, Mihalko D, Ide CF. Chronic exposure to high levels of atrazine alters expression of genes that regulate immune and growth-related functions in developing Xenopus laevis tadpoles. Environ Res. 2009; 109:379-389.
15. Kreutz LC, Barcellos LJ, Marteninghe A, Dos Santos ED, Zanatta R. Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings (Rhamdia quelen) to Aeromonas hydrophila challenge. Fish Shellfish Immunol. 2010; 29:694-697.
16. Mencoboni M, Lerza R, Bogliolo G, Flego G, Pannacciulli I. Effect of atrazine on hemopoietic system. *In Vivo*. 1992; 6:41-44.
17. Fournier M, Friborg J, Girard D, Mansour S, Krzystyniak K. Limited immunotoxic potential of technical formulation of the herbicide atrazine (AAtrex) in mice. Toxicol Lett. 1992; 60:263-274.
18. Filipov NM, Pinchuk LM, Boyd BL, Crittenden PL. Immunotoxic effects of short-term atrazine exposure in young male C57BL/6 mice. Toxicol Sci. 2005; 86:324-332.
19. Karrow NA, McCay JA, Brown RD, Musgrove DL, Guo TL, Germolec DR, White KL, Jr. Oral exposure to atrazine modulates cell-mediated immune function and decreases host resistance to the B16F10 tumor model in female B6C3F1 mice. Toxicology. 2005; 209:15-28.
20. Rajkovic V, Matavulj M, Johansson O. Combined exposure of peripubertal male rats to the endocrine-disrupting compound atrazine and power-frequency electromagnetic fields causes degranulation of cutaneous mast cells: A new toxic environmental hazard? Arch Environ Contam Toxicol. 2010; 59:334-341.
21. Dikić D, Zidovec-Lepej S, Remenar A, Bendelja K, Benković V, Horvat-Knežević A, Brozović G, Oršolić N. Effects of prometryne on apoptosis and necrosis in thymus, lymph node and spleen in mice. Environ Toxicol Pharmacol. 2009; 27:182-186.
22. Laws SC, Ferrell JM, Stoker TE, Cooper RL. Pubertal development in female Wistar rats following exposure to propazine and atrazine biotransformation by-products, diamino-S-chlorotriazine and hydroxyatrazine. Toxicol Sci. 2003; 76:190-200.
23. Rayner JL, Wood C, Fenton SE. Exposure parameters necessary for delayed puberty and mammary gland development in Long-Evans rats exposed in utero to atrazine. Toxicol Appl Pharmacol. 2004; 195:23-34.
24. Rooney AA, Matulka RA, Luebke RW. Developmental atrazine exposure suppresses immune function in male, but not female Sprague-Dawley rats. Toxicol Sci. 2003; 76:366-375.
25. Rowe AM, Brundage KM, Schafer R, Barnett JB. Immunomodulatory effects of maternal atrazine exposure on male Balb/c mice. Toxicol Appl Pharmacol. 2006; 214:69-77.
26. Blaylock BL, Holladay SD, Comment CE, Heindel JJ, Luster MI. Exposure to tetrachlorodibenzo-p-dioxin (TCDD) alters fetal thymocyte maturation. Toxicol Appl Pharmacol. 1992; 112:207-213.
27. Holladay SD. Prenatal immunotoxicant exposure and postnatal autoimmune disease. Environ Health Perspect. 1999; 107 (Suppl 5):687-691.
28. Theus SA, Tabor DR, Soderberg LS, Barnett JB. Macrophage tumoricidal mechanisms are selectively altered by prenatal chlordane exposure. Agents Actions. 1992; 37:140-146.
29. Domínguez-Gerpe L, Rey-Méndez M. Age-related changes in primary and secondary immune organs of the mouse. Immunol Invest. 1998; 27:153-165.
30. Dietert RR, Etzel RA, Chen D, Halonen M, Holladay SD, Jarabek AM, Landreth K, Peden DB, Pinkerton K, Smialowicz RJ, Zoetis T. Workshop to identify critical windows of exposure for children's health: Immune and respiratory systems work group summary. Environ Health Perspect. 2000; 108 (Suppl 3):483-490.
31. Pichler WJ, Tilch J. The lymphocyte transformation test in the diagnosis of drug hypersensitivity. Allergy. 2004; 59:809-820.
32. Ruiz VE, Sachdev M, Zhang S, Wen S, Moss SF. Isolating, immunophenotyping and *ex vivo* stimulation of CD4(+) and CD8(+) gastric lymphocytes during murine Helicobacter pylori infection. J Immunol Methods. 2012; 384:157-163.
33. Almajid FM. Lymphocyte activation test for diagnosis

of seronegative brucellosis in humans. Indian J Pathol Microbiol. 2011; 54:775-781.
34. Da Rocha Piemonte M, De Freitas Buchi D. Analysis of IL-2, IFN-gamma and TNF-alpha production, alpha5 beta1 integrins and actin filaments distribution in peritoneal mouse macrophages treated with homeopathic medicament. J Submicrosc Cytol Pathol. 2002; 34:255-263.
35. Mazzoccoli G, Sothern RB, Parrella P, Muscarella LA, Fazio VM, Giuliani F, Polyakova V, Kvetnoy IM. Comparison of circadian characteristics for cytotoxic lymphocyte subsets in non-small cell lung cancer patients versus controls. Clin Exp Med. 2012; 12:181-194.
36. Roxburgh CS, McMillan DC. The role of the in situ local inflammatory response in predicting recurrence and survival in patients with primary operable colorectal cancer. Cancer Treat Rev. 2012; 38:451-466.
37. Yang DQ, You LP, Song PH, Zhang LX, Bai YP. A randomized controlled trial comparing total glucosides of paeony capsule and compound glycyrrhizin tablet for alopecia areata. Chin J Integr Med. 2012; 18:621-625.
38. Tedgui A, Mallat Z. Cytokines in atherosclerosis: Pathogenic and regulatory pathways. Physiol Rev. 2006; 86:515-581.
39. Varin A, Gordon S. Alternative activation of macrophages: Immune function and cellular biology. Immunobiology. 2009; 214:630-641.
40. Turnbull IR, Gilfillan S, Cella M, Aoshi T, Miller M, Piccio L, Hernandez M, Colonna M. Cutting edge: TREM-2 attenuates macrophage activation. J Immunol. 2006; 177:3520-3524.
41. Pruett SB, Fan R, Zheng Q, Schwab C. Patterns of immunotoxicity associated with chronic as compared with acute exposure to chemical or physical stressors and their relevance with regard to the role of stress and with regard to immunotoxicity testing. Toxicol Sci. 2009; 109:265-275.
42. Yan DH, He Y, Wang H. Environmental characteristics of the atrazine in the waters in East Liaohe River Basin. Huan Jing Ke Xue. 2005; 26:203-208. (in Chinese)

(Received July 9, 2013; Revised August 27, 2013; Accepted August 28, 2013)