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Health hazards associated with dietary acrylamide exposure from the biscuit diet of growing female rat pups

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ABSTRACT

Acrylamide (AA) is a harmful substance widely found in infant and child biscuits; however, the health hazards of AA, especially endogenous AA, in the biscuit matrix is poorly understood. This study aimed to determine the effects of endogenous (0.11 mg/(kg bw·day)) and exogenous (1.31, 5.23, and 10.13 mg/(kg bw·day)) AA exposure from biscuit diet on the hematology, hormone levels, immune function, and liver and kidney damage in growing female rat pups. For the hematological indices, a quadratic reduction was observed in percentage of neutrophils (Neu%) and percentage of eosinophils (Eos%) in the leukograms and in mean corpuscular hemoglobin concentration and platelet in the erythrograms in all the AA-exposed groups. In terms of hormones, extremely remarkably elevations in estradiol (E_2) and growth hormone (GH) levels were associated with exogenous AA, and a significant increase in GH levels was noted in the endogenous AA group. Regarding immune function, endogenous and exogenous AA showed a dose-dependent immunotoxic effect on lysozyme (LYSO), nitric oxide (NO), immunoglobulin (Ig)G, and IgM. In particular, the lactate dehydrogenase (LDH) activity was significantly high in the exogenous medium dose (Exo-M) and exogenous high dose (Exo-H) groups, and the percentage of $CD3^+$ T cells in the blood and $CD8^+$ expression levels in the spleen were significantly elevated in the Exo-H group. For liver and kidney function, exogenous AA had a dose-dependent effect on alanine aminotransferases (ALT), aspartate transferases (AST), alkaline phosphatase (ALP), urea nitrogen (UREA), and creatinine (CREA-S). In addition to the dose-dependent effect on the pathological changes in the liver and kidneys, the endogenous AA group presented with hepatocellular steatosis, kidney inflammatory infiltrates, and glomerular and tubular atrophy. Overall, our findings suggested the dose-dependent harmful effect of endogenous and exogenous AA. Special attention should be paid to the damage caused by exposure to endogenous AA. Stringent AA intake guidelines and measures are required to minimize AA levels in the food matrix.

1. Introduction

Acrylamide (AA) is a well-known industrial chemical for organic synthesis and polymer production. Owing to its carcinogenic action in rodents, the International Agency for Research on Cancer has classified it as probably carcinogenic to humans (Group 2A) and a potential human carcinogen, neurotoxicant, and genotoxicant in 1994[1]. Since the Swedish National Food Administration first reported the presence of AA in foods in 2002, it has become a significant concern due to its presence in most thermally processed carbohydrate-rich foods[2]. Biscuits were identified by the Joint

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Expert Committee on Food Additives as one of significant sources of AA in daily diets worldwide, particularly in these foods cooked at temperatures above 120 °C[3].

People in Argentina consume nearly 13 kg of biscuits per person per year, the highest in the America and second only to Europe, which reaches 15 kg per capita in places such as Belgium and the Netherlands[4-5]. Investigations of AA exposure in different age groups in different countries found the highest levels in infant and young children[6], which were 2–3 times that in adults, and biscuits to be the main exposure source among food products[6-7]. In 2017, the European Commission further updated its regulatory measures by adopting different restrictions on AA levels in different food products. In particular, the level of AA in biscuits has been reduced from an indicative value of 500 μg/kg[8] to a baseline level of 350 μg/kg, and that infant biscuits has been restricted to 150 μg/kg[9]. However, a survey of AA levels in the Spanish biscuit market reported a range from 20 to 2 144 μg/kg, with 30% of the samples exceeding the benchmark level set by the EU[10]. A survey of AA levels in selected food products in the Slovenian market revealed that the benchmark AA level was exceeded in 49% of the samples, of which 57% were biscuits[11]. Similarly, the AA levels in biscuits for infants and young children collected in Turkey ranged from 12.3 to 1 270.0 μg/kg with a mean level of 233 μg/kg[12]. The widespread consumption of biscuits and the wide range of AA levels in these products above the baseline levels imply that biscuits pose a high risk to infant and child health.

Dietary AA can cause various harmful toxicities, such as genotoxicity, neurotoxicity, hepatoxicity, carcinogenicity[13-14], reproductive toxicity[15], and developmental toxicity. In addition to toxicity, health hazards, including dysfunction and disorders, cannot be ignored. A comprehensive hazard assessment that considers the interference with hormone levels in the body and the effect on organ function must be conducted to maintain health. However, demonstrations of the health hazards of dietary AA in growth-susceptible populations such as infants and toddlers are limited. The liver and kidneys are the main organs for metabolizing and are highly sensitive to hazardous substances[16]. Hazardous assessment of the liver and kidneys in infants and children is necessary because of their low tolerance to injury. Moreover, the spleen is an important organ of immunity and plays an important role in defense against the hazardous effects of foreign substances[17]. Thus, the liver, kidney, and spleen were selected as the major organs for AA-induced hazard in this study.

Previous studies examined the oral toxicity of exogenous AA exposure *via* 3 major routes using relevant rodent models: food, drinking water, and gavage[18-20]. High AA exposure is mainly due to dietary intake[21]. Among these 3 pathways, AA shows the highest bioavailability through dietary routes, reaching 32%–52%[14], and many bioavailable compounds are toxic. Hence, an evaluation must be conducted on the health risks of endogenous (self-generated) and exogenous AA intake *via* the dietary route in sensitive populations, especially in human biscuit matrices where AA exposure is high.

This study aimed to evaluate the potential hazards of AA exposure from a biscuit diet to growing female Wistar rat pups. The animals were divided into endogenous AA group and exogenous low-, medium-, and high-dose (Exo-L, Exo-M, and Exo-H) groups, and the health hazards were evaluated based on hematology, hormonal, immune function, and liver and kidney damage. This research was designed to assess the potential health hazards of endogenous and exogenous AA exposure to susceptible populations and provide a hazard characterization of food matrix AA relevant for hazard assessments by food regulatory authorities.

2. Materials and methods

2.1 Samples and chemicals

Biscuits regarded as almost AA-free (98.49 μg/kg) and endogenous AA (923.15 μg/kg) were commercially obtained. The AA-free infant biscuits were formulated with refined wheat flour, palm oil, and sodium bicarbonate, and the infant biscuits with endogenous AA were formulated with refined wheat flour, butter, and ammonium bicarbonate. Analytical standards for AA were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China).

2.2 Animals, diet, and experimental design

All animal studies were performed using 3-week-old female Wistar rats (50–60 g) (Beijing VL Laboratory Animal Co., Ltd., Beijing, China). All the experimental rats received an animal quality certificate (No. LL2023081601) and acclimatized for 3 days prior to experimentation. Each Wistar rat was maintained in a 12 h light-dark cycle at room temperature (20–25 °C) with 40%–70% relative humidity and an air exchange rate of 10–20 times/h and placed individually in metabolic cages (20 cm × 20 cm × 20 cm).

The endogenous dose was 0.11 mg/(kg bw·day) based on the endogenous AA content found in ingested commercial infant and toddler biscuits in our survey. Studies showed that exposure to 1 mg/(kg bw·day) produces mild or moderate toxicity[22-23], and exposure to 10 mg/(kg bw·day) produces significant target organ toxicity. In accordance with these published reports, exposures of 1.31, 5.23, and 10.13 mg/(kg bw·day) were selected as Exo-L, Exo-M, and Exo-H doses, respectively. The rats were fed for 15 days at the age of 3–5 weeks, representing the juvenile period before sexual maturity in rats[24]. For the assessment of AA exposure from infant biscuits, exogenous AA at different concentrations (0, 0.01, 0.04, and 0.08 mg/g) was mixed with the water required for the diet. The aqueous solution was then mixed with AA-free biscuits and liquid diet #L10015 (Research die, New Brunswick, NJ, USA) at a ratio of 5:4:1 to control the Exo-L, Exo-M, and Exo-H groups. The endogenous-group biscuits were mixed with water and a liquid diet in the same ratio to prepare the endogenous-group diet. The exposure values for each group are listed in Table 1. The rats showed signs of anorexia near the later stage (i.e., day 13–15 of the experiment). Therefore, the intake of biscuit diet was reduced.

Exposure values were calculated as follows:

$$\text{Exposure value}\,(\text{mg/(kg bw·day)}) = \frac{w_1 \times v_1 \times m_1}{m_2 \times m_3} \quad (1)$$

Where w_1 is AA mass fraction (mg/g), v_1 is water mass (g), m_1 is daily intake (g), m_2 is diet mass (g), and m_3 is body weight (kg).

The animals were randomly assigned to 1 of the 5 experimental diet groups (n = 8 per group) and fed for 15 days. The diet was placed in a liquid feed delivery bottle for the rats, the amount of food eaten was accurately recorded using a scale, and the AA intake was further calculated. The rats were allowed to freely drink water from a bottle. During this period, each rat received approximately 5–10 g of

Table 1
Exposure value of rats fed with biscuit diets containing endogenous and different doses of exogenous acrylamide for 15 days.

Exposure days	Exposure value (mg/(kg bw·day))				
	Control	Endogenous	Exo-L	Exo-M	Exo-H
1–3	0	0.07	0.94	3.77	7.55
4–10	0	0.12	1.35	5.38	10.77
11–12	0	0.16	1.84	7.35	12.99
13–15	0	0.10	1.07	4.27	8.55
1–15 (average)	0	0.11	1.31	5.23	10.13

specially made fresh feed at 9 a.m. and 4 p.m. every day. As the rats grew, the amount of food was adjusted according to their body weight every 7 days. The animals were weighed every 7 days, and the weight changes were recorded. The experiments were performed at the Laboratory Animal Center of Hunan University of Chinese Medicine, and the experimental procedures for animal care, euthanasia, and biological sample collection were approved by the Animal Care and Use Committee of Hunan University of Chinese Medicine (Approval LL2023081601). All the animals were fasted for 12 h at the end of the 15-day experiment.

2.3 Biological samples

After a 12 h fast, the rats were anesthetized by intraperitoneal injection of 10% (*m*/*V*) chloral hydrate one at a time. Whole blood was drawn from the abdominal aorta, and the rats were euthanized by decapitation. One part of each whole-blood sample was immediately stored in anticoagulant tubes at −4 ℃ to prevent stratification and later used for hematological analysis and immunophenotyping. The remainder of each blood sample was centrifuged at 3 000 × *g* for 15 min to collect serum and stored at –80 ℃ for biochemical, hormonal, immunotoxicity, and cytotoxicity assays.

Organs including the liver, kidneys, and spleen were harvested from eight rats in each group and then fixed in 10% (*V*/*V*) polyformaldehyde aqueous solution for histological and immunohistochemical analyses after wet weight measurement.

The organ indices of liver, kidney, and spleen were calculated as follows:

$$\text{Organ index}\,(\%) = \frac{m\,(\text{organ})}{m\,(\text{body})} \times 100 \quad (2)$$

Where *m* (organ) is the wet weight of the organ (g), and *m* (body) is the body weight of the rat (g).

2.4 Hematological evaluation of peripheral whole-blood samples

The hematological indices included white blood cell (WBC), percentage of neutrophils (Neu%), percentage of eosinophils (Eos%), percentage of lymphocytes (Lym%), percentage of basophils (Bas%), percentage of monocytes (Mon%), mean corpuscular hemoglobin concentration (MCHC), blood platelet (PLT), red blood cell (RBC), hemoglobin (HGB), platelet distribution width (PDW), percentage of platelet volume (PCT%), mean platelet volume (MPV), hematocrit (HCT%), mean cell volume (MCV), and mean hemoglobin volume (MCH). These measurements were performed using an automatic veterinary blood cell analyzer (BC-5000Vet; Shenzhen Mindray Animal Medical Technology Co., Ltd., Shenzhen, China).

2.5 Hormonal, immunotoxicity, and cytotoxicity assays of serum samples

Serum levels of estradiol (E_2), growth hormone (GH), immunotoxicity lysozyme activity (LYSO), nitric oxide (NO), immunoglobulin (Ig)G, and IgM were determined using rat ELISA kits (Shanghai Enzyme-Linked Biotechnology Co., Ltd., Shanghai, China) following the manufacturer's protocols.

Cytotoxic lactate dehydrogenase (LDH) activity was evaluated using a fully automated blood chemistry analyzer (BS-430/159; Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China).

Serum samples were used for the biochemical assays of liver and kidney functions, including total protein (TP), albumin (ALB-II), alanine aminotransferase (ALT), aspartate transferase (AST), alkaline phosphatase (ALP), urea nitrogen (UREA), and creatinine (CREA-S). These measurements were performed using the same blood analyzer as described above.

2.6 Immunophenotyping from peripheral whole-blood samples

For each sample, 100 μL of whole blood was added to tubes with antibodies (anti-rat $CD45^+$, anti-rat $CD3^+$, anti-rat $CD45RA^+$, rat $CD8a^+$, and anti-rat $CD4^+$, BioLegend Biotechnology Co., Ltd., Beijing, China) and incubated at room temperature for 30 min. After surface cell staining for 15 min at room temperature in the dark, the erythrocytes were lysed by incubation with lysis buffer for 10 min. The cells were centrifuged at 300 × *g* for 10 min and washed with PBS.

The details of the gating strategy are shown in Fig. S1. Immunofluorescence detection of lymphocyte subsets was performed using a BD FCAS CANTO II flow cytometer (BioLegend Biotechnology Co., Ltd., Beijing, China). The fluorescence detector 1 was set at a 488 nm bandpass filter to measure FITC (blue fluorescence), and the fluorescence detector 2 was set at a 640 nm bandpass filter to measure PE (red fluorescence). Data were acquired using the BD FACS Diva Version 6 Software system (BD Biosciences, New Jersey, USA) and analyzed using FlowJo v10 (Dollar Tree Inc., Michigan, USA).

2.7 Immunohistochemical evaluation of the spleen

Fixed spleens from 8 rats of each group were processed to prepare 5 μm sections in a cryostat. The sections were air dried, briefly prefixed in acetone, and stored at −70 ℃ until further processing. A panel of monoclonal antibodies ($CD4^+$ and $CD8^+$; Abcam Ltd., Cambridge, UK) was used, and the sections were stained with

hematoxylin and eosin (HE), examined, and observed (scale bar = 50 μm, VS200, Olympus, Tokyo, Japan). Statistical analysis of positive cells was performed using ImageJ 1.8.0 (National Institute of Health, Maryland, USA), and $CD4^+$ and $CD8^+$ cell expression levels were indicated by the average optical density (AOD).

2.8 Histopathological examination of liver and kidney

After fixation in polyformaldehyde, the liver and kidney samples were dehydrated in a gradient ethanol series (70%, 80%, 90%, and 100%) and xylene. The tissue was embedded in paraffin, cut into serial 3–5 μm sections, and stained with HE. Histopathological changes in the stained sections were examined and observed under an optical microscope (VS200; Olympus, Tokyo, Japan) (scale bar = 20 μm).

2.9 Data analysis

Data were statistically described as mean ± standard deviation (SD), and the analysis software used was IBM SPSS Statistics 23.0 with Graphpad Prism 9.5 for graphing.

3. Results and discussion

3.1 Body weight and organ indices

All the rats had a normal status throughout the experiment. No significant differences in body weight were recorded between the AA-exposed and control groups (Table S1). Different from previous studies, exposure to high AA doses (50 mg/(kg bw·day)) resulted in a significantly decreased appetite or weight loss[14,24]. The body weight of the rats showed a tendency to increase slowly according to the normal growth days, possibly because the daily meals are the quantitative diet that was not meant to be consumed *ad libitum*. A quantitative diet was used in this study to ensure that the basic daily nutritional needs and AA intake remained consistent across the rat groups.

Organ index changes have been used as reference criteria for the assessment of the toxicity of hazardous substances in animals. As shown in Table S1, the liver indices of the AA group were significantly lower than those of the control group ($P < 0.05$). The significant reduction in the liver index may have been caused by the AA-induced decomposition of liver tissue and blood cells, resulting in hepatotoxicity[25-26]. This finding suggests that the liver is highly sensitive to the effects of AA and is the target organ of AA toxicity. However, no significant differences were observed in the other organ indices, including those of the kidney and spleen. Similarly, no changes in organ indices including spleen and kidney were observed in previous studies of short-term administration of high-dose AA (5 mg/(kg bw·day))[27] and long-term administration of low-dose AA (2 mg/(kg bw·day))[14]. One of the major functions of the liver is to eliminate endogenous and exogenous compounds. Therefore, biotransformation must first occur in the liver to eliminate these xenobiotics[28].

3.2 Hematological evaluation

The hematopoietic system is one of the most sensitive systems for detecting the harmful effects of toxins[29]. AA exposure causes hematological toxic effects associated with oxidative stress and inflammation[30]. However, dietary exposure to AA is more extensive than environmental exposure. Therefore, the hematological parameters of individuals exposed to AA from a biscuit diet must be evaluated.

Table 2 shows the effects of endogenous and exogenous AA in biscuit diets for 15 successive days on the hematological variables of the leukograms and erythrograms of rats. Regarding the leukograms, a quadratic increase in WBC count was detected in the Exo-H group relative to that in the control group. Moreover, Neu% and Eos%

Table 2
Hematological indicators of rats fed with biscuit diets containing endogenous and different doses of exogenous acrylamide.

Parameter	Control	Endogenous	Exo-L	Exo-M	Exo-H	Linear	Quadratic
Leukogram							
WBC (10^9/L)	1.06 ± 0.30^{b}	1.10 ± 0.35^{b}	1.30 ± 0.59^{b}	1.33 ± 0.48^{b}	2.56 ± 1.05^{a}	0.677	0.90
Neu%	28.39 ± 4.62^{a}	16.28 ± 5.78^{b}	15.39 ± 7.09^{b}	14.58 ± 3.28^{b}	13.36 ± 2.93^{b}	0.67	0.90
Eos%	1.41 ± 0.63^{a}	0.65 ± 0.36^{b}	0.66 ± 0.27^{b}	0.56 ± 0.43^{b}	0.54 ± 0.42^{b}	0.62	0.88
Bas%	2.33 ± 2.34^{ab}	3.24 ± 1.69^{a}	1.39 ± 1.18^{bc}	0.41 ± 0.49^{c}	1.48 ± 1.11^{bc}	0.45	0.47
Lym%	71.98 ± 5.41^{b}	61.54 ± 5.37^{c}	78.66 ± 5.39^{a}	80.20 ± 4.85^{a}	76.79 ± 6.71^{ab}	0.35	0.35
Mon%	8.01 ± 3.22^{a}	6.19 ± 2.63^{ab}	4.71 ± 2.04^{b}	5.46 ± 2.44^{ab}	5.81 ± 2.38^{ab}	0.43	0.94
Erythrogram							
MCHC (g/L)	378.00 ± 4.34^{a}	366.50 ± 11.05^{b}	369.25 ± 3.54^{b}	367.88 ± 5.36^{b}	368.50 ± 8.94^{b}	0.59	0.80
PLT (10^9/L)	1 276.75 ± 127.36^{a}	1 140.63 ± 177.18^{b}	1 034.38 ± 82.27^{bc}	1 017.50 ± 120.9^{bc}	987.25 ± 127.77^{c}	0.09	0.86
RBC (10^{12}/L)	6.05 ± 0.58^{b}	6.60 ±0.37^{a}	5.94 ± 0.58^{b}	6.34 ± 0.37^{ab}	6.03 ± 0.24^{b}	0.03	0.13
HGB (g/L)	118.25 ± 11.26^{b}	130.75 ± 5.65^{a}	116.50 ± 9.24^{b}	123.75 ± 9.72^{ab}	120.38 ± 4.90^{b}	0.01	0.07
PDW (fL)	15.28 ± 0.13^{b}	15.45 ± 0.12^{a}	15.28 ± 0.13^{b}	15.25 ± 0.16^{b}	15.33 ± 0.18^{ab}	0.34	0.68
PCT%	0.67 ± 0.09^{b}	0.79 ± 0.08^{a}	0.77 ± 0.12^{a}	0.67 ± 0.08^{b}	0.70 ± 0.06^{ab}	0.75	0.82
MPV (fL)	6.79 ± 0.31^{a}	6.23 ± 0.23^{b}	6.73 ± 0.18^{a}	6.55 ± 0.21^{a}	6.78 ± 0.39^{a}	0.61	0.72
HCT%	32.33 ± 3.06^{ab}	34.64 ± 1.55^{a}	31.51 ± 2.40^{b}	33.65 ± 2.37^{ab}	32.69 ± 1.65^{ab}	0.00	0.02
MCV (fL)	53.46 ± 1.39^{ab}	52.49 ± 1.03^{b}	53.16 ± 1.74^{ab}	53.05 ± 1.45^{ab}	54.15 ± 0.97^{a}	0.58	0.69
MCH (pg)	19.59 ± 0.75^{a}	19.84 ± 0.52^{a}	19.63 ± 0.57^{a}	19.51 ± 0.72^{a}	19.94 ± 0.47^{a}	0.03	0.07

Note: Data shown represent the means ± SD of 8 rats at 1 time in each group experiments. Different uppercase letters in the same row represent significantly different ($P < 0.05$).

showed a quadratic reduction in all the AA-exposed groups compared with those in the control group. Lym% significantly increased in the different exogenous AA groups compared with that in the control group. For Bas% and Mon%, no regular change in statistical significance was observed between the AA-exposed groups and the control group. Leukograms are part of the immune system. A significant increase in WBC count and a significant decrease in Neu% and Lym% indicate the activation of the immune system by AA and the presence of inflammation. Similar results were obtained in previous studies[14]. Meanwhile, Eos% typically responds to acute infection. Inflammatory responses are also a hallmark of autoimmune diseases. Therefore, the damage to immune function from AA exposure cannot be ignored.

Regarding the erythrogram, a quadratic decrease in MCHC and PLT was observed in the AA-exposed groups compared with those in the control group. Decreases in MCHC and PLT levels indicate the presence of an iron deficiency anemia, which is closely associated with abnormal liver function. Moreover, AA decreases the level of hemoglobin by interacting with the sulfhydryl groups in the hemoglobin structure, causing the destruction of heme and disorders in the iron levels and leading to anemia[29]. Compared with different doses of exogenous AA, endogenous AA was more capable of significantly increasing the RBC count, HGB, PDW, and PCT% ($P < 0.05$) and significantly decreasing the MPV ($P < 0.05$) relative to the control group. Note the hazard differences between endogenous and exogenous AA, not just the quantity. Significantly elevated RBC count and HGB and PCT levels generally indicate the presence of chronic hypoxia or myeloproliferative disease. No significant differences in HCT%, MCV, or MCH were found between the AA-exposed groups. Given that hematopoietic function is a critical function of the kidneys, the spleen is vital in filtering blood and recovering RBC components[31]. Abnormalities in the hematological variables of leukograms and erythrograms may affect the functions of these organs.

3.3 Hormone levels

AA is a sensitive compound that induces hormonal disorders. Serum sex hormones contribute to the progression of AA-induced dysfunctions, such as impaired ovarian function, increased DNA damage in germ cells, and reduced fertility. E_2 and GH play important roles in the sexual and skeletal growth of female growing rat pups. Therefore, the E_2 and GH levels were used to assess the hormonal effects of AA exposure on growing female rat pups (Fig. 1).

Compared with those in the control group, the serum levels of E_2 and GH showed a dose-dependent increase in the AA-exposed group. Furthermore, the E_2 levels in all the exogenous AA groups exhibited an extremely significant increase ($P < 0.001$), but no significant difference was found in the endogenous group. Research on the reproductive toxicity of solvent-exposed AA showed that they can lead to elevated E_2 levels[32]. This phenomenon may be attributed to the damage caused by AA to Leydig cells, which accelerates the conversion of testosterone to E_2 by increasing aromatase activity in the liver[33]. Nevertheless, significantly elevated E_2 levels can interfere with normal developmental rates and accelerate uterine maturation, leading to precocious puberty. In addition, hormones and the immune system are complexly regulated, and an imbalance in E_2 levels may

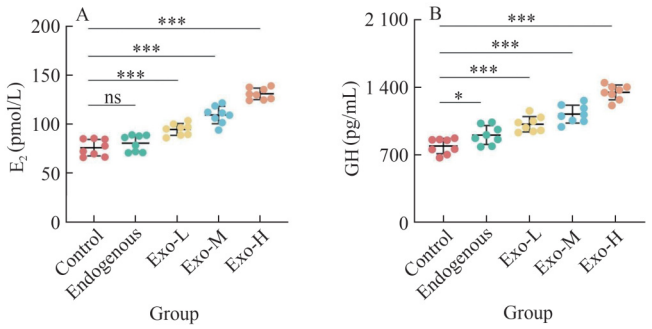

Fig. 1 Serum hormone levels of rats ($n = 8$) fed with biscuit diet containing endogenous and different doses of exogenous AA. (A) E_2; (B) GH. *$P < 0.05$; ***$P < 0.001$; ns indicates non-significance.

reduce immune cell function and trigger infections and tissue damage.

A significant increase in GH was observed in the growing rats exposed to all exogenous AA concentrations ($P < 0.001$). Different from the E_2 levels, the GH level in the endogenous group was significantly higher than that of control group ($P < 0.05$), suggesting that the endogenous AA group is more sensitive to GH than to E_2. High GH levels may be caused by liver and kidney insufficiency, as the slowing down of GH metabolism in the liver and kidneys results in elevated GH levels in the blood. Therefore, liver and kidney damage must be evaluated. In our study, although the body weight did not change, the liver index decreased possibly due to the increased phosphorylation and activation of hormone-sensitive lipases to stimulate liver cell lipolysis[34].

3.4 Immune function

3.4.1 Immunotoxicity and cytotoxicity assays

There were 5 indices related to immunotoxicity and cytotoxicity measured (Fig. 2). For immunotoxicity, the levels of 4 indices, LYSO, NO, IgG, and IgM, showed a dose-dependent increase with AA dose in all the AA-exposed groups. In particular, the 3 exogenous doses showed an extremely significant increase ($P < 0.001$) compared with the control group. Moreover, the endogenous group showed a significant increase in LYSO ($P < 0.05$), IgM, and IgG ($P < 0.01$) compared with the control group. No significant difference in NO levels was observed.

Increased LYSO levels reflect the severity of infections in the body and dose-dependently affect the immune function in congenital immune diseases[35]. As an intercellular messenger, an increase in NO levels indicates the activation of the immune function; immune system cells resist toxic substances by producing NO[36]. In an AA study using long-term low-dose solution matrices, the IgG and IgM levels increased exponentially[14]. These results indicated that AA intake from a solution or biscuit diet can produce immunotoxicity in rats. These indicators warned us of the immunotoxicity of endogenous AA in biscuit matrices, which may interfere with the immune system of rats and result in immune dysfunction. Elevated immunotoxicity indices are considered as markers of oxidative stress[37] and may induce adverse immune responses, further increasing the susceptibility to allergies.

Although no significant dose-dependent increase in LDH was observed, significant increases were recorded in the Exo-M and

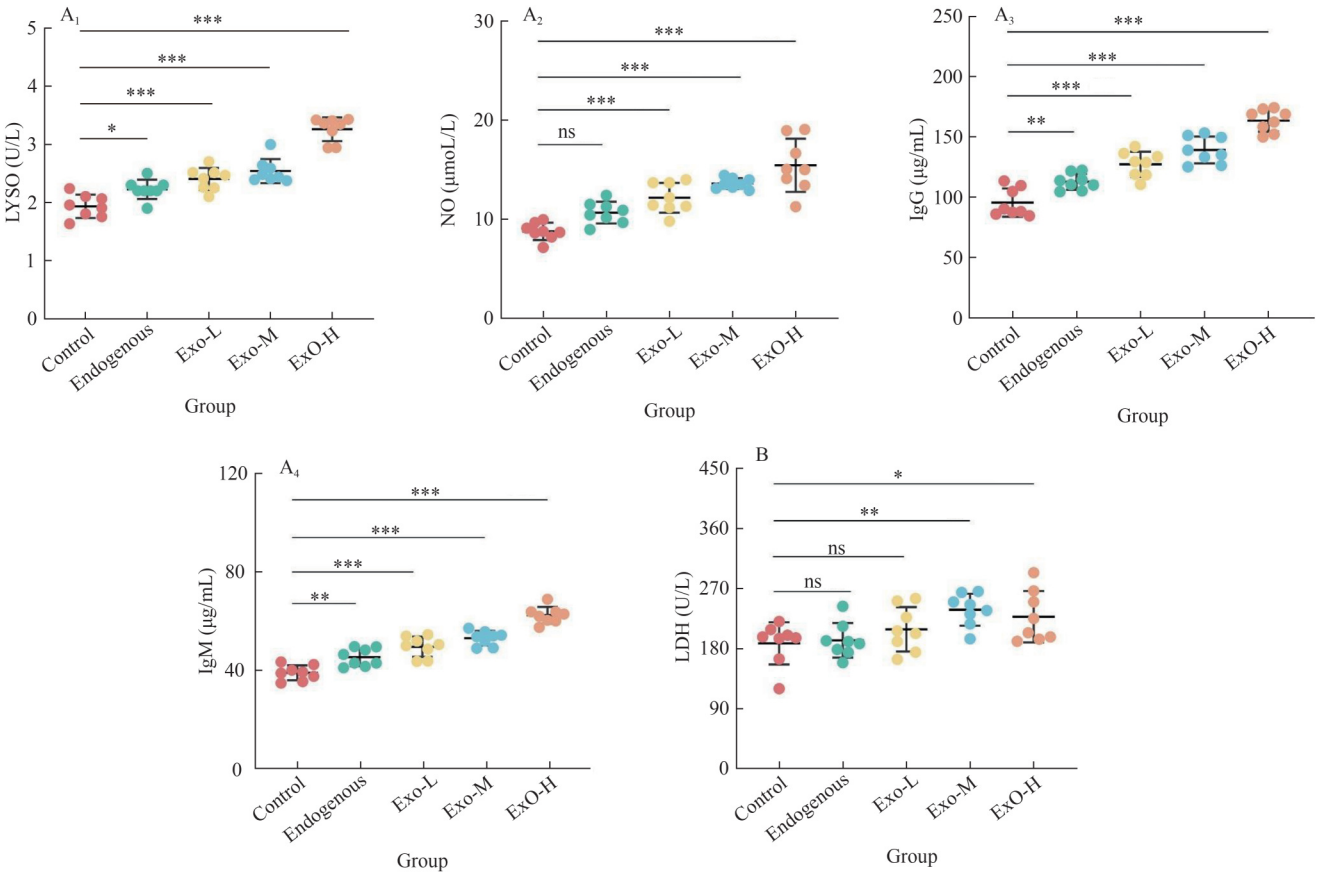

Fig. 2 Immunotoxicity and cytotoxicity indices of rats ($n = 8$) fed with biscuit diet containing endogenous and different doses of exogenous AA. (A) Immunotoxicity indicators (LYSO, NO, IgG, IgM). (B) cytotoxicity index (LDH). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns indicates non-significance.

Exo-H groups ($P < 0.01$, $P < 0.05$), respectively. LDH is an important cellular metabolic enzyme in organisms, and its increased activity in the blood is commonly seen in tissue injury, including liver and kidney damage[38]. Therefore, the effects of AA in biscuit diets on the liver and kidneys require further investigation.

3.4.2 *Immunophenotyping*

Given the significant increase in the serum levels of immune toxicity indicators, the immune function must be further evaluated through cellular immunity. The stabilization of lymphocyte subpopulations is necessary for the maintenance of normal immune function and is also an important indicator of cellular immunity[39]. Therefore, the stability of lymphocyte subpopulations was assessed by determining the percentage of $CD3^+$ T cells, $CD45RA^+$ B cells, $CD8a^+$ Tc cells, and $CD4^+$ Th cells in the peripheral blood by flow cytometry.

Relative to the control group, significant difference in immunophenotyping was only reflected in the percentage of $CD3^+$ T cells in the peripheral blood. In addition, no percentage of $CD45RA^+$ B cells, $CD8a^+$ Tc cells, or $CD4^+$ Th cells were found in the peripheral blood. Although no significant difference in the percentage of $CD45RA^+$ B cells was observed among the groups, it was elevated in all the AA groups. The lack of significant differences among the groups may be due to large changes in intragroup differences. In this case, we present a graph of percentage of $CD3^+$ T cells in each AA-exposed group in Fig. 3A and compare the percentage of $CD3^+$ T cells between the groups in Fig. 3B. The percentages of $CD45RA^+$ B cells, $CD8a^+$ Tc cells, and $CD4^+$ Th cells are shown in Figs. S2 and S3.

The percentage of $CD3^+$ T cells was significantly higher in the Exo-H group ($P < 0.01$) than in the control group (Fig. 3B). This finding suggests that the AA in the biscuit diet of the Exo-H group can disturb the stability of the lymphocyte subpopulations. Elevated the percentage of $CD3^+$ T cells are toxic to the rat cellular immune system and can have pathogenic roles in a range of inflammatory and autoimmune conditions related to acute and chronic liver and kidney diseases[40]. Moreover, immunotoxicity indicators and the percentage of $CD3^+$ T cells were significantly higher in the Exo-H group, suggesting that Exo-H has toxic effects on humoral and cellular immunity. We found an increase in the percentage of $CD45RA^+$ B cells in the AA groups, indicating that AA may enhance the immune activation of the peripheral system. The lack of significant differences in the percentages of $CD8a^+$ of Tc cells and $CD4^+$ Th cells may be attributed to the cumulative toxicity being insufficient to reach the threshold to produce a significant change over the 15-day exposure.

3.4.3 *Immunohistochemical evaluation*

$CD4^+$ and $CD8^+$ cell monoclonal antibodies indicate Th and Tc cells, respectively. A significantly high proportion of $CD3^+$ T cells was observed in the Exo-H group. Given that the spleen is the largest secondary lymphoid organ in the human body, we performed an immunohistochemical evaluation of two representative $CD4^+$ and

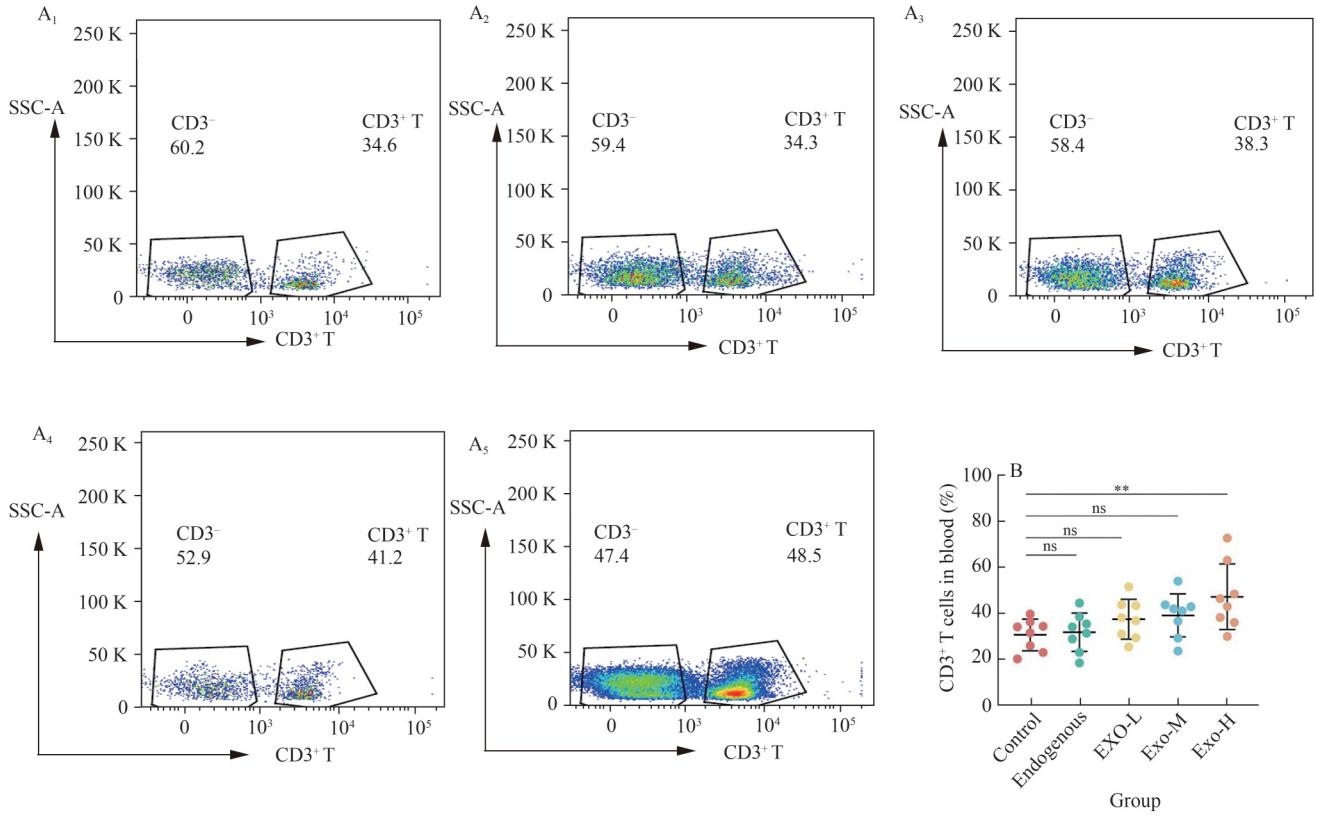

Fig. 3 Immunophenotyping ($CD3^+$ T cells) in peripheral blood of rats (n=8) fed with biscuit diet containing endogenous and different doses of exogenous acrylamide. (A) Representative positive and negative CD3 of T-cells graphs ((A_1) Control; (A_2) Endogenous; (A_3) Exo-L; (A_4) Exo-M; (A_5) Exo-H). (B) Mean value of $CD3^+$ T cells%. Significant different from the respective control value: ** $P < 0.01$; "ns" indicated non-significance.

$CD8^+$ T cells in the spleen to further investigate the effect of AA on the T cells in this organ. A representative photograph of each group and a quantitative examination of $CD4^+$ and $CD8^+$ cells in all the groups are shown in Fig. 4.

$CD4^+$ is a glycoprotein that appears yellow to brown with a normal distribution and intensity on the surface of Th and Tc cells in the control group. They are predominantly found in the periarterial lymphoid sheath (PALS) of the white medulla. As shown in Fig. 4A, no significant difference in $CD4^+$ immune cell AOD was observed between the AA-exposed and control groups.

Fig. 4B shows a subpopulation of cytotoxic lymphocytes, $CD8^+$ splenic lymphocytes, appearing as yellow to brown deposits found mainly around the PALS and red pulp (RP) in the control rats. Compared with that in the control group, $CD8^+$ AOD was significantly higher ($P < 0.05$) in the Exo-H group. In the other groups exposed to AA, no significant changes in $CD8^+$ AOD were recorded.

Alterations in the numbers of $CD4^+$ and $CD8^+$ T cells usually indicate imbalances in the immune system[41]. Our study only found significantly elevated percentage of $CD8^+$ cell in the Exo-H group. This finding suggests an imbalance in immune expression in the Exo-H group, which may lead to immune dysregulation[42] and produce reactions such as immunodeficiency and allergies. The number of $CD3^+$ T cells in the blood and $CD8^+$ T cells in the spleen was elevated in the Exo-H group, suggesting an imbalance in the immune system that may lead to immune system dysfunction. The dangers of splenic immune dysfunction are multifaceted and involves decreased immune function, dysregulated immunity triggering inflammatory and allergic reactions, hematological abnormalities, and other potential hazards. Immune system dysregulation increases the release of the inflammatory factors TNF-α and IL-6, leading to an inflammatory response[43]. If immune dysfunction leads to significant lesions in the spleen, then recovery cannot be achieved. Compared with the significant elevation of $CD4^+$ and $CD8^+$ expression found in a study of 90-day long-term low-dose AA exposure (1 and 2 mg/(kg bw·day))[14], the $CD4^+$ and $CD8^+$ expression was not significantly altered in the endogenous, Exo-L, and Exo-M groups in our work. This phenomenon may be due to differences in the duration of exposure. Nevertheless, the possible harm caused by AA cannot be ignored.

3.5 *Liver and kidney damage*

3.5.1 *Serum biochemical assays*

We measured multiple serum biochemical indices of liver and kidney function after endogenous and exogenous AA exposure from a biscuit diet (Fig. 5). Liver and kidney function indicators are clinical indicators of liver and kidney functions that can effectively reflect the degree of liver and kidney insufficiency. Except for TP and ALB-II, other serum liver function enzymes (ALT, AST, and ALP) and kidney function indices (UREA and CREA-S) increased with the exogenous AA exposure. For endogenous AA exposure, only TP showed significant difference. No differences in ALB-II levels were observed between the AA and control groups.

The levels of all these elevated liver function indicators were significantly higher in the Exo-H group ($P < 0.001$) and changed significantly ($P < 0.01$) or significantly ($P < 0.05$) in the Exo-M group

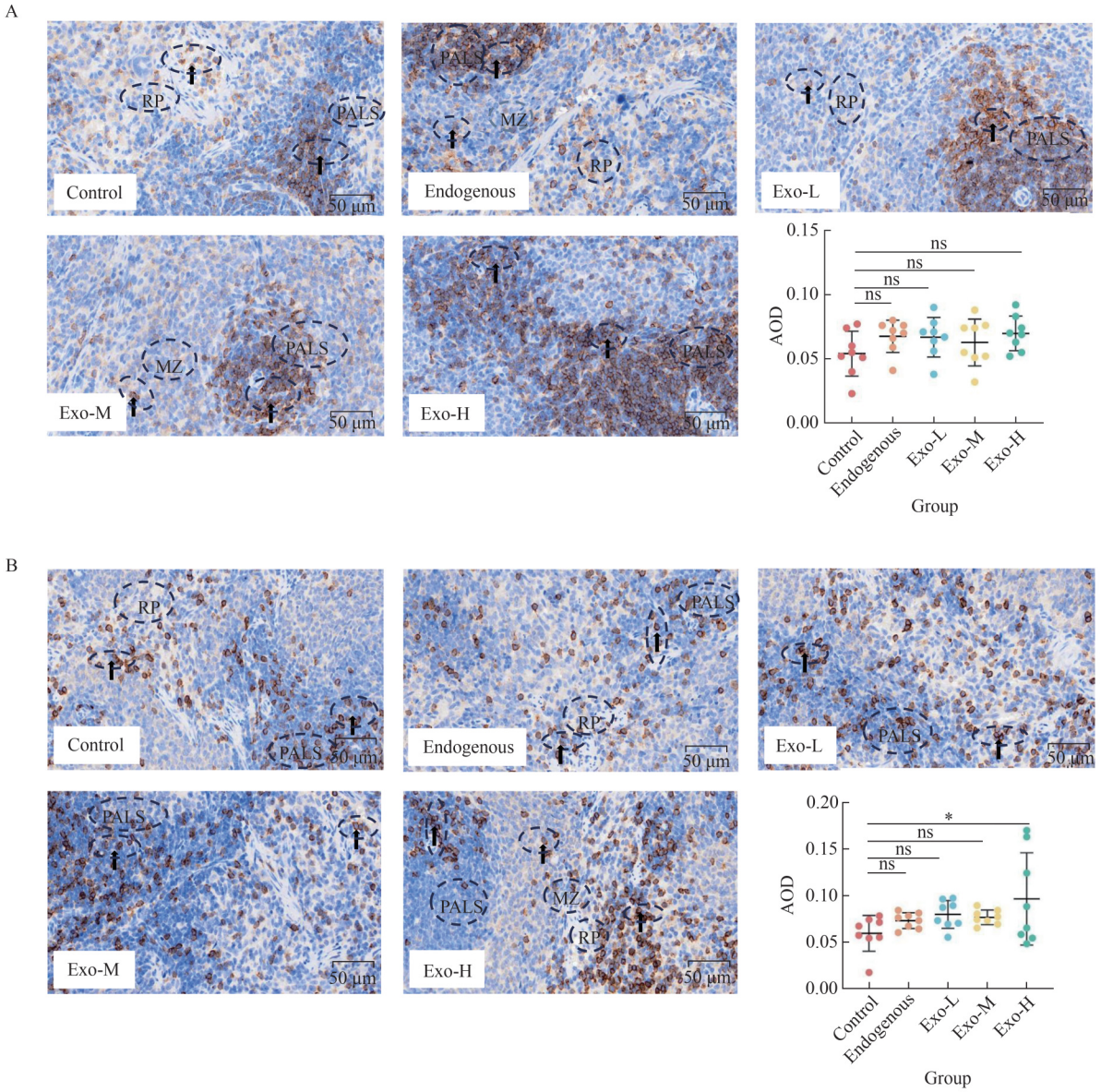

Fig. 4 (A) $CD4^+$ and (B) $CD8^+$ of immunohistochemical evaluation in spleen of rats ($n = 8$) fed with biscuit diet containing endogenous and different doses of exogenous AA. ↑ means positive cells; *$P < 0.05$; ns indicates non-significance.

relative to those in the control group. In the Exo-L group, only the AST levels showed significant ($P < 0.01$) changes among the liver function indices, indicating the possibility of mild liver injury. Nevertheless, high ALT, AST, and ALP levels are indicative of mixed liver injury and may trigger acute liver injury[44]. This finding indicates a severe mixed impairment in the Exo-H and Exo-M groups. The free radicals and oxidative stress induced by AA toxicity may lead to the rupture of cell membranes, which in turn disrupts the integrity of hepatocytes and ultimately leads to increased ALT and AST levels[45].

UREA and CREA-S were significantly higher in the Exo-H and Exo-M groups ($P < 0.001$) and also changed significantly in the Exo-L group ($P < 0.01$) relative to the control group. These indices are sensitive indicators of kidney function; if their expression levels are significantly elevated, the rats have severe renal insufficiency, which may be indicative of the development of acute kidney injury[46]. This finding indicates that the exogenous AA group may have kidney damage. A similar study found elevated urea in rats treated with 50 mg/(kg bw·day) AA, indicating kidney injury[47].

3.5.2 *Histopathological examination*

Pathology is one of the gold standards for a definitive diagnosis. The results are objective, real, and reliable because the changes are substantial. We investigated liver and kidney damage associated with AA exposure from a biscuit diet by histopathological observation. Fig. 6 shows the representative images of liver and kidney tissues, and Table 3 shows statistical data on histologic changes in each group. The rats in the control group showed no evident abnormalities or pathological changes in the histological structures of the liver and kidneys. The hepatocytes were neatly arranged and rich in cytoplasm; the glomeruli were regular in morphology and normal in size; the tubules were regularly arranged; and the epithelial cells were neatly arranged. In Fig. 6, hepatocellular steatosis (green arrow) was evident in the HE

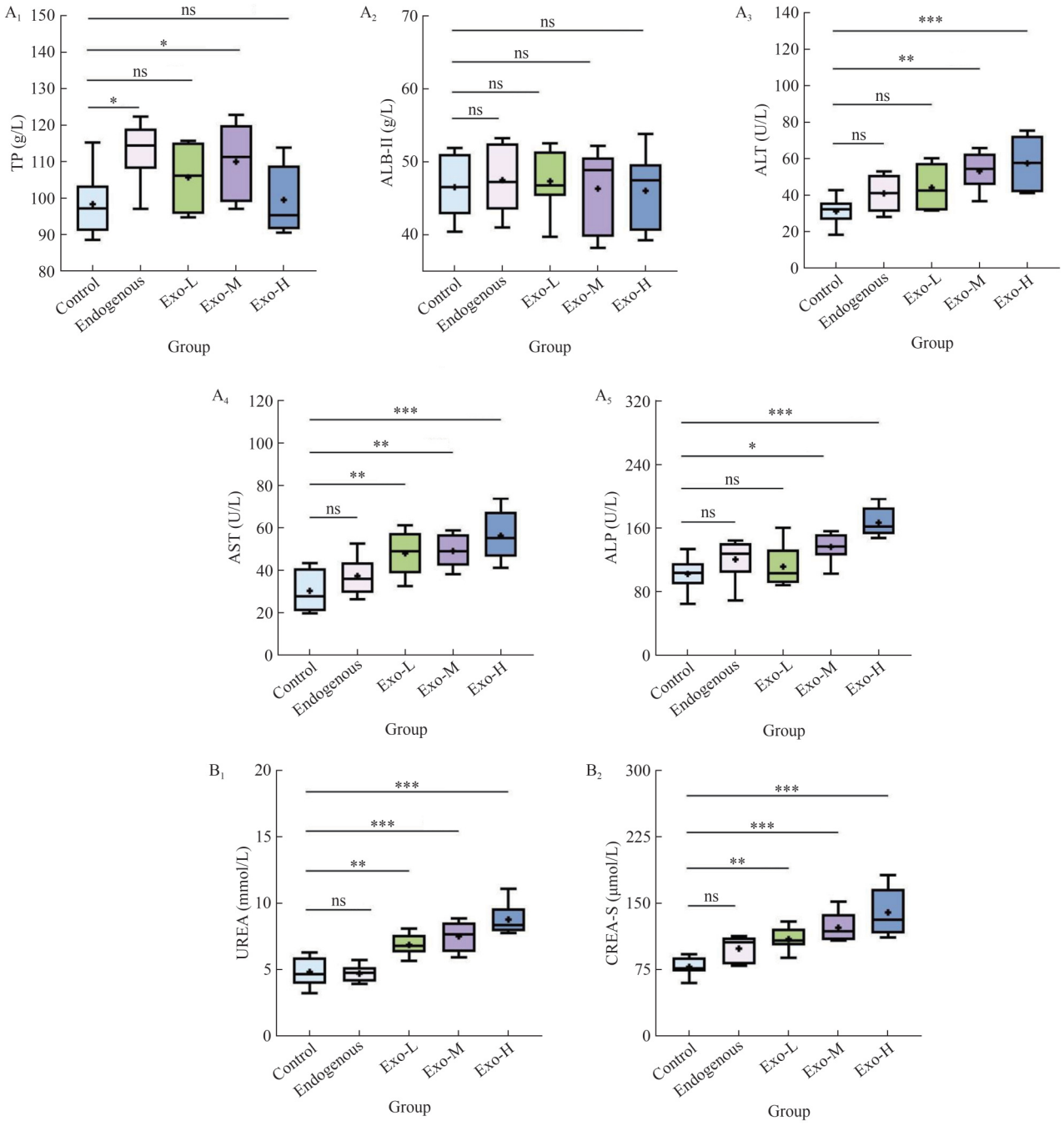

Fig. 5 Serum biochemistry of liver and kidney function of rats (n = 8) fed with biscuit diet containing endogenous and different doses of exogenous AA. (A) Serum liver function index (TP, ALB-II, ALT, AST, ALP). (B) Serum kidney function index (UREA, CREA-S). *P < 0.05; **P < 0.01; ***P < 0.001; ns indicates non-significance.

photograph of the endogenous and Exo-H groups; inflammatory cell infiltration (yellow arrow) and hepatocyte necrosis (black arrow) appeared in the HE photographs of the Exo-L and Exo-H groups; and inflammatory cell infiltration (yellow arrow) and hepatocellular edema (red arrow) were visible in the HE photographs of the Exo-M group. For kidney issues, inflammatory cell infiltration (yellow arrow) was evident in HE photographs of the endogenous and Exo-H groups, edema of epithelial cells (green arrow) appeared in HE photographs of Exo-H, and glomerular and tubular atrophy (blank arrow) were visible in the HE photographs of the endogenous and exogenous groups.

According to the statistical results in Table 3, the effect of endogenous AA on the liver and kidney appeared to be detrimental, including hepatocellular steatosis (1/8 + and 1/8 ++), kidney inflammatory cell infiltration (1/8 ++), and glomerular and tubular atrophy (2/8 ++). However, the pathological changes in the exogenous group were more severe than those in the endogenous group. Hepatocellular inflammatory infiltration (++), edema, and necrosis (+) were observed in the livers of all the exogenous groups, with the Exo-H group showing the most severe symptoms, hepatocellular steatosis (2/8 +), inflammatory cell infiltration (1/8 +), and edema and necrosis (1/8 +++). Similarly, Exo-H induced the most

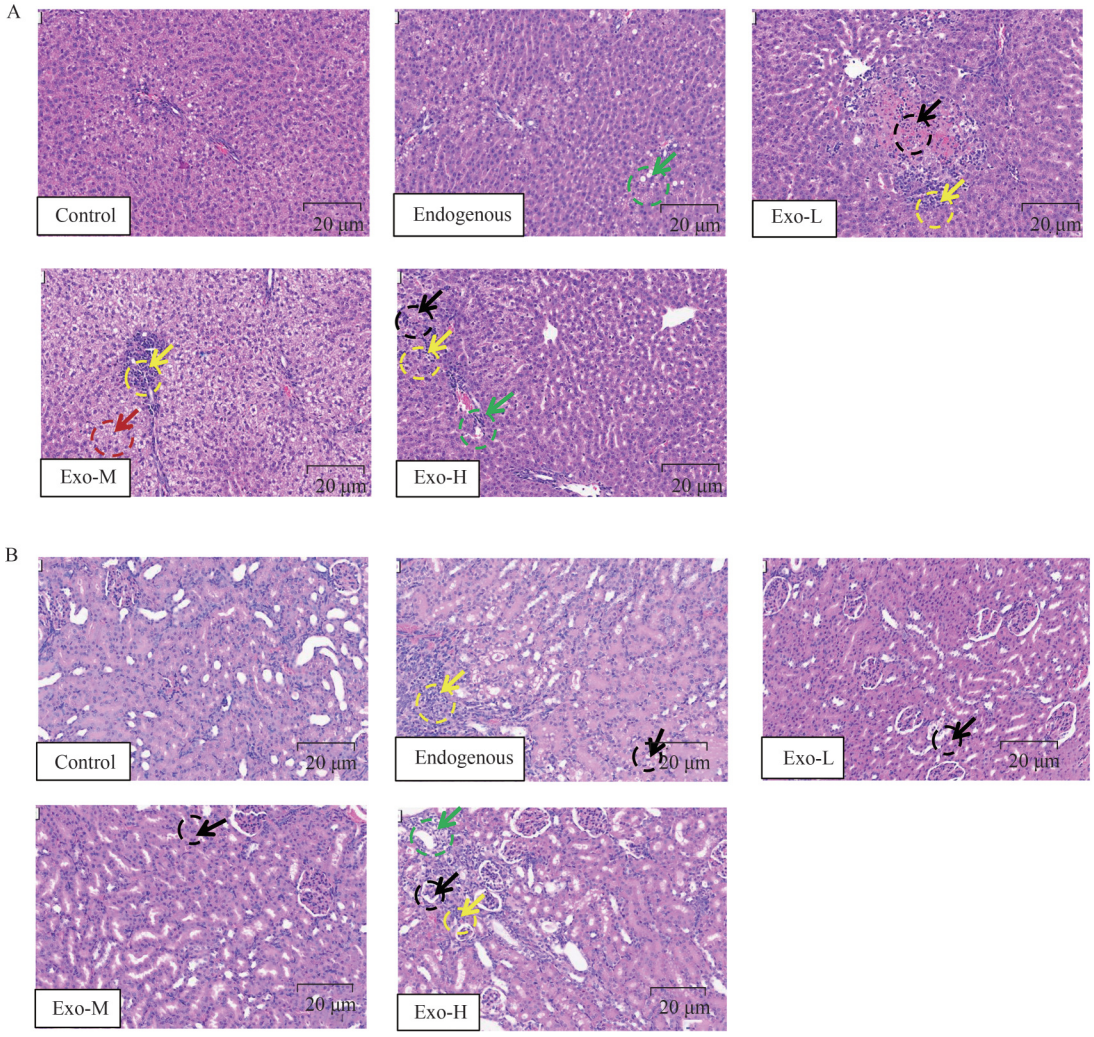

Fig. 6 Histopathological examinations of rats (n = 8) fed with biscuit diet containing endogenous and different doses of exogenous AA. (A) Liver pathology. Green arrow-hepatocellular steatosis; Yellow arrow-inflammatory cell infiltration; Red arrow-hepatocellular edema; Black arrow-hepatocellular necrosis. (B) Kidney pathology. Green arrow-edema of epithelial cells; Yellow arrow-inflammatory cell infiltration; Blank arrow-glomerular and tubular atrophy.

Table 3
Statistical results of histological liver and kidney changes in rats (n = 8) fed with biscuit diets containing endogenous and different doses of exogenous acrylamide.

Symptom	Liver							Kidney						
	Steatosis		Inflammatory cell infiltration		Edema and necrosis			Edema of epithelial cells		Inflammatory cell infiltration		Glomerular and tubular atrophy		
Rank	+	++	+	++	+	++	+++	+	++	+	++	+	++	+++
Control	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Endogenous	1/8	1/8	–	–	–	–	–	–	–	–	1/8	2/8	–	–
Exo-L	–	–	–	1/8	1/8	1/8	–	–	–	–	–	–	–	1/8
Exo-M	–	–	–	1/8	2/8	–	–	–	–	–	–	–	2/8	–
Exo-H	2/8	–	1/8	1/8	2/8	–	1/8	–	1/8	–	1/8	2/8	1/8	–

Note: "+" for mild lesions; "++" for moderate lesions; "+++" for severe lesions; "–" was not found.

severe pathological changes in the kidney, including epithelial cell edema (1/8 ++), inflammatory cell infiltration (1/8 ++), and glomerular and tubular atrophy (2/8 + and 1/8 ++). Exo-M showed only glomerular and tubular atrophy (2/8 ++), and Exo-L only showed glomerular and tubular atrophy (1/8 +++).

The pathological changes in the liver and kidneys were correlated with AA dose in the exogenous group, with the most severe observed in the Exo-H group. On the basis of the significantly elevated serum levels of indicators of liver and kidney function and immunotoxicity, the abnormalities in hepatic and kidney function and the immune system of the rats in the Exo-H group were determined. The pathological changes caused by endogenous AA may also lead to abnormal liver and kidney function. Other studies showed that the administration of solution AA (50 mg/(kg bw·day)) for 2 weeks caused similar liver and kidney damage[24], with oxidative stress and inflammation as the underlying pathological mechanisms[48]. Based on these findings, the AA concentrations in solution matrices are higher than those in biscuit matrices, yet they manifest similar toxic effects, possibly because the AA ingested through the food matrix has a higher bioavailability. The liver and kidneys are important organs for metabolism[49].

Liver metabolism studies revealed that the administration of 25 mg/kg solution matrix AA resulted in a significant decrease in CYP2E1 protein expression because the toxicity was mitigated through the biotransformation of AA to glycidylamide via cytochrome P450 and CYP2E1 enzymes[50]. For oxidative stress parameters in liver tissues, exposure to 50 mg/kg AA increased the MDA levels and significantly decreased the GSH levels[51]. Moreover, ROS overproduction induced by AA is associated with increased inflammatory processes[52-53]. High expression levels of the inflammation-associated genes IL-1β, TNF-α, and NF-κB were found in the liver of the AA-exposed group[54].

Furthermore, studies on 20 mg/kg AA-induced kidney toxicity in rats showed a dose-dependent increase in kidney MDA levels and a dose-dependent decrease in GSH activity[55-56]. Similarly, the IL-1β, TNF-α, and NF-κB levels were significantly higher in the AA group than in the control rats[56]. AA stimulates oxidative stress and induces ROS production in the kidneys, and elevated ROS levels promote the synthesis of cellular pro-inflammatory factors. Owing to the imperfect development of the liver and kidneys in infants and children during the growth period, liver and kidney damage during this stage leads to impaired protein synthesis and decreased immunity in the body, with symptoms of stunting and upper respiratory tract infections.

Further studies showed that rat intestinal permeability and serum ET-1 and *D*-lactate levels were significantly increased by 75% in the AA-treated group compared with the control group, suggesting that AA increases intestinal permeability and damage to the small intestinal mucosa[57]. HE staining of intestinal sections showed severe mucosal damage manifested by epithelial cell necrosis, wide villus spacing, and disrupted intestinal villi in the AA group[57]. The increased intestinal permeability and impaired barriers allow AA to enter the bloodstream and cause liver, kidney, and spleen damage. Accordingly, ways to modulate the intestinal barrier must be explored to effectively mitigate the toxicity of AA to target organs. This topic will be an important direction for our future research and key to protecting human health.

4. Conclusion

This study showed that endogenous AA (self-generated AA in biscuits) significantly affected the GH levels; increased the LYSO, IgG, and IgM levels; and induced immunotoxicity and hepatocellular steatosis in the liver and inflammatory cell infiltration and glomerular and tubular atrophy in the kidneys of growing female pups. In addition to these indicators of endogenous AA, exogenous AA significantly affected the E_2 levels, NO in immunotoxicity, LDH in cytotoxicity, and liver and kidney function indicators and caused dose-related acute liver and kidney injury. Overall, exogenous AA caused a dose-dependent damage. Our results indicated that endogenous AA in the biscuit matrix can damage the body in various ways, raising concerns about the intake of AA in the food matrix. Further studies are warranted to measure the metabonomic profiles of tissues and intestinal permeability for a comprehensive understanding of the molecular mechanism of dietary AA hazards, which is essential for prevention and treatment. Future research should strive to reduce AA by studying product processes, formulations, and microbial mitigation methods. In addition, timely updates of dietary recommendations and strict control of intake in susceptible populations, such as infants and young children, are effective means to ensure safety.

Conflict of interest

The authors have no competing interests to declare that are relevant to the content of this article.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2025.9250516.

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