

# New Mechanisms of Gastrointestinal Mucosal Injury and Bleeding Induced by Acute Unpleasant Exercise

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## Research

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# Abstract

It remains unclear whether acute unpleasant exercise (AUE) caused by foot shocks leads to gastrointestinal (GI) mucosal injury and bleeding. In this study, we investigated the involvement of inflammatory cytokines in AUE-induced GI mucosal injury/bleeding and oxidative stress by analyzing the expressions of pro-inflammatory (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and iNOS) and anti-inflammatory (IL-10) cytokines in the hypothalamus and duodenum after foot shocks using PCR. Results showed that the expressions of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and iNOS were significantly increased following the process, while IL-10 was not activated. These findings suggest that the activation of inflammatory response system (IRS) is closely related to GI mucosal injury/bleeding and oxidative stress induced by AUE caused by foot shocks.

## 1. Introduction

Stress is defined as a non-specific response of the body to the noxious stimulus. Physiological and psychological stressors threaten the homeostasis of the body(1). In recent years, acute unpleasant exercise (AUE) caused by foot shock has been considered as a stressor that can disturb homeostasis and affect the actions of many hormones in the body(2). This stress model has been shown to cause anemia and oxidative stress(3–5).

The hypothalamic pituitary adrenocortical (HPA) axis is a neuroendocrine system that plays a key role in stress response(6). Corticotropin releasing hormone (CRH), which is released from the paraventricular nucleus of the hypothalamus and response to many somatic stimuli, such as inflammation and hunger, is involved in the activation of HPA axis in the brain(7, 8). The activation of CRH regulates the secretion of adrenocorticotrophic hormone (ACTH) into the peripheral circulation, and then ACTH induces the secretion of glucocorticoids(9). CRH is associated with gastric secretion, colonic motility, and gastric pH level(10). The reduction of CRH and ATCH promoted jejunal contraction and gut motility by increasing Ghrelin(11). It is well known that the activation of HPA axis is closely related to the disturbance of gastrointestinal (GI) hormones and oxidative stress.

Cytokines are the regulatory proteins secreted by white blood cells and various other cells. They have numerous effects on immune system and inflammatory responses(12). During immune responses, activated macrophages, T cells, and natural killer cells produce proinflammatory cytokines, which in turn regulate the inflammatory response system (IRS). Then IRS activates HPA axis, most notably CRH and ACTH, stimulating the secretion of serotonin and catecholamines(13). A previous study showed that proinflammatory cytokines, such as IL-1 $\beta$ , IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), increased the release of CRH, suggesting the stimulation of HPA axis(14). The activation of iNOS in macrophages is regulated by cellular receptor molecules, such as Toll-like receptors and CD14, which play a critical role in the pro-inflammatory response of monocytes and macrophages via NF- $\kappa$ B pathway(15). The binding of IL-1 $\beta$  to TNF- $\alpha$  is an important mediator of iNOS, regulating the mRNA expression of iNOS(16, 17). Interleukin-10 (IL-10) is a key anti-inflammatory cytokine responsible for intestinal immune homeostasis, mainly through the inhibition of pro-inflammatory cytokines(18). When the intestinal immune homeostasis is

disturbed by stress, macrophages and T lymphocytes are activated to release pro-inflammatory cytokines, such as IL-1 $\beta$  and TNF- $\alpha$ (19). Recently, pro- and anti-inflammatory cytokines have been used as biomarkers of oxidative stress(20, 21).

Potent neutrophil-activated chemokines, such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , play crucial roles in AGE(22). The intestinal endotoxin induces intestinal barrier injury and activates innate immunity to produce pro-inflammatory cytokines including IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and iNOS. These plasma proteins destroy the intestinal epithelial barrier and increase the intestinal permeability(23–25). IL-10 is an anti-inflammatory cytokine that plays an important role in the protection of epithelial integrity and the regulation of mucosal immune system in small intestine(26).

Recent studies have shown that AUE caused by foot shocks is a novel acute model of stress. However, it remains unclear whether inflammatory cytokines participate in GI mucosal injury and bleeding. Therefore, this study aimed to explore the effects of this model on red blood cell (RBC) count, hemoglobin concentration and hematocrit, the expression of superoxide dismutase (SOD), and myeloperoxidase (MPO) activity. In addition, we examined the expressions of pro- and anti-inflammatory cytokines, including IL-1, IL-6, TNF- $\alpha$ , iNOS, and IL-10, in the hypothalamus and duodenum.

## **2. Materials And Methods**

### **2.1 Animals**

Twenty-four male Kunming mice ( $20 \pm 2$  g) were purchased from Fangyuanyuan breeding Farm (Beijing, China) and housed in an environment-controlled room ( $22 \pm 2$  °C, natural light / dark cycle) with free access to water and food. All animals were acclimated in a single room for at least one week. Mice were fasted for 24 h with ad lib access to water before experiments.

### **2.2 Protocols**

An instrument was designed to generate 1 mV electrical impulse by the Institute of Material Medica, Chinese Academy of Medical Science. This instrument was used to generate an animal model of depression by low voltage electricity. Mice were randomly divided into two groups: the control group and the experimental group. The experimental group was subjected to run and jump with the instrument for 0.5 h, and the control mice were placed into the instrument without electric stimulation. The experiments were performed between 8:00 and 13:00 to eliminate the effect of circadian rhythm on the experiment. In this research, all experiments were conducted according to the guidelines of National Institutions of Health for the Use and Care of Lab Animals and took following to the Minzu University of China's Ethics Review Board.

### **2.3 The measurement of RBC count, hemoglobin and hematocrit**

After the exercise, the whole blood was drawn from the eyeballs of all mice. A volume of 20  $\mu$ L whole blood was used for the measurement of RBC count, hemoglobin concentration and hematocrit using a Hematology analyzer (MEK-6318, made in Japan).

## **2.4 The measurement of MPO and SOD**

The blood samples were centrifuged for 15 min at 3000 rpm and the serum was collected for the detection of MPO and SOD activities (20  $\mu$ L for each measurement). The working solution was added into the 96-well Costar plate and the optical density (OD) was detected using enzyme labeling instrument. The activities of MPO and SOD were determined according to the standard curve.

## **2.5 Macroscopic and histological analysis of duodenum and stomach**

Mice were sacrificed after blood collection, and the stomachs and duodenum were harvested and fixed. The GI inner wall was identified with anatomical lens after fixed with 4% paraformaldehyde for 30 min. After the morphological observation of the duodenum and stomach, the samples were fixed with 4% paraformaldehyde, dehydrated, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Duodenal samples were collected from the same site and frozen at -80 °C for further analysis.

## **2.6 RNA isolation**

The total RNAs of the hypothalamus and duodenum were isolated using TRIzol Reagent. In detail, 50 mg organization was transferred to 1 ml TRIzol lysis. After adding 700  $\mu$ L 75% ethanol, the total RNAs were dissolved by 50  $\mu$ L water. The concentration and purity of the RNAs were quantitated by Quickdrop (Molecular Devices cat., USA). The reverse transcription of the RNAs was amplified by polymerase chain reaction (PCR). For cDNA synthesis, 10 ng cDNA was incubated with 0.2  $\mu$ M primers and 10  $\mu$ L 2  $\times$  RealStar Green Fast Mixture (Genestar, China). The reaction conditions were as follows: 2 min at 95 °C, then 95 °C for 15 s, followed by 41 cycles at 60 °C for 30 s. The oligonucleotide primer pairs were shown in Table 1. The relative mRNA levels were analyzed according to the  $2^{-\Delta\Delta C_t}$  method (27).

Table 1  
Gene specific primers for PCR.

Gene	Orientation	accession	Number	sequence
β-Actin	Forward			AGATCAAGATCATTGCTCCTCCT
	Reverse			CTCAGTAACAGTCCGCCTAGAA
IL-1β	Forward			GCAACTGTTCTGAACTCAACT
	Reverse			ATCTTTTGGGGTCCGTCAACT
IL-6	Forward			TAGTCCTTCCTACCCCAATTTCC
	Reverse			TTGGTCCTTAGCCACTCCTTC
IL-10	Forward			GCTCTTACTGACTGGCATGAG
	Reverse			CGCAGCTCTAGGAGCATGTG
TNF-α	Forward			GACGTGGAAGTGGCAGAAGAG
	Reverse			TTGGTGGTTTGTGAGTGTGAG
iNOS	Forward			GTTCTCAGCCCAACAATACAAGA
	Reverse			GTGGACGGGTCGATGTCAC

## Statistical Analysis

All the results were reported according to the SPSS 22.0. Statistical analysis was performed using a reported One-way ANOVA. \*P<0.05 was considered to be significant.

## 3. Results

### 3.1 GI mucosal injury and bleeding during AUE

The effects of AUE caused by foot shocks on the morphological changes of stomach and duodenum are shown in Fig. 1. The gastric mucosa and intestinal wall in the control group were smooth, uniform and translucent. Mice with AUE showed dark brown areas on the surface and the lesions. Histological analysis with H&E staining also matched with the macroscopic observations (Fig. 2). In addition, the fecal occult blood test was positive. These findings suggested that AUE induced GI mucosal injury and bleeding in mice.

### 3.2 Determination of RBC count, hemoglobin, hematocrit

The RBC count, the concentration of hemoglobin and hematocrit of the experimental group were significantly lower than those in the control group (P < 0.05), indicating that mice were anemic after the exercise (Fig. 3).

### 3.3 Plasma levels of SOD and MPO

As shown in Fig. 4, the activity of SOD was significantly decreased in the experimental group as compared to the controls, whereas the quantity of neutrophil MPO was significantly increased, suggesting an increase in MPO activity after AUE. These data implied that SOD and MPO might be involved in the exercise process.

### **3.4 Effects of Acute AUE on the expressions of cytokines in the hypothalamus**

To determine the expression levels of IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , and iNOS, the total RNAs were isolated from hypothalamus samples. As shown in Fig. 5, the expressions of cytokine genes were relatively low without stress. After exercise, the levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and iNOS were largely increased. No significant difference was observed on the change of IL-10 expression before and after exercise. iNOS gene showed a stronger response to the stress (Fig. 5E). The above results suggested that these cytokines in the hypothalamus participated in the AUE caused by foot shocks.

### **3.5 Effects of Acute AUE on cytokine gene expressions in the duodenum**

One-way ANOVA analysis showed that AUE significantly affected the expressions of IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , and iNOS in the duodenum. The levels of cytokines in the exercise group were significantly increased compared to the controls (Fig. 6).

## **4. Discussion**

The paraventricular nucleus of the hypothalamus contributes to stress responses induced by foot shocks(2). Foot shocks promotes escape behavior in mice, resulting in compelled acute exercise. Compelled acute exercise may induce non-specific immune responses in the body, including diarrhea, abdominal pain, even GI mucosal injury and bleeding. It is estimated that 30–65% of long-distance runners experience exercise-related GI mucosal injury(28). Compelled acute exercise causes oxidative stress and sports anemia(29), leading to erythrocyte damage(30), decreased SOD level and increased MPO activity in the serum. Moreover, pro- and anti-inflammatory cytokines (IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , and iNOS) may play a role in the initiation of GI mucosal injury/bleeding induced by AUE caused by foot shocks.

To investigate whether the model was related to GI mucosal injury and bleeding, we observed the morphological changes of gastric and duodenal mucosa after stress. Acute GI mucosal injury and bleeding following stress indicated that AUE was involved in this process. Previous studies confirmed the correlation between exercise-induced visceral hypoperfusion and GI diseases(31). It was consistent with the data obtained from animal models and patients, showing that GI cell damage was caused by AUE and ischemia(32). The decreased RBC count, hemoglobin concentration and hematocrit is a marker of anemia(33). Our results suggested that anemia was induced by exercise. The exercise-induced oxidative

stress was determined by the activities of SOD and MPO in the serum. Oxygenated free-radicals play a fundamental role in regulating the damage associated with GI mucosal injury and bleeding (34). The decreased serum level of SOD indicates the occurrence of GI mucosal injury and bleeding. MPO is a key player in oxidative process and the regulation of pro-inflammatory cytokines(31, 35). In our study, the increase of MPO matched with the decrease of SOD. The activity levels of SOD and MPO in the serum indicated that this model was involved in oxidative stress.

Physiological systems of the body respond to stress stimuli to promote defense and survival. In the present study, the animal model was considered as a special stressor that might cause GI mucosal injury/bleeding and oxidative stress. The release of inflammatory cytokines during stress induces the alterations in brain neuroendocrine functions. We explored the effects of pro- and anti-inflammatory cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , iNOS, and IL-10) on the hypothalamus in male mice. Results showed that the expressions of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and iNOS) after stress were significantly increased compared to the control group. After the activation of IL-1 $\beta$ , the elevated HPA activity is related to the increased release of norepinephrine (NA) and 5-hydroxytryptamine (5-HT) in the brain(36). It has been found that the level of IL-1 $\beta$  is significantly increased in the hypothalamus of rat with restraint stress(37). In addition, IL-6 and TNF- $\alpha$  produce corticotropin-releasing GABA by stimulating HPA axis(38). Hypothalamic paraventricular nucleus repairs colon injury by regulating the expressions of TNF- $\alpha$  and IL-1 $\beta$ (39). Clinical studies found that the activity of NOS in patients with celiac disease was higher than that in other patients(40). Animal studies showed that IL-1 $\beta$  was the key mediator of nitric oxide after endotoxin exposure. It promoted microglia to produce vasodilator and neuromodulator nitric oxide by increasing the biosynthesis of iNOS(41). Our findings were in line with previous studies, which suggested a mutual activation relationship of pro-inflammatory cytokines. Our data on the expression of IL-10 was also consistent with that in the excessive eccentric exercise model, in which the levels of IL-1 $\beta$  and IL-6 were increased, while the expression of IL-10 was decreased(42).

Next, we measured the expressions of well-known inflammatory markers in the duodenum. Pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and iNOS) were significantly increased as compared to the controls after stress. Recent evidence has shown that IL-1 $\beta$  increases the intestinal permeability(43). In intestinal cells, IL-1 $\beta$  partially increased the intestinal permeability by reducing the expression and redistribution of occludin(23). IL-6 is mainly distributed in intestinal monocytes and the activation of IL-6 promotes the proliferation and repair of intestinal epithelium after injury(44). It has been reported that TNF- $\alpha$  induces the apoptosis and inflammation of intestinal epithelial cells, and damages the intestinal mucosal barrier. TNF- $\alpha$  plays an essential role in regulating GI diseases and its expression in intestinal cells is closely correlated with intestinal barrier defect(45, 46). NOS produces excessive nitric oxide (NO) in the progression of various intestinal inflammatory diseases. The synthesis of NO by iNOS is associated with a variety of pathophysiological processes, including inflammation. Clinical data showed that the activity of iNOS in duodenal epithelial cells was increased in patients with celiac disease(47, 48). Consistently, we found that the pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and iNOS) in the duodenum were activated, resulting in GI mucosal injury/bleeding and the activation of immune responses in mice. IL-10 is a key anti-inflammatory cytokine produced by intestinal macrophages(49). As

shown in an in vitro study, IL-10 exhibited opposite effects on cellular functions when compared to TNF- $\alpha$ , IL-1 $\beta$ , and IL-6(45). Here, we found that IL-10 was not activated in the animal model, which was consistent with previous findings.

As shown in Fig. 7, IRS activated the hypothalamic CRH system, resulting in the secretion of ACTH into the peripheral circulation and the induction of glucocorticoids. Then the brain-gut axis activates mucosal mast cells, increases the expressions of pro-inflammatory cytokines, and promotes the activity of endocrine gland(9, 50). The intestinal homeostasis is maintained by the neural connections through the brain-gut axis, together with the regulation of reactive oxygen metabolites and pro-inflammatory cytokines, such as IL-1  $\beta$ , TNF- $\alpha$ , and IL-6. The increase of MPO activity and the decrease of SOD activity in inflamed mucosa is associated with gastric ulcers(34). A study of intestinal cells found that the overexpression of pro-inflammatory cytokines increased GI permeability and induced GI bleeding(35). In this study, the expressions of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and iNOS) in hypothalamus and duodenum were significantly increased following AUE. Our model of AUE induced-GI mucosal injury/bleeding was consistent with the results of previous studies.

## Conclusion

In conclusion, our results indicate that unpleased exercise caused by foot shocks is related to anemia and oxidative stress. A correlation was found between GI mucosal injury/bleeding and the expressions of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and iNOS) in the hypothalamus and duodenum. This is the first study to show that the activation of IRS is closely associated with GI mucosal injury/bleeding and oxidative stress caused by foot shocks induced AUE. This model may be used for future investigations on stress-induced GI mucosal injury/bleeding and oxidative stress.

## Declarations

### Acknowledgments

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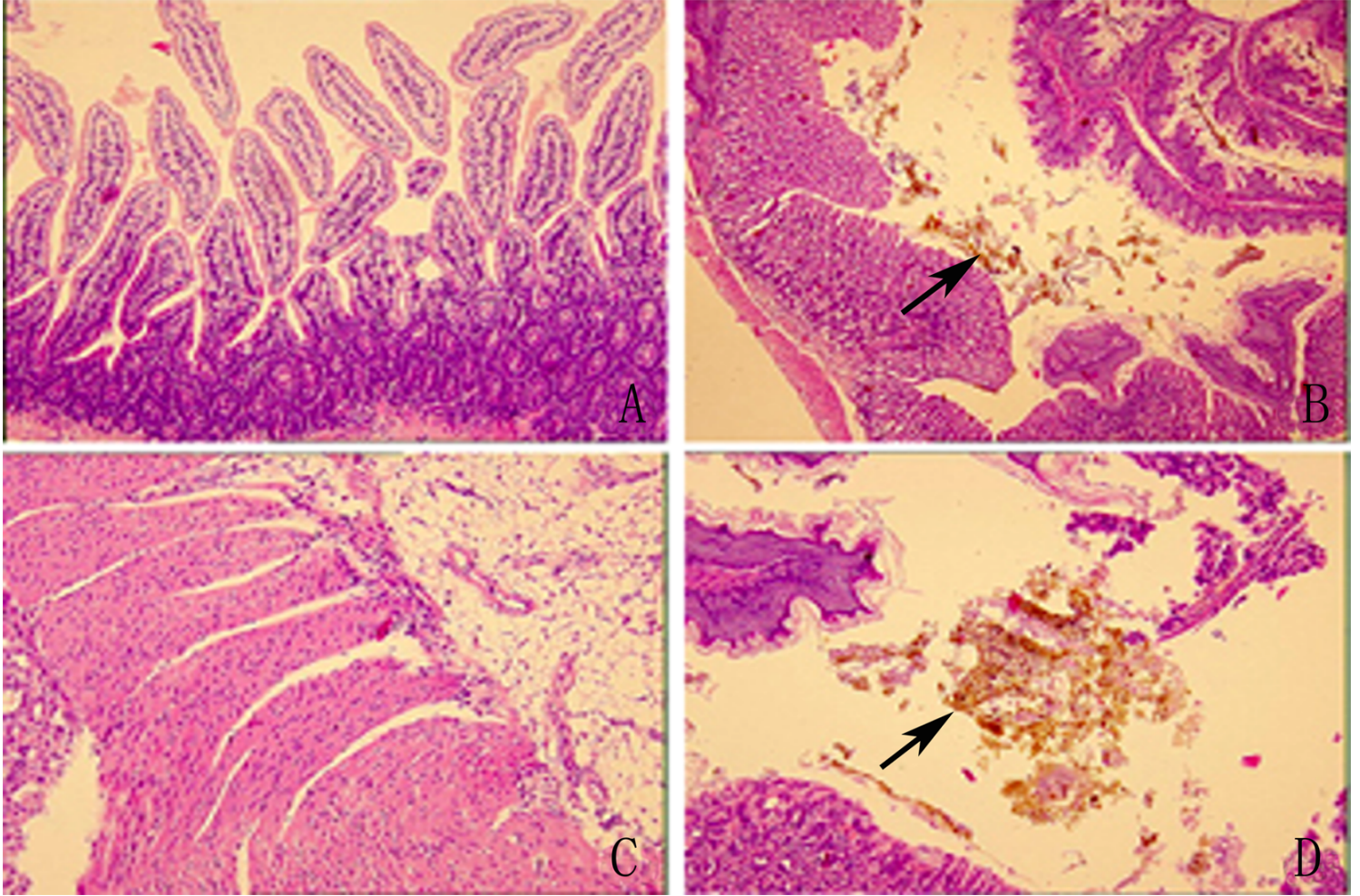
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## Figures



Figure 1

Effects of AUE on duodenum and stomach: (A) Stomach of Pre-AUE, (B) Stomach of After-AUE, (C) Duodenum of Pre-AUE, (D) Duodenum of After-AUE.



**Figure 2**

HE staining of duodenum and stomach: (A) Duodenum of Pre-AUE ( $\times 10$ ), (B) Duodenum of After-AUE ( $\times 10$ ), (C) Stomach of Pre-AUE ( $\times 10$ ), (D) Stomach of After-AUE ( $\times 10$ ).

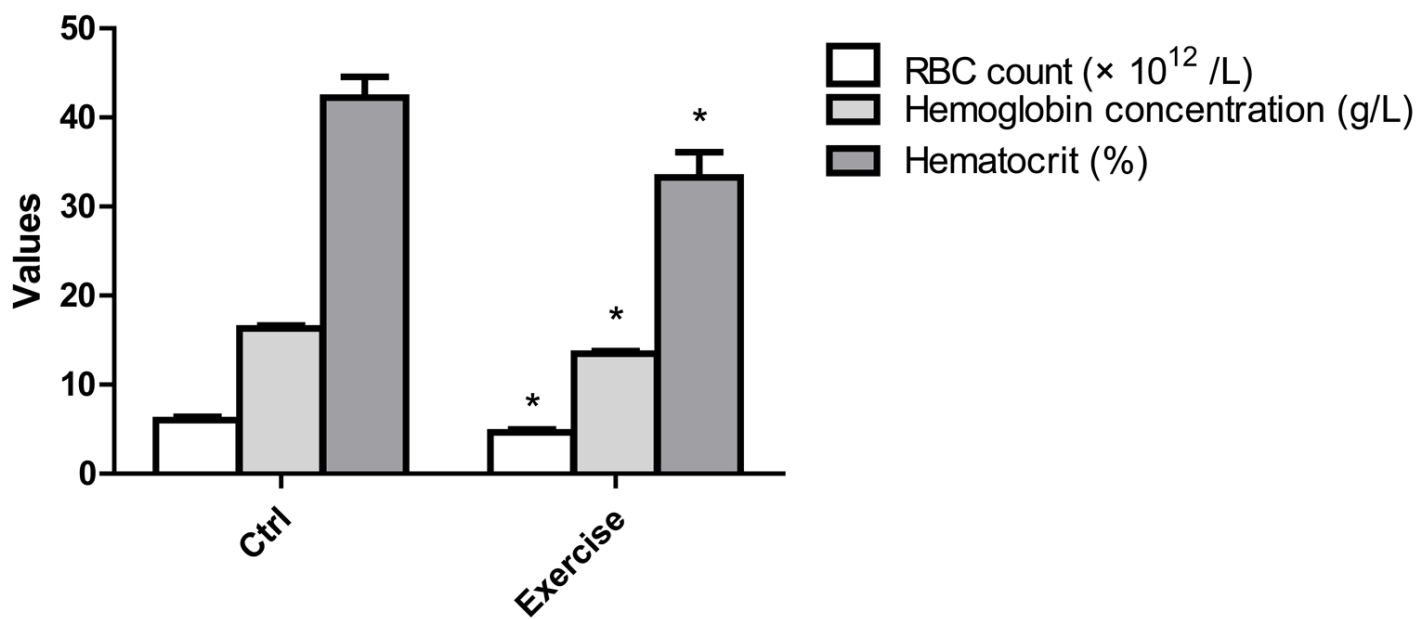


Figure 3

The effects of AUE on RBC, hemoglobin and hematocrit. All data are presented as mean  $\pm$  SD (n=12). \* P<0.05 vs. control group.

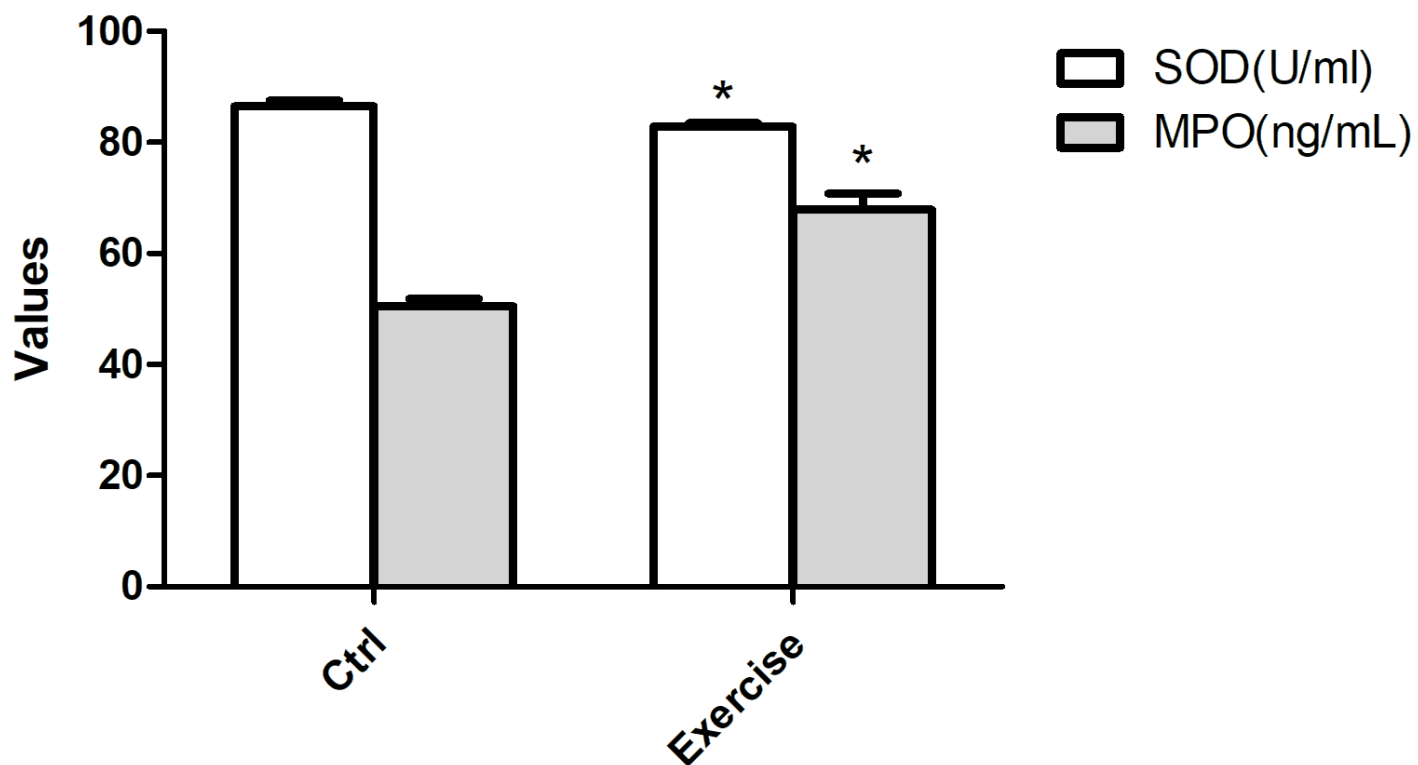
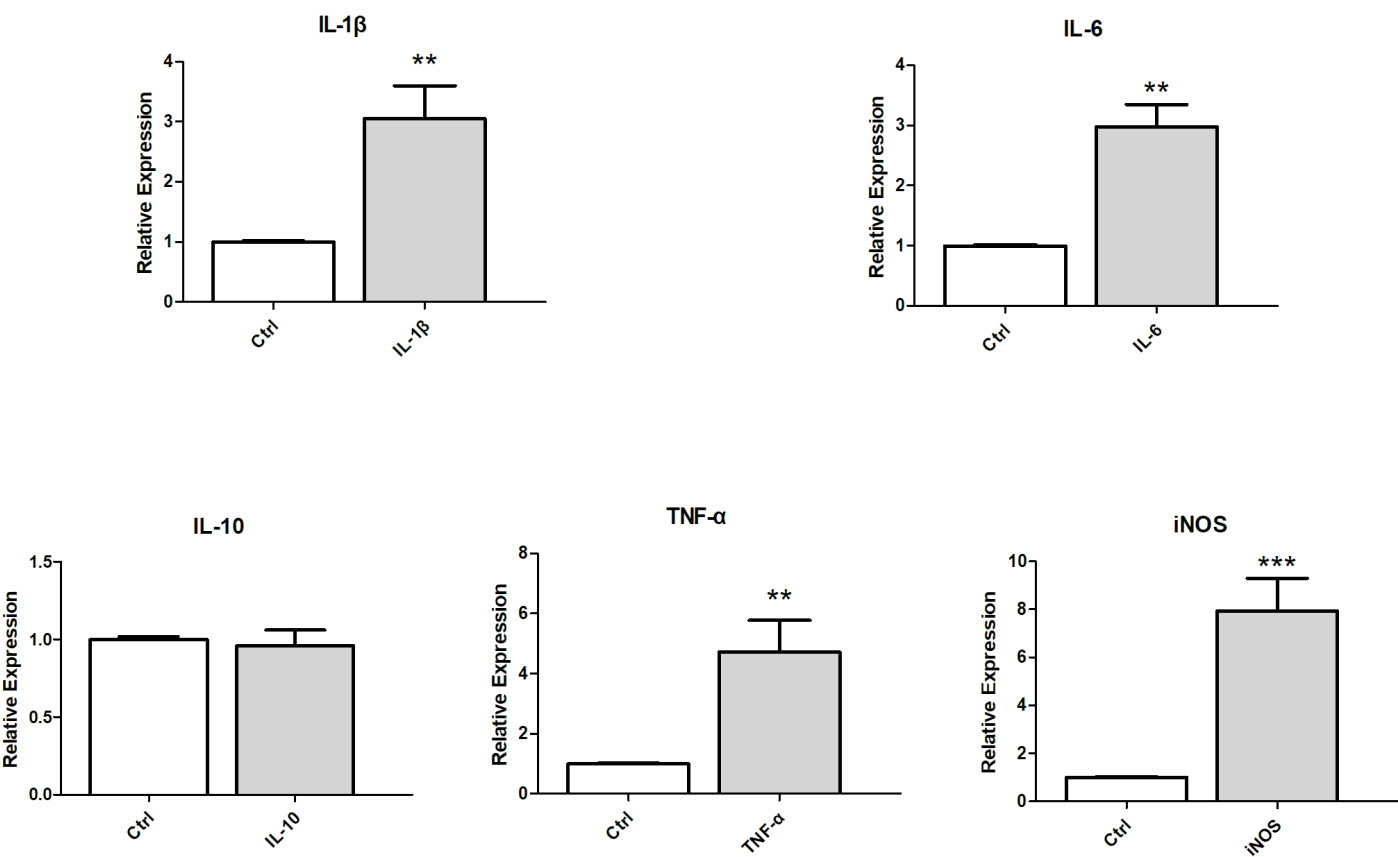


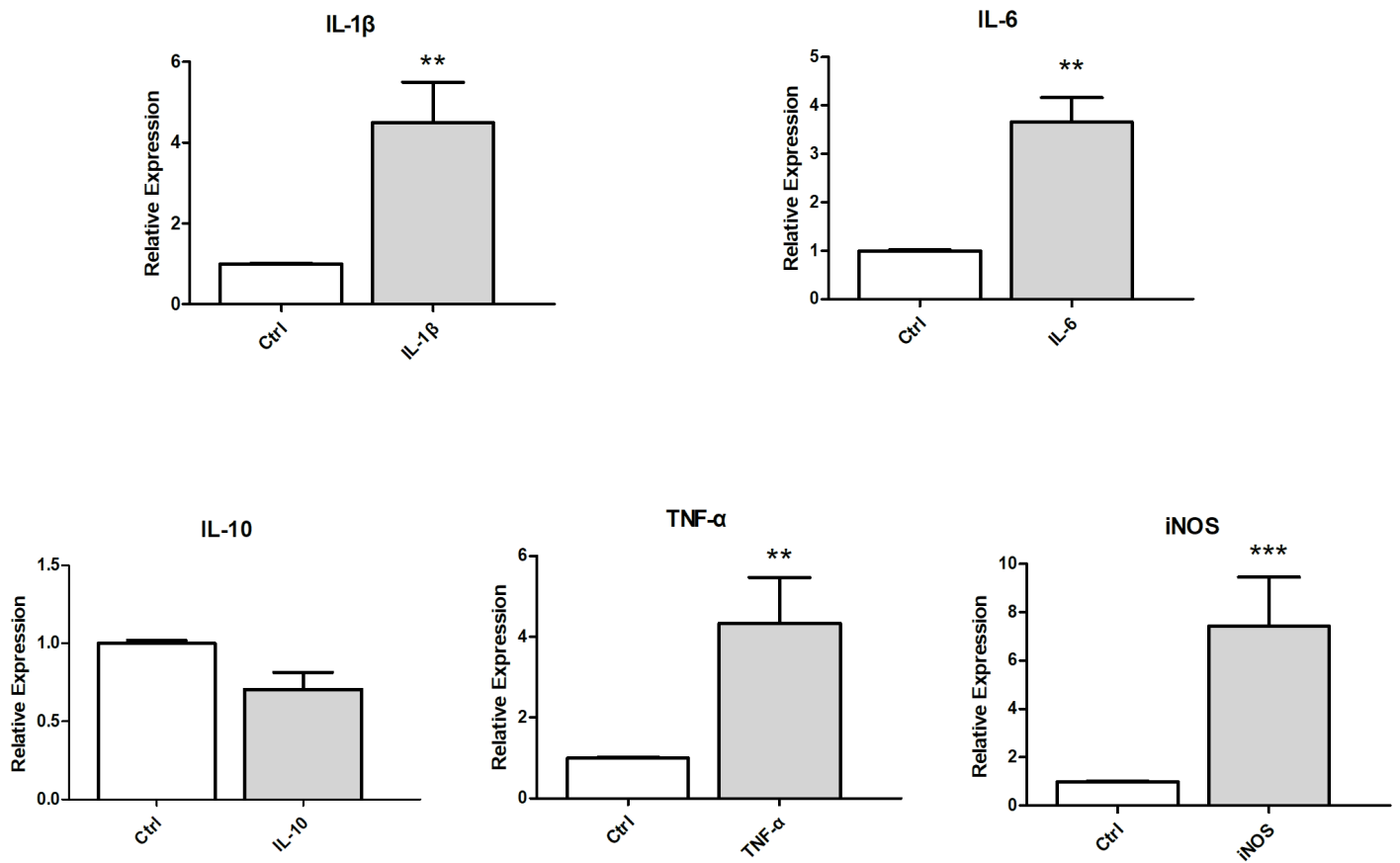
Figure 4

The effects of AUE on plasma SOD and MPO. All data are presented as mean  $\pm$  SD (n=12). \* P<0.05 vs. control group.



**Figure 5**

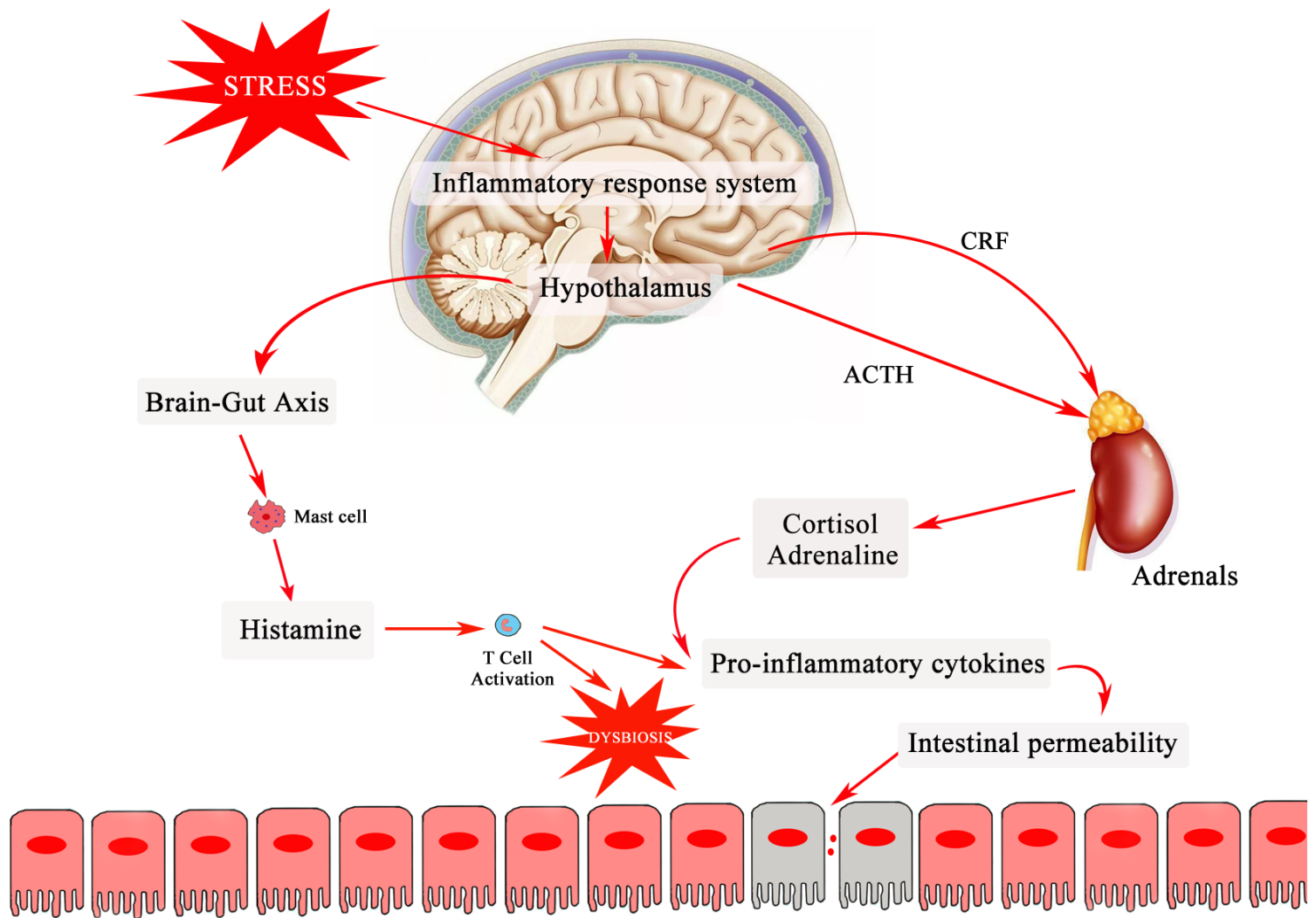
The levels of IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , and iNOS gene in hypothalamus of control and experimental group were detected by PCR. All data are presented as mean  $\pm$  SD (n=12). \* P<0.05 vs. control group.



**Figure 6**

The levels of IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , and iNOS gene in duodenum of control and experimental group were detected by PCR. All data are presented as mean  $\pm$  SD (n=12). \* P<0.05 vs. control group.





**Figure 7**

The mechanism of stress affecting brain, GI mucosa and GI permeability through inflammatory response system.