



## Effect of intestinal mucosal immunity induced by transport stress and the regulation mechanism of Suanzaoren decoction

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### ABSTRACT

The present study investigated the effects of transport stress on intestinal mucosal immunity of laboratory rats and the potential regulation mechanism of Suanzaoren Decoction (SZRD). Sprague Dawley rats were subjected to 35 °C and 60 revolutions per min (rpm) on a constant temperature shaker (2 hours/day) for 1, 2 or 3 days. Physiological, biochemical, morphological and immunological analysis were conducted before and after treatment to evaluate stress response. Testing lymphocyte subsets from mesenteric lymph nodes with Flow cytometry method and determining Th cell cytokines level from jejunum with ELISA were to investigate the regulation of SZRD on immunity status intestinal mucosa. Western blot analysis for TGF- $\beta$ /Smads signaling pathway was conducted to found the potential immunological mechanisms. According to our investigation, the mimicking transport treatment induced obvious stress responses with significant increases in serum corticosterone (CORT), serum glucose (GLU), creatine kinase (CK) and lactate dehydrogenase (LDH) levels ( $P < 0.01$ ), decreases in white blood cells and lymphocytes from Peripheral blood ( $P < 0.01$ ), and severe injury of jejunum, especially in S3d group. SZRD helps to restore mesenteric lymphocyte subsets changes and Th cell cytokines elevation ( $p < 0.05$ ,  $p < 0.01$ ), to suppress activation of TGF- $\beta$ /Smads signaling pathway caused by transport stress. So SZRD was helpful to maintain the body in equilibrium state.

**Key words:** transport stress, rat model, intestinal mucosal immunity, Suanzaoren Decoction, TGF- $\beta$ /Smads signal pathway.

### INTRODUCTION

Transport is considered a synthetic procedure that exposes livestock to a series of adverse stimuli responsible for several physiological responses. These stimuli include capture, collision and scrape, cold and heat, thirst and hunger, fear and so on [1]. Superposition of those various stimuli always influence animal growth, attenuate immunity, cause cell damage, and even lead to death.

People at home and abroad always used two methods to improve the transport stress: nutrition and mitigation. A.L. Schaefer relieved the transport stress of cattle by providing electrolyte drinking water [2]. Some scholars discovered some antipsychotic drugs (such as chlorpromazine, acepromazine and diazepam.) had good effect on transport stress on sheep [3]. However, it is not optimistic using antipsychotic drugs against transport stress, for which draws high attention with drug residue and is increasingly hard to get. In addition, studies have been reported that curcumin protected transport stress by reducing serum cortisol concentration and NO level of hippocampus, balancing BDNF expression in swine [4].

SZRD is from traditional Chinese medical work "Jingui Chamber", composed of Suanzaoren, Poria, Rhizoma

Anemarrhenae, Rhizoma Chuanxiong, and Radix Glycyrrhizae, is a classic prescription as a tranquilizing drug. Currently pharmacological studies about SZRD focused on its sedative and hypnotic effects. Our lab added and subtracted the original SZRD for protecting livestock from transport stress. This study was to investigate the effect of SZRD on the intestinal mucosal immunity against transport stress.

## EXPERIMENTAL SECTION

### Animal care and experimental groups

All experimental protocols were approved by the Committee for the Care and Use of Experimental Animals, China Agricultural University. Forty-two male Sprague Dawley rats weighing  $250 \pm 20$  g (Beijing Vital River Laboratory, Animal Technology Co., Beijing, P. R. China) were housed [25 °C, 60% relative humidity (RH)] for 7 days. On the 8th day, firstly twenty-four rats were chosen randomly to divide into the following four groups: control (C), 1-day stress, 2-day stress, and 3-day stress (S1d, S2d, and S3d) groups. Another 18 Sprague Dawley rats were randomly divided into the following three groups: control (C), transport stress model (T), SZRD medicine treatment (MT). Six rats in each group were housed in a controlled environment (25 °C, 60% RH); and provided free access to food and water.

### Treatment and sampling

Rats in stress (S1d, S2d, and S3d) groups were subjected to vibration at 60 rpm at 35 °C on a constant temperature shaker (DHZ-CA, TAICANG, China) from 9:00 to 11:00 daily to simulate transport stress for 1, 2, or 3 days. After treatment, all rats were given anesthesia by inhaling Ether absoluter (CH<sub>3</sub>CH<sub>2</sub>OCH<sub>2</sub>CH<sub>3</sub>, A.R. China), exsanguinated immediately after anesthesia, and then sacrificed. Blood samples were collected for the analysis of CORT, GLU, CK, LDH and ALP, as well as the number of leukocyte and lymphocyte. Sections of the jejunum were excised to fix in 10% buffered formalin phosphate for paraffin embedding, and to fix in 4% glutaraldehyde for electron microscopy. Rats in MT group were given SZRD (Suanzaoren, et al herbs purchased from Beijing Tongrentan, SZRD preparation accordance with our previous study, 1.2 g/kg body weight) for seven days. And then rats were repeated the 3-day stressed treatment in MT group and T group. Mesenteric lymph nodes (MLNs) of rats were separated for lymphocyte subsets analysis with flow cytometry. A 3-cm section of the jejunum was minced, snaps frozen in liquid nitrogen, and stored at -80 °C for Th cell cytokines with ELISA Assay.

### Biochemical indicator and morphological analysis

Serum CORT samples were detected using the commercially available enzyme linked immunosorbent assay (ELISA) CORT kit (ADI-900-097; Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's specifications. Concentrations of serum GLU, CK, LDH and ALP were detected by an automated biochemical analyzer (TBA-40FR, Toshiba, Tokyo, Japan), in which the performance rate method was used for CK, LDH, and ALP; the glucose oxidase method was used for GLU. Numbers of leukocyte and lymphocyte were counted by an automated blood cell analyzer (Mek-7222K, Nihon Konden, Tokyo, Japan) with electric-resistivity method.

Formalin-fixed samples were embedded in paraffin and transversely sectioned (5- $\mu$ m thickness). After deparaffinization and dehydration, some paraffin sections were stained with hematoxylin and eosin (Sigma, St. Louis, MO, USA) for microstructures of the jejunum observed using a BH2 Olympus microscope (DP71, Olympus, Tokyo, Japan) and analyzed using an Olympus Image Analysis System (version 6.0). The glutaraldehyde-fixed samples were washed in the same buffer and fixed for 1 h in cold 1% osmium tetroxide in cacodylate buffer. After dehydration in graded ethanol solutions, the preparations were embedded in Araldite (EPON812, Emicron, Shanghai, P. R. China). Ultra-thin sections were stained with saturated uranyl acetate in 50% ethanol and lead citrate and examined by transmission electron microscopy (JEM, 1230, JEOL, Tokyo, Japan).

### Flow Cytometric Analysis and ELISA Assay

To analyze the difference of lymphocyte subsets in MLNs, Triple-color flow cytometry with CD3, CD4, and CD8 was used to analyze the lymphocyte subsets with monoclonal antibody anti-rabbit CD3-FITC, CD4-APC and CD8-PE (BD, New York, USA). 10,000 cells were analyzed using a FACS Calibur™ flow cytometer (BD, New York, USA). CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> indicates  $\gamma\delta$  T cell, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> indicates CD4<sup>+</sup> T cells, CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup> indicates CTL, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> indicates Th memory cells, and CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>+</sup> indicates NK cells. Concentrations of IFN- $\gamma$ , IL-2, IL-4, and IL-6 from jejunum were measured by commercial ELISA kits (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

### Western Blot Analysis

Total proteins from jejunum were extracted using a total protein extraction kit (Biochain, Hayward, California, USA) and quantified using a BCA protein assay kit (Pierce, Rockford, USA). Proteins (20  $\mu$ g/sample) were separated by SDS-PAGE (Invitrogen, Inc, Carlsbad, USA) and transferred to nitrocellulose membranes (Pierce, Rockford, USA),

then hybridized with the specific antibodies (TGF- $\beta$ , T $\beta$ R-I, T $\beta$ R-II, SMAD2, p-SMAD2, SMAD3 and p-SMAD3. Cell Signaling Technology, Danvers, MA, USA). Blots were normalized by use of GAPDH to correct for differences in loading of the proteins. Densitometric values of immunoblot signals were obtained from three separate experiments using ImageJ (National Institutes of Health, NY, USA).

### Statistical analysis

All results are presented as the mean  $\pm$  SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) and post hoc tests using SPASS11.5 (SPSS, Inc. an IBM Company, Chicago, IL, USA). A P-value of  $<0.05$  was considered significant.

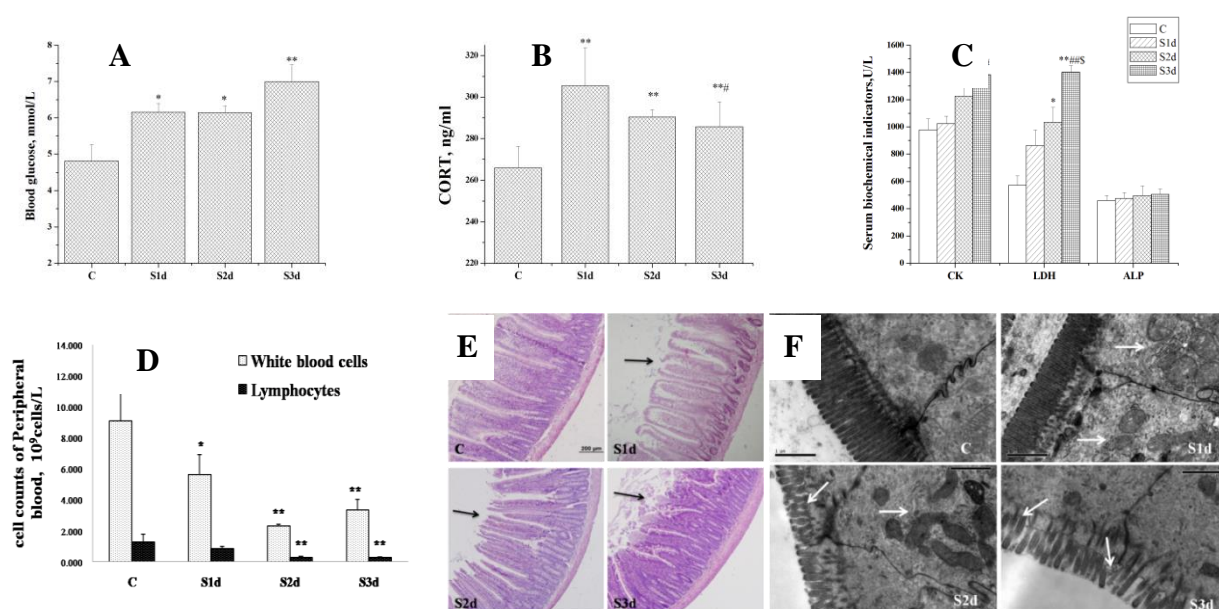
## RESULTS AND DISCUSSION

### Assessment of Stress Due to Modeling Transport Stress

Hypothalamic-pituitary-adrenal axis (HPA system) activation in stressful situations (e.g., transportation) always leads to the release of glucocorticoids (corticosterone in rodent, cortisol in human), which always provokes secondary responses including increased blood glucose [5]. In our study, mimicking transport stress caused significant elevation of serum GLU and CORT compared with control group ( $P<0.01$ ; Fig. 1A&B), which indicated that the HPA system was activated in response to the stimuli. But the following consecutive treatment decreased CORT level in S3d group compared with S1d group ( $P<0.05$ ; Fig.1B), which may due to the corticosterone-sensitive target cells enhanced negative feedback on the HPA axis [6].

The concentrations of CK and LDH in the S3d group increased evidently compared with the other groups ( $P < 0.05$ ,  $P<0.01$ ; Fig. 1C). Differences in ALP were not significant ( $P > 0.05$ ; Fig. 1C). Creatine kinase (CK), a cytoplasmic enzyme involved in energy metabolism, that is released into the blood in response to muscle damage, e.g., bruising and during vigorous exercise [7]. Lactate dehydrogenase (LDH), one of the main enzymes regulating glycolysis, is associated with stress and muscle fatigue (weariness) [8]. Increases of serum CK and LDH levels in the S3d group maybe because successive stressors more readily activated stress damage [9]. Both leukocyte and lymphocyte of peripheral blood in S2d and S3d group were decreased significantly ( $P<0.01$ ; Fig. 1D), indicating that continuous transport stress caused an immune suppression in peripheral blood [10].

**Figure 1. Mimicking transport treatment activated stress response and disturbance in rats**



**Figure 1. Mimicking transport treatment activated stress response and disturbance in rats**

(A) (B) Changes of serum CORT and GLU of rats before and after the vibration-controlled stress (60 rpm and 35 °C condition). (C) Changes of serum CK, LDH, and ALP levels induced by mimicking transport treatments. (E) Photomicrographs of hematoxylin- and eosin-stained sections of the rat jejunum from the control and stress groups. (F) Morphological alterations in the ultrastructure of the rat jejunal epithelium following mimicking transport treatment. C: control; S1d: 1-day stress group; S2d: 2-day stress group; S3d: 3-day stress group

Values represent the mean  $\pm$ SD,  $n=6$  rats for each group. \*/# $P<0.05$ , \*\*/## $P<0.01$

Photomicrographs of hematoxylin- and eosin-stained sections of the jejunum from control rats exhibited structural integrity. However, slight damage and edema to the intestinal villi were observed in S1d and S2d groups, severe

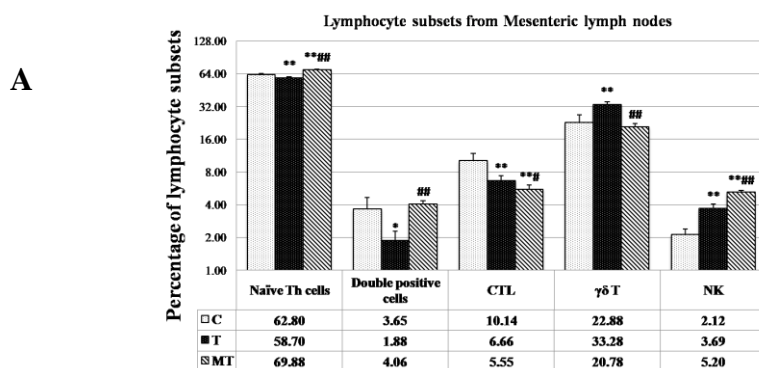
damage to the intestinal villi with desquamation at the tips of the intestinal villi and exposure of the lamina propria were examined in S3d group (Fig. 1E). Ultrastructure examination of the rat jejunum epithelium revealed that the microvilli were shortened and sparser, exhibited a breakage of mitochondrial cristae, chromatic agglutination, nucleus pyknosis, and deformation after transport-associated stress (Fig. 1F). Small intestine is not only the main place for digestion and absorption of nutrients, but also the body's largest mucosal barrier<sup>[11]</sup>. Jejunum is the longest section of the small intestine tissue. Activation of the HPA system caused by stress subsequently induced a reduction of intestinal blood flow, and thus hemorrhage, ischemia, and degeneration of the intestinal mucosa<sup>[12]</sup>. Intestinal epithelial cells act as a barrier against the penetration of microbial pathogens, cytotoxic agents, and other intestinal contents<sup>[13]</sup>. Once jejunum microvillus height became shorter and sparser and intestinal epithelial cells shed, permeability increased, which then augmented inflammatory and immune responses to infection and amplified disturbances in gut motor and sensory function that are known to accompany gut inflammation<sup>[14]</sup>.

### SZRD regulation of lymphocyte subsets in MLNs and Th cell cytokines in jejunum

The function of lymphocytes and the production of cytokines can be influenced by a variety of stressors<sup>[15]</sup>. And T lymphocytes play a variety of important roles in immune regulation, inflammation, and protective immune responses through lymphocyte subsets<sup>[16]</sup>. Compared with the control group, the changes of lymphocyte subsets in MLNs in model group were in varying degrees (Fig. 2A). Percentages of Naïve Th cells, Th memory cells and CTL cells from MLNs decreased significantly ( $P < 0.05$ ), percentages of  $\gamma\delta$  T cells and NK cells from MLNs increased significantly ( $P < 0.01$ ). Compared with the model group, SZRD could decreased percentages of Naïve Th cells, Th memory cells and CTL cells and increased  $\gamma\delta$  T cells and NK cells. Activated Naïve Th cells in the immune response process can differentiate into various effector T cell subsets, and thus play a corresponding role in humoral and cellular immunity<sup>[17]</sup>. Progressive or irregular decrease of Naïve Th cells marked that immune system was severely compromised. In other words, transport stress could induce the immune system damage, and SZRD protected the immune system by promoting Naïve Th cells proliferation. CTL cells play a very important role in the viruses, intracellular bacteria and tumor immune responses<sup>[18]</sup>. Decrease of CTL cells in model group indicated that the ability of dealing with viruses and bacterial infections declined, however, the regulation of SZRD on CTL cell is not significant. NK cells are the innate immune effector cells, which play a role in immune response before T and B cells<sup>[19]</sup>. The number of NK cell in MT group was increased significantly than control and T group, indicating that SZRD could promote proliferation and differentiation of NK cell. In addition, SZRD also could promote proliferation and differentiation of Th memory cell, but  $\gamma\delta$  T cells was inhibited.

According to our study, the concentrations of cytokines IFN- $\gamma$ , IL-2, IL-4, and IL-6 increased with different levels (Fig. 2B). Compared with the control group, in which the increase of IL-2 and IL-4 in model group was significant ( $P < 0.05$ ) in jejunum, the increase of IFN- $\gamma$  and IL-6 was extremely significant ( $P < 0.01$ ). Compared with the model group, SZRD reduced the Th cytokines expression ( $P < 0.05$ ;  $P < 0.01$ ). IFN- $\gamma$  and IL-2 help Naïve Th cells convert into Th1<sup>[20]</sup>, IL-4 and IL-6 contribute to conversion of Naïve Th cells into Th2<sup>[21]</sup>, in addition, IL-6 also help Naïve Th cells convert into Th17<sup>[22]</sup>. Elevation of those cytokines in T group indicated that transport stress stimulated Naïve Th cells to convert into Th1, Th2 and Th17 cells, which initiated cellular and humoral immune responses. SZRD could play a good role in regulating the equilibrium state, and rebuild the organism homeostasis.

Figure 2. SZRD regulated the lymphocyte subsets and Th cell cytokines following transport stress



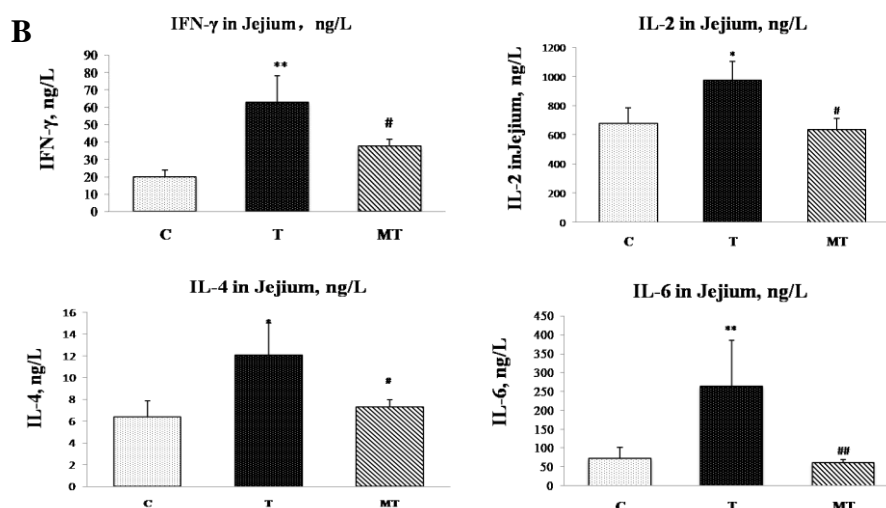


Figure 2. SZRD regulated the lymphocyte subsets and Th cell cytokines following transport stress (A) Transport stress induced the changes of lymphocyte subsets in MLNs and the effect of SZRD regulation. (B) Transport stress induced the changes of Th cell cytokines in jejunum and the effect of SZRD regulation

C: control; T: transport stress model group; MT: SZRD medicine treatment group. Values represent the mean  $\pm$  SD, n=6 rats for each group. \*/#P<0.05, \*\*/###P<0.01

### SZRD regulation of TGF- $\beta$ /Smads signaling pathway in jejunum

Nearly all immune cells, including B cells, T cells, dendritic cells and macrophages can secrete TGF- $\beta$ , and TGF- $\beta$  play a negative regulatory role on these cells for their differentiation and activation. So TGF- $\beta$  is regard as an effective immunosuppressant, related to autoimmune, inflammatory response and so on [23]. Process of TGF- $\beta$  signaling pathway is: firstly the complexes are formed by the ligand (TGF- $\beta$  family members) and the TGF- $\beta$  receptor (T $\beta$ R- I and T $\beta$ R- II), then Smads proteins are activated, and then the signal transduction is brought to the nucleus, lastly the function of transcription factors are successfully performed [24]. In our study, compared with the control group, the expression of T $\beta$ R- II, SMAD2 and p-SMAD2 were increased significantly (P < 0.05; P < 0.01), indicating that transport stress activated TGF- $\beta$  signaling pathway. Compared with the model group, the levels of T $\beta$ R- II and SMAD2 were decreased because of SZRD treatment, namely SZRD played a protective role in immunity by regulating TGF- $\beta$  signaling pathway.

Figure 3. SZRD regulated the TGF- $\beta$ /Smads signaling pathway following transport stress

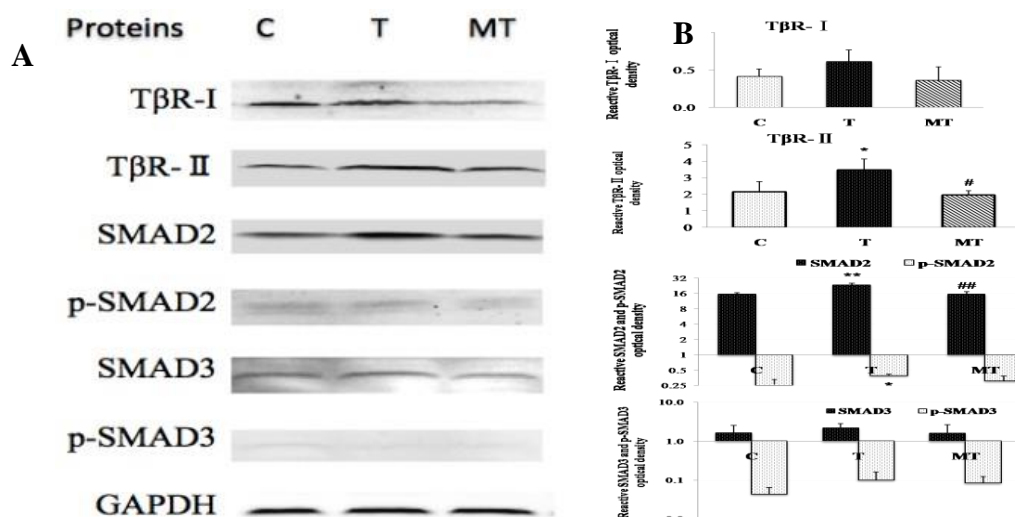


Figure 3. SZRD regulated the TGF- $\beta$ /Smads signaling pathway following transport stress

(A) Transport stress induced the activation of TGF- $\beta$ /Smads signaling pathway and the effect of SZRD regulation. (B) Quantitative analysis of immunoblot signals for (A). C: control; T: transport stress model group; MT: SZRD medicine treatment group

Values represent the mean  $\pm$  SD, n=6 rats for each group. \*/#P<0.05, \*\*/###P<0.01.

### CONCLUSION

Transport stress induced the immune system suppression, and SZRD was helpful to protect the mucosal immunity

by regulating mesenteric lymphocyte subsets, Th cytokine levels and TGF- $\beta$ /Smads signaling pathway.

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