sciendo

J Vet Res 68, 295-302, 2024
DOI:10.2478/jvetres-2024-0024

Effect on the splenocyte function of weaned piglets induced by continuous lipopolysaccharide injections

Tingyu Yang*, Guotong Zhao*, Wenlu Zhu, Wanting Yu, Yijie Jiang, Yunxiao Zhou, Yong Li✉

College of Animal Science and Technology, Jiangxi Agricultural University, Nanchang 330045, Jiangxi, China
liyong2912@163.com

Received: October 28, 2023 *Accepted: April 22, 2024*

Abstract

Introduction: When piglets are exposed to pathogens for a long period, the immune system organs, among them the spleen, play a major role in combating the stress caused by those pathogens. In the present study, the effect on splenocyte function was investigated in a model of weaned piglets in which stress was induced by multiple low doses of lipopolysaccharide (LPS). **Material and Methods:** Forty-eight 28-day-old piglets were divided into two groups: the LPS group and the control group. During the experimental period of thirteen days, the LPS group was intraperitoneally injected with LPS (100 μg/kg) once per day, and the control group was injected with the same volume of 0.9% sterile saline. On the 1st, 5th, 9th and 13th days, the piglets' spleens were collected for isolating splenocytes. The proliferation ability of splenocytes was evaluated by the cell-counting-kit 8 method. Flow cytometry was used to detect cell cycle stage and apoptosis, and the nitric oxide level of cell supernatant was also tested. **Results:** In the experimental group, the proliferation ability of splenocytes was enhanced, the proportion of cells in the G0/G1 phase was smaller, and cells were promoted to the S and G2/M phases. Meanwhile, apoptosis was suppressed and nitric oxide release upregulated. The results were significantly different between the LPS group and the control group on the 5th and 9th days. **Conclusion:** The difference between the results of one group and those of the other suggest that after the 5th LPS injection, multiple low doses of LPS activated splenocytes and restored the number of splenocytes, which maintained and possibly enhanced the regulation of the immune function of the spleen.

Keywords: weaned piglet, LPS, splenocytes, immune function.

Introduction

Pigs are one of the most economically important domestic animals in the world. During the weaning stage of piglets, stress problems often occur, which pose a great challenge to pig farm production (36). Stress is a set of non-specific responses that occur when the organism is stimulated by an internal or external stressor. Different stresses cause the body to produce specific and non-specific responses. Exogenous pathogens in the environment, such as bacteria and viruses, can infect the animal and cause an immune response. Under excessive stress, the immune system is activated, immune cells continuously release inflammatory cytokines in response to external stimuli, and immune allostasis is lost (38). Excessive stress can also result in heavy economic losses for the farmer; the stressed animal may exhibit a series of negative symptoms, including an increase in temperature and productivity-harming decreases in feeding rate and cessation of growth, and excessive stress may even lead to the animal's death (20). Moderate stress, in contrast, promotes the adaptation of the organism to the environment and improves tolerance to adverse stress (1, 4).

Lipopolysaccharide (LPS), a component of the cell surface in Gram-negative bacteria, is the main pathogenic factor that causes an inflammatory response. When Gram-negative bacteria lyse and die, LPS is released and recognised by toll-like receptor 4 (TLR4) (39). After binding to TLR4, LPS initiates signalling along a series of intracellular pathways, promoting the selection of bioactive factors such as interleukin (IL)-2, IL-10, interferon gamma (IFN-γ) and nitric oxide, and resulting in an inflammatory response (12, 28).

The spleen is a major immune organ, which is populated by many types of immune cells such as T cells, B cells and macrophages (33). As the central site of the immune response, the spleen plays a vital role in both innate and adaptive immunity which is crucial for protection against pathogens (6). Many of the regulators in splenic response involve splenocyte proliferation. The proliferation ability of splenocytes is thus the most direct

indicator of spleen immune function (41). Several active factors are involved besides the spleen in the immune response, such as nitric oxide (NO). Nitric oxide is an important messenger in the pathogenesis of inflammation, linking innate and adaptive immunity. When the immune function is activated, NO may mediate signalling, which is closely associated with the release of cytokines and T-cell differentiation (34, 38).

The relationship between stress and immune function is intricate. Moreover, the effect of stress-induced immune response varies depending on the species, pathway, duration and intensity (7). When an animal is faced with stress, the number and ratios of its blood leukocytes may change, secretion of cytokines may climb to abnormal levels and the ratio of subpopulation sizes after lymphocyte differentiation come into imbalance. These changes can significantly impact immune function (23). As an integral part of the immune function, splenocytes become involved when animals come under stress. However, relatively little research has been conducted on how splenocytes play a role in LPS-induced immune response. A stress model was established by continuous injection of LPS as presented by Zhou *et al.* (43). Such a model is also a popular one widely used in similar studies on stress and nutritional disturbances (37, 40). The aim of the study was to evaluate the effect on splenocyte function in a model of weaned piglets in which stress was induced by multiple low doses of LPS.

Material and Methods

Experimental animals. Forty-eight healthy weaned 28-day-old piglets (Landrace × Large White × Duroc weighing 6.65 ± 1.19 kg) were obtained from Jiangxi Aoyun Agricultural Development Co., Ltd. (Nanchang, Jiangxi, China). The piglets had free access to feed and water during the experimental period. After a three-day pre-feeding period to alleviate any stress responses, the piglets were randomly divided into an LPS group and a control group of 24 piglets each. The LPS group was intraperitoneally injected with 100 μg/kg body weight of LPS (*Escherichia coli* serotype 055: B5; Cat. No. L2880; Sigma-Aldrich, St. Louis, MO, USA) daily (17, 29). The control group was injected with the same volume of 0.9% sterile saline. The course of intraperitoneal injections lasted for 13 days. Six piglets were randomly selected from the LPS and control groups on the 1st, 5th, 9th and 13th days (d1, d5, d9 and d13). After the piglets were anaesthetised, euthanised and then dissected, the spleens were collected and immediately added to precooled sterile phosphate-buffered saline (PBS) for splenocyte isolation. All procedures in this experiment were approved by the Animal Care and Use Committee of Jiangxi Agricultural University (approval No. JXAULL-2022006).

Splenocyte isolation and cell culture. The piglets' spleens were washed twice with PBS, minced between the frosted edges of two microscopic slides, and then passed through a 200-mesh nylon screen. The cell suspension was collected and then centrifuged at 1,500 rpm for 5 min at 4°C. After removing the supernatant, the red blood cells (RBC) were lysed using RBC lysis buffer (R1010; Solarbio, Beijing, China) and kept on ice for 5 min. Subsequently, the splenocyte suspension was washed twice with PBS. Next, the splenocyte suspension was adjusted to a concentration of 5×10^6 cells/mL and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (11875; Solarbio) supplemented with 10% foetal bovine serum (13011-8611; Tianhang Biotechnology, Huzhou, China) and 1% penicillin-streptomycin (P1400; Solarbio) in a humidified incubator at 37°C with 5% CO_2.

Cell proliferation. A cell-counting-kit 8 (CCK-8) assay (abs50003; Absin, Shanghai, China) was used to assess cell proliferation according to the manufacturer's protocol. In this study, the effect of the mitogen concanavalin A (ConA C8110; Solarbio) on splenocytes was used to determine cell proliferation in both the LPS and control groups. Splenocytes were pipetted into 96-well plates and stimulated with ConA (5 μg/mL), or treated with the same volume of RPMI-1640 medium for 48 h. After culturing, splenocytes were treated with 10 μL of CCK-8 reagent and incubated for 4 h at 37°C. The absorbance of each well was measured at 450 nm optical density (OD) using a microplate reader (SpectraMax 190; Molecular Devices, Shanghai, China). The cell proliferation was calculated by the following formula:

$$\text{Cell proliferation} = (\text{OD (ConA)} - \text{OD (RPMI 1640)}) / \text{OD (ConA)}.$$

Cell cycle analysis. After being cultured for 48 h, the splenocytes were washed twice with PBS and fixed with ice-cold 70% ethanol at 4°C overnight. After the ethanol was removed and the splenocytes were washed with PBS again, the splenocytes were resuspended in 480 μL of staining solution, 10 μL of propidium iodide (PI) staining solution and 10 μL of ribonuclease A according to the cell cycle instructions (G1700; Servicebio, Hubei, China). The splenocytes were gently vortexed and then incubated for 30 min at 37°C protected from light. Finally, flow cytometry was performed (BD FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). Cell cycle distributions were analysed with FlowJo software (BD Biosciences). The proliferation index (PrI) was calculated according to the following equation:

$$\text{PrI} = (\text{S} + \text{G2/M}) / (\text{G0/G1} + (\text{S} + \text{G2/M})) \times 100\%,$$

where "S" indicates cells in the synthesis phase, "G2" cells in the gap 2 phase, "M" cells in the mitosis phase and "G0/G1" cells in the resting state or gap 1 phase.

Cell apoptosis analysis. After being cultured for 48 h, the splenocyte suspension was harvested, washed twice with PBS and adjusted to 1×10^5/mL in ice-cold binding buffer. A 100 μL volume of cell suspension was double stained with 5 μL of Annexin V-enhanced green fluorescent protein (EGFP) and 5 μL of PI (G1510; Servicebio), and then gently mixed and incubated for 10 min at room temperature in the dark. With all apoptotic cells becoming stained with EGFP, the

presence or absence of PI staining respectively indicated early or late apoptosis, and the total staining revealed total apoptosis. After the incubation step, 400 µL of binding buffer was added to resuspend the splenocytes, and the number of apoptotic cells was analysed by flow cytometry within 1 h. Cell apoptosis was evaluated subsequently using FlowJo software.

Nitric oxide analysis. The concentration of this compound in the cell supernatant was analysed with a nitric oxide assay kit (BC1475; Solarbio). The cell suspension was harvested and mixed with reagent 1 according to the manufacturer's protocol. After incubation at 37°C for 60 min, reagent 2 was added to each sample. The supernatant was removed after centrifugation at 3,500 rpm for 10 min at room temperature and placed into a 96-well plate. The mixed reagents were added to each well and the supernatant and reagent mixture was incubated for 10 min at room temperature. The absorbance was measured using a microplate reader at 550 nm.

Statistical analysis. All data were expressed as means ± standard deviation. Statistical differences between the LPS and control groups were determined by one-way ANOVA and a multiple-range test based on least significant differences using version 25 SPSS software (IBM, Armonk, NY, USA). Statistical significance was recognised at a P-value < 0.05. The corresponding graphs were created with Graph Prism 8.0 software (GraphPad, San Diego, CA, USA).

Results

Cell proliferation. The proliferation rate significantly increased in splenocytes treated with LPS from d9 to d13 (P-value < 0.01) compared with the control group, but there was no significant change on d1 or d5 (P-value > 0.05). The change in cell proliferation is shown in Fig. 1.

Cell cycle analysis. Compared with the control group, the splenocyte percentage in the resting phase and gap 1 phase from the LPS group decreased on d5, d9 and d13 (P-value < 0.01), the splenocyte percentage in the synthesis to gap 2 and mitosis phases from the LPS group increased on d5, d9 and d13 (P-value < 0.01), and the PrI in splenocytes treated with LPS also significantly rose on these days (P-value < 0.01). The percentage changes with time in experimental piglets are shown in Figs 2 and 3.

Cell apoptosis analysis. Staining of apoptotic cells was observed and is depicted in Fig. 4. Compared with the control group, the percentage of apoptosis was significantly lower in the LPS group on d1, d5, d9 and d13 (P-value < 0.01), as shown in Fig. 5.

Nitric oxide analysis. Levels of NO in the supernatant from splenocytes were determined. As shown in Fig. 6, the NO secretion was significantly elevated in splenocytes from the LPS group on d5 and d9 relative to the control group NO secretion (P-value < 0.01), but there were no significant differences between the two groups on d1 and d13 (P-value > 0.05).

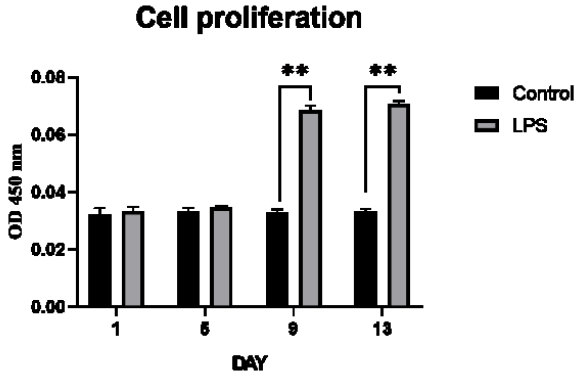

Fig. 1. Effect on splenocyte proliferation in weaned piglets induced by lipopolysaccharide (LPS) challenge. Data represent the mean ± standard deviation (n = 6 in each group)
** – highly significant difference (P-value < 0.01)

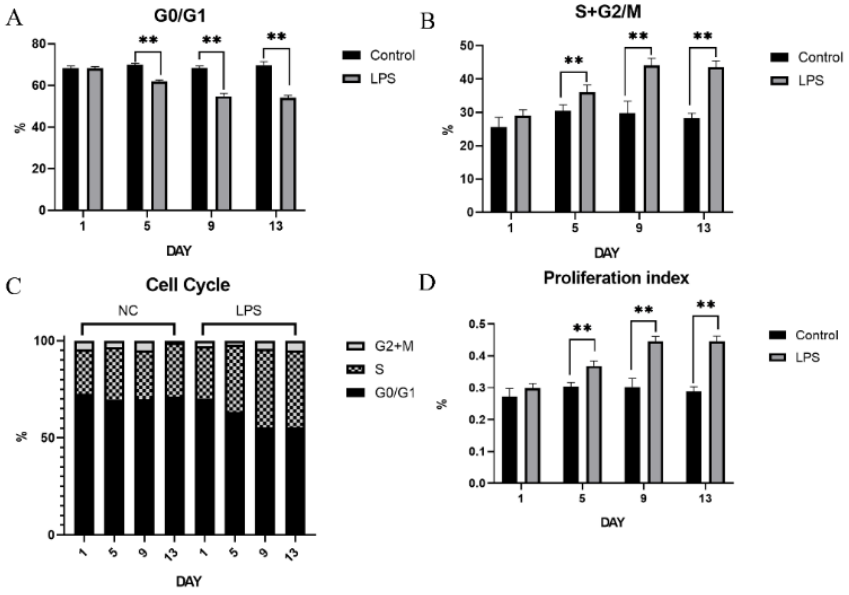

Fig. 3. Effect of lipopolysaccharide (LPS) challenge to weaned piglets on the cell cycle distribution and proliferation index (PrI) of splenocytes. A – percentage of splenocytes in the resting phase (G0) and gap 1 phase (G1); B – percentage of splenocytes in the synthesis (S), gap 2 (G2) and mitosis (M) phases; C – cell cycle phase distribution of splenocytes (NC – negative control group); D – Proliferation index. Data represent the mean ± standard deviation (n = 6 in each group)
** – highly significant difference (P-value < 0.01)

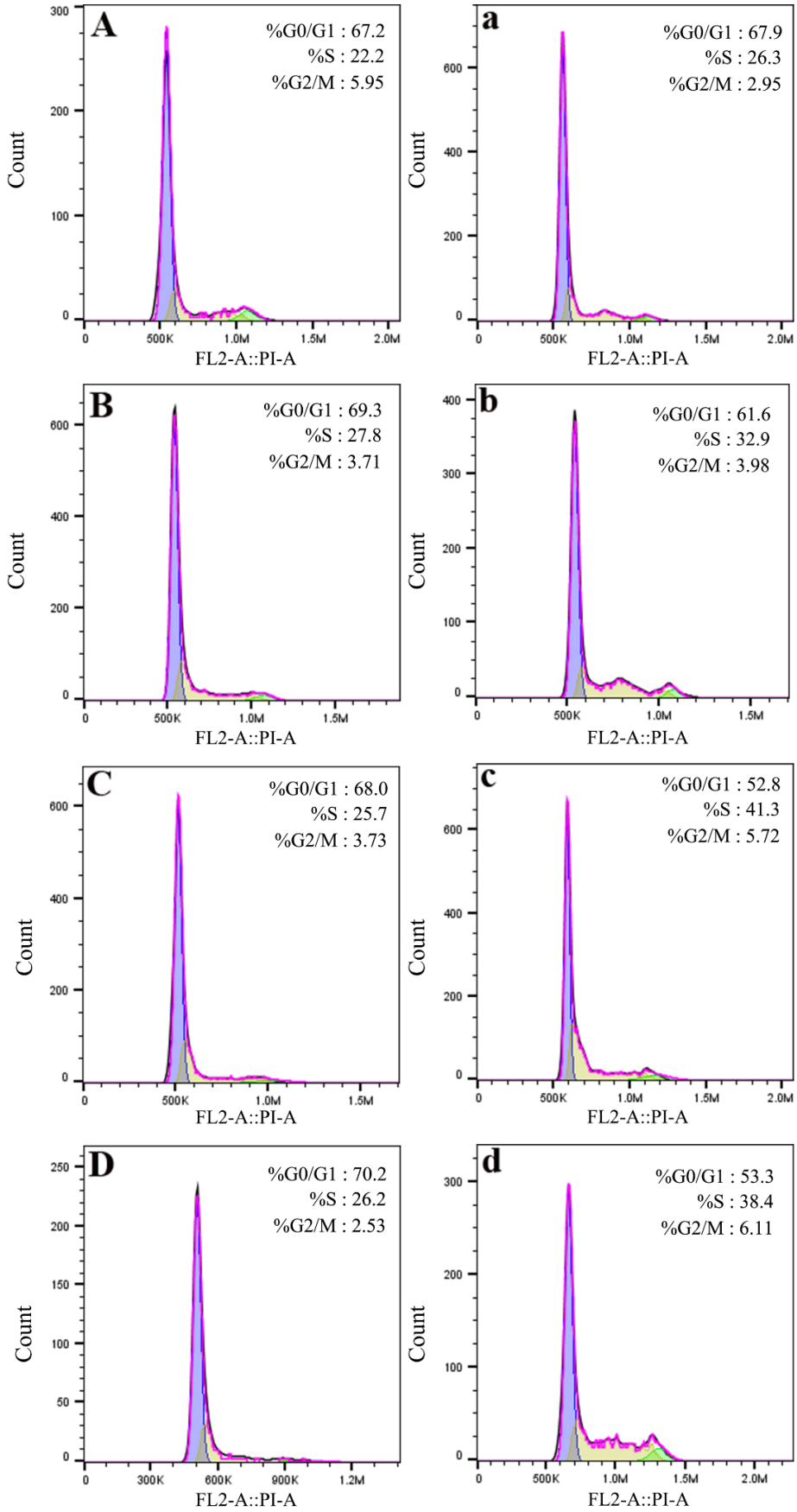

Fig. 2. Flow cytometry results of evaluation of weaned piglet splenocyte cell cycle distribution after lipopolysaccharide (LPS) challenge. A, B, C and D show the splenocytes of saline-treated piglets on day (d)1, d5, d9 and d13, respectively; a, b, c and d show the splenocytes of LPS-treated piglets on the same days; G0 – cells in resting phase; G1 – cells in gap 1 phase; S – cells in synthesis phase; G2 – cells in gap 2 phase; M – cells in mitosis phase; FL2-A – total relative fluorescent intensity in channel 2; PI-A – total fluorescent intensity from propidium iodide

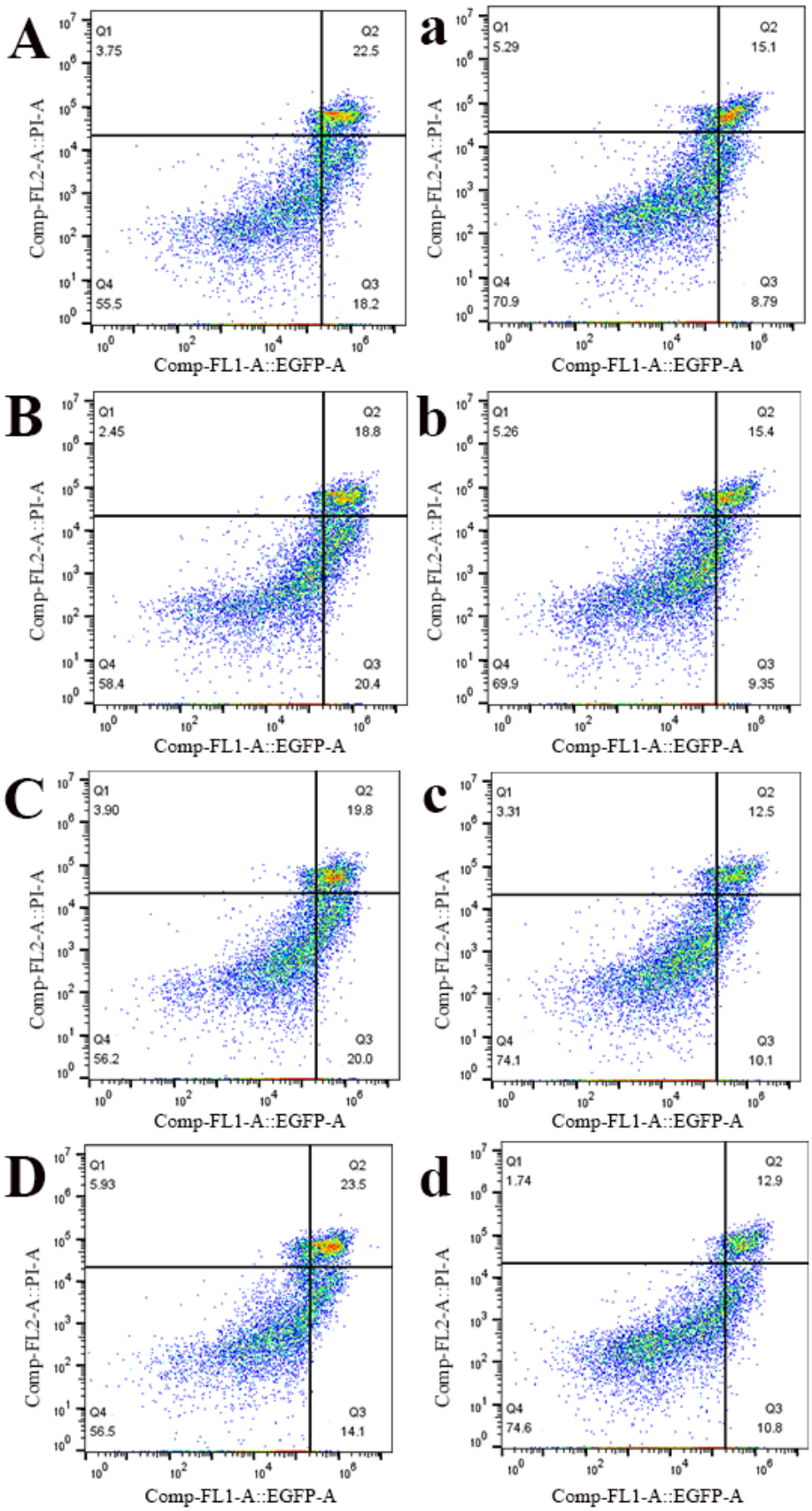

Fig. 4. Percentages of apoptosis in splenocytes detected using Annexin V-enhanced green fluorescent protein (EGFP)/propidium iodide staining by flow cytometry. Apoptosis was affected by lipopolysaccharide (LPS) challenge to weaned piglets. A, B, C and D show the splenocytes of saline-treated piglets on day (d)1, d5, d9 and d13, respectively; a, b, c and d show the splenocytes of LPS-treated piglets on the same days; Q – quadrant; Comp – compensated; FL1/2-A – total relative fluorescent intensity in channel 1/2; PI-A – total fluorescent intensity from propidium iodide; EGFP-A – total fluorescent intensity from enhanced green fluorescent protein

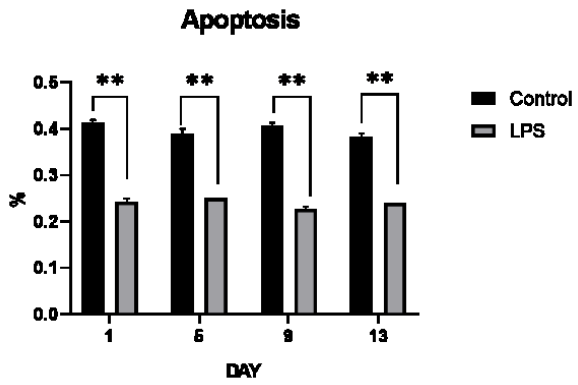

Fig. 5. Effect of lipopolysaccharide (LPS) challenge to weaned piglets on the percentage of apoptosis in splenocytes. Data represent the mean ± standard deviation (n = 6 in each group)
** – highly significant difference (P-value < 0.01)

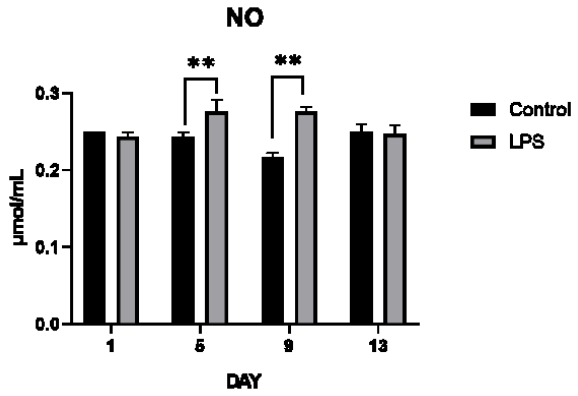

Fig. 6. Effect of lipopolysaccharide (LPS) challenge to weaned piglets on the nitric oxide secretion in splenocytes. Data represent the mean ± standard deviation (n = 6 in each group)
** – highly significant difference (P-value < 0.01)

Discussion

Populated by lymphocytes, macrophages and other immune cells, the spleen is an important site for generating the immune response to stimulation by antigens and is essential for total immune function (42). Lymphocytes are significant components of acquired immunity and serve as the primary effector cells of both cellular and humoral immunity. Splenic lymphocytes make up 25% of the body's lymphocytes (32). Much research has revealed that changes in immune function involve the activation and proliferation of lymphocytes. Thus, the ability of lymphocytes to proliferate and differentiate is an important indicator of cellular immunity (41). Lymphocyte mitogens, including ConA and phytohaemagglutinin, are non-specific for lymphocyte activation. Lymphocyte activity and immune response can be accurately assessed by adding mitogens to induce lymphocytes to proliferate *in vitro* (19). In the present study, the proliferation of splenocytes after ConA stimulation was investigated to elucidate the effect of continuous LPS induction. The results showed a significant increase in the transformation ability of splenocytes *in vitro* from the 5th day after the start of the LPS challenge. Proliferation ability is an important indicator reflecting the immune status of lymphocyte immunity (22). As an immune activator, LPS is the primary target of the immune system for recognising pathogens. It has been proved to mediate innate immunity by interacting with toll-like receptors on the surface of immune cells, releasing large amounts of cytokines while inducing the proliferation of lymphocytes as the primary specific immune response (12). The transformation ability of lymphocytes significantly increased and the immune system was activated in stressed pigs subjected to an LPS challenge (20). The microbial endotoxin induced the proliferation of pigs' T lymphocytes and promoted the creation of IL-2 (22). As important messengers, cytokines such as IL-2, tumour necrosis factor alpha and IFN-γ are released to induce further proliferation and activation of immune cells, as exemplified in an experiment by Ma *et al.* (21) in which they administered ulinastatin to pancreatitic rats and analysed their splenocytes.

The cell cycle was examined to further investigate the effect of LPS on splenocyte proliferation ability. In the cell cycle, DNA preparation is in the G1 phase, DNA synthesis is in the S phase, mitosis preparation is in the G2 phase, and mitosis occurs in the M phase (16). Many studies have shown that the G0/G1 phase is the initiation point of cell proliferation, while there may be a blockage in the G1/S phase. When the G1 phase is blocked, the cells cannot enter the S phase for DNA replication, which affects the proliferation of lymphocytes (8). Without any such blockage, as the proportion of cells in the G1 phase decreases and the proportion of those in the S phase to G2 phase increases, cells progress through the cycle, which leads to cell proliferation (18). In the present findings, the proportion of LPS-treated splenocytes in the G0/G1 phase significantly decreased and the proportions in the S to G2 phases significantly increased, as did the PrI. Splenocyte proliferation and DNA synthesis are closely related to mitosis, and when splenocytes are induced into the G2/M phase, the number of cells increases significantly (9). The number of immune cells is critical to effective immune response (41). Numerous studies have shown that an increase in cells in the G0/G1 phase delays the development of the spleen and suppresses its immune function. Moreover, an increase in cells in the S to G2/M phases also indicates its immune dysfunction (11, 16, 25, 30).

Apoptosis is a programmed cell death mode and an important physiological process to maintain allostasis (10). The level of splenocyte apoptosis may to some extent reflect the immune status of the organism. Our results showed that after LPS stimulation, the proportion of early and late apoptotic cells decreased significantly and the proportion of normal cells increased significantly, which consolidated the number of splenocytes and suggested that the splenocytes were activated. In physiological conditions, lymphocyte proliferation and apoptosis are balanced to maintain a stable number of these cells (24). The interplay of lymphocyte proliferation and apoptosis is closely related to initiating and terminating the immune response. Baumann *et al.* (2) suggested that activated lymphocytes are resistant to

apoptosis. Therefore, when the immune function is activated, the proliferation of immune cells is increased and apoptosis is inhibited. Many studies have shown that splenocyte apoptosis is suppressed when the immune status is activated. Conversely, immune suppression in the spleen is generally associated with excessive splenocyte apoptosis (15, 31). The experiment revealed a reduction in splenocyte apoptosis in piglets induced by LPS, and thus suggested that splenocyte viability increased and immune function was activated in piglets.

Nitric oxide is produced from L-arginine, catalysed by nitric oxide synthase (NOS), and plays a vital role in regulating vascular function, neurotransmission, inflammatory response and host defence (34). It can regulate immune function together with cytokines and its level reflects the development of diseases and the level of immunity (26). It was shown that most active immune cells, such as macrophages, monocytes and natural killer cells, can express inducible NOS (iNOS) and secrete NO. Moreover, the cytokines released in response to stress upregulate iNOS expression within immune cells and stimulate NO secretion, and this is the most critical pathway affecting NO expression (3). Hou *et al.* (13) found that the release of NO from intestinal epithelial cells increased after LPS stimulation in piglets, which caused damage to the intestinal mucosa. Jafari-Khataylou *et al.* (14) showed that NO was significantly increased in the serum of LPS-treated mice, while treatment with the hydroxyethylrutoside troxerutin significantly reduced NO levels and alleviated inflammatory symptoms. Tanaka *et al.* (35) isolated splenocytes from rats treated with LPS and found that LPS induced more inflammatory factors and NO production in those cells. In the present study, the NO release in the LPS group increased significantly on the 5th and 9th days, but did not change significantly on the 13th day. The induction of stress by LPS probably triggers macrophages to express NO in large quantities, but the compound's excessive concentration inhibited its sustained expression and protected the organism from its sustained effects (5). Nitric oxide has a direct cytotoxic effect on pathogens in non-specific immunity and also exerts a powerful immunomodulatory effect (34). A lower concentration of NO selectively induces the expression of IL-12, affecting lymphocyte proliferation and the balance of T helper 1 and 2 cells (27). Using iNOS knockout rats, Shen *et al.* (34) found that a deficiency of NO reduced the immune function of mice and made them more susceptible to pathogens. It was also demonstrated that NO was engaged in critical immunological processes such as apoptosis and autoimmune and infectious diseases (3).

Conclusion

Beginning on the 5th day of the experiment, splenocytes from the LPS group were significantly more activated than those from the control group. A decrease in the ratio of cells in the resting phase and gap 1 phase, an increase in the ratio of cells in the synthesis, gap 2 and mitosis phases, a decrease in splenocyte apoptosis, and an upregulation of NO on the 5th and 9th days were observed, which may be attributed to the effect of spleen immunomodulation. This demonstrates the spleen's cellular-level immune response to LPS induction in weaned piglets. In the future, further research is needed to investigate how splenocytes specifically regulate the action mode of the immune system at different stages of the piglet's life.

* These authors contributed equally to this work.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This research was funded by the National Natural Science Foundation of China (Grant Nos 32260872 and 31960688).

Animal Rights Statement: This experimental protocol was approved by the Animal Care and Use Committee of Jiangxi Agricultural University (Nanchang, Jiangxi, China) (approval No. JXAULL-2022006), and all procedures were conducted in accordance with the Ethical Guidelines for Animal Experiments.

References

1. Basile F., Capaccia C., Zampini D., Biagetti T., Diverio S., Guelfi G.: Omics insights into animal resilience and stress factors. Animals 2021, 11, 47, doi: 10.3390/ani11010047.
2. Baumann S., Krueger A., Kirchhoff S., Krammer P.: Regulation of T cell apoptosis during the immune response. Curr Mol Med 2002, 2, 257–272, doi: 10.2174/1566524024605671.
3. Bogdan C.: Nitric oxide and the immune response. Nat Immunol 2001, 2, 907–916, doi: 10.1038/ni1001-907.
4. Collier R.J., Baumgard L.H., Zimbelman R.B., Xiao Y.: Heat stress: physiology of acclimation and adaptation. Anim Front 2019, 9, 12–19, doi: 10.1093/af/vfy031.
5. Connelly L., Palacios-Callender M., Ameixa C., Moncada S., Hobbs A.J.: Biphasic regulation of NF-kappa B activity underlies the pro- and anti-inflammatory actions of nitric oxide. J Immunol 2001, 166, 3873–3881, doi: 10.4049/jimmunol.166.6.3873.
6. Crane G.M., Liu Y.C., Chadburn A.: Spleen: development, anatomy and reactive lymphoid proliferations. Semin Diagn Pathol 2021, 38, 112–124, doi: 10.1053/j.semdp.2020.06.003.
7. Dhabhar F.S., Saul A.N., Daugherty C., Holmes T.H., Bouley D.M., Oberyszyn T.M.: Short-term stress enhances cellular immunity and increases early resistance to squamous cell carcinoma. Brain Behav Immun 2010, 24, 127–137, doi: 10.1016/j.bbi.2009.09.004.
8. Dwijayanti D.R., Djati M.S., Rifa I.M.: The role of VipAlbumin® as an immunostimulatory agent for controlling homeostasis and proliferation of lymphoid cells. Cent Eur J Immunol 2016, 41, 31–38, doi: 10.5114/ceji.2016.58814.
9. Fan W., Zheng P., Wang Y., Hao P., Liu J., Zhao X.: Analysis of immunostimulatory activity of polysaccharide extracted from Yu-Ping-Feng *in vitro* and *in vivo*. Biomed Pharmacother 2017, 93, 146–155, doi: 10.1016/j.biopha.2017.05.138.
10. Fang J., Zhu P., Yang Z., Peng X., Zuo Z., Cui H., Ouyang P., Shu G., Chen Z., Huang C., Liu W.: Selenium ameliorates AFB_1–induced excess apoptosis in chicken splenocytes through death receptor and endoplasmic reticulum pathways. Biol Trace Elem Res 2019, 187, 273–280, doi: 10.1007/s12011-018-1361-7.

11. He Y., Mo Q., Hu Y., Chen W., Luo B., Wu L., Qiao Y., Xu R., Zhou Y., Zuo Z., Deng J., He W., Wei Y.: *E. adenophorum* induces cell cycle arrest and apoptosis of splenocytes through the mitochondrial pathway and caspase activation in Saanen goats. Sci Rep 2015, 5, 15967, doi: 10.1038/srep15967.
12. Hong Y., Lee J., Vu T.H., Lee S., Lillehoj H.S., Hong Y.H.: Exosomes of lipopolysaccharide-stimulated chicken macrophages modulate immune response through the MyD88/NF-kappaB signaling pathway. Dev Comp Immunol 2021, 115, 103908, doi: 10.1016/j.dci.2020.103908.
13. Hou Y., Yao K., Wang L., Ding B., Fu D., Liu Y., Zhu H., Liu J., Li Y., Kang P., Yin Y., Wu G.: Effects of α-ketoglutarate on energy status in the intestinal mucosa of weaned piglets chronically challenged with lipopolysaccharide. Br J Nutr 2011, 106, 357–363, doi: 10.1017/S0007114511000249.
14. Jafari-Khataylou Y., Emami S.J., Mirzakhani N.: Troxerutin attenuates inflammatory response in lipopolysaccharide-induced sepsis in mice. Res Vet Sci 2021, 135, 469–478, doi: 10.1016/j.rvsc.2020.11.020.
15. Kim H.B., Lee H.S., Kim S.J., Yoo H.J., Hwang J.S., Chen G., Youn H.J.: Ethanol extract of fermented soybean, Chungkookjang, inhibits the apoptosis of mouse spleen, and thymus cells. J Microbiol 2007, 45, 256–261.
16. Kudryavtseva N.N., Shurlygina A.V., Melnikova E.V., Tenditnik M.V., Bondar N.P., Panteleeva N.G., Smagin D.A., Kolesnikov N.N., Trufakin V.A.: Cell cycle arrest in the thymus and spleen in male mice under conditions of chronic social defeat stress: effects of diazepam. Bull Exp Biol Med 2011, 151, 411–414, doi: 10.1007/s10517-011-1343-7.
17. Laukova M., Vargovic P., Rokytova I., Manz G., Kvetnansky R.: Repeated stress exaggerates lipopolysaccharide-induced inflammatory response in the rat spleen. Cell Mol Neurobiol 2018, 38, 195–208, doi: 10.1007/s10571-017-0546-5.
18. Li T., Shi H.Y., Hua Y.X., Gao C., Xia Q., Yang G., Li B.: Effects of allicin on the proliferation and cell cycle of chondrocytes. Int J Clin Exp Pathol 2015, 8, 12525–12532.
19. Lin P., Ding B., Wu Y., Dong K., Li Q.: Mitogen-stimulated cell proliferation and cytokine production in major depressive disorder patients. BMC Psychiatry 2018, 18, 330, doi: 10.1186/s12888-018-1906-5.
20. Liu Y.L., Li D.F., Gong L.M., Yi G.F., Gaines A.M., Carroll J.A.: Effects of fish oil supplementation on the performance and the immunological, adrenal, and somatotropic responses of weaned pigs after an *Escherichia coli* lipopolysaccharide challenge. J Anim Sci 2003, 81, 2758–2765, doi: 10.2527/2003.81112758x.
21. Ma T., Kang C., Shao H., Qi Q., Hu W.: Protective effects of ulinastatin on proliferation and cytokine release of splenocytes from rats with severe acute pancreatitis. Eur Surg Res 2006, 38, 445–450, doi: 10.1159/000095087.
22. Mao X.F., Piao X.S., Lai C.H., Li D.F., Xing J.J., Shi B.L.: Effects of beta-glucan obtained from the Chinese herb *Astragalus membranaceus* and lipopolysaccharide challenge on performance, immunological, adrenal, and somatotropic responses of weanling pigs. J Anim Sci 2005, 83, 2775–2782, doi: 10.2527/2005.83122775x.
23. Martinez-Miro S., Tecles F., Ramon M., Escribano D., Hernandez F., Madrid J., Orengo J., Martinez-Subiela S., Manteca X., Ceron J.J.: Causes, consequences and biomarkers of stress in swine: an update. BMC Vet Res 2016, 12, 171, doi: 10.1186/s12917-016-0791-8.
24. Michalek R.D., Rathmell J.C.: The metabolic life and times of a T-cell. Immunol Rev 2010, 236, 190–202, doi: 10.1111/j.1600-065X.2010.00911.x.
25. Mitra S., Keswani T., Dey M., Bhattacharya S., Sarkar S., Goswami S., Ghosh N., Dutta A., Bhattacharyya A.: Copper-induced immunotoxicity involves cell cycle arrest and cell death in the spleen and thymus. Toxicology 2012, 293, 78–88, doi: 10.1016/j.tox.2011.12.013.
26. Nagy G., Clark J.M., Buzas E., Gorman C., Pasztoi M., Koncz A., Falus A., Cope A.P.: Nitric oxide production of T lymphocytes is increased in rheumatoid arthritis. Immunol Lett 2008, 118, 55–58, doi: 10.1016/j.imlet.2008.02.009.
27. Niedbala W., Wei X.Q., Campbell C., Thomson D., Komai-Koma M., Liew F.Y.: Nitric oxide preferentially induces type 1 T cell differentiation by selectively up-regulating IL-12 receptor beta 2 expression via cGMP. Proc Natl Acad Sci USA 2002, 99, 16186–16191, doi: 10.1073/pnas.252464599.
28. Niu X., Ding Y., Chen S., Gooneratne R., Ju X.: Effect of immune stress on growth performance and immune functions of livestock: mechanisms and prevention. Animals 2022, 12, 909, doi: 10.3390/ani12070909.
29. Nogueira J.E., Agostinho B.K.L., Mota C.M.D., Branco L.G.S.: Splenic anti-inflammatory reflex in immune tolerance. J Therm Biol 2019, 85, 102411, doi: 10.1016/j.jtherbio.2019.102411.
30. Peng X., Cui H., Deng J., Zuo Z., Lai W.: Histological lesion of spleen and inhibition of splenocyte proliferation in broilers fed on diets excess in selenium. Biol Trace Elem Res 2011, 140, 66–72, doi: 10.1007/s12011-010-8679-0.
31. Peng X., Zhang K., Bai S., Ding X., Zeng Q., Yang J., Fang J., Chen K.: Histological lesions, cell cycle arrest, apoptosis and T Cell subsets changes of spleen in chicken fed aflatoxin-contaminated corn. Int J Environ Res Public Health 2014, 11, 8567–8580, doi: 10.3390/ijerph110808567.
32. Sassi A., Mokdad Bzeouich I., Mustapha N., Maatouk M., Ghedira K., Chekir-Ghedira L.: Immunomodulatory potential of hesperetin and chrysin through the cellular and humoral response. Eur J Pharmacol 2017, 812, 91–96, doi: 10.1016/j.ejphar.2017.07.017.
33. Satoh E., Edamatsu H., Omata Y.: Acute restraint stress enhances calcium mobilization and proliferative response in splenic lymphocytes from mice. Stress 2006, 9, 223–230, doi: 10.1080/10253890601095794.
34. Shen J., Yu S.F., Peng M., Lai D.H., Hide G., Wu Z.D., Lun Z.R.: iNOS is essential to maintain a protective Th1/Th2 response and the production of cytokines/chemokines against *Schistosoma japonicum* infection in rats. PLoS Negl Trop Dis 2022, 16, e0010403, doi: 10.1371/journal.pntd.0010403.
35. Tanaka H., Nishikawa Y., Fukushima T., Taniguchi A., Fujita Y., Tsuda K., Inagaki N., Hosokawa M.: Lipopolysaccharide inhibits hepatic gluconeogenesis in rats: the role of immune cells. J Diabetes Investig 2018, 9, 494–504, doi: 10.1111/jdi.12729.
36. Tang X., Xiong K., Fang R., Li M.: Weaning stress and intestinal health of piglets: a review. Front Immunol 2022, 13, 1042778, doi: 10.3389/fimmu.2022.1042778.
37. Terenina E., Sautron V., Ydier C., Bazovkina D., Sevin-Pujol A., Gress L., Lippi Y., Naylies C., Billon Y., Liaubet L., Mormede P., Villa-Vialaneix N.: Time course study of the response to LPS targeting the pig immune gene networks. BMC Genomics 2017, 18, 988, doi: 10.1186/s12864-017-4363-5.
38. Wang L., Jing J., Yan H., Tang J., Jia G., Liu G., Chen X., Tian G., Cai J., Shang H., Zhao H.: Selenium pretreatment alleviated LPS-induced immunological stress via upregulation of several selenoprotein encoding genes in murine RAW264.7 cells. Biol Trace Elem Res 2018, 186, 505–513, doi: 10.1007/s12011-018-1333-y.
39. Wyns H., Plessers E., De Backer P., Meyer E., Croubels S.: *In vivo* porcine lipopolysaccharide inflammation models to study immunomodulation of drugs. Vet Immunol Immunopathol 2015, 166, 58–69, doi: 10.1016/j.vetimm.2015.06.001.
40. Yu J., Zheng C., Zheng J., Duan G., Guo Q., Zhang P., Wan M., Duan Y.: Development of intestinal injury and restoration of weaned piglets under chronic immune stress. Antioxidants 2022, 11, 2215, doi: 10.3390/antiox11112215.
41. Yu Y., Wang H., Meng X., Hao L., Fu Y., Fang L., Shen D., Yu X., Li J.: Immunomodulatory effects of cinobufagin on murine lymphocytes and macrophages. Evid Based Complement Alternat Med 2015, 2015, 835263, doi: 10.1155/2015/835263.
42. Zhang X., Xiao K., Qiu W., Wang J., Li P., Peng K.: The immune regulatory effect of boron on ostrich chick splenic lymphocytes. Biol Trace Elem Res 2021, 199, 2695–2706, doi: 10.1007/s12011-020-02392-5.
43. Zhou Y., Hu X., Zhong, S., Yu W., Wang J, Zhu W., Yang T., Zhao G., Jiang Y., Li, Y.: Effects of continuous LPS induction on oxidative stress and liver injury in weaned piglets. Vet Sci 2023, 10, 22, doi: 10.3390/vetsci10010022.