The beneficial effects of traditional Chinese medicine on antioxidative status and inflammatory cytokines expression in the liver of piglets

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Oxidative stress and inflammation seriously affected the growth and development of piglets. Traditional Chinese medicine (TCM) prescriptions has been used to prevent various diseases of piglets, including anti-inflammatory and antioxidant. Here, we identified the effects of Xiao-Jian-Zhong-Tang (XJZT) and Jingsananli-sepsis (JJS) on the oxidative stress and inflammatory in the liver of piglets. The piglets were fed with the basal diet (Control group), basal diet affixed with 10 g/kg XJZT (TCM I group), and basal diet affixed with 3 g/kg JJS (TCM II group), respectively. The serum was gathered on days 30 and 60 and the liver samples were also collected on day 60. Results showed that the TCM I and TCM II markedly increased the activities of the glutathione peroxidase (GSH-Px) and total antioxidant capacity (T-AOC), and reduced the levels of malonaldehyde (MDA), TNF-α, IL-6, and IL-8 in serum. In addition, compared to the control group, Nrf2, SOD-1, NQO-1, and HO-1 mRNA expression levels and the protein levels of Nrf2 and HO-1 were significantly increased while NF-κB, TNF-α, IL-6, and IL-8 mRNA expression levels and the phosphorylation levels of NF-κB and IκB-α were decreased in TCM I and TCM II groups. Collectively, these findings suggested that TCM I and TCM II could enhance anti-oxidative and anti-inflammatory capabilities in the liver of piglets via the Nrf2/NF-κB pathway, providing a basis for the functional exploration of TCM prescriptions.

KEYWORDS traditional Chinese medicine, piglet, antioxidant capability, inflammation, liver
Introduction

The climbing requirement for animal products in recent decades has led to the development of intensive animal production systems which has been demonstrated to produce stress responses in animals (1). Most human practices toward animals could lead to stress responses like climate change, social, environmental, and immunological stress, thereby causing oxidative stress (2–4). In consideration of global climate change, heat stress as a kind of oxidative stress brings about hundreds of millions of dollars of economic losses in the swine industry annually (5). Therefore, combating oxidative stress is absolutely crucial for the swine industry. Generally, there is an imbalance between the production of reactive oxygen species (ROS) and the biological ability to clear reactive intermediates (6). When out of control, the imbalance of ROS dynamic is hazardous to cellular macromolecules, posing toxic effects in the function and survival of numerous organs (7). Elevated levels of oxidative stress can impair milk production and reproductive performance of sows, which can affect not only the life of sows, but also the health of piglets (8). On the other hand, the occurrence of inflammation could be caused by increased ROS (9). Inflammation is an aspect of the immune response to injury and disease, whose process is closely related to the occurrence of oxidative stress (10). Thus, the two processes are crucial targets of developing therapeutics against numerous diseases.

TABLE 1 Composition and nutrient levels of basal diet (air-dry basis).

| Ingredients          | Content (%) | Analyzed composition, g/kg | Content (%) |
|----------------------|-------------|----------------------------|-------------|
| Maize                | 55.80       | DM                         | 89.21       |
| Soybean meal         | 16.30       | DE (MJ/kg)                 | 14.36       |
| Fermented soybean meal | 7.00       | Crude Protein (CP, %)     | 19.63       |
| Wheat middling       | 4.50        | Lysine                     | 1.32        |
| Fish meal            | 2.50        | Methionine                 | 0.43        |
| Dried porcine solubles | 2.50      | Methionine + Cystine       | 0.77        |
| Whey powder          | 6.25        | Threonine                  | 0.81        |
| Soy oil              | 1.65        | Calcium                    | 0.96        |
| Lysine               | 0.25        | Total phosphorus           | 0.60        |
| Methionine           | 0.10        | Total                      | 100.00      |
| Limestone            | 1.05        |                            |             |
| CaHPO4               | 0.80        |                            |             |
| NaCl                 | 0.30        |                            |             |
| Vitamin-mineral premix | 1.00      |                            |             |
| Total                | 100.0       |                            |             |

1The premix provides following per kilogram diet: Vitamin A 8 000 IU, Vitamin D 2 500 IU, Vitamin E 15 mg, nicotinic acid 20 mg, D-pantothenic acid 10 mg, riboflavin 4 mg, biotin 0.06 mg, folic acid 0.2 mg, thiamine 2 mg, choline chloride 500 mg, copper 165 mg, iron 110 mg, manganese 80 mg, zinc 330 mg, selenium 0.20 mg.
2Digestible energy is calculated value according to ingredients energy, the others are measured values.

Currently, traditional Chinese medicine (TCM) composed of natural plant derivative has been practiced to improve farm-animal health and prevent various diseases (11, 12). Compound preparations can potentially exert multiple effects in a distinct mechanism that is expected to reach more comprehensive effect by targeting multi pathways and multi targets (13). Xiao-Jian-Zhong-Tang (XJZT) consists of 10 medicinal herbs (Cassia Twig, Glycyrrhiza uralensis, Ziziphus zizyphus, Cynanchum otophyllum, Zingiber officinale Roscoe, Rhizoma Atractylodes, Atractylodes macrocephala, Poria cocos, Coptis chinensis Franch and Maltose) and is currently used to treat chronic liver diseases (14). Jingsananli-sepsis (JSS) composed of 8 medicinal herbs (Nepteta cataria L, Radix Saposhnikoviae, Notopterygium inciscum, Radix Angelicae pubescents, Radix bupleuri, Radix Peucedani, Poria cocos, and Glycyrrhiza uralensis), which have been used in the treatment of gripe, and fever with a long history (15). These may be related to their prescriptions composition consists with various types of antimicrobial,
immunoregulatory, anti-oxidative and anti-inflammatory active substances (16, 17). Many functional components in these two of TCM, including flavonoids, volatile oils, polysaccharides and organic acids have been demonstrated that were closely related to the immunity enhancement. The flavonoids have antioxidant activity of reduction of free radical formation and free radical scavenging (18). Volatile oil, a major active compound of many herbs, is known to inhibit oxidative stress, and inflammation (19). Additionally, polysaccharides and organic acids are also widely used in inhibits oxidative stress, bacterium and virus (20, 21).

Nuclear factor E2 related factor 2 (Nrf2) acts as a main regulatory factor in preserving cellular defense against oxidative stress. Evidence have been provided that it is responsible for the protection of liver injury and inflammation caused by oxidative stress in the way of regulating antioxidant proteins expression levels (14, 22). On the other hand, many in vivo and in vitro experiments have demonstrated that oxidative stress could activate NF-κB pathway. Additionally, the activation of NF-κB is thought to be a response to oxidative stress. This signaling pathway is a master regulator of inflammation and may the target of the anti-inflammatory effect of TCM. TCM are usually used to counteract diseases. Whereas studies on the effects of TCM with respect to their anti-inflammatory and anti-oxidative response are rather scanty. Hence, this study explored the potential effects of TCM prescriptions (10 g/kg XJZT and 3g/kg JSS) as feed additives on antioxidative status and inflammatory reaction in piglets, to shed light on the functional role of XJZT and JSS prescriptions.

Materials and methods

Animal treatments

All experimental procedures were performed by Jiangxi Agricultural University Animal Care and Use Committee. The experiment lasted for 60 days, and a total of thirty crossbred (Durox × Landrace × Yorkshire) piglets (weight 21.43 ± 2.86 kg) were randomly allotted to three dietary treatments groups on average according to initial body weight. The dietary treatments were basal diet (control group), 10 g/kg Xiao-Jian-Zhong-Tang (XJZT) combination with basal diet (TCM I group) and 3 g/kg Jingsananli-sepsis combination with basal diet (TCM II group). The composition and nutrient levels of basal diet are shown in Table 1. All raw materials for TCM I were bought from Changsheng pharmacy (Jiangxi, China) and TCM II was provided by The Spirit Jinyu Biological Pharmaceutical Co., Ltd. (Huhhot, Inner Mongolia, China). All dried herbs are crushed through a 2.5 mm screen. Composition and main active constituents of TCM I and TCM II are presented in Table 2.

| Table 3: Primers used in this study. |
|------------------------------------|
| **Gene** | **GeneBank Number** | **Primers Sequences (5′-3′)** | **Annealing temp (°C)** | **Amplification size (bp)** |
| Nrf2 | XM_005671982.1 | F: CCCATTCACAAAAGACAAACATTC R: GCTTTGCCCTTAGCTCATCTC | 58 | 72 |
| SOD-1 | NM_001190422.1 | F: GAGACCTGGGCAATGTGACT R: CTGCCCAAGTCATCTGGTT | 57 | 139 |
| NQO1 | NM_001159613.1 | F: CCAGCAGCGCCGGCAATCTG R: AGGTCCGACACGGCGACCTC | 66 | 160 |
| HO-1 | NM_001094027 | F: GCTGCCGGAATGAACAC R: GCTCTCTGACCTCCTC | 55 | 112 |
| NF-κB | NM_001048232.1 | F: CTGCGACAGGGAGACATGAA R: ACTACGCCAGAAAGGGATAT | 58 | 147 |
| TNF-α | NM_214022.1 | F: CCAATGGGCAGAGTGGGTATG R: TGAAGAGGACCTGGGAGTAG | 56 | 117 |
| IL-6 | NM_001252429.1 | F: TGCTCAGCTGCTTCCCTACC R: CAGGATTTTGGCCGGAGGATG | 58 | 132 |
| IL-8 | NM_213867.1 | F: TTGCATGCGCAGTGCTAAATA R: CTGTCGACACCTTCTGCCGCCA | 57 | 176 |
| IL-10 | NM_214041 | F: GGGCGCTGCTCAGCTTCTTG G: CCCCTGCTTGGAGCTTGCTA | 58 | 89 |
| GAPDH | NM_001206359 | F: ACTCACTCTTCACATTTGATGCT R: TGGTGCTAAGGCGAACATCA | 57 | 100 |

F: Forward primer; R, Reverse Primer.
Sample collection

Blood was obtained from the jugular vein in the collection tube on days 30 and 60. Serum was separated and stored at $-20^\circ\text{C}$ to detect the antioxidant indices and inflammatory cytokines. On the day 60 of the experiment, all piglets in each group were euthanized with sodium pentobarbital (40 mg/kg body weight). Livers were dissected immediately from all piglets. The blood of the liver was washed by precooled normal saline (0.9% NaCl, Beyotime, China), and the surface liquid was removed with filter paper, and then stored at $-80^\circ\text{C}$ for analysis after rapidly frozen in liquid nitrogen.

Determination of antioxidant indices and inflammatory cytokines in serum

The levels of T-AOC, SOD, GSH-PX, and MDA in serum were measured strictly based on the instructions of the kits (Nanjing Jiancheng Bioengineering Institute, China; T-AOC, A015-2-1; SOD, A001-3-1; GSH-PX, A005-1-2; MDA, A003-1-2). The levels of TNF-α, IL-6, IL-8, and IL-10 in serum were measured by enzyme linked immunosorbent assay kits (R&D, USA; TNF-α, MTA00B; IL-6, D6050; IL-8, D8000C; IL-10, M1000B) according to the instructions. The optical density of each well was read at 450 nm with an Absorbance Microplate Reader (SpectraMax, China).

Quantitative real-time PCR analysis

RT-qPCR assay was consistent with the method previously (23, 24). The primer sequences of Nrf2, SOD-1, NQO-1, HO-1, NF-κB, TNF-α, IL-6, IL-8, IL-10, and GAPDH are shown in Table 3.

Western blot analysis

Western blotting assay was performed in accordance with the procedure in a previous study (Dai et al., 2021). The primary antibodies were Nrf2 (Proteintech, 1:1000), HO-1, and GAPDH. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000, Proteintech). The bands were visualized using an enhanced chemiluminescence reagent (Thermo Scientific).
Determination of (A) TNF-α, (B) IL-6, (C) IL-8, and (D) IL-10 levels in serum on days 30 and 60.

Statistical analysis

Quantitative variables are expressed as the mean ± standard deviation (SD). All statistical analyses were calculated by one-way analysis of variance and the least significant difference test. P values < 0.05 was considered significant.

Results

Effects of TCM I and TCM II on antioxidant capabilities in serum

As shown in Figures 1A,B, T-AOC level in TCM I and TCM II groups was obviously higher than the control group (P < 0.05 or P < 0.001) on days 30 and 60. In addition, GSH-Px level was notably increased (P < 0.05) in TCM I group compared to the control group (Figure 1C). Furthermore, the T-AOC activity in TCM I and TCM II groups was markedly upregulated (P < 0.05) compared to the control group on day 60. However, MDA content was significantly decreased (P < 0.05) in TCM I and TCM II groups compared to the control group on day 60 (Figure 1D).

Effects of TCM I and TCM II on inflammatory cytokines in serum

As shown in Figures 2A–C, TNF-α, IL-6 and IL-8 levels in the TCM I and TCM II groups were significantly lower than the control group (P < 0.05 or P < 0.01) on days 30 and 60. Moreover, IL-8 level was markedly decreased (P < 0.01) in the TCM I group compared to the TCM II group on day 60. Additionally, IL-10 level was increased in TCM I and TCM II groups in comparison with the control group (Figure 2D).
Effects of TCM I and TCM II on mRNA levels of antioxidant-related genes and protein levels in liver

As described in Figure 3A, HO-1 and NQO-1 mRNA levels of liver in TCM and TCM II groups were higher than that in control group ($P < 0.05$ or $P < 0.01$). Otherwise, the mRNA level of SOD-1 was increased in TCM I and TCM II groups compared to the control group, whereas the differences were not significant. The protein levels of Nrf2 and HO-1 are presented in Figures 3B–D. Both Nrf2 and HO-1 protein levels in TCM I and TCM II groups were dramatically increased in comparison with the control group ($P < 0.05$ or $P < 0.01$).

Effects of TCM I and TCM II on mRNA levels of inflammatory cytokines and protein levels in liver

As shown in Figure 4A, NF-κB, IL-8, and TNF-α mRNA levels in TCM I group were significantly downregulated compared to control group ($P < 0.05$ or $P < 0.01$), while IL-10 mRNA level was significantly increased ($P < 0.01$). Additionally, Figure 4A shows that NF-κB and IL-6 mRNA levels in the TCM II group also dramatically declined in comparison with control group ($P < 0.01$ or $P < 0.001$). However, compared to the control group, IL-10 mRNA level was markedly up-regulated in TCM II group ($P < 0.001$). The protein expressions of IκB-α and NF-κB had no significant differences in TCM I and TCM II groups ($P > 0.05$) in comparison with the control group (Figures 4B,C). As shown in Figures 4E,F the protein expressions of the p-IκB-α and p-NF-κB in TCM I and TCM II groups were dramatically downregulated in comparison with the control group ($P < 0.01$ or $P < 0.001$).

Discussion

In intensive farming systems, piglets face numerous challenges like change in the nutritional source, feed contamination with mycotoxins, pathogenic micro-organisms, and some chemical agents, leading to excessive ROS accumulation that cause the occurrence of oxidative stress and inflammation (3, 25, 26). Accordingly, it is necessary to find functional products that can effectively protect piglets from
Effects of TCM I and TCM II on the mRNA levels of inflammatory cytokines and protein levels in liver. (A) mRNA levels of NF-κB, TNF-α, IL-6, IL-8, and IL-10. (B) Protein levels of IκB-α, NF-κB p65, p-IκB-α, and p-NF-κB. (C) Graph showing the protein level of IκB-α. (D) Graph showing the protein level of NF-κB p65. (E) Graph showing the protein level of p-IκB-α. (F) Graph showing the protein level of p-NF-κB.

oxidative stress and inflammation. Chinese herbal medicine is a unique medical resource in China. In most condition, many Chinese herbal medicines share the advantages of low toxicity, small side effects, low drug resistance and no residual, etc. (27, 28). Their application in livestock and poultry production not only enhanced the immune function of the body and improve disease resistance, but also promote the growth and development of animals. Due to the complicated process of oxidative stress and inflammation, the therapeutic effect of a single herb may not be ideal. XJZT and JSS are well-known traditional herbal medicines which have been used to alleviate oxidative stress and inflammation and improve immunity for a long time, containing with multiple antimicrobial, immunoregulatory, anti-oxidative and anti-inflammatory active substances (29–31). The flavonoids as a powerful antioxidant can scavenge free radicals from multiple targets, and they are safer and more effective than other antioxidants (19, 32). Additionally, polysaccharides and organic acids have abundant
biological activities, so they can also widely used in inhibits oxidative stress and inflammation (20, 21). Therefore, this study investigated the effects of dietary supplementation of XJZT or JJS on the anti-oxidative capacity and inflammatory response in the liver of piglets.

Oxidative stress refers to a state of imbalance between oxidation and antioxidation in which numerous oxidized intermediates are produced. The impulse induction of protective antioxidant enzymes can exert profitable effects on the ability of the body to maintain homeostasis. T-AOC represents the enzymatic and non-enzymatic antioxidant defense systems. Superoxide anion radicals (O$_2^-$) can be scavenged by T-SOD and peroxides and hydroxyl radicals produced in the process of cell metabolism can be eliminated by GSH-Px, thereby protecting the body from oxidative damage (33, 34). Additionally, the degree of lipid peroxidation could be reflected by MDA content, so its level is proportional to the degree of cell damage (35). Previous reports have shown that subcutaneous lipopolysaccharide injection and supplement ampelopsin in diet for pigs improved the anti-oxidative capacity in plasma (36, 37). In this study, we found that supplementation with XJZT and JJS increased GSH-Px and T-AOC levels but decreased MDA content in plasma, indicating that XJZT and JJS supplementation in piglets could save tissues from lipid peroxidation. This benefit might be due to the reduction of the antioxidative system burden by dietary total flavonoids, polysaccharides, and phenolicsand. Additionally, changes in the levels of these antioxidant indexes (SOD, GSH-Px, and MDA) have affinities with Nrf2 expression level. Moreover, Nrf2 binds to antioxidant response elements such as HO-1 and NQO1 to prevent oxidative damage (38). Our results illustrated that XJZT and JJS could significantly increase Nrf2, SOD-1, HO-1, and NQO-1 mRNA levels and Nrf2 and HO-1 protein levels. These results demonstrated that XJZT and JJS against oxidative damage by activating the Nrf2 signal pathway in liver.

Additionally, there is a strong relationship between oxidative stress and inflammation. As a redox-sensitive transcription factor, NF-κB expression level can be promoted by oxidative stress, whose level can mediate the transcription of numerous inflammatory genes (39). TNF-α occupies an important position in the inflammatory responses which is responsible for plenty of cytokines and chemokines production (40, 41). IL-10 acts as an anti-inflammatory cytokine with important immunoregulatory functions in the way of restraining the inflammatory cytokines expression levels like TNF-α, IL-6 and IL-1 (40). In our study, the XJZT and JJS decreased the concentrations of TNF-α, IL-6, and IL-8 and increased IL-10 level in the plasma. NF-κB is of great importance for controlling the expression levels of inflammatory response related proteins. Under normal conditions, there is covalently binding between NF-κB and 1κB in the cytoplasm. After receiving immune stimulation signals such as TNF-α and lipopolysaccharides, IκB is phosphorylated and degraded, followed by NF-κB translocation to the nucleus, promoting transcription of inflammatory genes like IL-1β, and IL-6. In this study, XJZT and JJS were significantly decreased the mRNA expression levels of NF-κB, IL-6, IL-8, and TNF-α, but remarkably increased the IL-10 mRNA expression. Furthermore, the results showed that XJZT and JJS could decrease the protein levels of IκB-α and NF-κB, and remarkably decrease the p-IκB-α and p-NF-κB protein levels in liver. These results illustrated that the XJZT and JJS inhibited the production of pro-inflammatory mediators in livers through down-regulating the NF-κB signal pathway.

Conclusion

Our data confirmed that 10 g/kg XJZT and 3 g/kg JJS prescriptions exhibited strong hepatoprotective effect on the liver of piglets, which have a strong relationship with their anti-oxidative and anti-inflammatory capabilities by activating the Nrf2/NF-κB pathway. Accordingly, 10 g/kg XJZT and 3 g/kg JJS prescriptions can act as potential materials for drug and functional food development to prevent oxidative stress and inflammatory of piglets.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Experimental Animal Care and Use Committee of Jiangxi Agricultural University.

Author contributions

XW, YW, and FY contributed to conception and design of the study. YM, AH, and TX organized the database. FW, GZ, and YY performed the statistical analysis. HC wrote the first draft of the manuscript. XG, AH, and TX wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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