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Full Length Article

Effect of Piglet Weaning Age on mRNA and Protein Expression of Heat Shock Proteins

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Abstract

The study objective was to determine the effects of weaning age on piglet *HSPs*. Piglets were randomly assigned to four groups (n=6 per group) that were weaned at 14, 21, 28, or 35 days of age. *HSP27, HSP70, and HSP90* mRNA levels and proteins were measured in kidney, myocardium, and longissimus dorsi muscle. In the kidney, *HSP70* mRNA and protein levels were significantly higher in piglets weaned at 14 and 21 days compared to piglets weaned at 35 days (p <0.05), and *HSP90* expression was significantly lower in piglets weaned at 21 days compared to other groups (p < 0.05). In myocardium, weaning at 21 and 28 days resulted in significantly higher *HSP70* mRNA expression compared to weaning at 14 and 35 days (p < 0.05). Myocardial *HSP90* mRNA expression in piglets weaned at 28 days was significantly lower compared to piglets weaned at 14 days, and weaning at 14 days resulted in significantly higher myocardial *HSP70* expression compared to weaning at 35 days (p < 0.05). In longissimus dorsi muscle, weaning at 21 days resulted in significantly lower expression of *HSP70* mRNA compared to weaning at 14 days of age (p < 0.01), and piglets weaned at 21 days had significantly lower expression of HSP90 and HSP27 compared to piglets weaned at 35 days (p < 0.05). These results indicate that expression of HSP genes and protein in piglets was affected by weaning age, and the HSP expression pattern was different among different tissues. Changes in HSP expression may be the reason for differences in growth performance and immune and antioxidant function variations in piglets.

Keywords: *HSPs*; Kidney; Longissimus dorsi muscle; Myocardium; Piglets; Weaning age

Introduction

Weaning is a highly stressful life event in piglets. In pigs, weaning may cause intestinal and immune system dysfunction which results in reduced growth and feed intake, and an overall decline in health (Campbell *et al.*, 2013). Weaning age affects the propagation performance of sows, but also affects piglet immune responses, in part due to postnatal development of the immune system (Niekamp *et al.*, 2007; Smith *et al.*, 2008). As piglet weaning age is delayed, the average weight gain and feed intake per day increases, along with the gain per feed amount (Xun *et al.*, 2017). Currently, the European Union recommends that the minimum weaning age of pigs should be increased to 28 days to address animal welfare concerns, and the weaning age for organic pork production in the United Kingdom (UK) is 42 days (Li *et al.*, 2016).

Heat shock proteins (*HSPs*) are an important part of the stress response, a complex process of adaption to stress. The effects of stress on *HSP* expression is of great interest in both basic biology and medicine (Li *et al.*, 2012). Heat shock proteins are a heterogeneous and evolutionarily conserved family of proteins with high sequence homology. HSPs are intracellular chaperones that play a crucial role in protein structure and folding during cellular stress (Musiał and Zwolińska, 2011). Heat shock proteins are grouped by size, structure, and function into five major families: *HSP60, HSP70*, *HSP90*, *HSP110*, and small *HSPs* (Lindquist and Craig, 1988). Heat shock proteins of different sizes have differing functions. For example, *HSP70*s participate in protein synthesis and *HSP90*s play a role in regulating cellular protein activity (Zuehlke and Johnson, 2010; Wu *et al.*, 2013).

Research on the expression of *HSPs* in piglets weaned at different ages is scarce. According to previous research, *HSP27* exerts antiapoptotic effects via the mitochondria, and *HSP70* prevents caspase activation downstream of cytochrome c release in the mitochondria (Samali *et al.*, 2001). In addition, previous research suggests that *HSP90* is an abundant cytosol protein that forms dimers, exists in two isoforms, and has several functions, such as stabilizing proteins against heat stress (α-*HSP90* and β-*HSP90*) (Garnier *et al.*, 2001). However, the effects of weaning age on these activities are unclear, so we probed the influence of piglet weaning age on *HSP* expression in kidney, myocardium, and longissimus dorsi muscle.

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Materials and Methods

Animal Treatments

Piglets (Duroc × Landrace × Yorkshire) were randomly assigned to four treatment groups (6 per group) and weaned at 14, 21, 28 and 35 days after birth. All experimental procedures were conducted according to the guidelines of the regional Animal Ethics Committee.

Feeding Management

All piglets had unrestricted access to sow milk and water until the time of weaning, and were supplied with nutritionally complete, two-stage creep feed beginning at 8 days of age. The piglet diet composition and nutrient levels was referred as previously reported (Li *et al.*, 2016). Piglet health was monitored and recorded daily.

Sample Collection

Piglets were sacrificed at the age of 42 days, after a 12 h fast. Kidney, myocardium, and longissimus dorsi muscle samples were collected and immediately placed in liquid nitrogen (-80°C) for storage.

Reverse Transcription and Polymerase Chain Reaction

Previously described procedures for reverse transcription and polymerase chain reactions (PCR) were used, but are briefly described below (Monteiro *et al.*, 2016). TRIzol reagent was used to extract total RNA from kidney, myocardium, and longissimus dorsi muscle according to the manufacturer's directions (Invitrogen Life Technologies Biotech Co., Ltd., USA). RNA integrity was determined by visualizing intact 18S and 28S ribosomal RNA after electrophoresis (1.2% TAE agarose gel). Total RNA concentration was determined spectrophotometrically (260 nm) (Astra Gene II, location). RNA purity was determined by calculating the A260/A280 ratio; all RNAs used in experiments were between 1.8~2. Isolated RNA was stored at -80°C.

Reverse transcription (RT) was accomplished using the following reaction mixture: 2 μg of RNA, 10 m*M* deoxynucleotide triphosphates (dNTPs), 10 μM random hexamer primers, RNase inhibitor (40 U), Moloney murine leukemia virus reverse transcriptase (M-MLV, 200 U), and 5× RT buffer. RNA was denatured (70°C for 5 min) then placed on ice for 5 min with random hexamer primers and dNTP prior to reverse transcription. The reactions were subsequently incubated for 1 h at 37°C, denatured for 5 min at 95°C, and cooled to 4°C. The cDNAs were stored at -20°C until quantification using PCR.

PCR (25 μL) was performed using the following reaction ingredients: 2 μL cDNA, Taq DNA polymerase (1.5 U), dNTPs (10 mmol/L), 10×PCR Buffer, forward and reverse primers (1.0–30.0 μmol/L). *HSP70*, *HSP90*, *HSP27*, and glyceraldehyde-3-phosphate dehydrogenase primers were prepared based on porcine sequences located in GenBank (Table 1).

Contamination with genomic and environmental DNA during the RT and PCR procedures was monitored using the appropriate controls. Optimization of the PCR conditions and normalization for intra-assay variation was accomplished using a pooled sample of equal quantities of total RNA from each sample. The number of PCR cycles was optimized to ensure termination of the reaction within the linear range for quantitation. All samples were run at the same time and the reactions were repeated at least 3 times. The Gene Amp PCR System 9600 (Perkin Elmer, USA) was used for both the RT and PCR.

PCR products were separated by electrophoresis and analyzed with Image Lab software (Bio-Rad Co. Ltd., USA). The abundance of mRNA expression was also determined using the ratio of the target gene mRNA to GAPDH mRNA.

Western Blotting analysis

All procedures for western blotting were described previously (Li *et al.*, 2016). Protein extracts were made from kidney, myocardium and longissimus dorsi muscle by centrifuging at 5000 × g for 10 min after adding radio immuno precipitation assay (RIPA) buffer (Solarbio, China). Protein extracts (36 μL) were denatured (4×loading buffer and boiling for 5 min), then separated by 10% SDS-PAGE. Total protein concentration was measured using a spectrophotometer (595 nm) (Beijing Purkinje General Co. Ltd., China). Isolated protein was stored at -80°C.

After separation by electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Thermo Fisher Scientific, China). Membranes were blocked for 2 h at room temperature with buffer containing 5% fat-free dry milk. After regular washing, membranes were incubated overnight at 4°C with monoclonal antibodies to *HSP70* (Abcam, Cambridge, UK, dilution 1:4000), *HSP90* (Abcam, Cambridge, UK, dilution 1:3000), or *HSP27* (Abcam, Cambridge, UK, dilution 1:500). To remove unbound antibodies, membranes were subsequently washed (1x for 15 min, then 4x for 5 min). After incubation with secondary antibody (Anti-rabbit or Anti-rat IgG conjugated with horseradish peroxidase; Abcam, Cambridge, UK; dilution 1:4500), membranes were washed 5 times (as indicated above). Antibody binding was detected with enhanced chemi luminescence (LumiGlo substrate, PIERCE Super Signal West Pico Trial Kit, China) followed by exposure to X-ray film. Films were scanned and analyzed using Image Lab software (Bio-Rad Co. Ltd., USA).

Table 1: The primers used in this study

Genes	Product length (bp)	Sequence	Reference
GAPDH	285	F:5'-TACATGGTCTACATGTTCCAGTATG -3' R:5'- CAGGAGGCATTGCTGACAATCTTG -3'	AF017079
HSP70	152	F:5'- GCCCTGAATCCGCAGAATA -3' R:5'- TCCCCACGGTAGGAAACG -3'	AT3G12580
HSP90	206	F:5'- AATCGCCCAGTTGATGTCG -3' R:5'-TGTCCACTATCGTGAGGGTCC -3'	SPAC926.04c
HSP27	200	F:5'- CCGGTGTTTCACTCGAAAATACA -3' R:5'- GCTTTTCCGACTTTCCAGCTTCT -3'	Dmel_CG4466

F: forward; R: reverse

Statistical Analysis

Groups were compared by one-way analysis of variance using SSPS software (IBM, USA) (Statistical Product and Service Solutions, China). The mean values ± standard error of the means are presented. Differences were considered significant when $p < 0.05$.

Results

Relative Expression of HSP mRNA in Kidney

As shown in Fig. 1, kidney *HSP70* mRNA expression was significantly higher in piglets weaned at 14 and 21 days compared to piglets weaned at 35 days ($p < 0.05$). Weaning at 21 days resulted in significantly lower expression of kidney *HSP90* mRNA compared to weaning at 14 days, and significantly higher relative expression of *HSP90* mRNA compared to weaning at 28 and 35 days ($p < 0.05$). Weaning at different ages had no significant effects on kidney *HSP27* mRNA ($p > 0.05$).

Relative Expression of HSP mRNA in Myocardium

As shown in Fig. 2, piglets weaned at 21 and 28 days had significantly increased myocardial *HSP70* mRNA levels compared to piglets weaned at 14 and 35 days ($p < 0.05$). Weaning at 28 days resulted in significantly lower relative expression of myocardial *HSP90* mRNA compared to weaning at 14 days, and significantly higher relative expression compared to weaning at 35 days ($p < 0.05$). Weaning at different times had no significant effect on myocardial *HSP27* mRNA ($p > 0.05$).

Relative Expression of HSP mRNA in Longissimus Dorsi Muscle

As shown in Fig. 3, *HSP70* mRNA expression in longissimus dorsi muscle was significantly lower in piglets weaned at 21 days compared to piglets weaned at 14 days ($p < 0.01$). Weaning at different times had no significant effect on longissimus dorsi muscle *HSP90* mRNA ($p > 0.05$). Weaning at 28 and 35 days of age resulted in significantly lower relative expression of longissimus dorsi muscle *HSP27* mRNA compared to weaning at 14 and 21 days ($p < 0.01$).

Expression of HSP Protein in Kidney

As shown in Fig. 4, kidney HSP70 protein in piglets weaned at 35 days was significantly lower compared to piglets weaned at earlier times ($p < 0.05$). Piglets weaned at 21 days had significantly lower kidney HSP90 protein compared to piglets weaned at other times ($p < 0.05$). Weaning at different times had no significant effects on kidney *HSP27* protein ($p > 0.05$).

Expression of HSP Protein in Myocardium

As shown in Fig. 5, piglets weaned at 14 days had significantly higher expression of myocardial HSP70 than piglets weaned at 35 days of age ($p < 0.05$). Weaning at different times had no significant effect on myocardial *HSP90* and *HSP27* protein levels ($p > 0.05$).

Expression of HSP Protein in Longissimus Dorsi Muscle

As shown in Fig. 6, piglets weaned at 14 and 21 days had significantly elevated *HSP70* expression in longissimus dorsi muscle compared to piglets weaned at 28 and 35 days ($p < 0.05$). Piglets weaned at 21 days had significantly lower expression of *HSP90* and *HSP27* in longissimus dorsi muscle compared to piglets weaned at 35 days ($p < 0.05$).

Discussion

Weaning at 21 days resulted in significantly elevated myocardial *HSP70* mRNA compared to weaning at 14 days ($p < 0.05$). However, weaning at 21 days of age also resulted in substantially lower expression of *HSP70* mRNA in the longissimus dorsi muscle compared to weaning at 14 days ($p < 0.01$). Previous research showed that piglets weaned at 21 and 28 days after birth had increased *HSP27* and *HSP70* expression in the stomach and duodenum 6-12 h after weaning, as well as an increase 24-48 h after weaning in the jejunum and ileum (David *et al.*, 2002). This suggests that *HSP* expression in piglets is affected by weaning date and that it varies by tissue type.

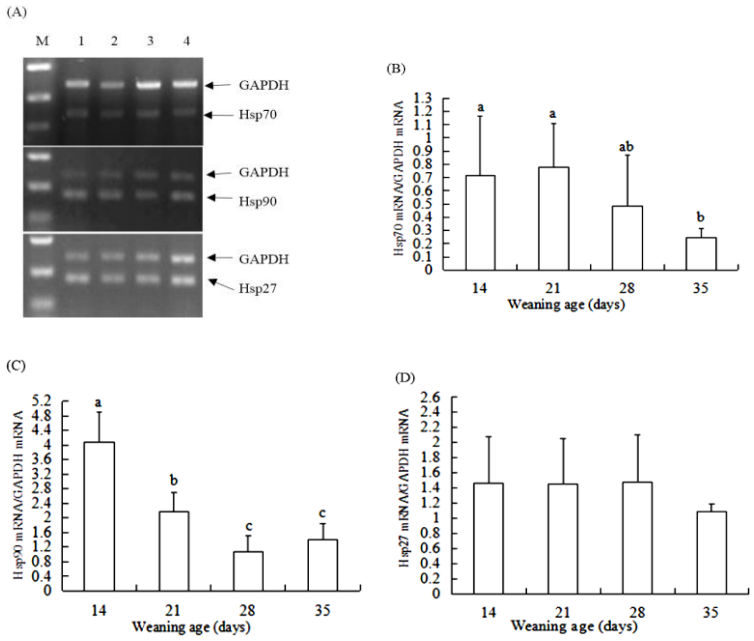

Fig. 1: Relative expression of *HSP70* mRNA, *HSP90* mRNA, and *HSP27* mRNA in piglet kidneys at different weaning ages. (A) Representative electrophoresis photos. M: DNA ladder (DL2000), 1-4: electrophoresis photos of *HSPs* mRNA in piglets weaned at different ages (14, 21, 28, 35 days), respectively; (B-D) relative expression of liver *HSP70* mRNA, *HSP90* mRNA and *HSP27* mRNA. Date are expressed as means ± standard error of the mean, different lowercase letters above error bars indicates significant difference between breeds ($p < 0.05$), and different capital letters indicates significant difference between breeds ($p < 0.01$)

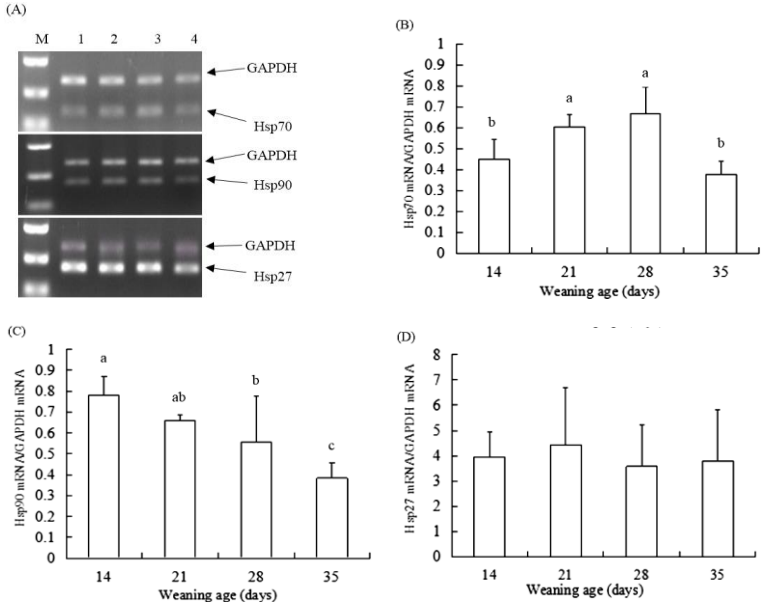

Fig. 2: Relative expression of *HSP70* mRNA, *HSP90* mRNA, and *HSP27* mRNA in in piglet myocardium weaned at 14, 21, 28, and 35 days of age. (A) Representative electrophoresis photos. M: DNA ladder (DL2000), 1-4: electrophoresis photos of *HSPs* mRNA in piglets weaned at different ages (14, 21, 28, 35, days), respectively; (B-D) relative expression of myocardial *HSP70* mRNA, *HSP90* mRNA and *HSP27* mRNA respectively

Piglets weaned at 21 days after birth had significantly elevated *HSP70* expression in kidney and longissimus dorsi muscle compared to piglets weaned at 35 days ($p<0.05$). In contrast, weaning at 21 days resulted in significantly lower *HSP90* expression in the kidney and longissimus dorsi muscle compared to weaning at 35 days ($p < 0.05$).

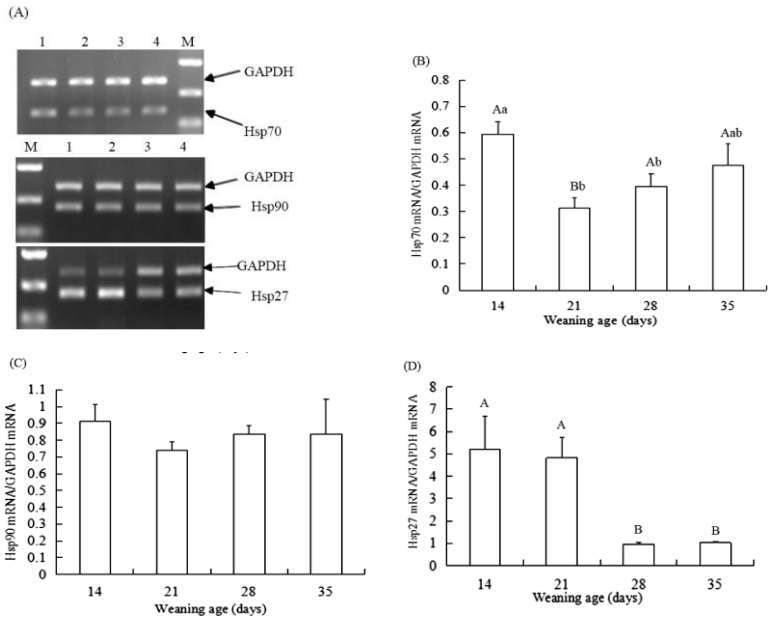

Fig. 3: Relative expression of *HSP70* mRNA, *HSP90* mRNA, and *HSP27* mRNA in piglet longissimus dorsi muscle at different weaning ages. (A) Representative electrophoresis photos. M: DNA ladder (DL2000), 1-4: electrophoresis photos of *HSPs* mRNA in piglets weaned at different ages (14, 21, 28, 35, days), respectively; (B) relative expression of longissimus dorsi muscle *HSP70* mRNA, *HSP90* mRNA, and *HSP27* mRNA

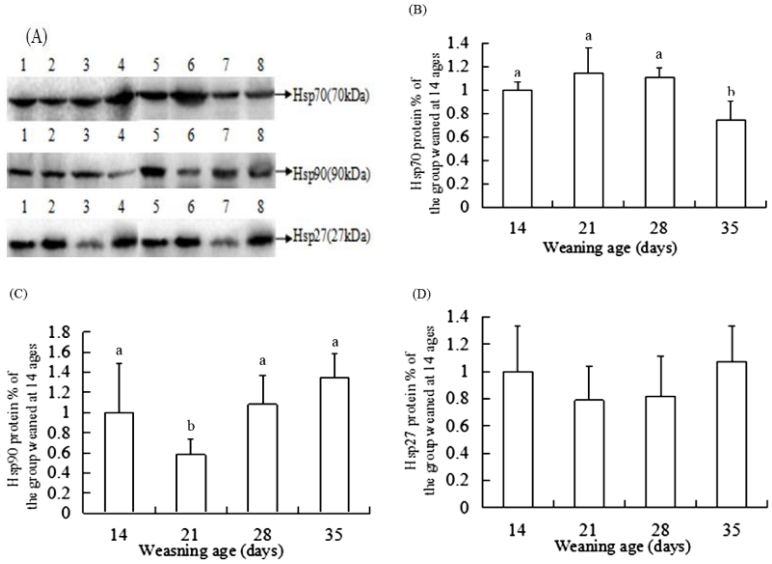

Fig. 4: Expression of *HSP27*, *HSP70*, and *HSP90* in piglet kidneys at different weaning ages. (A) Western blot HSP protein immunoreactive bands. 1-4: Western blotting photos of 14, 21, 28, and 35 days of age weaning groups, 5-8: A group of repeat; (B-D) Expression of *HSP70*, *HSP90*, and *HSP27* in piglet kidneys

This suggests that *HSP70* and *HSP90* have different structural or functional roles. Previous research indicates that *HSP70* is involved in protein synthesis, folding, and translocation (Zhong *et al.*, 2010). Furthermore, after cellular stress, which causes protein denaturation, *HSP70* inhibits protein aggregation and stimulates refolding of damaged proteins (Park *et al.*, 2001). On the other hand, *HSP90* proteins act as chaperones, supporting the stability and activity of various proteins with diverse functions (Nollen and Morimoto, 2002; Zuehlke and Johnson, 2010). Unlike *HSP70*, several *HSP90* subclasses require specific co-chaperones for recruitment into the chaperone complex, and co-chaperones areimportant for *Hsp90* substrate recognition *in vivo* (Didenko *et al.*, 2012).

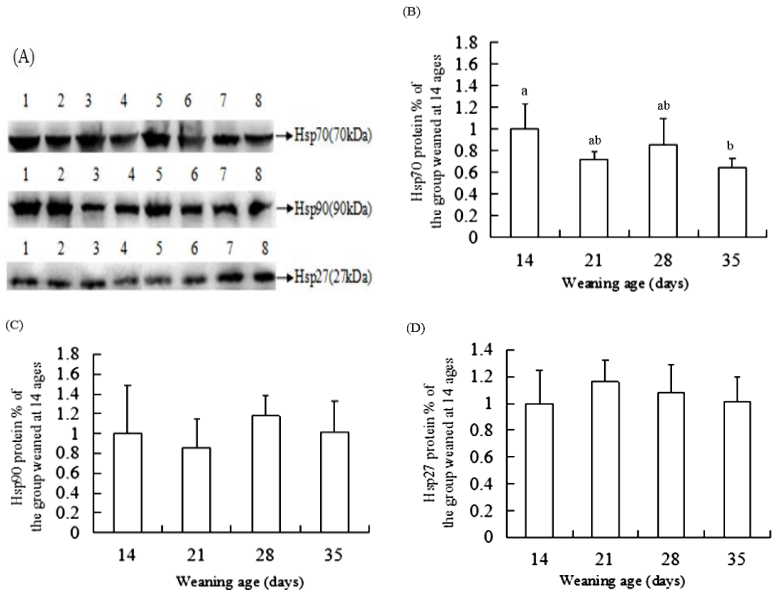

Fig. 5: Expression of *HSP27*, *HSP70*, and *HSP90* in piglet myocardium at different weaning ages. (A) Western blot HSP protein immunoreactive bands. 1-4: the Western blotting photos of 14, 21, 28, and 35 days of age weaning group orderly, 5-8: A group of repeat; (B-D) Expression of *HSP70*, *HSP90* and *HSP27* in myocardium

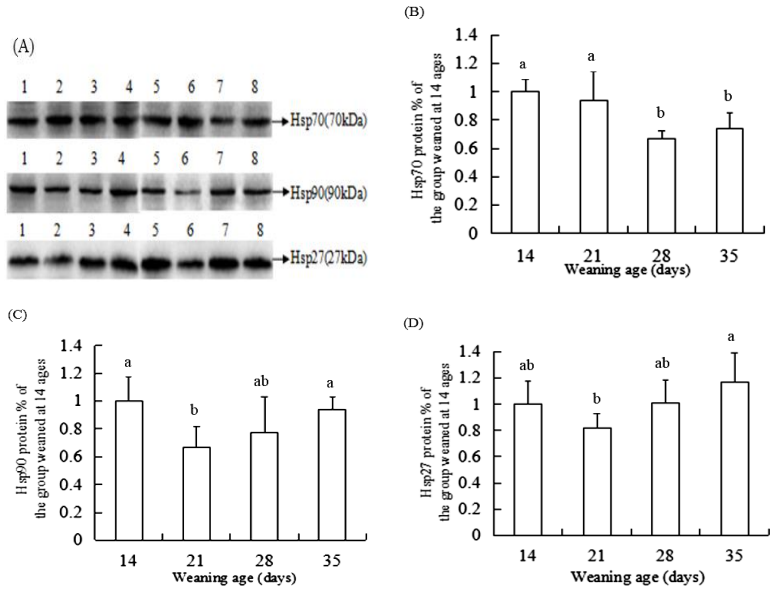

Fig. 6: Relative expression of *HSP27*, *HSP70* and *HSP90* in piglet longissimus dorsi muscle at different weaning ages. (A) Western blot HSP protein immunoreactive bands. 1-4: the Western blotting photos of 14, 21, 28, and 35 days of age weaning group orderly, 5-8: A group of repeat; (B-D) relative expression of longissimus dorsi muscle *HSP70*, *HSP90* and *HSP27* protein in piglets weaned at different ages (14, 21, 28, 35, days)

Kidney *HSP*90 mRNA expression was significantly higher in piglets weaned at 21 days compared to piglets weaned at 35 days ($p < 0.05$). This contrasts with kidney *HSP90* protein, where expression was higher in piglets weaned at 35 days ($p < 0.05$). Similarly, weaning at 21 days resulted in significantly higher expression of *HSP27* mRNA in the longissimus dorsi muscle compared to weaning at 35 days ($p < 0.01$), whereas *HSP27* protein expression was lower in piglets weaned at 35 days of age ($p < 0.05$). This may be because protein expression provides negative feedback to mRNA expression. This feedback system is suspected to eventually result in reduced overall levels of heat shock protein in the organism (Bendtsen *et al.*, 2015).

The change in piglet growth performance, immune function, and antioxidant function after weaning may be due to variations in *HSP* expression (Zhu *et al.*, 2012; Sun *et al.*, 2016a, b). As constitutively expressed proteins, *HSPs* play a fundamental role in maintaining the stability of other cellular proteins (Gullo and Teoh, 2004). Therefore, considerable attention has been focused on studying feed supplementation as a means of increasing *HSP* expression in animals. For example, Zhong (Zhong *et al.*, 2011) proposed that supplementation with glutamine can improve intestinal growth and morphology by increasing *HSP70* expression in weaning piglets. In addition, increased intestinal growth and *HSP70* was observed in weaning pigs when their corn and soybean meal diet was supplemented with L-arginine or N-carbamylglutamate (Wu *et al.*, 2010).

Expression of either *HSP27* mRNA or protein in kidney and myocardial tissue was unaffected by piglet weaning date. Zhang's study showed that levels of *HSP27* did not change significantly in transported pigs when compared to control pigs, though a similar (non-significant) trend was observed that paralleled the trend in *HSP90* levels (Zhang *et al.*, 2011). In our study, the expression of *HSP27* also did not change significantly in pigs suffering weaning stress at different ages, but there were similar trends in *HSP90* levels in kidney and longissimus dorsi muscle. The molecular mechanism for these findings in piglets needs further investigation.

Conclusion

The expression of *HSP* genes and proteins was affected by piglet weaning age. *HSP* expression patterns were different in different tissues, and the change in *HSPs* may be the reason for variations in growth performances, immune function, and antioxidant function in piglets.

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