

Over-expression of HSP70 induces apoptosis of intestinal epithelial cells in heat-stressed pigs: A proteomic approach

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Research

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Abstract

Background

Heat stress (HS)-induced intestinal epithelial cell apoptosis may play a pivotal role in intestinal barrier function injury in animals. However, the underlying molecular mechanism by which HS induces apoptosis of intestinal epithelial cells is still poorly understood.

Methods

The trial uses a prospective study. Firstly, a eukaryotic expression vector for the HSP70 gene was constructed and transfected into intestinal porcine epithelial cells (IPEC-J2) and then analyzed with a functional proteomics approach followed by liquid-chromatography-tandem mass spectrometry (LC-MS/MS) identification. Next, heat stress treatment of IPEC-J2 cells and pigs were performed. Immunoblotting and flow cytometry were used to examine the protein changes and cellular apoptosis.

Results

246 differentially expressed proteins (DEPs) were identified in HSP70-overexpressed IPEC-J2 cells, functional annotation suggested that most of the DEPs were related to ECM-receptor interaction, focal adhesion, and apoptosis. Moreover, apoptosis rate was increased in IPEC-J2 cells transfected with porcine HSP70 overexpressing plasmid accompanied with the expression of apoptosis-related proteins, Caspase-3, PARP, and Bax were up-regulated, whereas Bcl-2 protein expression was down-regulated. Interestingly, increased Caspase-3, PARP, and Bax with decreased Bcl-2 were observed in IPEC-J2 cells under heat stress conditions. In an *in vivo* porcine model, HS-induced cellular apoptosis in the duodenum, cecum, and colon, as well the up-regulation of HSP70 in the intestinal tissues.

Conclusions

HSP70 may play a regulatory role in cellular apoptosis within IPEC-J2 cells. Taken together, our findings provide new insights for understanding the molecular mechanisms of cellular apoptosis associated with HSP70.

Background

Rising global temperatures have been accompanied with heat stress (HS) during a long period of hyperthermic weather and special geographical location directly or indirectly impacts on humans and animals health [1]. HS, as well as pathogens, can damage the intestinal epithelia barrier function and induce inflammatory bowel disease (IBD), which potentially result in large-scale economic losses to the pig-farming industry [2]. Heat stress proteins (HSP) were first observed as heat-stimulated proteins in

Drosophila [3] and they are classified into six families namely small HSP (15–30 kDa), HSP40, HSP60, HSP70, HSP90, and HSP100 [4]. Among them, HSP70-based molecular chaperone participate in various cellular functions including protein folding, trafficking, membrane translocation, and cellular apoptosis [5, 6]. An understanding of the HSP70-associated mechanisms causing IBD is crucial, because most of the emerging diseases confined to the gut lead to increased zoonotic disease risk that threatens public health.

The temperatures greater than human normal body temperature, (from 41.6 °C to 42 °C), can result in cell death (from apoptosis) in a few hours [7]. The mild hyperthermia may induce apoptosis which is prevented in thermotolerant cells [8, 9]. HS-induced human endothelial cells apoptosis via the mitochondrial pathway in form of mitochondrial p53 translocation in ROS dependent and ensure Ca^{2+} dyshomeostasis, while Bax mitochondrial translocation, as the upstream events casing apoptosis taken place [10]. HS-induced cellular apoptosis and survival maintenance through the mitochondrial apoptotic pathway Bax/Caspase9/Caspase3 and apoptosis-inhibiting Bcl-2 in the trophoblast cells [11]. Moreover, HS induces HSP70 expression and the production of pro-inflammatory cytokines, which modulates the immune function in intestine of swine [12, 13]. In addition, the expression of extracellular HSP70 induces the production of TNF- α and IL-6 through the activation of Toll-like receptor 4 (TLR4) signaling pathway in mouse bone marrow-derived mast cells [14]. In particular, when HSP70 fused with- or in complex with human hantavirus nucleocapsid protein, it greatly enhances both Th1 and Th2 responses in mice [15]. Based on above evidences, the interaction mechanism of HSP70 induce apoptosis in intestinal epithelial cells of heat-stressed pigs requires further investigation.

In recent years, quantitative proteomic studies were used to investigate the underlying molecular response in porcine cells and tissues, including mesenchymal stem cells [16], intestines [17], pulmonary alveolar macrophages [18], and porcine circovirus type 2 (PCV2) infected PK-15 cells [19]. Nevertheless, no systematic study combined a proteomic analysis was performed to investigate the mechanism of HSP70 expression and cellular apoptosis in intestinal epithelial cells of heat-stressed pigs. Thus, in the present study, a functional proteomics approach was combined with LC-MS/MS to measure the interaction of HSP70 induced epithelial cell survival in heat-stressed pigs.

Methods

IPEC-J2 cells were obtained from the Collection of Cell Lines in the College of Veterinary Medicine, Guangdong Ocean University, China.

Construction of porcine HSP70 expressing vectors and transfection into IPEC-J2 cells

Porcine HSP70 encoding sequences were ligated into mammalian expression vector pcDNA3.1/V5-His-TOPO TA, procured from Invitrogen (Shanghai, China). The plasmid DNAs were extracted with an endotoxin-free Plasmid Maxi Kit (Qiagen, Valencia, CA, USA). IPEC-J2 cells were incubated in a 6-well tissue culture plate in culture medium (DMEM-F12: Gibco, Grand Island, NY, USA) supplemented with 100 mg/mL streptomycin, 100 U/mL penicillin, and 10% fetal bovine serum at a concentration of 1×10^6

cells/mL (5% CO₂, 95% air, 100% humidity). IPEC-J2 cells were grown in plate at 85–95% confluence and then transfected with plasmid DNA encoding porcine HSP70 or empty vector as a control by Lipofectamine[®]-3000 (Invitrogen, Shanghai, China).

Proteins preparation, iTRAQ labeling, and strong cation exchange chromatography

In brief, total proteins were extracted from IPEC-J2 cells transfected with plasmid DNA encoding porcine HSP70 or empty using a total protein extraction kit (Biochain, Hayward, CA, USA) and quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA). Next, the protein from test and control group was digested [20], and then labeled using the 8-plex iTRAQ reagent, according to the manufacturer's instructions (Applied Biosystems). Test samples, the protein from IPEC-J2 cell transfected with HSP70 encoding plasmid were labeled as 116 (HSP70-1), 117 (HSP70-2), and 118 (HSP70-3), while control samples were labeled as 113 (Empty-1), 114 (Empty-2), 115 (Empty-3) (three replicates for each sample). The labeled peptides were fractionated by strong cation exchange (SCX) chromatography and the detailed steps was described by Han et al [21].

LC - ESI MS/MS and data analysis

The protocols of experiments were performed on a Q Exactive mass spectrometer coupled to Easy nLC (Thermo Fisher Scientific, Shanghai, China). The steps are the same as mentioned by Wu et al [22]. Using the top 10 most abundant precursor ions as the MS/MS data modification. Subsequently, the detailed steps about identification and quantification of proteins as well as the determination of the DEPs in the IPEC-J2 cells transfected with pHSP70 and Empty DNA plasmids were same and presentation in our previous research [23]. Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) databases were used to facilitate the biological interpretation and pathway analysis of the identified proteins in this study using the online tool, DAVID (<http://david.abcc.ncifcrf.gov>).

HS treatment of IPEC-J2 cells

IPEC-J2 cells were incubated into a 6-well tissue culture plates in culture medium (DMEM-F12: Gibco, Grand Island, NY, USA) at a concentration of 1×10^6 cells/mL (5% CO₂, 95% air, 100% humidity). One plate was incubated at 42°C (5% CO₂, 95% air, 100% humidity) for 2 h and then incubated at 37°C for recovery for 1.5 h, with sampling at 0, 0.5, 1, and 1.5 h (heat stress-treated cells). Meanwhile, another culture plate was incubated at 37°C (5% CO₂, 95% air, 100% humidity) without any treatment (control cells).

Immunoblotting

Immunoblotting was performed as described previously by An et al. [24]. Briefly, total protein from IPEC-J2 cells transfected with pHSP70 and Empty DNA plasmid groups, or from intestine with heat stress treatment was extracted using a total protein extraction kit (Biochain, Hayward, CA, USA), and quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA). Thereafter, proteins were separated on SDS-PAGE gels, and then transferred to polyvinylidene fluoride membranes (Millipore, Darmstadt, Germany).

Blocking the membranes in 5% fat-free milk/TBST solution at room temperature for 2 h and then incubated overnight at 4°C with primary antibodies, anti-PPM1B (1:2000, Abcam), anti-CGNL1 (1:1500, Abcam), anti-Caldesmon1 (1:1500, Abcam), anti-Caspase3 (1:1500, Cell Signaling Technology), anti-Caspase7 (1:1500, Cell Signaling Technology), anti-PARP (1:2000, Cell Signaling Technology), anti-Bax (1:1000, Cell Signaling Technology), anti-Bcl2 (1:2000, Cell Signaling Technology), and anti- β -actin (1:1,000, Proteintech, China; loading control). Subsequently, the membranes were incubated with the corresponding secondary antibodies conjugated to horseradish-peroxidase-conjugated at room temperature for 2 h. A chemiluminescence method was used to visualize immunoreactive bands and the positive bands were visualized by enhanced chemo-luminescence (Tanon, Shanghai, China). The bands were analyzed semi-quantitatively via densitometry using Gel-Pro Analyzer v4.0 software (Meyer Instruments, Houston, TX, USA), and the relative protein expression levels were normalized to β -actin.

Flow cytometry assay

IPEC-J2 cells grown into 6-well plates were transfected with pHSP70 and Empty DNA plasmid, respectively. Afterward, the cells were harvested for cellular apoptosis analysis with Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. The apoptotic results were analyzed using the same method as described previously by Ma et al [11].

Animals ethics statement and sample collection

Twenty-four crossbred pigs (Duroc \times Local Luchuan pig) weighing 15 ± 1 kg were obtained from the farm in the Guangdong province of China. The animals experiment and protocols were approved by the Guangdong Ocean University Animal Ethics Committee (Permit No. 206–1108). The pigs were randomly assigned to two groups (test and control, 12 barrows per groups). They were housed in three pens (1.5 m² per pig) with two females and two males in each pen, and habituated for 1 week. Pigs allotted to HS treatment were kept at $35 \pm 1^\circ\text{C}$ (carried out in an artificial climate chamber) in a controlled climate room. Control pigs were kept at an ambient temperature of $20 \pm 2^\circ\text{C}$, and the relative humidity of the two groups was kept at approximately 85%. The experimental design was carried out as described previously [23]. All pigs were euthanized using a head-only electric stunning tong apparatus on the morning of sampling days 1, 7, 14, and 21 after the start of the experimental protocol, followed by manual exsanguination. Duodenum, cecum, and colon were immediately removed after slaughter, and used for cellular apoptosis analysis.

Cellular apoptosis analysis

Serial 6- μm -thick sections were treated with DNase to fragment the DNA (positive control) and 50 μL of the prepared terminal deoxynucleotidyl transferase reaction mixture was added to each slide. The slides were incubated for 60 min at 37°C , rinsed with deionized water, and washed with 3% bovine serum albumin (BSA) + 0.1% Triton[®] X-100 in phosphate-buffered saline (PBS) for 5 min. Following another rinse with $1 \times$ PBS, 50 μL of the Click-iT[®] Plus TUNEL reaction cocktail was added to each slide, ensuring

that the solution was spread evenly over the surface. The slides were incubated for 30 min at 37°C in the dark. The Click-iT® Plus TUNEL reaction cocktail was then removed, after which the slides were washed with 3% BSA in PBS for 5 min, and then rinsed with 1 ×PBS. Cellular apoptosis was examined under a fluorescence microscope (Anjue Medical Equipment Co., Ningbo, China). The optical density of apoptotic cells per unit area was measured using Image Pro Plus 6.0.

Statistical analysis

All results in this study are represented as mean ± SEM. Student's t-test was used to compare differences of protein expression between the pHSP70 and pEmpty transfected IPEC-J2 cells groups and calculate P values. Proteins with at least two unique peptides and FDR of < 0.01 was qualified for further quantification data analysis. Fold change of ≥ 1.3 or ≤ 0.77 was set as the threshold to identify differently expressed proteins. Differences in expression of HSP70 and apoptosis-related proteins, Caspase-3, PARP, Bax, and Bcl-2 in cells were analyzed using one-way ANOVA. *indicates $p < 0.05$ and ** $p < 0.01$.

Results

Ectopic expression of porcine HSP70 in IPEC-J2 cells

We used a PCR technique to isolate cDNA fragments of the HSP70 gene from porcine peripheral blood mononuclear cells (PBMCs) and cDNA fragments were inserted into the PMD18-T vector (TaKaRa, Beijing, China). The pcDNA3.1/V5-His-TOPO vector with a His-tag at the C-terminus was ligated with porcine HSP70 (pHSP70) cDNA gene sequences, yielding pcDNA3.1/V5-HSP70-His (Fig. 1A, B). DNA plasmids of pHSP70 and the Empty vector were transfected into IPEC-J2 cells. Immunoblotting analysis showed that the HSP70 levels were significantly increased in transfected IPEC-J2 cells ($p < 0.01$). Thus, a eukaryotic expression vector for the HSP70 gene was successfully constructed (Fig. 1C, D).

iTRAQ proteomic analysis to identified proteins in IPEC-J2 cell transfected with pHSP70

To further investigate the underlying functions and features of HSP70 in IPEC-J2 cells, a quantitative proteomic method, iTRAQ analysis coupled with LC-MS/MS was used to identify and quantify the potential changes of the IPEC-J2 cells transfected with pHSP70 DNA plasmid. A total of 3546 proteins were identified, and a MS comparison of the protein expression identified 246 proteins that were up- or down-regulated ($p < 0.05$), as calculated by MaxQuant [25]. Hundred and forty-four proteins were down-regulated, and hundred and two proteins were highly upregulated. Among the upregulated proteins, higher expression levels of HSP70 were detected (2.56-fold). A partial list of the up- and downregulated proteins, their respective peptides, corresponding P values, and relevant biological processes are shown in Tables 1 and 2. All the DEPs are provided in the Supplementary Table.

Table 1
Significantly up-regulated proteins transfected pHSP70 in IPEC-J2 cells

No.	Accession	Protein name	Ratio	Peptides	p-value
1	A0A0B8S070	Heat shock 70 kDa protein 6 (HSP70B')	2.56	1	0.009
2	F1S5K0	Protein phosphatase, Mg ²⁺ /Mn ²⁺ dependent 1B (PPM1B)	1.76	1	0.001
3	Q06AS8	GBAK	1.75	4	0.000
4	F1RUV5	Pyruvate carboxylase	1.72	1	0.011
5	A7WLI1	Tetraspanin	1.64	3	0.018
6	F1SGG0	Tensin 2	1.63	1	0.000
7	F1RZ52	Tripartite motif-containing protein 26	1.60	1	0.003
8	A0A287AUT1	Plexin B2	1.53	1	0.002
9	F1RMU2	TATA-box binding protein associated factor 7	1.52	1	0.004
10	A0A287A1C8	Cingulin like 1(CGNL-1)	1.46	1	0.008
11	A0A287BSA8	Ribosomal protein	1.48	1	0.018
12	K9IWI1	Protein tyrosine phosphatase type IVA 1	1.45	3	0.046
13	Q1RPR5	Integrin beta	1.43	1	0.001
14	D0G6Y0	Hydroxysteroid (17-beta) dehydrogenase 12 (Fragment)	1.41	1	0.006
15	K7GSP8	PAK1 interacting protein 1	1.40	1	0.002

Table 2
Significantly down-regulated proteins transfected pHSP70 in IPEC-J2 cells

NO.	Accession	Protein name	Ratio	Peptides	p-value
1	F1RWI8	Secernin 2	0.82	1	0.025
2	A0A287ASR2	Coiled-coil domain containing 88A	0.82	2	0.017
3	A0A287BRE7	Pleckstrin homology and RhoGEF domain containing G3	0.82	1	0.027
4	Q767L6	Flotillin-1	0.82	5	0.000
5	A0A287AKP8	Family with sequence similarity 83 member H	0.82	2	0.036
6	A0A286ZSG3	Catalase	0.82	4	0.047
7	A0A286ZPA6	BCAR1, Cas family scaffolding protein	0.82	2	0.009
8	Q9GL64	Transcription factor A (Fragment)	0.82	1	0.007
9	B5APV0	Actin-related protein 2/3 complex subunit 5	0.82	5	0.005
10	F1S4Z2	Charged multivesicular body protein 4B	0.81	3	0.010
11	A0A287A8E8	PHD finger protein 1	0.81	1	0.018
12	A0A286ZJI6	Kinesin-like protein	0.81	2	0.020
13	E0YLM4	Mesencephalic astrocyte-derived neurotrophic factor	0.81	5	0.004
14	A0A286ZUH0	Caldesmon-1	0.80	1	0.043
15	A0A287AGZ2	Caspase 7	0.74	1	0.033

To understand the molecular/functional classes and subcellular annotations of the DEPs, the UniProtKB/Swiss-Prot and TrEMBL protein databases, as well as GO database were used to analyzed the underlying biological function of the 246 DEPs. Our results showed that both upregulated and downregulated proteins in IPEC-J2 cells transfected with pHSP70 and Empty DNA plasmid were localized in the same cellular organelles and extracellularly, however, at different ratios (Fig. 2A, B).

We used the DAVID database to determine the genes associated with differentially abundant proteins and found that the DEPs can be categorized as: involved in biological processes, molecular functions, and cellular components. The biological processes of the upregulated and downregulated proteins were primarily associated with cellular processes, single-organism processes, metabolic processes, biological regulation, response to stimuli, cellular component organization or biogenesis, and localization (Fig. 3A). The cellular components of the DEPs include cells, organelles, membrane, macromolecular complex, extracellular regions, membrane-enclosed lumen, and cell junctions (Fig. 3B). The molecular functions of the proteins included binding, catalytic activity, structural/molecular activity, molecular function regulator,

transporter activity, and molecular transducer activity (Fig. 3C). KEGG pathway enrichment analysis was performed to ascertain the biological signaling pathway of HSP70 in IPEC-J2 cells. The top fourteen most significantly enriched pathways identified by this analysis include arrhythmogenic right ventricular cardiomyopathy (ARVC), ECM-receptor interaction, dilated cardiomyopathy, focal adhesion, and apoptosis (Fig. 3D). A protein-protein interaction (PPI) network of the 246 DEPs was constructed by STRING (Fig. 4). The PPI network analysis revealed that most of the DEPs were highly connected. Two selected DEPs, PPM1B and CGNL-1, participated in various biological processes, such as inflammation and signal transduction, which may modulate signaling pathways and cellular function in IPEC-J2 cells.

Three candidate proteins, PPM1B, CGNL-1, and Caldesmon-1, were selected for validation of the proteomic results. The immunoblotting analysis showed that the expression of PPM1B and CGNL-1 were significantly upregulated in IPEC-J2 cells transfected with pHSP70 DNA plasmid compared with cells transfected with Empty DNA plasmid ($p < 0.05$ and 0.01 , respectively), whereas the level of Caldesmon-1 was obviously downregulated ($p < 0.05$) (Fig. 5A, B). In sum, these results are consistent with data obtained from iTRAQ findings.

Overexpression of HSP70 induces cellular apoptosis in in vitro and in vivo models

Proteomics results showed that HSP70 was related to cellular apoptosis in IPEC-J2 cells. To further investigate the interaction between HSP70 and cellular apoptosis, as shown in Fig. 6, we noticed that four cellular apoptosis-related proteins, including Caspase-3, PARP, Bcl-2, and Bax, were significantly changed in IPEC-J2 cells transfected with pHSP70 DNA plasmid compared with cells transfected with Empty DNA plasmid ($p < 0.05$ and 0.01 , respectively). The expression of pro-apoptotic protein Caspase-3, PARP, and Bax were increased while the anti-apoptotic protein Bcl-2 was decreased in IPEC-J2 cells transfected with pHSP70 DNA plasmid compared to cells transfected with Empty DNA plasmid. Further, we detected the cellular apoptotic rate of IPEC-J2 cells by flow cytometry. The assay revealed that the percentage of apoptotic cells was much higher in IPEC-J2 cells transfected with pHSP70 DNA plasmid than that in control IPEC-J2 cells (Fig. 6C, D). Next, we examined the levels of HSP70 in IPEC-J2 cells challenged by HS for 2 h and allowed to recover for up to 1.5 h. The results clearly showed that the expression of HSP70 was significantly up-regulated ($p < 0.01$) in IPEC-J2 cells under HS and recovery condition (Fig. 7A, B). Moreover, immunoblotting analysis revealed that HS treatment promoted the expression levels of apoptosis-related proteins, Caspase-3, PARP, and Bax, and decreased the levels of Bcl-2 (Fig. 7C-F).

We further elucidated the effect of HS treatment on HSP70 production and apoptosis in porcine *in vivo* intestinal cell model. The distribution of intestinal apoptotic cells after heat stress was detected by the TUNEL method. According to TUNEL assay, heat stress group had significantly higher ratio of apoptotic cells to total cells in duodenum and cecum on day 7 ($p < 0.05$) (Fig. 8A-D). On the other hand, HS markedly increased the ratio of apoptotic cells to total cells on days 7 and 14 in colon ($p < 0.01$) (Fig. 8E, F). Furthermore, the expression of HSP70 in colon was significantly upregulated in HS pigs compared to that in control group ($p < 0.05$ and 0.01 , respectively) (Fig. 8G, H).

Discussion

In recent years, there has been an increased interest in searching the potential effect of heat stress on host-apoptosis interaction. A study in baboon model revealed that HS-induced widespread apoptosis in spleen, thymus, and small bowel [26]. In *in vitro* studies, there were evidences showing that apoptosis can be promoted by application of heat stress [27, 28]. In this study, we have found for the first time that over-expression of HSP70 could induce apoptosis of intestinal epithelial cell in pigs. This finding reveals that HSP70 might be involved in the regulation of heat stress induced related disease, thus further developing the underlying functions of HSP70.

HSP70, one of the highly conserved molecular chaperone, plays a critical role in maintaining cellular protein homeostasis [29, 30]. Intracellular HSP synthesis is very susceptible to abnormal environmental stressors, such as exposure to high temperature, chemicals, bacteria, viruses, and heavy metals. This synthesis plays a vital role in protecting cells against the abnormal environmental stress conditions. A previous study has shown that oxidative stress induced by benzene and its homologues can substantially induce HSPs expression in cells [31]. In this study, it was found that heat stress dramatically induced HSP70 expression in *in vitro* cultured IPEC-J2 cells and in the duodenum of pigs. Previous research has also demonstrated that the expression of HSPs were related to the phosphoinositide-3-kinases (PI3K)/protein kinase B (Akt) signaling pathway like HSP27 can interact with PI3K/Akt and subsequently inhibit apoptosis [32]. *In vitro* cultured lung fibroblasts, exposure to high temperature treatment can activate the PI3K/Akt signaling pathway and leading to high expression of HSP70. Furthermore, when inhibited the activation of PI3K/Akt by siRNA the high HSP70 expression was also disappeared [33]. Therefore, further studies are needed to elucidate the role of PI3K/Akt signaling pathway in heat stress induced HSP70 expression.

Apoptosis efficiently contributes to intracellular pathogen removal by eliminating the favorable intracellular survival mechanisms. The relationship between invading pathogen and its host cells is a complex interaction, in which the pathogen strives to survive and replicate [34]. A former study has shown that HSP70 would suppress apoptosis by directly associating with Apaf-1 and blocking the assembly of a functional apoptosome [35]. Caspase-3, caspase-7, and Bax are the calpain-cleaved substrates. Calpains cleave and inactivate caspase-3, -7 in biochemical *ex vivo* apoptosis assays and during Ca^{2+} ionophore-induced apoptosis *in vitro* [36]. However, our results show that heat stress induces apoptosis in intestinal epithelia not only in *in vivo* experiment, but also in *in vitro* cultured cells that were exposed to heat stress for 2 h. Meanwhile, HSP70 overexpression induced the apoptosis of IPEC-J2 cells as well the upregulation of caspase-3, PARP, and the ratio of Bax to Bcl-2. These findings suggesting that HSP70 may plays a vital role in heat stress induced apoptosis of intestinal epithelial cells.

KEGG enrichment analysis showed that apoptosis is one of the most significantly enriched pathway, our proteomic results showed that the caspase-7 and poly [ADP-ribose] polymerase (PARP) were downregulated in IPEC-J2 cells transfected with pHSP70 plasmid. Stable isotope labeling analysis revealed that apoptosis-related proteins, including caspase-3, caspase-7, and Bax-alpha protein were

downregulated in PK-15 cells infected with PCV-2 [19]. These findings are consistent with our proteomic results. The reason maybe is those apoptosis-related proteins (both pro- and anti-apoptotic proteins) are either activated or inactivated by calpains. In the proteomics results, calpain-cleaved products of apoptosis-related proteins are not identified by LC – MS/MS, resulting in their apparent reduced expression levels. Furthermore, calpains activate caspase-7 in B cell apoptosis [37]. Indeed, the function of calpains in apoptosis is puzzling, and appears to be highly dependent on the specific cell type and apoptotic stimulus [38]. This phenomenon is linked to their seemingly different preferences for individual substrates among experimental systems.

Cell adhesion molecules are typically single-pass transmembrane receptors [39]. It reported that the cell adhesion molecules including the immunoglobulin super family of cell adhesion molecules (IgCAMs), cadherins, integrins, and the C-type lectin-like domain proteins (CTLDs) [40]. Integrins, as one of the major classes of receptors, mediate the interactions between the extracellular matrix (ECM) and collagen, fibrinogen, fibronectin, and vitronectin [41]. Previous research revealed that integrins act essential links between the extracellular environment and the intracellular signaling pathway, which play a role in cell fate, such as apoptosis, differentiation, survival, and transcription [42]. Integrins can activate the RhoA/RhoKinase and MAPK signaling pathways and participate in wall remodeling changes by binding to extracellular ligands, such as fibronectin, laminin, and matrix metalloproteinase-2 [43, 44]. A previous study showed that GEGs in the aneurysm wall tended to take part in the process of extracellular matrix receptor interaction, focal adhesion and cellular communication [45]. In the proteomic analysis results, integrin beta and integrin subunit alpha 6 were upregulated approximately 1.2-fold in IPEC-J2 cells transfected with pHSP70 plasmid. Hence, we assume that alteration of these proteins might be involved in HSP70-mediated biological functions in IPEC-J2 cells.

Here, proteomics and bioinformatics were combined to identify proteins that are differentially expressed in IPEC-J2 cells transfected with pHSP70 DNA plasmid. A total of 246 DEPs were identified, including those involved in regulation of cellular proliferation, cellular assembly and organization, and signaling. Furthermore, as cells were exposed to heat stress, overexpression of HSP70 ensued, further leading to increased Caspase-3, PARP, and ratio of Bax to Bcl-2. Moreover, *in vivo* studies revealed that heat stress significantly increased the ratio of apoptosis in intestinal epithelial cells and the expression of HSP70. Taken together, these findings suggest that HSP70 expression induces apoptosis in the intestine of heat-stressed pigs. Thus, our study provides an important clue concerning the molecular mechanism underlying the development of apoptosis in the intestines due to heat stress.

Conclusions

Over-expression of HSP70 was observed in IPEC-J2 cells transfected with pHSP70 DNA plasmid and the iTRAQ-based quantitative proteomics approach showed that HSP70 may extensively influence the expression of proteins associated with molecular response such as signaling pathways, cell adhesion, and apoptosis in IPEC-J2 cells. Apoptosis-related proteins, Caspase-3, PARP, Bax, and Bcl-2 expression were significantly activated in IPEC-J2 cells transfected with porcine HSP70 plasmid. Furthermore, heat

stress-induced HSP70 overexpression and cellular apoptosis was observed in the intestine of pigs. Taken together, our expression profiles provide a novel insight into the molecular mechanisms of HSP70 induced apoptosis in IPEC-J2 cells.

Abbreviations

Bcl-2, B-cell lymphoma 2; BSA, Bovine serum albumin; DEPs, Differentially expressed proteins; FAK, Focal adhesion kinase; GO, Gene ontology; HS, Heat stress; HSP70, Heat shock protein 70; IBD, Inflammatory bowel diseases; IPEC-J2, Intestinal porcine epithelial cells; iTRAQ, Isobaric tag for relative and absolute quantification; KEGG, Kyoto encyclopedia of genes and genomes; LC MS/MS, Liquid-chromatography-tandem mass spectrometry; PBS, Phosphate-buffered saline; PPI, Protein-protein interaction; TLR4, Toll-like receptor 4; TNF- α , Tumor necrosis factor- α .

Declarations

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Authors' contributions

JL and YY designed the experiments, performed the data analysis, created figures, and edited the manuscript. TY, BF, LW, and CH were responsible for sample collection. XL, ZY, and XM analyzed the enrichment analysis and participated in writing the manuscript. AMA and RG revised the manuscript. XJ provided the financial support and revised the manuscript. All authors have read and approved the final version of the manuscript.

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Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

No ethics approval was required for this study that did not involve patients or patient data.

Consent for publication

All authors consent to publication.

Competing interests

No potential conflict of interest was reported by the authors in this study.

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Figures

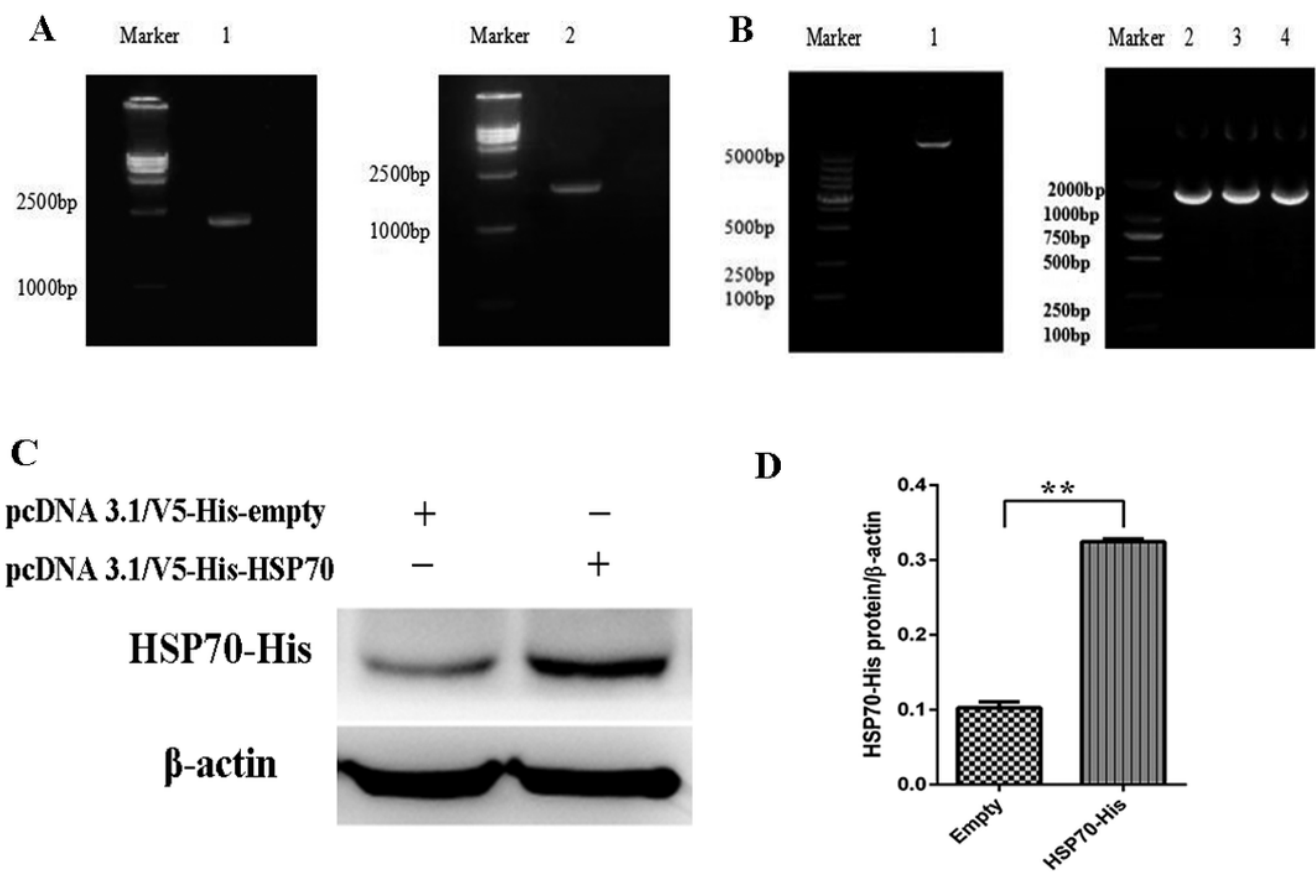


Figure 1

The construction of HSP70 ectopic expression system and HSP70 overexpression in IPEC-J2 cells. A PCR product of HSP70 plasmid. B PCR product of pcDNA 3.1/V5-His-HSP70. C-D Densitometric analysis

generated for western blots of HSP70 expression. **indicates $p < 0.01$.

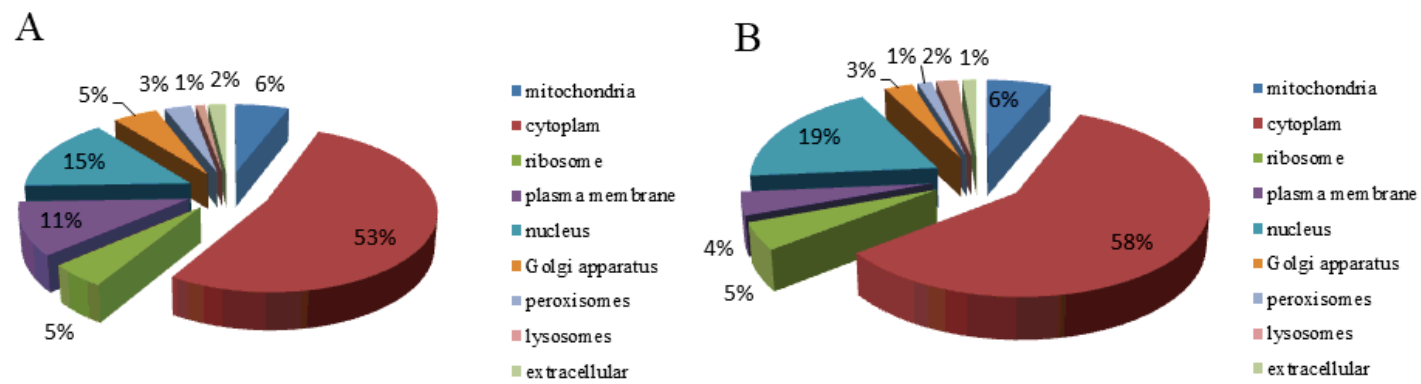


Figure 2

Location of the proteins with differential expression ($p < 0.05$) between pHSP70-transfected and Empty-transfected IPEC-J2 cells. A Up-regulated proteins. B down-regulated proteins.

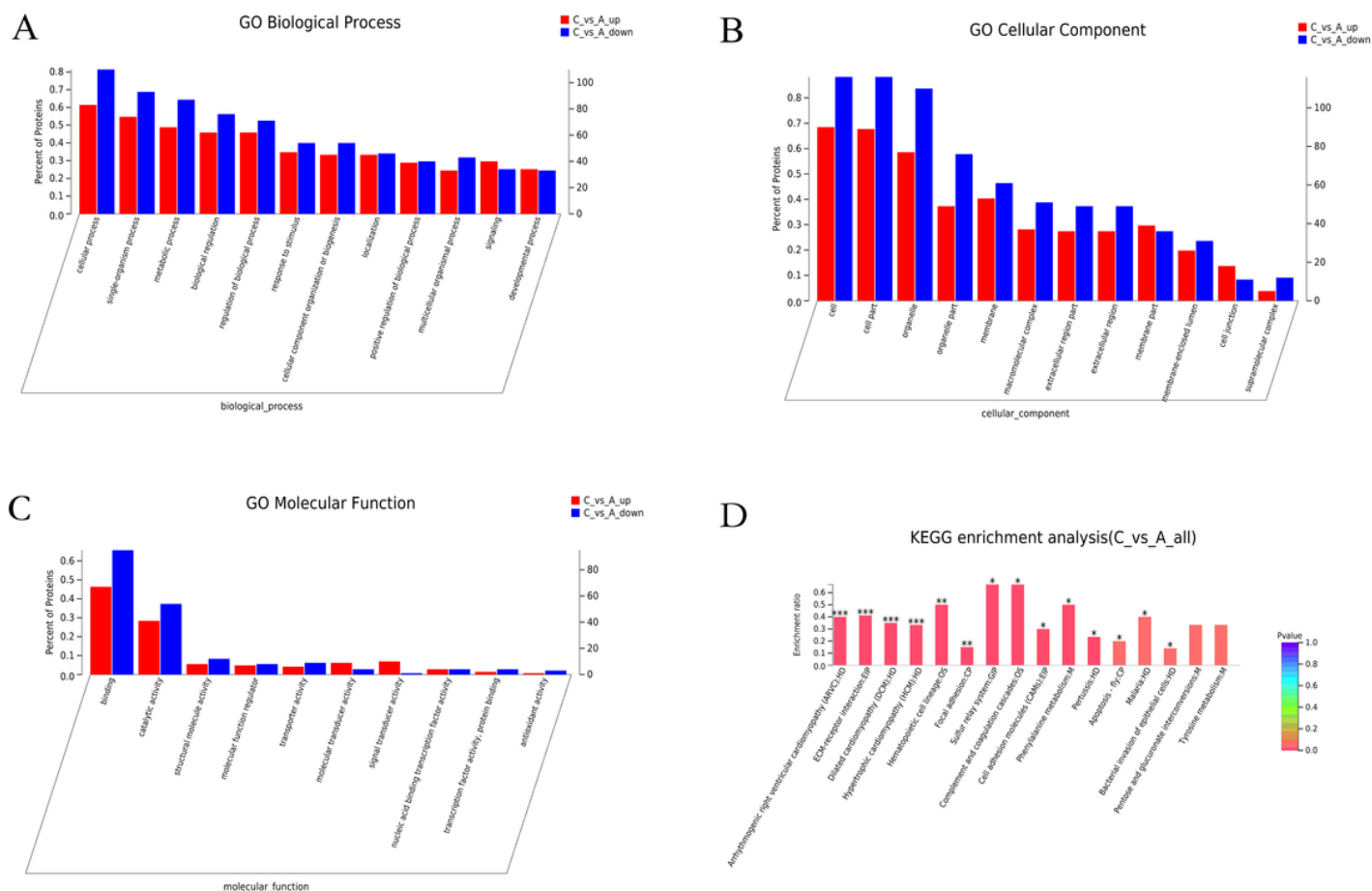


Figure 3

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of differentially expressed proteins. A Biological process-based analyses showed that a majority of the proteins were involved in cellular processes and single-organism processes. B Cellular component-based analyses showed that the main components were cells and organelles. C Molecular function-based analyses showed a significant number of proteins associated with binding, catalytic activity, and structural molecule activity. D The top terms of KEGG pathway for all differentially expressed proteins (DEPs), including arrhythmogenic right ventricular cardiomyopathy (ARVC), ECM-receptor interaction, and dilated cardiomyopathy. (C refers to pHSP70 and A refers to Empty in the illustrated graphs).

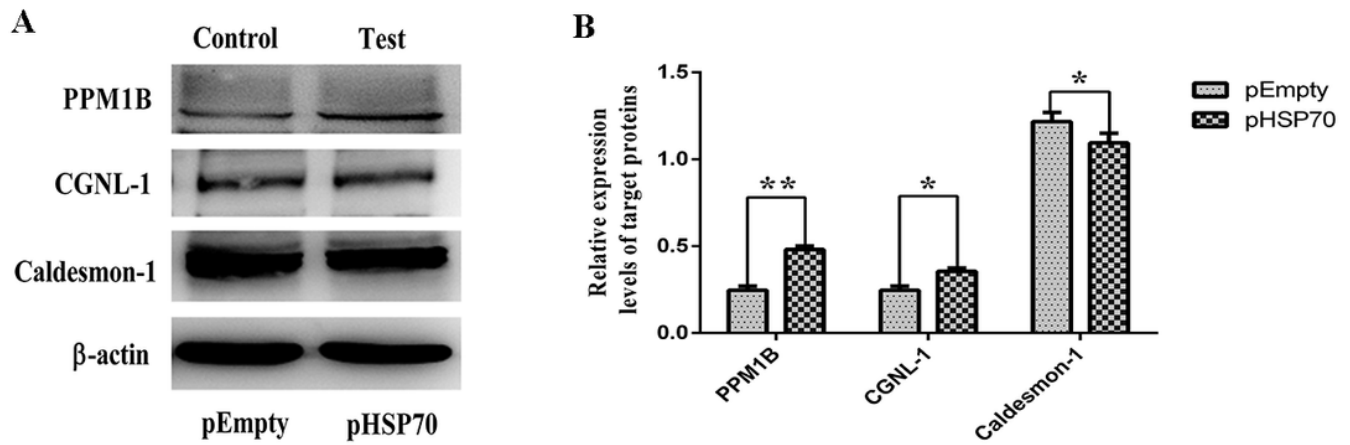


Figure 5

Gene expression analysis of transfected cells. A Western blotting validation of selected proteins that are differentially expressed between Empty and pHSP70 transfected into IPEC-J2 cells, respectively. The expression levels of PPM1B, CGNL-1, and Caldesmon-1 illustrated by western blotting analysis. B Quantitative assessment of protein expression using densitometric analysis. The bars represent the standard deviation of three independent experiments. *indicates $p < 0.05$, ** $p < 0.01$.

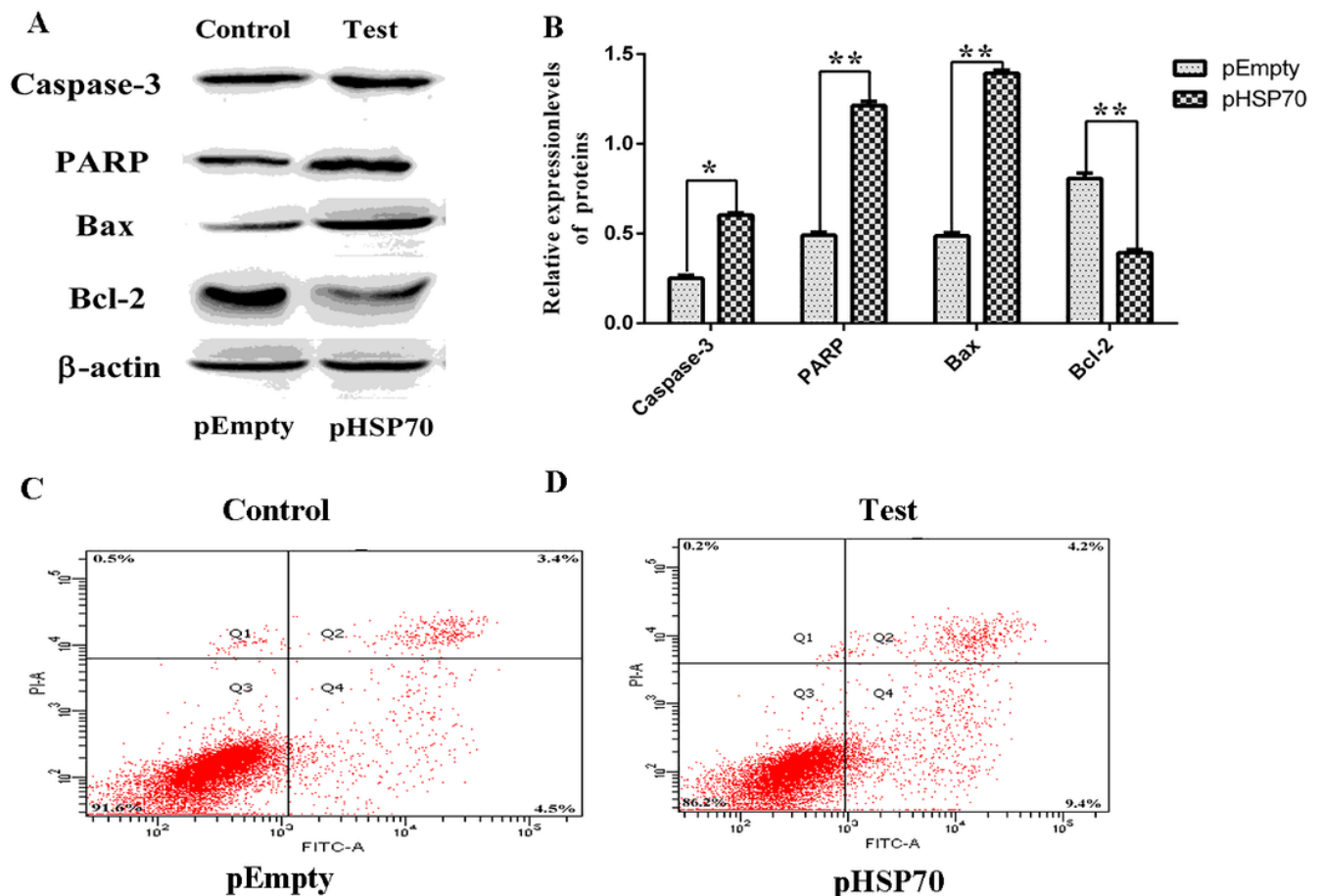


Figure 6

HSP70 overexpression promotes cellular apoptosis in IPEC-J2 cells. A-B The expression levels of Caspase-3, PARP, Bax, and Bcl-2 in IPEC-J2 cells transfected with porcine HSP70 (pHSP70) and Empty DNA plasmids by western blotting. C-D Effect of HSP70 transfection treatment on the apoptotic rate of IPEC-J2 cells detected by flow cytometry. The bars represent the standard deviation of three independent experiments. *indicates $p < 0.05$, ** $p < 0.01$.

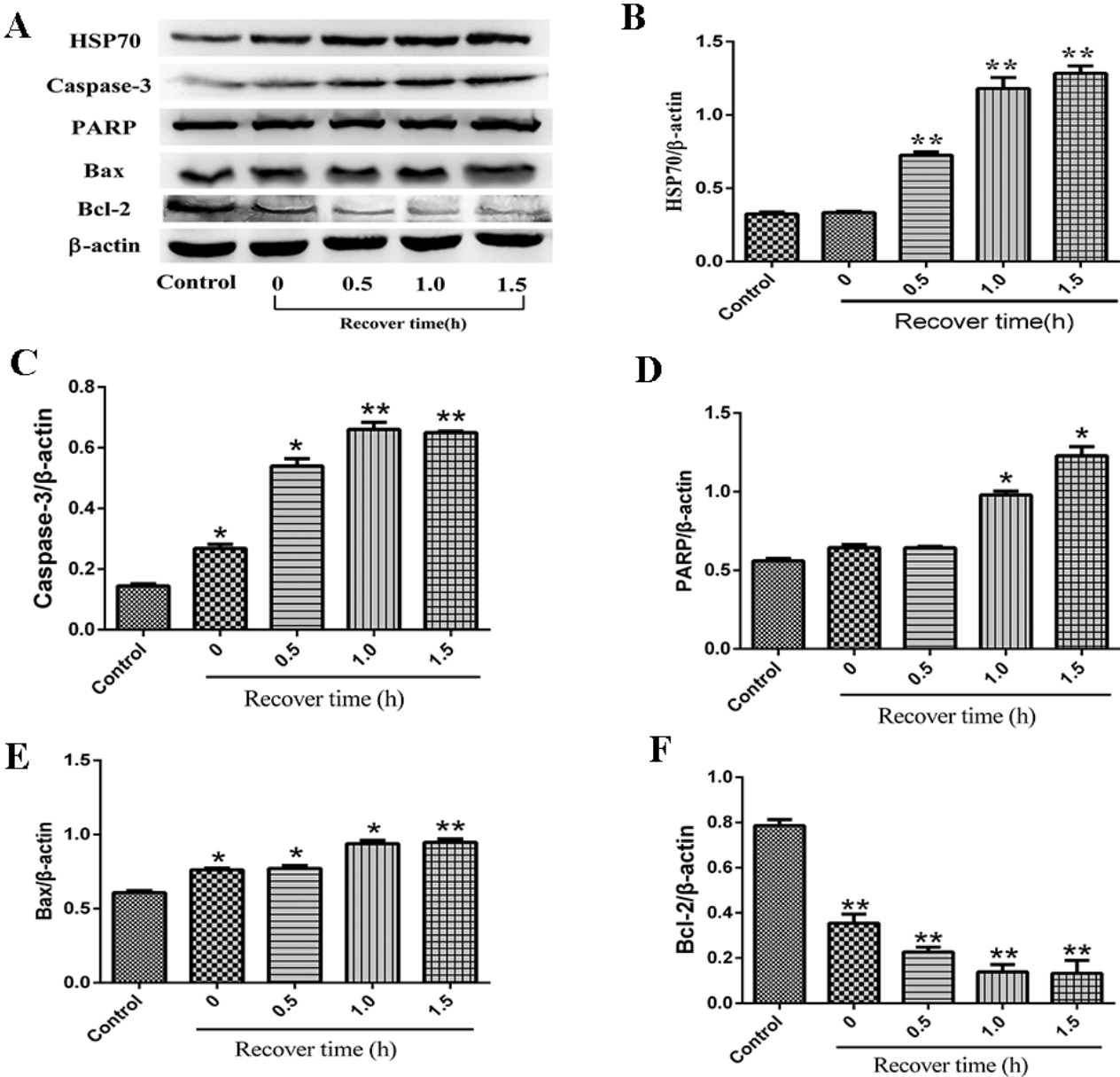


Figure 7

Validation of HSP70 expression and cellular apoptosis-related proteins in IPEC-J2 cells by western blotting. A-F The expression levels of HSP70 and apoptosis-related proteins, Caspase-3, PARP, Bax, and Bcl-2 in IPEC-J2 cells under heat stress treatment. Quantitative assessment of protein expression using densitometric analysis. The bars represent the standard deviation of three independent experiments. *and

**denote a significant difference between heat stress timepoint and control cells. *indicates $p < 0.05$, ** $p < 0.01$.

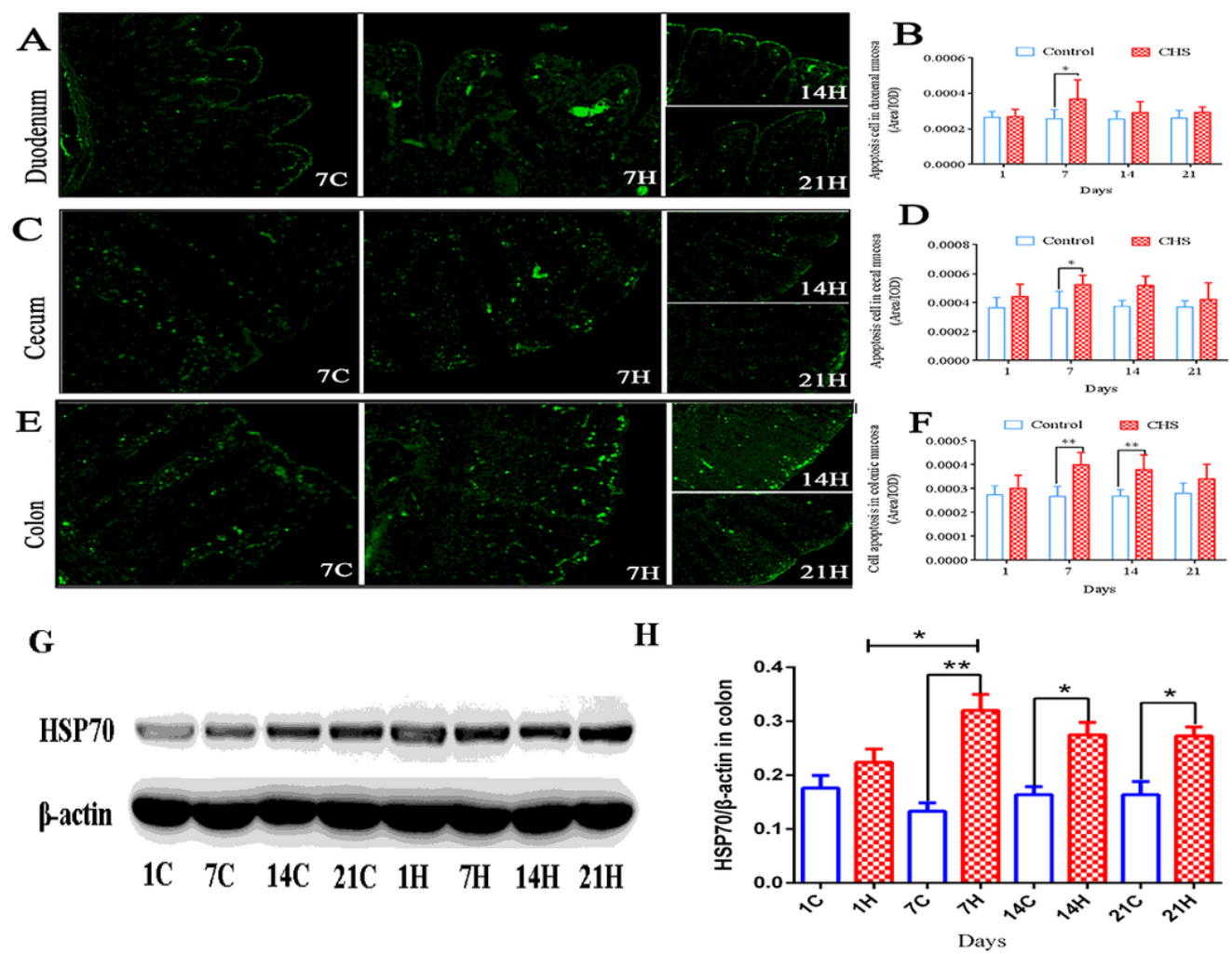


Figure 8

Cellular apoptosis and HSP70 expression analysis in intestine of heat stress and control pigs. A-B, C-D, and E-F Apoptosis in duodenum, cecum, and colon of control pigs and heat stressed pigs detected at day 7, 14, and 21 after heat stress begun and the ratio of apoptotic cells. G-H: HSP70 expression in colon of control pigs and heat-stressed pigs detected at day 7, 14, and 21. Apoptotic cells in the basal layer of the epithelium displayed as optical density per unit area and analyzed by Image Pro Plus 6.0. The asterisk “*” and double-asterisk “**” denote a significant difference between stressed and control pigs on the same day. *indicates $p < 0.05$, ** $p < 0.01$.

Supplementary Files

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