Chronic corticosterone exposure induces liver inflammation and fibrosis in association with m⁶A-linked post-transcriptional suppression of heat shock proteins in chicken

Yue Feng^{1,2} · Yun Hu^{1,2} · Zhen Hou^{1,2} · Qinwei Sun^{1,2} · Yimin Jia^{1,2,3} · Ruqian Zhao^{1,2,3}

Received: 3 April 2019 / Revised: 22 August 2019 / Accepted: 4 September 2019 / Published online: 19 November 2019
© Cell Stress Society International 2019

Abstract

Our previous study had shown that chronic corticosterone (CORT) exposure causes excessive fat deposition in chicken liver, yet it remains unknown whether it is associated with inflammation and fibrosis. In general, heat shock proteins (HSPs) are activated in response to acute stress to play a cytoprotective role, and this activation is associated with m⁶A-mediated post-transcriptional regulation. However, changes of HSPs and the m⁶A methylation on their mRNAs in response to chronic CORT treatment in chicken liver have not been reported. In this study, chronic CORT exposure induced inflammation and fibrosis in chicken liver, associated with significantly modulated expression of HSPs that was significantly upregulated at mRNA level yet downregulated at protein level. Concurrently, m⁶A methyltransferases METTL3 content was upregulated together with the level of m⁶A methylation on HSPs transcripts. The m⁶A-seq analysis revealed 2–6 significantly ($P < 0.05$) hypermethylated m⁶A peaks in the mRNA of 4 different species of HSPs in CORT-treated chicken liver. HSP90B1 transcript had 6 differentially methylated m⁶A peaks among which peaks on exon 16 and exon 17 showed 3.14- and 4.72-fold of increase, respectively. Mutation of the 8 predicted m⁶A sites on exon 16 and exon 17 resulted in a significant ($P < 0.05$) increase in eGFP-fused content of HSP90B1 exon 16 and exon 17 fragment in 293 T cells, indicating a possible role of m⁶A in post-transcriptional regulation of HSPs. In conclusion, chronic CORT exposure induces inflammation and fibrosis in chicken liver along with an increase in the levels and m⁶A methylation of several HSPs mRNAs; HSPs levels were however reduced under the indicated conditions. Results presented suggest that the reduction in HSPs levels may be associated with m⁶A methylation in CORT-exposed chickens.

Keywords Chicken liver · Inflammation · Fibrosis · Heat shock proteins · N⁶-methyladenosine

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12192-019-01034-7>) contains supplementary material, which is available to authorized users.

✉ Yimin Jia
yiminj@njau.edu.cn

- ¹ MOE Joint International Research Laboratory of Animal Health & Food Safety, Nanjing Agricultural University, Nanjing 210095, People's Republic of China
- ² Key Laboratory of Animal Physiology & Biochemistry, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, People's Republic of China
- ³ Quality and Safety Control, Jiangsu Collaborative Innovation Center of Meat Production and Processing, Nanjing 210095, People's Republic of China

Introduction

Modern intensive poultry production attracts accusations of poor welfare concerning exposure of chickens to high temperature, insistent humidity, and over density, which causes chicken long-term exposure to these stressors (Cohen et al. 2011; Dawkins et al. 2004; Liu and Zhao 2014). Long-term stress is a high-risk factor for nonalcoholic fatty liver disease (NAFLD) in rodents (Cheng et al. 2017; Czech et al. 2013; Fu et al. 2010) and zebrafish (Cinaroglu et al. 2011). NAFLD ranges from excessive fat deposition in hepatocytes (steatosis) to steatohepatitis, which can progress to fibrosis, cirrhosis, and ultimately, hepatocellular carcinoma (Cohen et al. 2011). Inflammation is a characteristic feature of NAFLD, which is associated with the development of fibrosis, cirrhosis, and hepatocellular carcinoma (Seki and Schwabe 2015). Previous studies indicated that chronic corticosterone (CORT) administration could cause excessive fat deposition

in chicken livers (Hu et al. 2018; Lin et al. 2006a; Lin et al. 2006b). However, it remains unknown whether CORT-induced fatty liver is accompanied with inflammation and fibrosis in the chicken.

In mammals, glucocorticoid response and heat shock proteins (HSPs) play a critical role in regulation of the biphasic, pro-inflammatory and anti-inflammatory, immune response (Stolte et al. 2009). Activated hypothalamic–pituitary–adrenal axis induces the release of cortisol from the adrenal cortex which is bound to glucocorticoid receptor (GR) to inhibit nuclear factor- κ B (NF- κ B) nuclear translocation and inflammatory cytokine production through direct and indirect genomic effects and non-genomic mechanism (Rhen and Cidlowski 2005). Additionally, GR function is regulated by coordinated action of the HSP90 and HSP70 chaperone cycles (Kirschke et al. 2014). HSP90 is required for GR to enhance cortisol affinity and become active (Picard et al. 1990), while HSP70 inactivates GR through protein aggregation. Furthermore, HSPs could regulate cellular immune response by itself. Extracellular HSP70 acts as damage-associated molecular pattern and induces pro-inflammatory cytokine release, such as interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor (TNF- α), in THP-1 cells (Hulina et al. 2018) and human peripheral blood monocytes (Ferat-Osorio et al. 2014). Whereas intracellular HSP70 interacting with IKK- γ impairs NF- κ B signaling (Ran et al. 2004). Also, Chen et al. (2002) reported that HSP90 is an essential stability factor of IKK signalosome to inhibit NF- κ B signaling. On the other hand, other reports indicate that HSPs protect against pulmonary, renal, dermal and intestinal fibrosis in mice through inhibition of aberrant transforming growth factor (TGF)- β signaling (Kitamura et al. 2011; Noh et al. 2012; Tanaka et al. 2010; Tomcik et al. 2014), which is a central mediator of fibrogenesis. However, less is known whether HSPs are involved in hepatic inflammation and fibrosis by CORT treatment in chicken.

N⁶-methyladenosine (m⁶A) is the most prevalent and internal modification occurring in RNAs, which plays an important role in RNA splicing, degradation, and translation (Maity and Das 2016). The METTL3-METTL14 protein complex is the enzymatic core of the m⁶A writer, while Wilms' tumor-associated protein (WTAP) and other factors regulate the methylation process (Liu et al. 2014). Demethylases fat mass and obesity-associated protein (FTO) and AlkB Homolog 5 (ALKB5) are erasers that remove m⁶A marks from RNAs (Zheng et al. 2013). Additionally, the m⁶A reader proteins (e.g., YTHDF family proteins) recognize the m⁶A code to regulate both mRNA stability and translation (Wang et al. 2015; Zhou et al. 2015). Previous studies demonstrated that HSPs are modulated by m⁶A RNA methylation at the post-transcriptional level when cells (Yu et al. 2018; Zhou et al. 2015) and sheep (Lu et al. 2019) are subjected to acute heat

shock. However, it is still unknown whether m⁶A RNA methylation regulates HSPs gene expression in the liver of chickens chronically treated with CORT.

In this study, we aimed to know whether chronic CORT administration could induce hepatic inflammation and fibrosis in juvenile chickens, and to determine whether such effects are related to the translational regulation of HSPs through m⁶A modifications.

Materials and methods

Ethics statement

All the experiments were approved by the Animal Ethics Committee of Nanjing Agricultural University (31672512). The sampling procedures were as per the "Guidelines on Ethical Treatment of Experimental Animals" (2006) No. 398 set by the Ministry of Science and Technology, China.

Animals and treatment

Twenty-four male chickens (at 8 weeks of age) were selected from Poultry Institute of Yangzhou, Jiangsu, China, and were randomly divided into control (CON) and corticosterone (CORT) groups (12 in each group), being subjected to either vehicle or corticosterone treatment with daily subcutaneous injection of solvent (15% ethanol) or CORT (C2505, Sigma-Aldrich, USA) in a dose of 4.0 mg/kg body mass for 7 days (twice per day, 9:00–10:30 and 21:00–22:30), according to Lin et al. 2004. At 9 weeks of age, all the chickens were weighed and killed by rapid decapitation which is considered to be acceptable for euthanasia of birds according to the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals: 2013 Edition. Blood samples were taken and plasma samples were separated and stored at -20°C . The liver (without the gall bladder) samples were rapidly frozen in liquid nitrogen and kept at -80°C for further analysis.

Aspartate aminotransferase, alanine aminotransferase, and interleukin 1 β concentration in serum

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were measured using an automatic biochemical analyzer (7020, Hitachi, Tokyo, Japan), following the manufacturer's instructions. Serum contents of IL-1 β were measured by using the IL-1 β ELISA Kit according to the manufacturer's instructions (RGB-60013C, Rigor Bioscience, Beijing, China). A microplate reader (Synergy BioTek, USA) was used to measure the absorbance of each well at 450 nm. All assays were done in duplicates. The

detection limit of the assay was 7.5–120 ng/L. The intra-assay and inter-assay CVs were 4.5% and 8.4%, respectively.

Histological analysis of liver tissue

Fresh liver tissue was fixed with 4% paraformaldehyde and paraffin-embedded, and then the sections of liver were stained with hematoxylin eosin (HE) and Sirius red.

Total RNA isolation and real-time PCR

Total RNA was isolated from 30-mg liver samples by using TRIzol Reagent (3101, Shanghai Pufei Biotechnology Co., Ltd., China), and 1 µg of RNA was reverse-transcribed into cDNA by using a HiScript® II Reverse Transcriptase kit (R233-01, Vazyme, China) according to the manufacturer's protocol. Two microliters of diluted cDNA (1:20, v:v) was used for real-time quantitative PCR by using a Mx3000P

Real-Time PCR System (Stratagene, USA). All primers (Table 1) were synthesized by Genewiz Biotech (Suzhou, China). 18S rRNA was chosen as a reference gene and was not affected by CORT treatment. Data were analyzed by using the method of $2^{-\Delta\Delta CT}$.

Protein extraction and western blot assay

Total protein was extracted from 60-mg frozen liver samples as previously described (Hu et al. 2015). The protein concentrations were measured by using BCA Protein Assay kit (No.23225, Thermo Scientific, USA) according to the manufacturer's instructions. 40 µg of proteins were used for electrophoresis on a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membrane was blocked in 4% milk for 2 h, then incubated with primary antibodies overnight and secondary antibodies 2 h. Western blot analysis for GR (Antibody was customized by Genecreate, China, diluted

Table 1 Nucleotide sequences of primers

Target genes	Primer sequences (5'to 3')	
TLR1	F: ACCCGTTCAAGTGTTCTGTG	R: TTCCGCTCAAGTCTTCTGG
TLR2	F: GGTGTTCTGTTCATCCTCATC	R: GTTGGAGTCGTTCTCACTGTAGG
TLR3	F: TCAGTACATTTGTAACACCCCGCC	R: TCAGTACATTTGTAACACCCCGCC
TLR4	F: ATCTTTCAAGGTGCCACATC	R: GGATATGCTTGTTCACCA
TLR5	F: ACTCCCTTCCTTCCACATCT	R: GTTTGCGAGCCAGTTTCTCTCT
TLR7	F: TTCTGGCCACAGATGTGACC	R: CCTTCAACTTGGCAGTGCAG
MyD88	F: AGCGTGGAGGAGGACTGCAAGAAG	R: CCGATCAAACACACACAGCTTCAG
NLRP3	F: GGTTTACCAGGGGAAATGAGG	R: TTGTGCTTCCAGATGCCGT
IL-1β	F: CAAACTGCTGCGGAGGC	R: AGGACTGTGAGCGGGTGTAG
IL-4	F: CTGCCTGGATGCTGTTCT	R: TGCCCTGTCTCAGTGCTTT
IL-8	F: CAAGCCAAACACTCCT	R: TTTAGGGTGGATGAAGT
IL-10	F: GCTGAGGGTGAAGTTTGAG	R: CAGGTGAAGAAGCGGTGA
IL-12	F: GGGAACAGAACTGAAAGG	R: GCTGATAATCTCGTGGG
IL-17α	F: ACATGGGAAGGTGATACGGC	R: TGGGTTAGGCATCCAGCATC
IFN-α	F: ATCTGCTGCTCACGCTCCTTCT	R: GGTGTTGCTGGTGTCCAGGATG
IFN-β	F: GCCTCCAGCTCCTTCAGAATACG	R: CTGGATCTGGTTGAGGAGGCTGT
P65	F: AGGACTTAAATGGCAGGAGA	R: GCTGTTCGTAGTGGTAAGTCT
COL1A1	F: ACCTCAGCAAGAACCCCAAG	R: CTCACCGCCGTACTCAAAC
COL1A2	F: GCGGTTTCTACTGGATTGA	R: AGCGAGACGGCTTATTTG
TGFβ1	F: GCAAAGTGGTCTGACCG	R: ACGAAGAAGATGCTGTGGC
α-SMA	F: AAGCACCCTGAATCCCAAAG	R: CCAGAGTCAAGCACAATCCCT
MMP2	F: CGATGCTGTCTACGAGTCCC	R: TAGCCCCTATCCAGGTTGCT
TIMP2	F: CGACGTAGTGATCCGAGCAA	R: ACTGGATTCTGCTGATGGGG
HSP60	F: GGACGGAAAGGTGTAATC	R: TTCAAGAGCTGGAAGTATG
HSP70	F: CGTCAGTGCTGTGGACAAGAGTA	R: CCTATCTCTGTTGGCTTCATCCT
HSP90AA1	F: GAGTTTGACTGACCCGAGCA	R: CAGCTGATGACTCCCAAGCA
HSP90B1	F: GCCAGTTTGGTGTGGCTTT	R: TTAGCCAGTCGTGTGCGATT
GAPDH	F: ATCGTCAAGGCTGAGAACGGG	R: ATCTGCCCATTTGATGTTGCT

1:1000), p65 (sc-372, Santa Cruz, UK, diluted 1:200), NLRP3 (13158, Cell Signaling Technology, USA, diluted 1:1000), TIMP1 (BS1697, Bioworld, USA, diluted 1:1000), TIMP2 (BS1366, Bioworld, USA, diluted 1:1000), MMP2 (BS1236, Bioworld, USA, diluted 1:1000), HSP60 (As a gift from Northeast Agricultural University, diluted 1:1000), HSP70 (As a gift from Northeast Agricultural University, diluted 1:1000), HSP90 α (As a gift from Northeast Agricultural University, diluted 1:1000), HSP90 β (BS1851, Bioworld, USA, diluted 1:1000), HSP90B1 (BS1155, Bioworld, USA, diluted 1:1000), METTL3 (AB98009, Abcam, USA, diluted 1:1000), METTL14 (AB98116, Abcam, USA, diluted 1:1000), WTAP (BS2062, Bioworld, USA, diluted 1:1000) was carried out according to the manufacturer's instructions. Tubulin- β (AP0064, Bioworld, USA, diluted 1:5000) was selected as an internal control. Images were captured by VersaDoc 4000MP system (Bio-Rad, USA) and the band density was analyzed with Quantity One software (Bio-Rad, USA).

m⁶A sequencing

m⁶A-IP and library preparation were performed according to the reported protocol (Dominissini et al. 2012). Briefly, poly-A-purified RNA was fragmented and incubated with m⁶A primary antibody for 2 h at 4 °C. The mixture was then immunoprecipitated by incubation with Protein A beads (21348, Thermo Fisher) for 2 h at 4 °C. Captured RNA was washed 3 times, then eluted with m⁶A nucleotide solution and purified by a RNA clean and concentrator kit (R1013, Zymo Research, USA). The m⁶A-sequencing and data analysis were performed by Novogene (Beijing, China).

m⁶A mini-gene construction

The m⁶A mini-gene was constructed based on a pEGFP-C1 vector by Applied Biological Materials Inc. (Nanjing, China). Exon16 and exon17 of HSP90B1 containing 8 m⁶A motifs (DRACH) was cloned in-frame into the mini-gene following the coding region of EGFP by ClonExpress®II One Step Cloning Kit (C112-01, Vazyme, China). Synonymous point mutations were introduced to disrupt m⁶A RAC core motif without changing any codons (Supplemental 1). HEK293TI cells maintained at 37 °C in a humidified incubator containing 5% CO₂. Twenty-four hours prior to transfection, cells were grown in medium supplemented with 10% FBS at a suitable density in 6-well plates. Before transfection, the culture medium was replaced with fresh DMEM. The transfection was conducted with a mixture of Lipofectamine® 2000 Transfection Reagent (11668-019, Thermo Scientific, US) and Opti-MEM (31985, Thermo Scientific, USA) according

to the manufacturer's protocol. Whole cell lysates were extracted and the protein concentration was determined by using Pierce BCA Protein Assay kit (No.23225, Thermo Scientific, USA) according to the manufacturer's instruction. Thirty-microgram whole cell proteins were loaded on a 10% SDS-PAGE gel for electrophoresis. Mouse monoclonal anti-GFP tag antibody (G096, Applied Biological Materials Inc., China, diluted 1:1000) was used for western blot.

Statistical analysis

All data are presented as means $\pm$ SEM and the differences between groups were analyzed using independent-samples *t* test with SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). The differences were considered statistically significant when $P < 0.05$.

Results

Body weight, liver weight, liver index, and serum AST, ALT, and IL-1 β levels in CORT-treated chickens

It was shown that chronic CORT administration decreased the body weight of chickens as compared with untreated group ($P < 0.05$, Table 2). Liver weight and liver index were however significantly increased in the treated group ($P < 0.05$, Table 2). In addition, serum AST, ALT, and IL-1 β were also elevated the CORT group ($P < 0.05$, Table 2).

Encoding proinflammatory agents in livers of CORT treated chickens

Microscopic evaluation of HE-stained (Fig. 1a) liver section revealed that the chronic CORT-treated exhibited higher infiltration cells compared with untreated control. We also observed significant increase ($P < 0.05$) in the mRNA levels of proinflammatory cytokines IL-1 β , IL-8, and IL-12 in the

Table 2 The body weight, liver weight, liver index, and serum AST, ALT, and IL-1 β levels

Parameters	Control	CORT	<i>P</i> value
Initial body weight (g)	576.15 $\pm$ 5.49	581.54 $\pm$ 4.92	0.47
Final body weight (g)	656.23 $\pm$ 8.67	617.77 $\pm$ 7.04	< 0.01
Liver weight (g)	14.49 $\pm$ 0.39	18.61 $\pm$ 0.79	< 0.01
Liver index (%)	2.21 $\pm$ 0.05	3.00 $\pm$ 0.10	< 0.01
Plasma ALT (U/L)	3.54 $\pm$ 0.45	5.17 $\pm$ 0.51	0.02
Plasma AST (U/T)	248.58 $\pm$ 11.10	295.92 $\pm$ 13.20	0.01
Plasma IL-1 β (ng/L)	14.56 $\pm$ 2.53	24.61 $\pm$ 3.59	0.04

Fig. 1 mRNA and protein levels of inflammatory related genes. **a** Histological images of inflammatory cell infiltration (H&E staining technique, black arrows); **b**, **c** mRNA levels of inflammatory related

genes; **d** p65 and NLRP3 protein content. CON = control; CORT = corticosterone. Values are expressed as the mean $\pm$ SEM; * p < 0.05 (n = 6)

Fig. 2 mRNA and protein levels of fibrosis related genes. **a** Sirius red staining of collagen (black arrows). The red area indicated by the arrow is collagen; **b** Liver fibrosis-related mRNA expression; **c** Liver fibrosis-

related protein content. CON = Control; CORT = Corticosterone. Values are expressed as the mean $\pm$ SEM; * p < 0.05 (n = 5)

livers of CORT-treated chickens over control group (Fig. 1b). Transcript toll like receptors (TLR) 1, 2, 3, 4, MyD88 (adaptor protein required for TLR signaling), and NLRP3 (inflammasome constituent) were all significantly upregulated ($P < 0.05$) in the CORT-administered group (Fig. 1c). In addition, protein content of p65 and NLRP3 were also significantly up-regulated ($P < 0.05$, Fig. 1d) compared with control.

mRNA and protein levels of fibrosis-related genes in livers of CORT-treated chickens

Collagen fiber content as detected by Sirius red staining (Fig. 2a) was increased in the CORT group. In addition, mRNA levels of fibrosis-related genes, COL1A1, COL1A2, TGF β 1, and α -SMA ($P < 0.05$), were significantly increased by CORT administration (Fig. 2b). Whereas the protein levels of metalloproteinase inhibitor TIMP1 and TIMP 2 were significantly elevated ($P < 0.05$) in the CORT-treated group, those of the matrix metalloproteinase MMP2 was lower in the experimental group ($P < 0.05$, Fig. 2c).

mRNA and protein levels of HSPs genes in livers of CORT-treated chickens

The expression of hepatic HSP60, HSP70, HSP90 α , and HSP90B1 were significantly increased ($P < 0.05$) at mRNA levels in the CORT group (Fig. 3a); however, the protein levels of HSP60, HSP70, HSP90 α , and HSP90B1 were significantly decreased ($P < 0.05$) in the CORT group (Fig. 3b).

Protein content of m⁶A methyltransferase and profiling of m⁶A in HSPs transcripts

METTL3, METTL14, and WTAP are characterized as the methyltransferase core complex. Our results showed that the protein content of METTL3 ($P < 0.05$) was significantly increased in the CORT group, while no significant difference was observed in METTL14 or WTAP (Fig. 4a). Furthermore, we detected the m⁶A modification in HSPs genes through m⁶A-seq. It was found that the m⁶A sites of HSP60, HSP70, HSP90 α , and HSP90B1 transcripts mainly distribute on exons. In addition, it was observed that the m⁶A sites were most enriched around stop codons in HSP70 and HSP90B1 transcripts. We have identified 3 of 4 m⁶A peaks were significantly increased in HSP60 transcript ($P < 0.05$), 1 of 7 m⁶A peaks in HSP70 transcript ($P < 0.05$), 1 of 4 m⁶A peaks in HSP90 α transcript ($P < 0.05$), and 6 of 6 m⁶A peaks in HSP90B1 transcript ($P < 0.05$, Fig. 4b–e).

Functional verification of m⁶A modification in vitro

Since the major difference of m⁶A sites were identified in exon 16 to exon 17 of HSP90B1 transcript, we used the

Fig. 3 mRNA and protein levels of HSPs genes in liver. **a** HSPs mRNA expression; **b** HSPs protein content. CON = Control; CORT = Corticosterone. Values are expressed as the mean $\pm$ SEM; * $p < 0.05$ ($n = 6$)

mini-gene experiment to verify the function of m⁶A sites. All predicted m⁶A sites (8 in total) of HSP90B1 transcripts in exon 16 and exon 17 were synonymous point mutated (Fig. 5a) and cloned to a mini-gene vector in frame with EGFP. It was found that both of mRNA and protein levels of EGFP was significantly increased by synonymous point mutation in m⁶A sites ($P < 0.05$, Fig. 5b, c).

Discussion

Previous reports demonstrated that chronic CORT treatment causes hepatic lipid accumulation and progresses to NAFLD in mice (Sun et al. 2013) and chicken (Hu et al. 2017). If fatty liver is accompanied with stress and inflammation, up to 30% of people with NAFLD will develop non-alcoholic steatohepatitis (NASH) (Rinella 2015; Younossi et al. 2016); If the liver function is further damaged by increased hepatic fibrosis, up to 20% of people with NASH die of liver cirrhosis

Fig. 4 Protein content of m⁶A methyltransferase and profiling of m⁶A in HSPs transcripts. **a** Protein content of methyltransferase core complex. Values are expressed as the mean ± SEM; **p* < 0.05 (*n* = 6); **b–e** m⁶A

modification in HSPs genes through m⁶A-seq, **p* < 0.05. CON = Control; CORT = Corticosterone

(Rinella 2015). In this study, we found that chronic CORT treatment could cause liver injury and increase inflammatory cytokines and fibrotic biomarkers in the liver of chicken. Here, we may cautiously suppose that chronic stress, especially increased glucocorticoid, is a risk factor in the progression of fatty liver to liver cirrhosis.

In this study, we found that the protein contents of hepatic HSP60, HSP70, HSP90α, and HSP90B1 were all significantly decreased in CORT-treated chicken, which were associated with hepatic inflammation and fibrosis. HSP60 is a mitochondrial chaperonin that is essentially required for protein folding in mitochondria (Ostermann et al. 1989). However, recent research has indicated that HSP60 can activate macrophages and dendritic cells and can alter adaptive immune response (Rajaiah and Moudgil 2009). In addition, HSP60 can promote cytokine production through toll-like receptors and other ligands of innate immune receptors (Rajaiah and Moudgil 2009). In contrast, HSP70 collaborates with HSP90α to cope with excessive stress response through inhibiting NF-κB signaling (Ran et al. 2004; Chen et al. 2002). Furthermore, HSP90B1, an HSP90 paralogue that is found in the endoplasmic reticulum, has been considered as an essential immune chaperone to regulate both innate and adaptive immunity (Schild 2000). It seems that there are paradoxical results for HSPs protein levels in our study, and we supposed that acute stress could induce HSPs to repair the misfolding proteins to relieve cell injury, while chronic stress may cause sustained

cell damage, and then the decreased HSPs may lose the protective function which induces hepatic inflammation and fibrosis. Sun et al. was reported that HSP70 inhibit NF-κB activation and cytokine production in murine Kupffer cells (Sun et al. 2005). Since liver is an organ with multitudinous cell components, further study should be performed to research the interaction between Kupffer and stellate cells.

Intriguingly, we found that mRNA levels of hepatic HSPs were significantly increased in CORT-treated chicken, which was inconsistent with their protein levels. It seems that there may be a post-transcriptional mechanism involved in regulation of HSPs' translation by chronic CORT treatment. m⁶A is a prevalent post-transcriptional modification in mammalian mRNA impacting a variety of physiological events (Yue et al. 2015). m⁶A mRNA methylation are dynamically and reversibly regulated by m⁶A “writers” (METTL3, METTL14, WTAP) and the “eraser” FTO. In this study, we found that CORT treatment enhances the protein concentrations of hepatic METTL3 and WTAP in chicken; in addition, m⁶A-seq analysis revealed that m⁶A peaks of HSPs are mainly enriched on exons and near stop codons. Until now, there are limited information is available about the function of m⁶A modification in exons. Xiao et al. (2016) revealed that m⁶A reader YTHDC1 promotes exon inclusion to regulate targeted mRNA splicing. Whereas knockdown of METTL3 in mouse embryonic stem cells discovered that less than 100 exons containing an m⁶A site are alternatively spliced (Dominissini et al.

Fig. 5 Functional verification of m⁶A modification in vitro. **a** The mini-gene experiment to verify the function of m⁶A sites for HSP90B1; **b** EGFP mRNA expression after synonymous point mutation in m⁶A sites;

c EGFP protein content after synonymous point mutation in m⁶A sites. CON = Control; CORT = Corticosterone. Values are expressed as the mean $\pm$ SEM; * p < 0.05 (n = 3)

2012; Geula et al. 2015; Ke et al. 2017). Furthermore, previous research found that internal m⁶A residues increase the in vitro translation efficiency of dihydrofolate reductase mRNA (Heilman et al. 1996). For m⁶A located near stop codons, it was discovered that m⁶A residues in the last exons play an important role in regulating proximal alternative polyA choice (Ke et al. 2015) and mRNA stability (Ke et al. 2017). This m⁶A-containing region (Last exon) was cloned to a mini-gene vector in-frame with GFP, and they found that mutate the A in DRACH motif can increase mRNA half-life compared with wild type. Our results of synonymous mutation in exon DRACH motif were consistent with Ke's report. It was suggested that m⁶A modifications near the last exon of HSP90B1 may have a similar mechanism to reduce the stability of mRNA.

In conclusion, chronic corticosterone administration induces post-transcriptional suppression of heat shock proteins with m⁶A modification in chicken liver. This finding would provide insights into the new regulatory mechanisms of HSPs-mediated liver lipid accumulation to inflammation and fibrosis transformation situations in chicken.

Funding information This work was supported by the National Key Research and Development Program of China (2016YFD0500502), the National Natural Science Foundation of China (31672512) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Compliance with ethical standards All the experiments were approved by the Animal Ethics Committee of Nanjing Agricultural University (31672512). The sampling procedures were as per the "Guidelines on Ethical Treatment of Experimental Animals" (2006) No. 398 set by the Ministry of Science and Technology, China.

References

- Abu-Elsaad NM, Serrya MS, El-Karef AM, Ibrahim TM (2016) The heat shock protein 90 inhibitor, 17-AAG, attenuates thioacetamide induced liver fibrosis in mice. *Pharmacol Rep* 68:275–282
- Ambade A, Catalano D, Lim A, Mandrekar P (2012) Inhibition of heat shock protein (molecular weight 90 kDa) attenuates proinflammatory cytokines and prevents lipopolysaccharide-induced liver injury in mice. *Hepatology* 55:1585–1595
- Archer AE, Rogers RS, Von Schulze AT, Wheatley JL, Morris EM, McCoin CS, Thyfault JP, Geiger PC (2018) Heat shock protein 72

- regulates hepatic lipid accumulation. *Am J Physiol Regul Integr Comp Physiol* 315:R696–R707
- Asea A, Kabingu E, Stevenson MA, Calderwood SK (2000) HSP70 peptidibearing and peptide-negative preparations act as chaperokines. *Cell Stress Chaperones* 5:425–431
- Chen G, Cao P, Goeddel DV (2002) TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. *Mol Cell* 9: 401–410
- Cheng F, Ma C, Wang X, Zhai C, Wang G, Xu X, Mu J, Li C, Wang Z, Zhang X (2017) Effect of traditional Chinese medicine formula Sinisan on chronic restraint stress-induced nonalcoholic fatty liver disease: a rat study. *BMC Complement Altern Med* 17:203
- Cinaroglu A, Gao C, Imrie D, Sadler KC (2011) Activating transcription factor 6 plays protective and pathological roles in steatosis due to endoplasmic reticulum stress in zebrafish. *Hepatology* 54:495–508
- Cohen JC, Horton JD, Hobbs HH (2011) Human fatty liver disease: old questions and new insights. *Science* 332:1519–1523
- Czech B, Neumann ID, Müller M, Reber SO, Hellerbrand C (2013) Effect of chronic psychosocial stress on nonalcoholic steatohepatitis in mice. *Int J Clin Exp Pathol* 6:1585
- Dawkins MS, Donnelly CA, Jones TA (2004) Chicken welfare is influenced more by housing conditions than by stocking density. *Nature* 427:342
- Di Naso FC, Porto RR, Fillmann HS, Maggioni L, Padoin AV, Ramos RJ, Mottin CC, Bittencourt A, Marroni NA, de Bittencourt PI Jr (2015) Obesity depresses the anti-inflammatory HSP70 pathway, contributing to NAFLD progression. *Obesity* 23:120–129
- Dominissini D, Moshitch-Moshkovitz S, Schwartz S, Salmon-Divon M, Ungar L, Osenberg S, Cesarkas K, Jacob-Hirsch J, Amariglio N, Kupiec M et al (2012) Topology of the human and mouse m⁶A RNA methylomes revealed by m⁶A-seq. *Nature* 485:201–206
- Ferat-Osorio E, Sanchez-Anaya A, Gutierrez-Mendoza M, Bosco-Garate I, Wong-Baeza I, Pastelin-Palacios R, Pedraza-Alva G, Bonifaz LC, Cortes-Reynosa P, Perez-Salazar E et al (2014) Heat shock protein 70 down-regulates the production of toll-like receptor-induced pro-inflammatory cytokines by a heat shock factor-1/constitutive heat shock element-binding factor-dependent mechanism. *J Inflamm* 11: 19
- Fu J-H, Sun H-S, Wang Y, Zheng W-Q, Shi Z-Y, Wang Q-J (2010) The effects of a fat-and sugar-enriched diet and chronic stress on nonalcoholic fatty liver disease in male Wistar rats. *Dig Dis Sci* 55:2227–2236
- Geula S, Moshitch-Moshkovitz S, Dominissini D, Mansour AA, Kol N, Salmon-Divon M, Hershkovitz V, Peer E, Mor N, Manor YS et al (2015) Stem cells. m⁶A mRNA methylation facilitates resolution of naive pluripotency toward differentiation. *Science* 347:1002–1006
- Heilman KL, Leach RA, Tuck MT (1996) Internal 6-methyladenine residues increase the in vitro translation efficiency of dihydrofolate reductase messenger RNA. *Int J Biochem Cell Biol* 28:823–829
- Hu Y, Sun Q, Li X, Wang M, Cai D, Li X, Zhao R (2015) In Ovo injection of betaine affects hepatic cholesterol metabolism through epigenetic gene regulation in newly hatched chicks. *PloS one* 10:e0122643
- Hu Y, Sun Q, Liu J, Jia Y, Cai D, Idriss AA, Omer NA, Zhao R (2017) In ovo injection of betaine alleviates corticosterone-induced fatty liver in chickens through epigenetic modifications. *Sci Rep* 7:40251
- Hu Y, Sun Q, Hu Y, Hou Z, Zong Y, Omer NA, Abobaker H, Zhao R (2018) Corticosterone-induced lipogenesis activation and lipophagy inhibition in chicken liver are alleviated by maternal betaine supplementation. *J Nutr* 148:316–325
- Hulina A, Grdic Rajkovic M, Jaksic Despot D, Jelic D, Dojder A, Cepelak I, Rumora L (2018) Extracellular Hsp70 induces inflammation and modulates LPS/LTA-stimulated inflammatory response in THP-1 cells. *Cell Stress Chaperones* 23:373–384
- Kampinga HH, Hageman J, Vos MJ, Kubota H, Tanguay RM, Bruford EA, Cheetham ME, Chen B, Hightower LE (2009) Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress Chaperones* 14:105–111
- Ke S, Alemu EA, Mertens C, Gantman EC, Fak JJ, Mele A, Haripal B, Zucker-Scharff I, Moore MJ, Park CY et al (2015) A majority of m⁶A residues are in the last exons, allowing the potential for 3' UTR regulation. *Genes Dev* 29:2037–2053
- Ke S, Pandya-Jones A, Saito Y, Fak JJ, Vagbo CB, Geula S, Hanna JH, Black DL, Darnell JE Jr, Darnell RB (2017) m⁶A mRNA modifications are deposited in nascent pre-mRNA and are not required for splicing but do specify cytoplasmic turnover. *Genes Dev* 31: 990–1006
- Kirschke E, Goswami D, Southworth D, Griffin PR, Agard DA (2014) Glucocorticoid receptor function regulated by coordinated action of the Hsp90 and Hsp70 chaperone cycles. *Cell* 157:685–1697
- Kitamura H, Yamamoto S, Nakase H, Matsuura M, Honzawa Y, Matsumura K, Takeda Y, Uza N, Nagata K, Chiba T (2011) Role of heat shock protein 47 in intestinal fibrosis of experimental colitis. *Biochem Biophys Res Commun* 404:599–604
- Lin H, Decuypere E, Buyse J (2004) Oxidative stress induced by corticosterone administration in broiler chickens (*Gallus gallus domesticus*) 1. Chronic exposure. *Comparative Biochem Physiol Part B, Biochem Mol Biol* 139:737–744
- Lin H, Sui S, Jiao H, Buyse J, Decuypere E (2006a) Impaired development of broiler chickens by stress mimicked by corticosterone exposure. *Comp Biochem Physiol A Mol Integr Physiol* 143:400–405
- Lin H, Sui SJ, Jiao HC, Buyse J, Decuypere E (2006b) Impaired development of broiler chickens by stress mimicked by corticosterone exposure. *Comp Biochem Physiol A Mol Integr Physiol* 143:400–405
- Lin S, Choe J, Du P, Triboulet R, Gregory RI (2016) The m⁶(A) methyltransferase METTL3 promotes translation in human cancer cells. *Mol Cell* 62:335–345
- Liu W, Zhao J (2014) Insights into the molecular mechanism of glucose metabolism regulation under stress in chicken skeletal muscle tissues. *Saudi J Biol Sci* 21:197–203
- Liu J, Yue Y, Han D, Wang X, Fu Y, Zhang L, Jia G, Yu M, Lu Z, Deng X et al (2014) A METTL3-METTL14 complex mediates mammalian nuclear RNA N⁶-adenosine methylation. *Nat Chem Biol* 10:93–95
- Lu Z, Ma Y, Li Q, Liu E, Jin M, Zhang L, Wei C (2019) The role of N⁶-methyladenosine RNA methylation in the heat stress response of sheep (*Ovis aries*). *Cell Stress Chaperones*
- Mahmoud KZ, Edens FW, Eisen EJ, Havenstein GB (2004) Ascorbic acid decreases heat shock protein 70 and plasma corticosterone response in broilers (*Gallus gallus domesticus*) subjected to cyclic heat stress. *Comparative Biochem Physiol Part B, Biochem Mol Biol* 137:35–42
- Maity A, Das B (2016) N⁶-methyladenosine modification in mRNA: machinery, function and implications for health and diseases. *FEBS J* 283:1607–1630
- Najafi P, Zulkifli I, Jajuli NA, Farjam AS, Ramiah SK, Amir AA, O'Reilly E, Eckersall D (2015) Environmental temperature and stocking density effects on acute phase proteins, heat shock protein 70, circulating corticosterone and performance in broiler chickens. *Int J Biometeorol* 59:1577–1583
- Noh H, Kim HJ, Yu MR, Kim WY, Kim J, Ryu JH, Kwon SH, Jeon JS, Han DC, Ziyadeh F (2012) Heat shock protein 90 inhibitor attenuates renal fibrosis through degradation of transforming growth factor-beta type II receptor. *Laboratory investigation; a journal of technical methods and pathology* 92:1583–1596
- Ostermann J, Horwich AL, Neupert W, Hartl FU (1989) Protein folding in mitochondria requires complex formation with hsp60 and ATP hydrolysis. *Nature* 341:125–130
- Picard D, Khursheed B, Garabedian MJ, Fortin MG, Lindquist S, Yamamoto KR (1990) Reduced levels of hsp90 compromise steroid receptor action in vivo. *Nature* 348:166–168

- Rajaiah R, Moudgil KD (2009) Heat-shock proteins can promote as well as regulate autoimmunity. *Autoimmun Rev* 8:388–393
- Ran R, Lu A, Zhang L, Tang Y, Zhu H, Xu H, Feng Y, Han C, Zhou G, Rigby AC (2004) Hsp70 promotes TNF-mediated apoptosis by binding IKK gamma and impairing NF-kappa B survival signaling. *Genes Dev* 18:1466–1481
- Rhen T, Cidlowski JA (2005) Antiinflammatory action of glucocorticoids—new mechanisms for old drugs. *N Engl J Med* 353:1711–1723
- Rinella ME (2015) Nonalcoholic fatty liver disease: a systematic review. *Jama* 313:2263–2273
- Schild H (2000) Rammensee HG: gp96—the immune system's Swiss army knife. *Nat Immunol* 1:100–101
- Seki E, Schwabe RF (2015) Hepatic inflammation and fibrosis: functional links and key pathways. *Hepatology* 61:1066–1079
- Stolte EH, Chadzinska M, Przybylska D, Flik G, Savelkoul HF, Verburg-van K, BM. (2009) The immune response differentially regulates Hsp70 and glucocorticoid receptor expression in vitro and in vivo in common carp (*Cyprinus carpio* L.). *Fish Shellfish Immunol* 27:9–16
- Sun D, Chen D, Du B, Pan J (2005) Heat shock response inhibits NF-kappaB activation and cytokine production in murine Kupffer cells. *J Surg Res* 129:114–121
- Sun X, Luo W, Tan X, Li Q, Zhao Y, Zhong W, Sun X, Brouwer C, Zhou Z (2013) Increased plasma corticosterone contributes to the development of alcoholic fatty liver in mice. *Am J Physiol Gastrointest Liver Physiol* 305:G849–G861
- Svensson PA, Asea A, Englund MC, Bausero MA, Jernas M, Wiklund O, Ohlsson BG, Carlsson LM, Carlsson B (2006) Major role of HSP70 as a paracrine inducer of cytokine production in human oxidized LDL treated macrophages. *Atherosclerosis* 185:32–38
- Tanaka K, Tanaka Y, Namba T, Azuma A, Mizushima T (2010) Heat shock protein 70 protects against bleomycin-induced pulmonary fibrosis in mice. *Biochem Pharmacol* 80:920–931
- Tomcik M, Zerr P, Pitkowski J, Palumbo-Zerr K, Avouac J, Distler O, Becvar R, Senolt L, Schett G, Distler JH (2014) Heat shock protein 90 (Hsp90) inhibition targets canonical TGF-beta signalling to prevent fibrosis. *Ann Rheum Dis* 73:1215–1222
- Wang X, Zhao BS, Roundtree IA, Lu Z, Han D, Ma H, Weng X, Chen K, Shi H, He C (2015) N(6)-methyladenosine Modulates Messenger RNA Translation Efficiency. *Cell* 161:1388–1399
- Whitley D, Goldberg SP, Jordan WD (1999) Heat shock proteins: a review of the molecular chaperones. *J Vasc Surg* 29:748–751
- Xiao W, Adhikari S, Dahal U, Chen YS, Hao YJ, Sun BF, Sun HY, Li A, Ping XL, Lai WY et al (2016) Nuclear m(6)A reader YTHDC1 regulates mRNA splicing. *Mol Cell* 61:507–519
- Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M (2016) Global epidemiology of nonalcoholic fatty liver disease—meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 64:73–84
- Yu J, Li Y, Wang T, Zhong X (2018) Modification of N6-methyladenosine RNA methylation on heat shock protein expression. *PLoS one* 13:e0198604
- Yue Y, Liu J, He C (2015) RNA N6-methyladenosine methylation in post-transcriptional gene expression regulation. *Genes Dev* 29:1343–1355
- Zheng G, Dahl JA, Niu Y, Fedorcsak P, Huang CM, Li CJ, Vagbo CB, Shi Y, Wang WL, Song SH et al (2013) ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. *Mol Cell* 49:18–29
- Zhou J, Wan J, Gao X, Zhang X, Jaffrey SR, Qian SB (2015) Dynamic m(6)A mRNA methylation directs translational control of heat shock response. *Nature* 526:591–594
- Zulkifli I, Najafi P, Nurfarahin AJ, Soleimani AF, Kumari S, Aryani AA, O'Reilly EL, Eckersall PD (2014) Acute phase proteins, interleukin 6, and heat shock protein 70 in broiler chickens administered with corticosterone. *Poult Sci* 93:3112–3118

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.