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Physiology

Heat challenge influences serum metabolites concentrations and liver lipid metabolism in Japanese quail (Coturnix japonica)

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ABSTRACT. High temperature induces various metabolic disturbances in animals. However, no comprehensive information is currently available on the metabolic pathway affected by high environmental temperature. The present study examined metabolite content in the serum of heat challenged quails using metabolomic analysis. In the present study, female quails with normal laying rate at 20 weeks kept in standard condition (control group) or exposed to 34°C 4 hr per day (12:00 to 16:00 hr)(heat group) for 10 consecutive days. The metabolomic analysis identified 165 metabolites in the serum, and significant differences were observed in the serum for 7 metabolites between two groups. An analysis by MetaboAnalyst, a web-based metabolome data tool, indicate that high temperature affect ketone body metabolism, butyrate metabolism, arginine and proline metabolism. Furthermore, histological examination of liver indicates a heat challenge induced abnormal lipid metabolism. Triglyceride and cholesterol level in the liver increased, however cholesterol level decreased in the serum. Genes related to lipid metabolism significantly increased in the liver after heat challenge. The present study demonstrated that high temperature cause liver damage, thus lipid metabolic was affected. Protect liver under high temperature could be one solution for coping with high temperatures in summer.

KEY WORDS: heat challenge, Japanese quail, lipid metabolism, liver, serum metabolite

High temperature in summer is one of the major factors restrict poultry industry. Heat stress is a prime consideration in poultry production systems and affects animal health and productivity [6], which causes huge economic loss. In poultry industry, heat stress can reduce egg laying number [8], decrease ovarian weight [19] and hierarchical follicles number [23].

The change of physiological and biochemical characteristics during heat stress have been reported, for example, heat stress increased apical glucose transport in the chicken jejunum [3], acute heat stress induced superoxide radical production in chicken skeletal muscle [16]. Increased the vasoconstrictor responses to 5-HT by a mechanism that involved extracellular Ca^{2+} influx through calcium channels [22]. Heat stress removal on function of hepatic mitochondrial respiration, ROS production and lipid peroxidation in broiler chickens [26]. And affect gene expression of brain-gut neuropeptides in broiler chickens (*Gallus gallus domesticus*) [12].

Few researches reported on metabolites change under high temperature, and then they found multiple pathways affected by heat stress including glucose, amino acid, and lipid metabolism [7]. Environmental high temperature may affects multiple tissues, the organism alter its physiology to maintain homeostasis. As metabolite content should reflect the net results of physiological changes, it may provide an insight into the metabolic disturbance under high temperature.

Liver plays a central role in metabolism. Liver regulates glycogen storage and decomposition of red blood cells. Liver also plays as an accessory digestive gland that produces bile, an alkaline compound that helps the breakdown of fat. In sexually matured birds, the elevated level of estrogen indirectly regulates the rapid growth of vitellogenic oocytes by potentiating the synthesis of yolk precursor proteins in the liver [21]. It is reasonable to hypothesize that the liver will be responsive to high temperature due to its central role in maintaining the overall metabolism of the organism.

The objective of the present study was to investigate metabolic change in heat challenged quails. Gas chromatography mass spectrometry (GC/MS) was used to analyze low molecular weight metabolites in heat-challenged quails. Their livers were investigated for understanding the reason of metabolic disturbance.

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MATERIALS AND METHODS

Animals and experimental design

Female Japanese quails were hatched and raised in a breeding colony at Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology. Quails were provided with commercial quail food (Quail Cosmos, Tahara, Japan) and water, and were allowed to feed *ad libitum*. Quails were housed in metal cages in a controlled environment (lights on, 05:00 to 19:00 hr; temperature, 23 ± 2°C; humidity, 50 ± 10%; air exchanged 20 times hourly).

At the age of 20 weeks, 18 healthy female quails with normal laying rate were selected and divided into control and heat group. For the heat group females were exposed to 34°C for 4 hr per day (12:00 to 16:00 hr) during 10 consecutive days in Biomulti incubator (LP-80CCFL-6CTAR, NK System, Tokyo, Japan). At day 10th of treatment, quails were killed by decapitation after heat treatment. Blood was obtained from carotid artery and separated by centrifugation at 3,000 × g for 30 min at 4°C, serum was separated and stored at −20°C for metabolic analysis and triglyceride and cholesterol measurement.

Liver was removed and measured weight. Middle lobe of fresh liver samples were fixed in 4% paraformaldehyde for 24 hr. The fixed samples were transferred through a graded series of ethanol and xylene and then embedded in paraffin wax. Other part of livers were collected and stored at −80°C for RT-PCR, triglyceride and cholesterol measurement.

The ethical committee of animal experimentation of Tokyo University of Agriculture and Technology permitted all experimental protocols.

Non-targeted analysis of low molecular weight metabolites in serum

A serum metabolomics analysis was performed using GC/MS as described previously [24] with some modifications. In brief, a sample of 50 μl of serum was mixed with 5 μl of 1 mg/m*l* 2-isopropylmalic acid (Sigma-Aldrich, Tokyo, Japan) in distilled water as an internal standard, and 250 μl of methanol–chloroform–water (2.5:1:1) mixture. Then samples were lyophilized, and added with 40 μl of 20 mg/m*l* methoxyamine hydrochloride (Sigma-Aldrich), dissolved in pyridine for oximation. After mixing, the samples were shaken for 90 min at 30°C. Next 20 μl of N-methyl N-trimethylsilyl-trifluoroacetamide (GL Science, Tokyo, Japan) was added for trimethylsilylation, and the mixture was incubated at 37°C for 45 min. The sample was subjected to GC/MS (GCMS QP2010-Ultra; Shimadzu, Kyoto, Japan). The Shimadzu Smart Metabolites Database (Shimadzu) was used to identify metabolites. Samples were normalized by a pooled sample from control group. A metabolic pathway analysis was performed using MetaboAnalyst [25]. Metabolites that significantly diffed between two groups were subjected to an enrichment analysis (http://www.metaboanalyst.ca/faces/upload/EnrichUploadView.xhtml).

Histological analysis

Paraffin-embedded samples were serially sectioned (5 μm thick) onto 3-aminopropyl-triethoxysilane (APES)-coated slides and stained with hematoxylin–eosin (HE). The stained sections were observed and photographed under a microscope (Keyence BX-51, Osaka, Japan)

Triglyceride and cholesterol in serum and liver

One hundred milligram of liver from each quail was put into centrifuge tube containing 0.2 m*l* saline, homogenized on ice, added with 0.8 m*l* chloroform-methanol (1:1), then mixed well and stayed at 4°C for 18 hr, followed by centrifugation with 3,500 × g for 10 min at 4°C. Three layers were clearly separated, and the bottom layer of lipid phase was gently collected and stored at 4°C for triglyceride and cholesterol measurement.

Triglyceride of serum and liver was detected by LabAssay^{TM} Triglyceride kit (Wako, Osaka, Japan). The cholesterol was detected by LabAssay^{TM} Choleserol kit (Wako).

RNA extraction, reverse transcription (RT) and quantitative PCR

Total RNA was extracted from liver using Isogen reagent kit (Nippon Gene, Tokyo, Japan). The concentration and purity of the isolated total RNA was determined spectrophotometrically at 260 and 280 nm wave length with a Nanodrop (Thermo Fisher Scientific, Wilmington, DE, U.S.A.). The total RNA (1 μg) was reversely transcribed to cDNA with an Omniscript® Reverse Transcription kit (Takara, Tokyo, Japan) with Oligo-dT primers (Takara) according to the manufacturer's protocol.

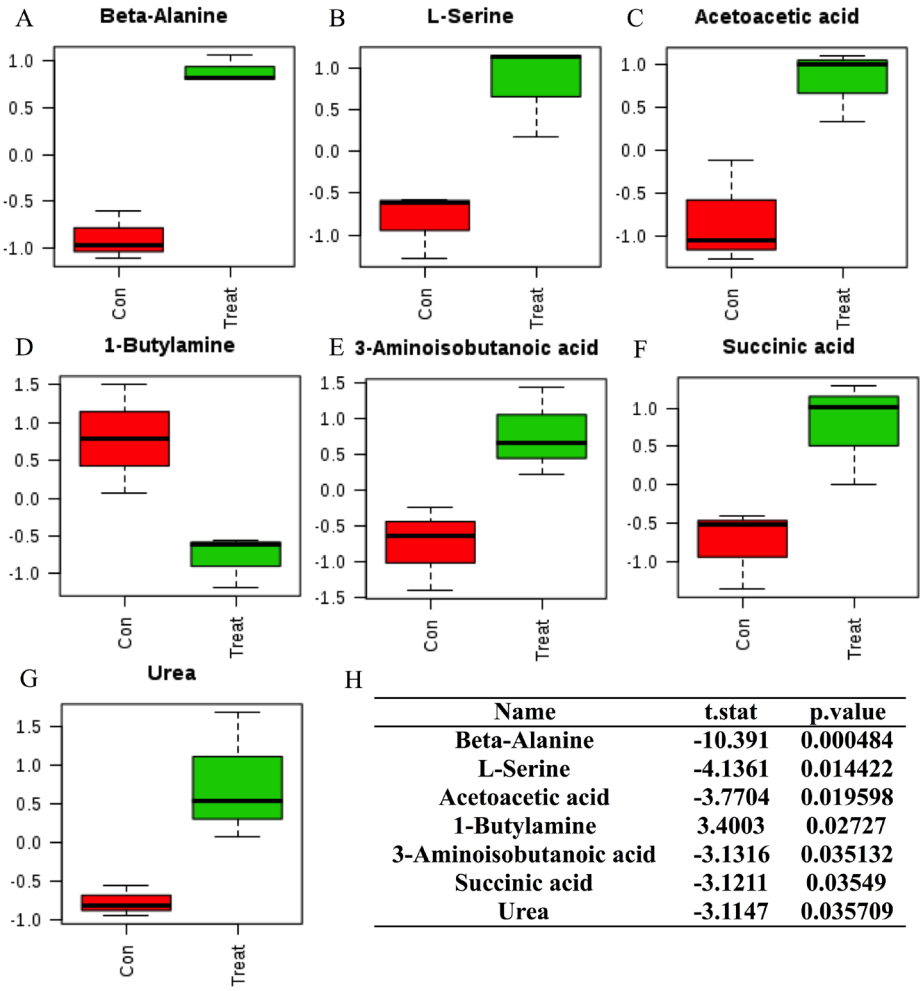
Target genes and the housekeeping gene (GAPDH) were quantified by real-time PCR using an ABI 7500 Fast and a commercial kit (SYBR Premix Ex Taq^{TM} II, Takara). The specificity of the PCR product was verified by a melting curve and by agarose gel electrophoresis. The relative concentration of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method [20]. Ct values from control samples were used as calibrators. The gene-specific primers were designed on the basis of chicken mRNA sequences using Primer Version 5.0 (Table 1).

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) and were analyzed by *t*-test. Statistical analysis was performed with GraphPad Prism software (GraphPad Software, San Diego, CA, U.S.A.). Differences were considered statistically significant when the $P<0.05$.

Table 1. Primers used for real-time PCR analysis

Genes	Primer sequences	Size of PCR product (bp)	Annealing Temperature (°C)
PPARa	F: 5′-TTGTCGCTGCCATCATTTGC-3′ R: 5′-CTGGCAGTCGTGTTGAGAGAG-3′	189	60
SREBF	F: 5′-GCCTTTGTCTTCCTCTGCCT-3′ R: 5′-ATGCTTCTTCCAGGACCAGC-3′	152	60
AGPAT2	F: 5′-GCTCATCACATACCTCGGGG-3′ R: 5′-CCTGGACAGCGAGGTGAAAT-3′	194	60
AGPAT5	F: 5′-TGCTGTTTCTAGGTGGCCTG-3′ R: 5′-ACCCACAGGCAGCCAATTAA-3′	184	59.8
GAPDH	F: 5′-TGATGCTCCCATGTTCGTGA-3′ R: 5′-TAAGACCCTCCACGATGCC-3′	149	60



H

Name	t.stat	p.value
Beta-Alanine	-10.391	0.000484
L-Serine	-4.1361	0.014422
Acetoacetic acid	-3.7704	0.019598
1-Butylamine	3.4003	0.02727
3-Aminoisobutanoic acid	-3.1316	0.035132
Succinic acid	-3.1211	0.03549
Urea	-3.1147	0.035709

Fig. 1. Seven serum metabolites in control group (Con) and heat group (Treat). A: Beta-Alanine, B: L-Serine, C: Acetoacetic acid, D: 1-Butylamine, E: 3-Aminoisobutanoic acid, F: Succinic acid, G: Urea. H: t. stat and *P* value of each metabolites ($P<0.05$, *t*-test).

RESULTS

Metabolites and metabolic pathway affected by heat challenge

A total of 165 metabolites in the serum were identified using the non-target analysis, and heat challenge significantly affected the concentration of 7 metabolites (Fig. 1). A total 6 metabolites were significantly higher in the heat-challenged group than in the control group (Beta-Alanine, L-Serine, Acetoacetic acid, 3-Aminoisobutanoic acid, Succinic acid and Urea), whereas one metabolite was lower in the heat-challenged group (1-Butylamine).

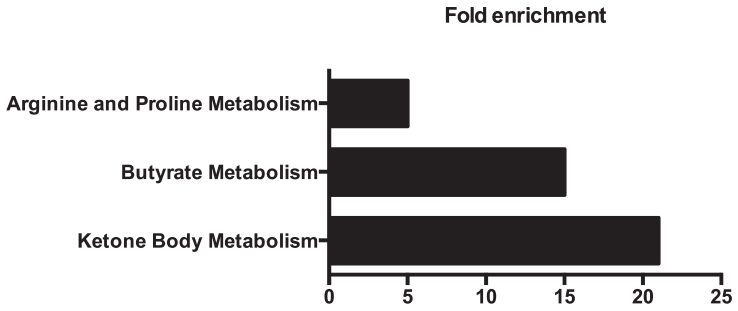


Fig. 2. Metabolic pathway affected in the serum of quails under heat challenge.

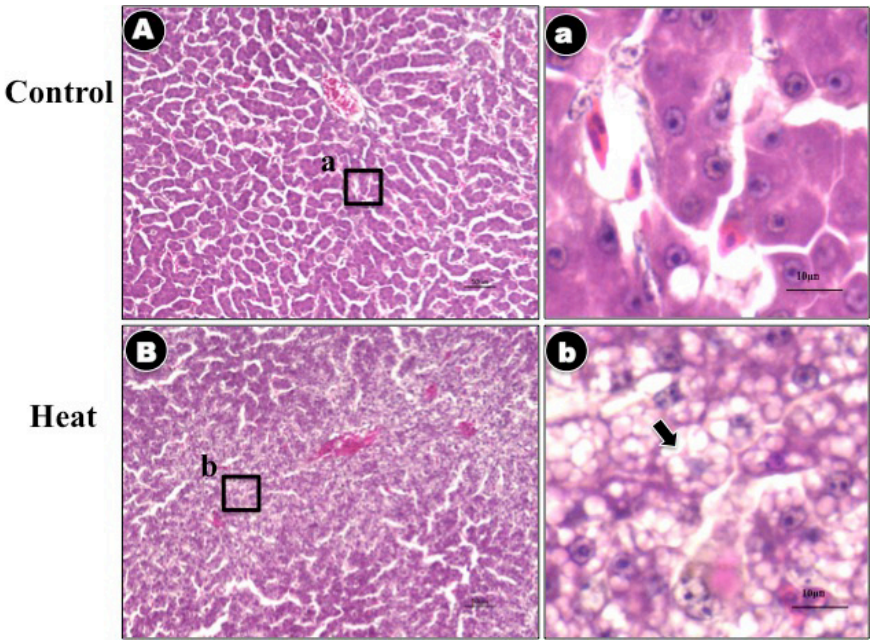


Fig. 3. HE staining of the sections derived from Japanese quail liver tissues, control group (A and a), heat group (B and b), bar=50 μm in A and B, bar=10 μm in a and b. Arrow indicate lipid deposition in hepatocytes in heat group.

The MetaboAnalyst analysis indicated that ketone body metabolism, butyrate metabolism, arginine and proline metabolism were affected in the serum of heat-challenged quails (Fig. 2).

Histopathology analysis of liver

To investigate the effect of heat challenge on liver structure, morphological change in quail livers was examined. As shown by microscope (Fig. 3), no obvious change was observed in the control liver (Fig. 3A and 3a). On the other hand, high ambient temperature induced lipid deposition in hepatocytes. The typical morphological changes of lipid steatosis were detected in the heat livers (Fig. 3B and 3b). This is the histologic appearance of hepatic macrovesicular steatosis (fatty change). The lipid accumulates in the hepatocytes as vacuoles. These vacuoles have a clear appearance with HE staining.

Serum and liver triglyceride and cholesterol level

Serum and liver triglyceride and cholesterol were detected. Both serum triglyceride and cholesterol lower in heat group (Fig. 4A and 4B), and cholesterol significantly lower in heat challenged quails (P<0.05). However, triglyceride and cholesterol in the liver showed significantly higher in heat group (Fig. 4C and 4D)(P<0.05).

Lipid metabolic gene expression in liver

The mRNA transcript levels of *PPARα* and *AGPAT5* significantly higher in heat challenged liver comparing with those in control group (P<0.05) (Fig. 5A and 5D), *SREBF* and *AGPAT2* also higher in heat challenged liver, however did not have significant difference (P>0.05).

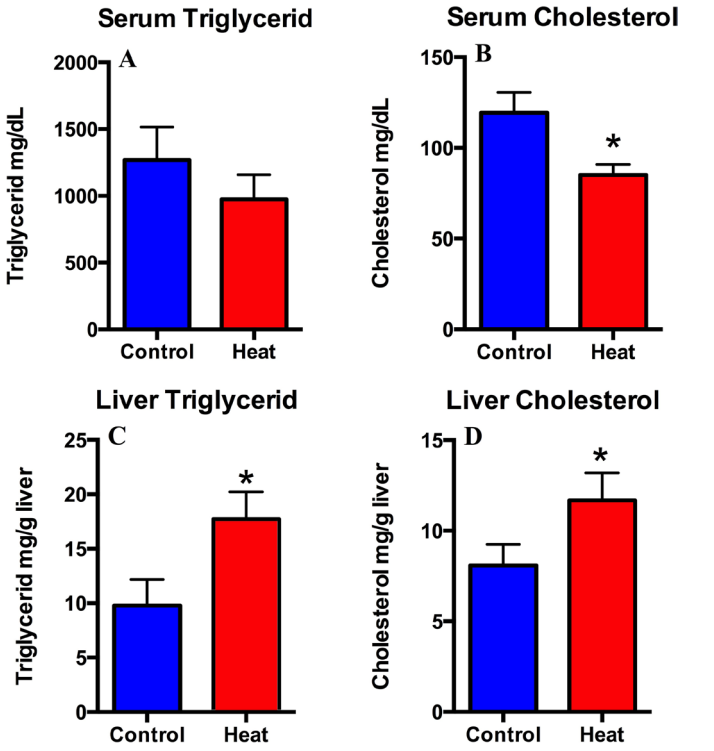


Fig. 4. Serum and liver triglyceride and cholesterol level. Serum triglyceride (A), serum cholesterol (B), liver triglyceride (C) and liver cholesterol (D) in control (Control) and heat treated (Heat) groups. Each bar represents the mean ± SEM. *Significantly different from the values at control (P<0.05, t-test), n=9/group.

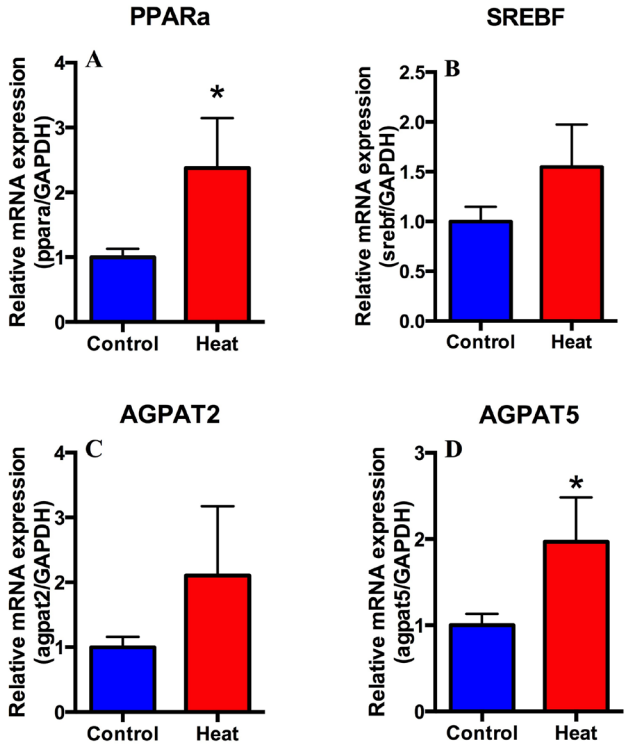


Fig. 5. Expression of lipid metabolic gene expression in liver in control (Control) and heat treated (Heat) groups. Each mRNA was normalized to the *GAPDH* mRNA expression level in the same preparation, and the mean of each experimental control was assigned a value of 1.0. A: liver peroxisome proliferator activated receptor alpha (*PPARα*) mRNA; B: liver sterol regulatory element binding factor (*SREBF*) mRNA; C: liver 1-acylglycerol-3-phosphate O-acyltransferase 2 (*AGPAT2*) mRNA; D: liver 1-acylglycerol-3-phosphate O-acyltransferase 5 (*AGPAT5*) mRNA. The values shown are the mean ± SEM. *Significantly different from the values at control (P<0.05, t-test), n=9/group.

DISCUSSION

The present study explored metabolite contents in the serum of quail under 10 days heat challenge. The results analyzed by MetaboAnalyst analysis revealed that (1) serum beta-alanine, l-serine, acetoacetic acid, 3-aminoisobutanoic acid, succinic acid and urea increased by heat challenge, (2) ketone body metabolism, butyrate metabolism, arginine and proline metabolism were affected by heat challenge. Increase of triglyceride and cholesterol level and lipid metabolic gene expression in liver indicates that heat challenge may cause disturbance in some metabolic pathway such as lipid metabolism in liver.

In the present study, acetoacetic acid significantly increased in heat-challenged quail, which suggest the rate of synthesis exceeds the rate of utilization. Ketone bodies, namely, acetone, acetoacetic acid, and β-hydroxybutyrate, are small lipid-derived molecules that serve as a circulating energy source for tissues in times of fasting or prolonged exercise [1]. During fasting, muscle and liver stores of glycogen are depleted first. Then, fatty acids are mobilized from adipocytes and transported to the liver for conversion to ketone bodies. Ketone bodies are transported from the liver to the extrahepatic tissues and converted into acetyl-CoA, which then enters the citric acid cycle and is utilized for energy production. The relative proportion of ketone bodies present in blood may vary from 78% (β-hydroxybutyric acid) to 20% (acetoacetic acid) and 2% (acetone) [10]. In normal conditions, the ketone bodies are produced constantly by the liver and utilized by peripheral tissues.

The increase of acetoacetic acid is commonly detected in the serum of diabetic humans and rodents, which are more susceptible to heat-related illnesses and exogenous insulin rescues this phenotype [17, 22]. Furthermore, thermal therapy improves insulin sensitivity in diabetic and obese rodents [4, 9].

PPARα gene expression increased in heat challenged quail liver, since *PPARα* is a key transcriptional regulator of lipid metabolism and activated by a lipid surge to induce lipid catabolism. It plays an important role in the formation of fatty liver [15]. *PPARα* can also reduce inflammation, improve liver oxidative stress, and prevent liver tissue damage [13]. Present results indicate serum triglyceride and cholesterol level decreased after heat challenge, which agreed by Fujita *et al.*, showed *PPARα* agonist can

significantly reduce plasma triglyceride content, improve liver steatosis, and increase insulin sensitivity [2, 14].

Another gene, *AGAPT5* increased in the liver after heat. Acylglycerophosphate acyltransferases/lysophosphatidic acid acyltransferases (*AGPAT*) are a homologous family of enzymes that catalyze the formation of phosphatidic acid (PtdOH). Parks *et al.* [18] conducted antisense oligonucleotides (ASOs) to silence Agpat5 expression in liver and adipose tissue, and identify *AGPAT5* as a gene associated with plasma insulin levels and insulin resistance, inhabitation of *AGPAT5* could significantly improve glucose tolerance after high-fat or high-fructose feeding. *PPARα* can increase insulin sensitivity, however *AGPAT5* cause insulin resistance, the triglyceride and cholesterol level increased in live, but cholesterol level decreased in serum may due to lipid metabolic disturbance in liver after 10 days heat challenge.

The liver of mammals and birds are different [11]. In avian species, lipids and especially triglycerides stores in adipocytes, hepatocytes and growing follicles [5]. When hepatic lipogenesis exceed the capacity of very low density lipoprotein (VLDL) secretion, triglycerides accumulated in the liver, and cause a steatosis [5]. One of the reasons contribute to the decreased ovarian and follicular weight might be liver damage and lipid metabolic dysfunction under high environmental temperature, the synthesis of yolk precursor proteins in the liver were decreased.

High temperature may influence many aspects of animal. It takes further researches to clarify the mechanism under this situation. The present study demonstrated that high temperature influence nutrition absorption. Ketone body metabolism affect may be due to the change of insulin regulation. Consequently hepatic steatosis, hepatic metabolism dysfunction, especially lipid (triglyceride and cholesterol) may occur. The present results may suggest prevent in damage of liver under high temperature, increasing nutrition intake could be one solution for against problems caused by summer high temperature in poultry farm.

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REFERENCES

1. Berg, J. M., Tymoczko, J. and Stryer, L. 2012. Oxidative Phosphorylation. Biochemistry, WH Freeman, New York.
2. Fujita, N., Matsushita, T., Ishida, K., Kubo, S., Matsumoto, T., Takayama, K., Kurosaka, M. and Kuroda, R. 2011. Potential involvement of SIRT1 in the pathogenesis of osteoarthritis through the modulation of chondrocyte gene expressions. *J. Orthop. Res.* **29**: 511–515. [Medline] [CrossRef]
3. Garriga, C., Hunter, R. R., Amat, C., Planas, J. M., Mitchell, M. A. and Moretó, M. 2006. Heat stress increases apical glucose transport in the chicken jejunum. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **290**: R195–R201. [Medline] [CrossRef]
4. Gupte, A. A., Bomhoff, G. L., Swerdlow, R. H. and Geiger, P. C. 2009. Heat treatment improves glucose tolerance and prevents skeletal muscle insulin resistance in rats fed a high-fat diet. *Diabetes* **58**: 567–578. [Medline] [CrossRef]
5. Hermier, D. 1997. Lipoprotein metabolism and fattening in poultry. *J. Nutr.* **127** Suppl: 805S–808S. [Medline] [CrossRef]
6. Humphrey, T. 2006. Are happy chickens safer chickens? Poultry welfare and disease susceptibility. *Br. Poult. Sci.* **47**: 379–391. [Medline] [CrossRef]
7. Jastrebski, S. F., Lamont, S. J. and Schmidt, C. J. 2017. Chicken hepatic response to chronic heat stress using integrated transcriptome and metabolome analysis. *PLoS One* **12**: e0181900. [Medline] [CrossRef]
8. Kirunda, D. F., Scheideler, S. E. and McKee, S. R. 2001. The efficacy of vitamin E (DL-α-tocopheryl acetate) supplementation in hen diets to alleviate egg quality deterioration associated with high temperature exposure. *Poult. Sci.* **80**: 1378–1383. [Medline] [CrossRef]
9. Kokura, S., Adachi, S., Manabe, E., Mizushima, K., Hattori, T., Okuda, T., Nakabe, N., Handa, O., Takagi, T., Naito, Y., Yoshida, N. and Yoshikawa, T. 2007. Whole body hyperthermia improves obesity-induced insulin resistance in diabetic mice. *Int. J. Hyperthermia* **23**: 259–265. [Medline] [CrossRef]
10. Kumar, V. and Gill, K. D. 2018. Basic Concepts in Clinical Biochemistry: A Practical Guide, Springer, Germany.
11. Lee, C. H., Kamijima, M., Li, C., Taneda, S., Suzuki, A. K. and Nakajima, T. 2007. 3-Methyl-4-nitrophenol metabolism by uridine diphosphate glucuronosyltransferase and sulfotransferase in liver microsomes of mice, rats, and Japanese quail (Coturnix japonica). *Environ. Toxicol. Chem.* **26**: 1873–1878. [Medline] [CrossRef]
12. Lei, L., Hepeng, L., Xianlei, L., Hongchao, J., Hai, L., Sheikhahmadi, A., Yufeng, W. and Zhigang, S. 2013. Effects of acute heat stress on gene expression of brain-gut neuropeptides in broiler chickens. *J. Anim. Sci.* **91**: 5194–5201. [Medline] [CrossRef]
13. Ma, C. H., Chiua, Y. C., Wu, C. H., Jou, I. M., Tu, Y. K., Hung, C. H., Hsieh, P. L. and Tsai, K. L. 2018. Homocysteine causes dysfunction of chondrocytes and oxidative stress through repression of SIRT1/AMPK pathway: A possible link between hyperhomocysteinemia and osteoarthritis. *Redox Biol.* **15**: 504–512. [Medline] [CrossRef]
14. Matsuzaki, T., Matsushita, T., Takayama, K., Matsumoto, T., Nishida, K., Kuroda, R. and Kurosaka, M. 2014. Disruption of Sirt1 in chondrocytes causes accelerated progression of osteoarthritis under mechanical stress and during ageing in mice. *Ann. Rheum. Dis.* **73**: 1397–1404. [Medline] [CrossRef]
15. Minashima, T., Zhang, Y., Lee, Y. and Kirsch, T. 2014. Lithium protects against cartilage degradation in osteoarthritis. *Arthritis Rheumatol.* **66**: 1228–1236. [Medline] [CrossRef]
16. Mujahid, A., Yoshiki, Y., Akiba, Y. and Toyomizu, M. 2005. Superoxide radical production in chicken skeletal muscle induced by acute heat stress. *Poult. Sci.* **84**: 307–314. [Medline] [CrossRef]
17. Niu, C. S., Lin, M. T., Liu, I. M. and Cheng, J. T. 2003. Role of striatal glutamate in heatstroke-induced damage in streptozotocin-induced diabetic rats. *Neurosci. Lett.* **348**: 77–80. [Medline] [CrossRef]
18. Parks, B. W., Sallam, T., Mehrabian, M., Psychogios, N., Hui, S. T., Norheim, F., Castellani, L. W., Rau, C. D., Pan, C., Phun, J., Zhou, Z., Yang, W. P., Neuhaus, I., Gargalovic, P. S., Kirchgessner, T. G., Graham, M., Lee, R., Tontonoz, P., Gerszten, R. E., Hevener, A. L. and Lusis, A. J. 2015. Genetic architecture of insulin resistance in the mouse. *Cell Metab.* **21**: 334–347. [Medline] [CrossRef]
19. Rozenboim, I., Tako, E., Gal-Garber, O., Proudman, J. A. and Uni, Z. 2007. The effect of heat stress on ovarian function of laying hens. *Poult. Sci.* **86**: 1760–1765. [Medline] [CrossRef]
20. Schmittgen, T. D. and Livak, K. J. 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* **3**: 1101–1108. [Medline]

[CrossRef]
21. Schneider, W. J., Osanger, A., Waclawek, M. and Nimpf, J. 1998. Oocyte growth in the chicken: receptors and more. *Biol. Chem.* **379**: 965–971. [Medline]
22. Siddegowda, Y. K. B., Leo, M. D. M., Kumar, D., Hooda, O. K., Prakash, V. R. and Mishra, S. K. 2007. Influence of heat stress on the reactivity of isolated chicken carotid artery to vasoactive agents. *Exp. Physiol.* **92**: 1077–1086. [Medline] [CrossRef]
23. Star, L., Nieuwland, M. G., Kemp, B. and Parmentier, H. K. 2007. Effect of single or combined climatic and hygienic stress on natural and specific humoral immune competence in four layer lines. *Poult. Sci.* **86**: 1894–1903. [Medline] [CrossRef]
24. Takemoto, S., Tomonaga, S., Funaba, M. and Matsui, T. 2017. Effect of long-distance transportation on serum metabolic profiles of steer calves. *Anim. Sci. J.* **88**: 1970–1978. [Medline] [CrossRef]
25. Xia, J. and Wishart, D. S. 2011. Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst. *Nat. Protoc.* **6**: 743–760. [Medline] [CrossRef]
26. Yang, L., Tan, G. Y., Fu, Y. Q., Feng, J. H. and Zhang, M. H. 2010. Effects of acute heat stress and subsequent stress removal on function of hepatic mitochondrial respiration, ROS production and lipid peroxidation in broiler chickens. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **151**: 204–208. [Medline] [CrossRef]