UNIVERSITI PUTRA MALAYSIA

HEAT SHOCK PROTEINS AS MODIFYING FACTORS IN PHYSIOLOGICAL STRESS RESPONSES IN POULTRY

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FP 2011 1

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By

ABDOREZA SOLEIMANI FARJAM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of Requirements for the Degree of Doctor of Philosophy

January 2011

DEDICATION

This Tiny Window to the Puzzle of Mother Nature is Dedicated to the Spirit of the Universe

Abstract of thesis presented to the Senate of Universisti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

HEAT SHOCK PROTEINS AS MODIFYING FACTORS IN PHYSIOLOGICAL STRESS RESPONSES IN POULTRY

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Chairman: Professor Zulikifli Idrus, PhD

Faculty: Agriculture

Five experiments were conducted to investigate the effects of genetic and neonatal manipulation on physiological stress response and their roles in thermotolerance parameters such as plasma corticosterone concentration (CORT), heterophil to lymphocyte ratio (HLR), heat shock protein 70 (Hsp70) and glucocorticoid receptor (GR) expression, body temperature, performance and *Salmonella enteritidis* resistance in poultry.

In Experiment I, two trials were conducted to compare the thermotolerance of the red jungle fowl (RJF) (*Gallus gallus spadiceus*), village fowl (VF) and commercial broilers (CB) at (i) a common age (30 days old) and (ii) a common body weight (930±30 g) when exposed to 36 ±1 ^{o}C for 3 h. In both trials, irrespective of the stage of heat treatment the RJF had lower HLR, higher CORT concentration and higher Hsp70 expression than the VF and CB. Unlike the RJF, heat exposure significantly elevated the body temperature, HLR, CORT concentration and Hsp70 expression of CB.

Experiment II was carried out to further ascertain the functional roles of CORT and Hsp70 in acquisition of thermotolerance through neonatal feed restriction. A total of 180 day-old broiler chicks were subjected to one of the following four feeding regimens: *ad libitum* (control); 60% feed restriction on days 4, 5 and 6 (FR60); 60% feed restriction on days 4, 5 and 6 +1500 mg/kg metyrapone (FR60M); 60% feed restriction on days 4, 5 and 6 +1500 mg/kg quercetin (FR60Q). To elicit heat stress from day 35 to 42, all chicks were exposed to 37±1°C for 3 h daily. The results suggest that subjecting chicks to FR60, as measured by HLR, FCR and weight gain, can enhance their ability to withstand, high ambient temperature and Hsp70 induction did not appear to play a profound role in the acquisition of thermotolerance.

To gain more insights on the roles of Hsp70, the Hsp70 response and *S. enteritidis* colonization were investigated in neonatally feed restricted broiler chickens subjected to heat stress later in life in Experiment III. Chicks were divided into three feeding regimens: *ad libitum* (control); 60% feed restriction on days 4, 5 and 6 (FR60); 60% feed restriction on days 4, 5 and 6 +1500 mg/kg quercetin (FR60Q). On d 35, all chickens were individually inoculated with 1 mL *S. enteritidis* (1.5×10^8 cfu/bird) and exposed to 37 ±1°C for 3 h daily. After heat exposure, the FR60 and FR60Q birds showed a significantly lower *S. enteritidis* colonization and lower Hsp70 expression than control birds. The least colonization was observed in the FR60Q group (1.38 $\log_{10}$ cfu/g in spleen and 1.96 $\log_{10}$ cfu/g in cecal content) and the highest in the control group (2.1 $\log_{10}$ cfu/g in spleen and 4.42 $\log_{10}$ cfu/g in cecal content). This clearly demonstrated that neonatally feed restricted chicks developed their thermotolerance ability and a greater ability to respond to the *S. enteritidis* invasion.

In Experiment II we emphasised on relationship between CORT and Hsp70 responses under heat stress. To elaborate more on this relationship, Experiment IV was conducted to determine the relationship between Hsp70 expression in the heart and brain and CORT modulated by ascorbic acid and α-tocopherol supplementation in quails subjected to social isolation. Fifty three day old male Japanese quails were randomly assigned to each one of the six feeding regimens for 3 days as follows: (i) Basal diet (control); (ii) Basal diet+1500 mg/kg metyrapone (BM); (iii) Basal diet+30 mg/kg corticosterone (BCO); (iv) Basal diet +250mg/kg ascorbic acid (BC); (v) Basal diet +250mg/kg α-tocopherol (BE); (vi) Basal diet +250mg/kg ascorbic acid and 250mg/kg α-tocopherol (BCE). The birds were subsequently subjected to social isolation stress for 2 hours. Two hours of isolation stress elevated CORT significantly in the control and BE but not in the BC, BCE and BM birds. Isolation stress increased Hsp70 expression of the brain and heart in the control and BM birds. However, Hsp70 expression was not significantly altered after isolation stress by supplementation of ascorbic acid, α-tocopherol or their combination. Although CORT did not increase after isolation stress, Hsp70 expression significantly increased both in the heart and brain. Moreover, exogenous corticosterone supplementation did not result in elevation of Hsp70 expression.

The last Experiment (Experiment V) was conducted to investigate the long term impact of neonatal feed restriction on the HPA axis response and hippocampal Hsp70 expression in senescent quail exposed to acute heat challenge. Equal numbers of male Japanese quails (*Coturnix coturnix japonica*) were subjected to one of the following two feeding regimens: *ad libitum* (control); 60% feed restriction on days 4, 5 and 6 (FR). At the age of 21 (young) and 270 (old) days, four groups of 10 quails

each from the control or FR group were randomly selected and blood and hippocampus samples were collected representing the different stages of heat treatment: Basal (no heat treatment), 1 h heat challenge (43°C), 1 h recovery and 2 h recovery. With the use of real-time PCR and EIA, the expression of regulatory genes in the hippocampus and CORT were examined. Aging resulted in a higher CORT, lower Hsp70 and GR expression in control birds, while in senescent FR birds only Hsp70 expression was attenuated following heat challenge. It is interesting to note that regardless of the age, there was a significantly lower basal GR expression in FR than control birds. Moreover, Hsp70 expression was upregulated in senescent FR birds during the recovery period and basal condition compared to control.

Together, it can be concluded that selective breeding for phenotypic traits has resulted in tremendous alterations in the physiology of CB and concomitantly the ability to withstand high ambient temperature as compared to the RJF and VF. In other words, selective breeding consciously or unconsciously reduced physiological stress response thresholds in CB, producing more stress susceptible phenotypes rather than stress resistant ones. It is also apparent that genetic differences in body size and age *per se* may not determine breed or strain variations in response to heat stress. Neonatal modification seems to modify this stress response pattern leading to improved FCR and enhanced weight gain and resistance to *S. enteritidis* colonization. The Hsp70 and CORT alone are probably insufficient for inducing thermotolerance in chicks during neonatal modification and further studies are needed to investigate the participation of other genes and mechanisms during maturation of the thermoregulatory system. With regard to the relationship between Hsp70 and CORT, it is hypothesized that although Hsp70 expression may have been

modulated by the effect of CORT in oxidative stress or glucocorticoid receptor association with heat shock proteins, it may also have been regulated mainly by ACTH functions in the HPA axis. It is also evident that the effect of neonatal modification on HPA axis response may last life-long. Thus, it is revealed to be a functional approach to reduce the allostatic load and restore homeostasis more efficiently in senescent birds leading to the development of adaptive, healthy and resilient phenotypes.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PROTEIN PENGKEJUT HABA SEBAGAI FAKTOR MODIKASI DALAM FISIOLOGI RESPONS STRES DALAM POULTRI

Oleh

ABDOREZA SOLEMANI FARJAM

Januari 2011

Pengerusi : Profesor Zulkifli Idrus, PhD

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Lima eksperimen telah dijalankan untuk menyiasat kesan-kesan manipulasi genetik dan epigenetik ke atas fisiologi respons stres dan peranannya dalam parameter-parameter termo-toleransi seperti kepekatan plasma kortikosteron (CORT), kadar heterofil kepada limfosit (HLR), protein pengkejut haba 70 (Hsp70) dan ekspresi reseptor glukokortikoid (GR), suhu badan, prestasi dan pertahanan *Salmonella enteritidis* dalam poultri.

Dalam Eksperimen I, dua percubaan telah dijalankan untuk membandingkan termo-toleransi pada ayam hutan merah (RJF) (*Gallus gallus spadiceus*), ayam kampong (VF) dan komersial ayam pedaging (CB) di antara (i) suatu umum usia (berumur 30 hari) dan (ii) suatu umum berat badan (930±30 g) semasa dikenakan kepada 36±1 ^{o}C untuk 3 jam. Dalam kedua-dua percubaan, terlepas dari tahap rawatan haba, RJF mempunyai lebih rendah HLR, lebih tinggi kepekatan CORT dan lebih tinggi ekspresi Hsp70 daripada VF dan CB. Tidak seperti RJF, penggenaan haba

singifikasinya meningkatkan suhu badan, HLR, kepekatan CORT dan ekspresi Hsp70 daripada CB.

Eksperimen II telah dijalankan untuk memastikan fungsi CORT dan Hsp70 memainkan peranan dalam toleransi-haba melalui sekatan makan pada ayam neonat. Seratus lapan puluh anak ayam pedaging yang baru lahir telah dibahagikan kepada salah satu daripada empat rawatan: makan secara *ad libitum* (kawalan); 60% sekatan makan pada hari 4, 5 and 6 (FR60); 60% sekatan makan pada hari 4, 5 and 6 +1500 mg/kg metyrapone (FR60M); 60% sekatan makan pada hari 4, 5 and 6 +1500 mg/kg quercetin (FR60Q). Bagi menghasilkan stres haba dai hari 35 hingga 42, semua anak ayam telah dikenakan kepada 37±1°C untuk 3 jam/hari. Keputusam menunjukkan ayam yang diberi rawatan FR60, dengna pemeriksaan HLR, FCR dan weight gain, didapati meningkatkan kebolehan penahanan kepada suhu ambien yang tinggi dan induksi Hsp70 tidak memainkan peranan kepentingan dalam toleransi-haba.

Bagi mendapatkan lebih data mengenai peranan Hsp70, kita memeriksa respon Hsp70 dan kolonisasi *S. enteritidis* dalam ayam pedaging neonat yang disekat makan semasa dikena stres haba pada hidup selanjutnya dalam Eksperimen III. Anak-anak ayam telah dibahagikan kepada tiga rawatan: makan secara *ad libitum* (kawalan); 60% sekatan makan pada hari 4, 5 and 6 (FR60); 0% sekatan makan pada hari 4, 5 and 6 +1500 mg/kg quercetin (FR60Q). Pada hari 35, semua ayam individu telah diinokulasikan dengan 1 mL *S. enteritidis* (1.5×10^8 cfu/ekor) dan dikenakan kepada 37±1°C untuk 3 jam/hari. Selepas dikenakan dengan haba, ayam FR60 dan FR60Q menunjukkan lebih rendah kolonisasi *S. enteritidis* dan lebih rendah ekspresi Hsp70 daripada ayam kawalan. Kolonisasi yang paling sedikit telah didapati dalam

kumpulan FR60Q (1.38 $\log_{10}$ cfu/g dalam limpa and 1.96 $\log_{10}$ cfu/g dalam kandungan sekum), paling tinggi dalam kumpulan kawalan (2.1 $\log_{10}$ cfu/g dalam limpa and 4.42 $\log_{10}$ cfu/g dalam kandungan sekum). Hal ini jelas menunjukkan bahawa sekatan makan pada ayam neonat telah menghasilkan termo-toleransi dan mempunyai lebih kebolehan untuk respon kepada serangan *S. enteritidis.*

Dalam Eksperimen II kita menekankan perhubungan antara respon CORT dan Hsp70 dibawah stres haba. Bagi perhubungan yang lebih terperinci, Eksperimen IV telah dijalankan untuk menentukan perhubungan antara espresi Hsp70 dalam jantung dan otak dan CORT diubah oleh supplemen askorbik asid dan α-tocopherol dalam ayam puyuh yang dikena sosial pengekangan. Ayam puyuh jantan yang berumur 53 hari telah dibahagikan secara rawak kepada setiap satu daripada enam rawatan selama tiga hari seperti berikut: (i) diet basal (kawalan); (ii) diet basal + 1500 mg/kg metyrapone (BM); (iii) diet basal + 30 mg/kg kortikosteron (BCO); (iv) diet basal + 250 mg/kg askorbik asid (BC); (v) diet basal + 250 mg/kg α-tocopherol (BE); (vi) diet basal + 250 mg/kg askorbik asid dan 250 mg/kg α-tocopherol (BCE). Ayam-ayam itu seterusnya telah dikenakan kepada sosial stres pengekangan selama 2 jam. Stres pengekangan selama dua jam telah meningkatkan plasma CORT signifikasinya dalam ayam kawalan dan kumpulan BE tetapi tidak di ayam BC, BCE dan BM. Stres pengekangan meningkatkan ekspresi Hsp70 dalam otak dan jantung dalam ayam kawalan dan ayam BM. Namun, ekspresi Hsp70 adalah tidak signifikan berubah selepas stres pengekangan oleh suplemen askorbik asid, α-tocopherol atau kombinasinya. Although CORT did not increase after isolation stress, Hsp70 expression significantly increased both in heart and brain. Moreover, exogenous corticosterone supplementation did not result in elevation of Hsp70 expression.

Eksperimen terakhir telah dijalankan untuk memeriksa kesan jangka panjang sekatan makan pada ayam neonat ke atas respon axis HPA dan espresi hippocampal Hsp70 dalam senescent ayam puyuh yang dikena segera cabaran haba. Nombor anak ayam yang sama telah dikenakan ke atas salah satu dua rawatan berikut: makan *ad libitum* (kawalan); 60% sekatan makan pada hari 4, 5 dan 6 (FR). Pada umur 21 (muda) dan 270 (tua) hari, empat kumpulan masing-masing dari 10 ayam puyuh kawalan atau FR telah dipilih secara rawak dan sampel darah dan hippokampus mereka telah dikumpulkan sebagai mewakili tahap cabaran haba yang berbeza: Basis (tiada cabaran haba), 1 jam cabaran haba (43°C), 1 jam pemulihan dan 2 jam pemulihan. Dengan menggunakan real-time PCR dan EIA, kami menyemak ekspresi gen pengatur di dalam hippocampus dan plasma CORT. Penuaan menyebabkan peningkatan CORT, penurunan ekspresi Hsp70 dan GR dalam ayam kawalan, sedangkan pada ayam senescent FR hanya espresi Hsp70 telah dilemahkan berikut cabaran haba. Sangat menarik untuk dicatat bahawa terlepas dari usia, basis ekspresi GR adalah signifikasinya lebih rendah dalam ayam FR berbanding kawalan. Selain itu, ekspresi Hsp70 adalah di atas regulasi pada ayam FR senescent selama tempoh pemulihan dan keadaan basis dibandingkan dengan kawalan.

Bersama-sama, dapat disimpulkan bahawa domestikasi dan pembiakan selektif untuk sifat fenotipik telah menyebabkan perubahan yang luar biasa dalam fisiologi CB dan bersamaan kemampuan untuk menahan suhu persekitaran yang tinggi berbanding dengan RJF dan VF. Batas stres perilaku respon telah ditinggikan dengan mengorbankan pengurangan stres batas respon fisiologi selama domestikasi. Hal ini juga jelas bahawa perbezaan genetik pada saiz tubuh dan usia *per se* tidak dapat menentukan jenis atau strain variasi sebagai respons terhadap stres haba.

Pengubahsuaian epigenetik tampaknya mengubah pola respon stres terkemuka untuk meningkatkan FCR dan miningkatkan pertambahan berat dan pertahanan terhadap kolonisasi *Salmonella enteritidis*. Hsp70 dan CORT sendiri adalah mungkin tidak cukup untuk menghasilkan termo-toleransi dalam anak ayam semasa pengubahsuaian epigenetik dan penelitian lebih lanjut diperlukan untuk menyiasat penyertaan gen lain dan mekanisma selama pematangan sistem termo-regulasi. Berkenaan dengan hubungan antara Hsp70 dan CORT, maka hipotesis bahawa Hsp70 sebahagian besar dipengaruhi oleh beberapa bahagian yang lebih tinggi dari paksi HPA daripada CORT sendiri. Hal ini juga terlihat bahawa kesan pengubahsuaian epigenetik pada respon paksi HPA boleh berlangsung seumur hidup. Jadi, itu mengungkapkan menjadi pendekatan cara kerja untuk mengurangkan beban allostatik dan memulihkan homeostasis lebih efisien pada ayam berumur menyebabkan pembangunan adaptasi, phenotipe yang sihat dan tabah.

ACKNOWLEDGEMENTS

At the outset, I would like to express my sincere appreciation to my dedicated committee members: Prof. Dr. Zulkifli Idrus, Prof. Dr. Abdul Rahman Omar and Prof. Dr. Raha Abdul Rahim for their kind guidance and invaluable support, supervision and friendship. The contributions of Prof. Dr. Mohd Hair Bejo, Assoc. Prof. Dr. Shaik Mohamed Amin Babjee and Assoc. Prof. Dr. Ahmad Mujahid in developing and practicing my research techniques are highly appreciated.

I would like to extend my deep and sincere appreciation to Universiti Putra Malaysia who provided me the graduate research fellowship and enabled me to continue my study in UPM. They have changed my life to the right way.

My special, humble and gracious appreciation is offered to Mr. Punusami, Poultry Unit of the Department of Animal Science for his skills and enthusiastic cooperation during my non-ending farm experiments over and over. His dedication to his work is something that I will always admire.

I also would like to thank all of my past professors from University of Tehran in Iran; they have taught me well and I will be forever grateful. I also must thank my closest helpful kind active ever after friend Mahdi Ebrahimi. Also my dearest friends in Malaysia; Arash Javanmard, Syed Reza Hashemi, Amir Meimandipour, Kurosh Khaledi, Mahdi Moamer, Behnam Kamali, Morteza Karami, Iman Noraee, Tang Siew Ching, Riad Abas, Habibollah Naderi, Syed Mohammad Mirzadeh and back home in Tehran; Naser Ghara and Mahdi Glaider. They have kept me going and have

never allowed me to forget to have a little bit of fun. I would like to thank esteemed postgraduate students in our department namely Lai Pui Wah, Zing Fung Cheng, Heisam, Wing Kit, Abdullah Samii, Mokhtar Mohajer, Reza Tohidi, Syed Mohammad Hashemi, Mohammad Houshmand for their cooperation and moral support during my study. The author owed to those who have made things run smoothly throughout the study.

I would like to express my profound gratitude to my family, for their love, guidance, support and encouragement. I thank my elder brother Alireza Basiji for instilling in me the value of an education. He taught me that talking about great things is not enough and I also need to do that to leave a positive legacy. Thank you for motivating me to stray from the flock on occasion and find my own way. I would especially like to thank my parents for being the best role models of a mother and father. There are no words to express my feelings of thank to you. None of this, absolutely none of it would be possible without you. You are every element I need to survive. Masome Khaton, Mohammad Comando, Ali Shelman and Hasan Gambo, I am forever indebted to you for being always by my side and last but not least I am indebted to the one who breathed when I breathed, smiled and cheered when I need it, the one who was by my side every second, my great dearest TarGol forever after.

I certify that a Thesis Examination Committee has met on 7 January 2010 to conduct the final examination of Abdoreza Soleimani Farjam on his Doctor of Philosophy thesis entitled "Heat shock proteins as modifying factors in physiological stress responses in poultry" in accordance with Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the degree of Doctor of Philosophy (PhD).

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This thesis was submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institutions.

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ABDOREZA SOLEIMANI FARJAM

Date: 7 January 2011

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