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Biochemical and molecular investigation of thermal manipulation protocols during broiler embryogenesis and subsequent thermal challenge

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Abstract

Background: The aim of the current study was to evaluate the effect of different thermal manipulation (TM) protocols during embryogenesis on thermotolerance acquisition parameters during subsequent thermal challenge (TC) at posthatch day 28. A total of 1500 fertile chicken eggs were divided randomly into five treatments (300 eggs each): control was maintained at 37.8 °C and 56 % relative humidity (RH) whereas, TM₁, TM₂, TM₃ and TM₄ were subjected to 38.5, 39, 39.5 and 40 °C for 18 h and 65 % RH daily during embryonic days ED 12–18. Hatched chicks from each treatment group allocated randomly into two sub-treatment groups (thermo-neutral, naïve (TN) and thermal challenge (TC). At day 28 of age, chicks subjected to TC by adjusting room temperature to 42 °C for 6 h while naïve chicks kept under regular conditions (22 ± 1 °C and 50–60 % RH). Chick's response to TC evaluated by determination of plasma T₃, T₄, corticosterone, total proteins, albumin, selected enzymes and some electrolytes at the beginning (0 h) and after 1, 3 and 5 h of TC in TM and TN chicks. Furthermore, pectoral and thigh muscles mRNA expression of Atrogin-1, CK, avUCP, DIO3, DIO2 were evaluated in TC and TN sub-treatment groups.

Results: TM induced a significant reduction in free T₃ and elevation in total proteins and albumin in plasma with significant down-regulation of Atrogin-1 and DIO2 and significant up-regulation of DIO3 mRNA expression in muscle of TM chicks compare to control. During TC at day 28, decrease in the concentrations of plasma free T₃, total proteins and albumin with increase in T₄ have been detected in control and TM chicks. TC induced up-regulation of Atrogin-1 and DIO3 with down-regulation of DIO2 gene expression in muscles of all TC chicks.

Conclusion: The present study indicated that, TM improved thermotolerance acquisition by decreasing basal metabolic rate and muscle injury during thermal stress. Basal metabolic rate decreased via reduction of plasma T₃ concentration with up and down regulation of expression of DIO3 and DIO2, respectively in muscles. Muscle injury protected by stimulation of protein biosynthesis and down-regulation of Atrogin-1 expression.

Keywords: Thermal manipulation, Embryogenesis, Heat stress, Gene expression, Muscles, Plasma, Biochemistry

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Background

The detrimental effects of high ambient temperature on broiler performance have been extensively investigated [1, 2]. Several management protocols proposed to improve the thermotolerance acquisition in birds. Thermal manipulation (TM) at early and late stages of broiler embryogenesis is one of the most modern management model applied to improve the thermotolerance acquisition in broilers chickens [3–11]. TM using several temperatures (38.5, 39.0, 39.5 and 41.0 °C) for different durations (2, 3, 6, 12 and 24 h) during the incubation period has been investigated [4–11]. Blood biochemistry such as serum concentrations of glucose, ions, proteins, thyroid hormones, some enzyme activities and corticosterone considered as useful biomarkers of stress response [1, 12]. The ALT and CK activities increased in heat stressed chickens compare to control [13] whereas, serum total protein and albumin concentrations reduced in TM local chicks compare to control [14]. TM induced significant long-term effects on thyroid hormone metabolism by decrease the muscle mRNA expression of DIO3 and increase DIO2 mRNA expression in the liver [15]. Furthermore, a significant increase in muscle mRNA levels of Hsp70 during embryogenesis and during TC in posthatch chicks has been reported [3]. However, the effect of TM during embryogenesis and subsequent TC at posthatch day 28 on biochemical parameters and mRNA expression of stress related genes is not completely elucidated. In addition, the exact molecular and cellular bases for the improvement of the thermotolerance acquisition of the thermally manipulated chicks during thermal challenge are not fully understood.

Therefore, the objectives of the current study were to evaluate (a) the effect of TM at 38.5 °C, 39 °C, 39.5 °C and 40 °C for 18 h and 65 % RH daily during ED12–18 on selected biochemical parameters in plasma (free and bound thyroid hormones, total proteins, albumin, corticosterone, ALT, AST, ALP, CK, GGT, calcium, magnesium, sodium, potassium and chloride) and differential mRNA expression of Atrogin-1, CK, avUCP, DIO3, DIO2 in muscles of broiler chickens (b) a TM chick's ability to cope with TC at posthatch day 28 as reflected on the above mentioned biochemical parameters when compared with control birds.

Methods

Incubating and hatching management

All experimental procedures and management conditions used in this study were approved by the King Faisal University Animal Care and Use Committee (KFU-ACUC). One thousand and seven hundred fertile Ross 315 broiler eggs obtained from one breeder flock (Riyadh, Saudi Arabia) were incubated in semi-commercial incubators

(type OVA-Easy 380 Advance Series II, Brinsea, Sandford, UK). One thousand and five hundred normal eggs selected for an initial weight 62 ± 2 g. The selected eggs were then divided into five incubation treatment groups (300 each): control group was maintained at 37.8 °C 56 % relative humidity (RH) throughout the incubation period; TM₁ was subjected to TM at 38.5 °C for 18 h and 65 % RH daily during ED12–18; TM₂ was subjected to TM at 39 °C for 18 h and 65 % RH daily during ED12–18; TM₃ was subjected to TM at 39.5 °C for 18 h and 65 % RH daily during ED12–18 and TM₄ was subjected to TM at 40 °C for 18 h and 65 % RH daily during ED12–18. At hatching, chicks were distributed in floor pens at 32 °C and the temperature was gradually decreased to 21 °C at day 24 and maintained at 21 °C until day 35. Water and feed supplied to the chicks *ad libitum*. After feather drying, the one-day old chicks transferred to Veterinary Research Station, King Faisal University, Saudi Arabia for conduction of the field experiment.

Thermal manipulation and heat challenge

Chicks from each treatment group divided randomly into two sub-treatment groups, Thermo-neutral (TN) and thermally challenged (TC). At post hatched day 28, forty chicks, randomly selected from the TC group were thermally challenged by adjusting room temperature to 42 °C for 6 h, respectively. TN chicks kept under regular condition (22 ± 1 °C and 50–60 % RH) in a separated room. At the beginning (0 h) and after 1, 3 and 5 h of TC, whole blood was drawn from the wing (brachial) vein from 5 chicks per treatment group and placed in an EDTA containing tubes. Identical samplings and measurements also conducted using the TN chicks as control. The harvested plasma were used for determination of free and bound thyroid hormones (T₃, T₄), corticosterone, selected enzyme activities (AST, ALT, ALP, CK, CGT), protein pattern (total protein and albumin) and electrolytes concentrations (Ca⁺, Mg⁺, Na⁺, Cl).

Biochemical analysis of samples

Plasma was isolated by centrifugation of whole blood samples at 5,000 g for 10 min. Isolated plasma samples were then stored at -20 °C until further analysis. Commercial ELISA kits of Biocheck, Foster city, CA, USA were used for determination of total T₃ (BC-1005), free T₃ (BC-1006), total T₄ (BC-1007), free T₄ (BC-1008) and corticosterone on ELISA reader (Absorbance Microplate Reader ELx 800TM BioTek®, USA). The results calculated according to the manufacturer's instruction. In addition, commercial kits of Reactvos biolab, Spain were used for determination of calcium, magnesium, potassium, sodium and chloride on EasyLyte Plus analyser (Medica corporation, Bedford, MA, USA). The commercial kits of Arab company for medical

diagnostics (ARCOMEX), Jordan, were used for determination of the concentrations of total proteins and albumin and activities of ALP and CK whereas commercial diagnostic kits of Biosystem, Spain were used for estimation of ALT and AST activities on chemistry analyser and the results were calculated according to the manufacturer's instruction.

RNA isolation and semi-quantitative real time RT-PCR analyses

Pectoral and thigh muscles mRNA expressions of Atrogin1, Creatine kinase (CK), avian Uncoupling protein (avUCP), Deiodinase, iodothyronine, type III (DIO3), Deiodinase, iodothyronine, type II (DIO2) were evaluated using the semi-quantitative real time RT-PCR analyses. Pectoral and thigh muscles samples collected from 200 chicks (5 chicks from each treatment group per time point) at day 28 of age. Total RNA extracted from the muscle tissues, homogenated using the TRIzol/choloroform/isopropanol method followed by the removal of supernatants. The RNA pellet dissolved in diethylpyrocarbonate treated RNase free water (Ambion, Austin, TX, USA). DNA removed using DNase I kit (Ambion) and the RNA samples checked for concentration and purity (260:280 nm absorbency). RNA (2 µg) was reverse transcribed to cDNA in a reaction mixture using iScriptcDNA synthesis Kit BIO-RAD, Hercules, CA, USA). The cDNA used for semi-quantitative real-time RT-PCR using the CFX96 Touch™ Real time PCR (BIO-RAD). The semi-quantitative real time RT-PCR performed by the ssoAdvanced™ SYBR Green Supermix kit (BIO-RAD). Briefly, the 20 µl reaction mix was prepared from 10 µl of master mix; 2 µl forward primer (10 pmol); 2 µl revers primer (10 pmol); 2 µl cDNA of the sample and 4 µl of nuclease-free water. Cycling parameters were 50 °C for 2 min, 95 °C for 15 min, 40 cycles of 95 °C for 10 s, followed by 30 s at 57 °C and 72 °C for 10 s with final melting at 95 °C for 20 s. Duplicates from each cDNA analyzed, fluorescence emission detected and relative quantification calculated automatically.

Primers designing

Sequences of broiler Atrogin1, Creatine kinase (CK), Avian uncoupling protein (avUCP), deiodinase, iodothyronine, type III (DIO3), deiodinase, iodothyronine, type II (DIO2), and GAPDH were obtained from the gene bank (accession no: NM_00103956, NM_205507.1, AF433170.2, NM_001122648, NM_204114.3, NM_204305.1). Using these sequences, primers designed for each gene for the semi-quantitative real time RT-PCR analyses. All primers were designed using IDT Primer quest software (<http://eu.idtdna.com/Primerquest/Home/Index>). The sequences of the primers showed in Table 1.

Table 1 Primers for semi-quantitative real time RT-PCR analyses

Gene	Sequence (5'- 3')
GAPDH	GTGTTATCATCTCAGTCCCTCAG GGTCATAAGACCCCTCCACAATG
Atrogin1	CGCAAACGGCTAATCCTATCT CTTCCGTGGGTAACACCTTATG
CK	TCTGGCACAACGACAACAA CCGGAAGACCTCCTTCATATTTTC
avUCP	GCCCCGAACCTCCATCATTAA GGGACGTTGTCTGTGCATCAG
DIO3	GCAGAAGCTGGACTTCTTCA CTGCCGAAGTTGAGGATGAG
DIO2	GGCTGACTTTCTGTTGGTCTAC CTTCTGATTCTGTGCTTCTT

Statistical analysis

All statistical analyses performed using IBM SPSS statistics 21 software for Mac OS (IBM software, Chicago, USA). Data for the selected biochemical parameters, changes in control and TM treatment groups were analyzed by using One-way analysis of variance (ANOVA) followed by all-pairs Bonferroni test was used to compare different parameter and analysis of covariance (ANCOVA) for repeated measures, with the within-subject factors "treatment groups" and "time" (four time points (0, 1, 3 and 5 h of TC). Differences considered significant at $P < 0.05$.

Results

The effects of TC (42 °C for 6 h; posthatch day 28) during 4 time points (at the beginning (0 h) and after 1, 3 and 5 h of TC on plasma free and bound thyroid hormones (T_3 and T_4) of broiler chicks subjected to different TM protocols at ED 12–18 showed in Table 2. The summarized data indicated that, a significant decrease ($p < 0.05$) in free T_3 concentration without changes in free T_4 level has been reported in plasma of TM chicks compare to controls. The decrease of free T_3 concentration in TM chicks does not influenced by changes in TM protocols (TM1-4). During TC at day 28, a significant decrease in plasma free T_3 and increase in free T_4 have been observed in control and TM chicks. Although, TC induced reduction in free T_3 concentration of all TC chicks, this decrease was higher in TM birds compare to control. However, the concentrations of free T_4 in plasma of TM birds were comparable to those of TN, control birds in spite of TC. TM and TC did not affect the concentrations of bound T_3 and T_4 throughout the experimental period.

The data of Table 3 showed the effect of TC (42 °C for 6 h; posthatch day 28) during 4 time points (at the beginning and after 1, 3 and 5 h of TC) on total proteins, albumin and corticosterone of broiler chicks subjected to different TM protocols at ED 12–18. These findings indicated that, TM induced a significant increase in plasma total proteins and albumin of TM chicks compared to TN chicks, control. The increase of total proteins and albumin values in TM chicks does not

Table 2 The effect of TC (42 °C for 6 h; posthatch day 28) during 4 time terminals (at the beginning and after 1, 3 and 5 h of TC) on plasma free and bound thyroid hormones (T₃ and T₄) of broiler chicks subjected to different TM protocols at ED 12–18 (n = 5)

Parameters	Groups	Treatments				
		Control	TM ₁	TM ₂	TM ₃	TM ₄
Free T ₃	TN	3.63 ± 0.16 ^{aw}	2.66 ± 0.29 ^{bw}	2.70 ± 0.11 ^{bw}	2.65 ± 0.13 ^{bw}	2.60 ± 0.16 ^{bw}
	TC ₁	2.43 ± 0.28 ^{ax}	1.33 ± 0.16 ^{bx}	1.32 ± 0.29 ^{bx}	1.79 ± 0.25 ^{bx}	1.65 ± 0.28 ^{bx}
	TC ₃	2.47 ± 0.20 ^{ax}	1.47 ± 0.14 ^{bx}	1.40 ± 0.31 ^{bx}	1.79 ± 0.15 ^{bx}	1.67 ± 0.16 ^{bx}
	TC ₅	2.81 ± 0.30 ^{ax}	1.41 ± 0.14 ^{bx}	1.42 ± 0.27 ^{bx}	1.72 ± 0.12 ^{bx}	1.81 ± 0.23 ^{bx}
	Bound T ₃	TN	4.05 ± 0.01 ^{aw}	3.90 ± 0.29 ^{aw}	3.75 ± 0.35 ^{aw}	3.68 ± 0.54 ^{aw}
Bound T ₃	TC ₁	3.57 ± 0.51 ^{aw}	3.68 ± 0.78 ^{aw}	3.57 ± 0.23 ^{aw}	3.79 ± 0.24 ^{aw}	3.54 ± 0.28 ^{aw}
	TC ₃	3.50 ± 0.53 ^{aw}	3.62 ± 0.78 ^{aw}	3.96 ± 0.03 ^{aw}	3.96 ± 0.59 ^{aw}	3.65 ± 0.60 ^{aw}
	TC ₅	3.94 ± 0.40 ^{aw}	3.89 ± 0.89 ^{aw}	3.76 ± 0.07 ^{aw}	3.79 ± 0.33 ^{aw}	3.67 ± 0.91 ^{aw}
Free T ₄	TN	0.21 ± 0.12 ^{aw}	0.29 ± 0.14 ^{aw}	0.34 ± 0.12 ^{aw}	0.30 ± 0.13 ^{aw}	0.38 ± 0.18 ^{aw}
	TC ₁	0.69 ± 0.10 ^{ax}	0.69 ± 0.16 ^{ax}	0.40 ± 0.13 ^{ax}	0.50 ± 0.12 ^{ax}	0.51 ± 0.13 ^{ax}
	TC ₃	0.52 ± 0.13 ^{ax}	0.58 ± 0.14 ^{ax}	0.59 ± 0.21 ^{ax}	0.58 ± 0.17 ^{ax}	0.56 ± 0.11 ^{ax}
	TC ₅	0.54 ± 0.12 ^{ax}	0.51 ± 0.13 ^{ax}	0.56 ± 0.11 ^{ax}	0.56 ± 0.11 ^{ax}	0.55 ± 0.11 ^{ax}
	Bound T ₄	TN	2.56 ± 0.13 ^{aw}	2.19 ± 0.45 ^{aw}	2.87 ± 0.61 ^{aw}	2.65 ± 0.42 ^{aw}
Bound T ₄	TC ₁	2.56 ± 0.46 ^{aw}	2.88 ± 0.17 ^{aw}	2.67 ± 0.32 ^{aw}	2.86 ± 0.55 ^{aw}	2.62 ± 0.20 ^{aw}
	TC ₃	2.02 ± 0.57 ^{aw}	2.71 ± 0.47 ^{aw}	2.71 ± 0.23 ^{aw}	2.33 ± 0.51 ^{aw}	2.78 ± 0.25 ^{aw}
	TC ₅	2.98 ± 0.54 ^{aw}	2.30 ± 0.34 ^{aw}	2.94 ± 0.16 ^{aw}	2.95 ± 0.01 ^{aw}	2.45 ± 0.41 ^{aw}

Control 37.8 °C, TM₁ Thermal manipulation at 38.5 °C for 18 h, TM₂ Thermal manipulation at 39 °C for 18 h, TM₃ Thermal manipulation at 39.5 °C for 18 h, TM₄ Thermal manipulation at 40 °C for 18 h, TC Thermal challenged

^{a-b} within rows, means ± SD with different superscripts differ significantly (P < 0.05)

^{w-x} Between naive and TC chicks within a parameter, means ± SD with different superscripts differ significantly (P < 0.05)

influenced by changes in TM protocols (TM1-4). TC at day 28 induced significant reduction in total proteins and albumin in TM and control chicks. Although, TC induced reduction in total proteins and albumin concentrations of all TC chicks, this decrease was higher in

control birds than TM birds. TM and TC did not affect the concentration of corticosterone throughout the experimental period (Table 3).

The effect of TC (42 °C for 6 h; posthatch day 28) during 4 time points (at the beginning and after 1, 3 and

Table 3 The effect of TC (42 °C for 6 h; posthatch day 28) during 4 time terminals (at the beginning and after 1, 3 and 5 h of TC) on plasma protein pattern and cortisol of broiler chicks subjected to different TM protocols at ED 12–18 (n = 5)

Parameters	Groups	Treatments				
		Control	TM ₁	TM ₂	TM ₃	TM ₄
Total proteins	TN	28.4 ± 0.3 ^{aw}	37.5 ± 1.1 ^{bw}	36.0 ± 1.3 ^{bw}	35.0 ± 1.4 ^{bw}	37.1 ± 1.4 ^{bw}
	TC ₁	21.9 ± 0.1 ^{ax}	27.2 ± 1.0 ^{bx}	27.5 ± 0.9 ^{bx}	26.6 ± 1.1 ^{bx}	26.8 ± 1.6 ^{bx}
	TC ₃	22.7 ± 0.2 ^{ax}	27.4 ± 1.1 ^{bx}	27.5 ± 1.1 ^{bx}	26.7 ± 1.0 ^{bx}	27.5 ± 1.2 ^{bx}
	TC ₅	22.6 ± 1.5 ^{ax}	29.6 ± 0.6 ^{bx}	28.0 ± 1.0 ^{bx}	28.1 ± 1.0 ^{bx}	28.2 ± 0.8 ^{bx}
	Albumin	TN	17.5 ± 0.6 ^{aw}	20.2 ± 1.1 ^{bw}	20.2 ± 1.0 ^{bw}	20.3 ± 1.2 ^{bw}
Albumin	TC ₁	12.0 ± 0.2 ^{ax}	15.5 ± 0.5 ^{bx}	15.4 ± 0.3 ^{bx}	14.0 ± 0.7 ^{bx}	14.8 ± 0.4 ^{bx}
	TC ₃	12.1 ± 0.2 ^{ax}	15.2 ± 0.8 ^{bx}	15.9 ± 0.7 ^{bx}	14.7 ± 1.0 ^{bx}	14.9 ± 1.0 ^{bx}
	TC ₅	12.4 ± 0.1 ^{ax}	15.1 ± 0.7 ^{bx}	14.7 ± 0.4 ^{bx}	14.6 ± 1.7 ^{bx}	15.1 ± 2.3 ^{bx}
Corticosterone	TN	0.21 ± 0.03 ^{aw}	0.21 ± 0.02 ^{aw}	0.22 ± 0.01 ^{aw}	0.22 ± 0.03 ^{aw}	0.20 ± 0.02 ^{aw}
	TC ₁	0.21 ± 0.00 ^{aw}	0.20 ± 0.01 ^{aw}	0.21 ± 0.01 ^{aw}	0.22 ± 0.05 ^{aw}	0.21 ± 0.03 ^{aw}
	TC ₃	0.21 ± 0.01 ^{aw}	0.21 ± 0.11 ^{aw}	0.21 ± 0.01 ^{aw}	0.21 ± 0.12 ^{aw}	0.21 ± 0.01 ^{aw}
	TC ₅	0.22 ± 0.05 ^{aw}	0.21 ± 0.05 ^{aw}	0.20 ± 0.01 ^{aw}	0.21 ± 0.05 ^{aw}	0.22 ± 0.01 ^{aw}

Control 37.8 °C, TM₁ Thermal manipulation at 38.5 °C for 18 h, TM₂ Thermal manipulation at 39 °C for 18 h, TM₃ Thermal manipulation at 39.5 °C for 18 h, TM₄ Thermal manipulation at 40 °C for 18 h, TC Thermal challenged

^{a-b} within rows, means ± SD with different superscripts differ significantly (P < 0.05)

^{w-x} Between naive and TC chicks within a parameter, means ± SD with different superscripts differ significantly (P < 0.05)

5 h of TC) on plasma ALT, AST, GGT and CK activities of broiler chicks subjected to different TM protocols at ED 12–18 is summarized in Table 4. The data indicated that, neither TM nor TC at day 28 resulted in changes in the activities of these enzymes throughout the experimental period (Table 4).

The data of Table 5 showed the effect of TC (42 °C for 6 h; posthatch day 28) during 4 time points (at the beginning and after 1, 3 and 5 h of TC) on the concentrations of calcium, magnesium, sodium, potassium and chloride of broiler chicks subjected to different TM protocols at ED 12–18. This data indicated that, during TC at day 28, the concentrations of these elements were comparable in all treatments including the control. Furthermore, TM did not affect the concentrations of these elements in all treatments.

The effect of TC (42 °C for 6 h; posthatch day 28) during 4 time terminals (at the beginning and after 1, 3 and 5 h of TC) on relative mRNA expression of Atrogin-1, cKM, avUCP, DIO3 and DIO2 in the muscles of broiler chicks subjected to different TM protocols at ED 12–18 are shown in Table 6. This data indicated that, TM induced a significant down-regulation of Atrogin-1 and DIO2 and significant up-regulation of gene expression of DIO3 in muscle of TM chicks compared to TN chicks, control without any significant changes among TM protocols (TM1-4). TC induced up-regulation of

gene expression of Atrogin-1 and DIO3 and down-regulation of gene expression of DIO2 in control and TM chicks. Although, TC induced up-regulation of Atrogin-1 and DIO3 and down-regulation of DIO2 expression in muscle of all TC chicks, the up-regulation of Atrogin-1 and DIO3 and down-regulation of DIO2 expression in muscle of TM birds were higher than that of control. TM and TC did not affect the gene expression of CKM and avUCP in muscles of investigated chicks.

Discussion

The importance of thyroid gland hormones in adaptation to heat stress is related to the central role that thyroid hormones play in the regulation of metabolic rate of birds [16]. This effect has been demonstrated by thyroid hormone administration, which stimulates heat production [17] and by surgical or chemical thyroidectomy of chicken, which produces a decrease in metabolic rate and body temperature [18]. The current findings indicated that TM during embryogenesis was able to affect the thermoregulatory events of broiler chickens, which resulted in a lowered free T₃ in TM chicks compared with control. In addition, TC at posthatch day 28 reduced free T₃ in TM and control birds. It is well known that, the epigenetic heat adaptation involves changes in blood contents, hormonal and metabolic reaction that enhances heat endurance [14]. Parallel to the current

Table 4 The effect of TC (42 °C for 6 h; posthatch day 28) during 4 time terminals (at the beginning and after 1, 3 and 5 h of TC) on selected enzymes activities of broiler chicks subjected to different TM protocols at ED 12–18 (n = 5)

Parameters	Groups	Treatments				
		Control	TM ₁	TM ₂	TM ₃	TM ₄
ALT	TN	5.5 ± 1.7 ^{aw}	6.5 ± 1.9 ^{aw}	5.5 ± 0.7 ^{aw}	7.0 ± 1.4 ^{aw}	6.5 ± 0.7 ^{aw}
	TC ₁	6.0 ± 2.4 ^{aw}	8.5 ± 2.1 ^{aw}	8.0 ± 1.9 ^{aw}	9.5 ± 1.5 ^{aw}	8.5 ± 1.5 ^{aw}
	TC ₃	7.5 ± 0.4 ^{aw}	9.0 ± 1.7 ^{aw}	7.5 ± 0.7 ^{aw}	8.5 ± 0.7 ^{aw}	8.5 ± 1.6 ^{aw}
	TC ₅	5.5 ± 2.1 ^{aw}	6.0 ± 1.6 ^{aw}	8.5 ± 1.5 ^{aw}	9.0 ± 1.6 ^{aw}	9.5 ± 1.7 ^{aw}
AST	TN	80.5 ± 0.7 ^{aw}	75.8 ± 4.1 ^{aw}	76.5 ± 3.5 ^{aw}	83.5 ± 2.3 ^{aw}	83.5 ± 6.4 ^{aw}
	TC ₁	80.5 ± 6.4 ^{aw}	76.5 ± 7.6 ^{aw}	80.0 ± 1.4 ^{aw}	83.5 ± 2.0 ^{aw}	81.0 ± 8.5 ^{aw}
	TC ₃	85.0 ± 2.8 ^{aw}	70.0 ± 1.2 ^{aw}	77.0 ± 1.4 ^{aw}	72.0 ± 2.9 ^{aw}	76.0 ± 4.2 ^{aw}
GGT	TN	10.5 ± 3.5 ^{aw}	11.5 ± 0.7 ^{aw}	11.5 ± 0.7 ^{aw}	12.5 ± 0.7 ^{aw}	11.0 ± 1.4 ^{aw}
	TC ₁	11.5 ± 0.7 ^{aw}	11.5 ± 0.7 ^{aw}	11.8 ± 0.6 ^{aw}	10.5 ± 2.1 ^{aw}	12.5 ± 0.7 ^{aw}
	TC ₃	11.5 ± 1.0 ^{aw}	12.0 ± 0.7 ^{aw}	12.5 ± 0.7 ^{aw}	12.0 ± 1.4 ^{aw}	12.5 ± 0.7 ^{aw}
	TC ₅	11.5 ± 0.9 ^{aw}	9.5 ± 2.1 ^{aw}	12.5 ± 0.5 ^{aw}	10.0 ± 1.2 ^{aw}	11.0 ± 0.9 ^{aw}
CK	TN	1276 ± 79 ^{aw}	1277 ± 100 ^{aw}	1394 ± 98 ^{aw}	1239 ± 99 ^{aw}	1293 ± 141 ^{aw}
	TC ₁	1202 ± 80 ^{aw}	1314 ± 108 ^{aw}	1182 ± 150 ^{aw}	1135 ± 161 ^{aw}	1205 ± 138 ^{aw}
	TC ₃	1310 ± 100 ^{aw}	1410 ± 100 ^{aw}	1253 ± 138 ^{aw}	1239 ± 163 ^{aw}	1285 ± 124 ^{aw}
	TC ₅	1239 ± 95 ^{aw}	1307 ± 150 ^{aw}	1257 ± 96 ^{aw}	1205 ± 144 ^{aw}	1265 ± 90 ^{aw}

Control 37.8 °C, TM₁ Thermal manipulation at 38.5 °C for 18 h, TM₂ Thermal manipulation at 39 °C for 18 h, TM₃ Thermal manipulation at 39.5 °C for 18 h, TM₄ Thermal manipulation at 40 °C for 18 h, TC Thermal challenged

^{a-d} within rows, means ± SD with different superscripts differ significantly (P < 0.05)

^{wz} Between naïve and TC chicks within a parameter, means ± SD with different superscripts differ significantly (P < 0.05)

Table 5 The effect of TC (42 °C for 6 h; posthatch day 28) during 4 time terminals (at the beginning and after 1, 3 and 5 h of TC) on selected plasma electrolytes concentrations of broiler chicks subjected to different TM protocols at ED 12–18 (n = 5)

Parameters	Groups	Treatments				
		Control	TM ₁	TM ₂	TM ₃	TM ₄
Calcium	TN	13.5 ± 0.7 ^{aw}	13.0 ± 1.4 ^{aw}	12.8 ± 0.3 ^{aw}	12.5 ± 0.7 ^{aw}	11.9 ± 2.6 ^{aw}
	TC ₁	12.6 ± 0.6 ^{aw}	12.9 ± 1.8 ^{aw}	12.7 ± 0.5 ^{aw}	11.5 ± 3.3 ^{aw}	12.0 ± 0.0 ^{aw}
	TC ₃	13.8 ± 1.2 ^{aw}	13.0 ± 1.4 ^{aw}	13.5 ± 0.7 ^{aw}	11.9 ± 1.8 ^{aw}	12.4 ± 0.5 ^{aw}
	TC ₅	12.5 ± 0.8 ^{aw}	12.0 ± 1.2 ^{aw}	13.5 ± 3.0 ^{aw}	12.8 ± 0.8 ^{aw}	13.0 ± 2.2 ^{aw}
Magnesium	TN	4.1 ± 1.4 ^{aw}	4.0 ± 1.1 ^{aw}	3.8 ± 1.5 ^{aw}	4.9 ± 1.6 ^{aw}	4.2 ± 1.6 ^{aw}
	TC ₁	3.9 ± 1.1 ^{aw}	3.5 ± 1.3 ^{aw}	4.2 ± 1.4 ^{aw}	3.2 ± 1.5 ^{aw}	3.9 ± 1.1 ^{aw}
	TC ₃	3.7 ± 1.3 ^{aw}	4.1 ± 1.2 ^{aw}	3.7 ± 1.1 ^{aw}	3.7 ± 1.3 ^{aw}	3.2 ± 1.6 ^{aw}
	TC ₅	4.0 ± 1.1 ^{aw}	4.6 ± 1.8 ^{aw}	4.0 ± 1.6 ^{aw}	4.7 ± 1.1 ^{aw}	5.4 ± 1.8 ^{aw}
Sodium	TN	146.3 ± 5.9 ^{aw}	147.9 ± 1.6 ^{aw}	142.5 ± 3.5 ^{aw}	142.4 ± 0.8 ^{aw}	146.5 ± 6.4 ^{aw}
	TC ₁	142.5 ± 3.5 ^{aw}	137.7 ± 1.7 ^{aw}	139.3 ± 2.0 ^{aw}	138.5 ± 2.1 ^{aw}	142.5 ± 5.8 ^{aw}
	TC ₃	139.6 ± 5.2 ^{aw}	140.7 ± 2.1 ^{aw}	141.5 ± 0.7 ^{aw}	141.7 ± 6.6 ^{aw}	140.0 ± 4.2 ^{aw}
	TC ₅	138.0 ± 6.9 ^{aw}	143.6 ± 3.7 ^{aw}	138.1 ± 5.0 ^{aw}	142.2 ± 9.5 ^{aw}	143.6 ± 3.6 ^{aw}
Potassium	TN	9.7 ± 1.3 ^{aw}	10.4 ± 1.4 ^{aw}	10.5 ± 0.2 ^{aw}	9.8 ± 1.3 ^{aw}	9.4 ± 1.4 ^{aw}
	TC ₁	10.3 ± 1.5 ^{aw}	9.4 ± 1.1 ^{aw}	10.9 ± 1.0 ^{aw}	9.4 ± 1.6 ^{aw}	9.8 ± 1.4 ^{aw}
	TC ₃	9.5 ± 1.6 ^{aw}	10.7 ± 1.3 ^{aw}	10.3 ± 1.2 ^{aw}	9.7 ± 1.1 ^{aw}	11.7 ± 1.4 ^{aw}
	TC ₅	9.2 ± 1.3 ^{aw}	9.8 ± 1.3 ^{aw}	8.8 ± 2.6 ^{aw}	8.8 ± 1.4 ^{aw}	9.9 ± 1.8 ^{aw}
Chloride	TN	113.0 ± 1.8 ^{aw}	112.5 ± 2.1 ^{aw}	114.0 ± 1.1 ^{aw}	110.5 ± 2.9 ^{aw}	107.5 ± 7.5 ^{aw}
	TC ₁	116.5 ± 3.7 ^{aw}	109.5 ± 4.8 ^{aw}	116.0 ± 1.4 ^{aw}	113.0 ± 1.8 ^{aw}	112.0 ± 3.1 ^{aw}
	TC ₃	118.5 ± 3.9 ^{aw}	116.0 ± 1.4 ^{aw}	116.0 ± 1.4 ^{aw}	115.5 ± 2.1 ^{aw}	114.5 ± 2.1 ^{aw}
	TC ₅	114.5 ± 0.5 ^{aw}	112.0 ± 0.9 ^{aw}	112.0 ± 0.7 ^{aw}	114.5 ± 3.5 ^{aw}	115.0 ± 1.4 ^{aw}

Control 37.8 °C, TM₁ Thermal manipulation at 38.5 °C for 18 h, TM₂ Thermal manipulation at 39 °C for 18 h, TM₃ Thermal manipulation at 39.5 °C for 18 h, TM₄ Thermal manipulation at 40 °C for 18 h, TC Thermal challenged

^{a-d} within rows, means ± SD with different superscripts differ significantly ($P < 0.05$)

^{w-z} Between naïve and TC chicks within a parameter, means ± SD with different superscripts differ significantly ($P < 0.05$)

findings, the concentration of T₃ reduced in heat acclimation treated than control broilers [19]. In consistent with the present study, TC (41°C/6 h on days 3, 7 and 42 of age) caused a reduction in T₃ level in TC chicks compared with TN chicks [3]. The current finding indicated that TC at posthatch day 28 increased T₄ level in TC chicks compared with TN chicks. This finding come in accordance with the previous results [3] which demonstrated that, TC (41°C/6 h on days 3, 7 and 42 of age) caused an increase in T₄ level in TM chicks compared with TN chicks. The current findings also indicate that, TM at ED (12–18) induced significant reduction in T₃ level without changes in T₄ level in TM birds compare to control. This finding agrees with previous report [3] with regard to T₃.

TM resulted in significant increases in plasma total proteins and albumin of TM chicks compared to control chicks. Although, TC at posthatch day 28 induced a significant reduction of both plasma total proteins and albumin concentrations in TM and control birds, the concentrations of these proteins in TM birds were still higher than that of control. This evidenced that, TM

modulates the thermoregulation process during embryogenesis and posthatching stages. Previously, significant reduction in plasma total protein and albumin was observed when embryos were exposed to high incubation temperature than the control [20]. In addition, it is well known that, heat stress leads to delay the synthesis of most proteins except heat shock proteins [21]. Serum total protein and albumin concentrations were significantly decreased in one day hatched local Egyptian chicks subjected to TM (40 °C; RH 55 %; ED 14–17) compared to control [14]. As reported in the current study, the increment of total proteins and albumin concentrations in TM birds can considered as a sort of protection of muscle mass against injury induced by TC.

Publications about the effect of heat stress on enzymes activities are contradictory, possibly due to the large variability of the enzymes [22, 23]. The current findings indicated that, TM did not change the activities of examined enzymes activities throughout the experimental period. Similarly, subsequent TC at posthatch day 28 did not affect the enzyme activities of TM and control birds. According to the obtained results, heat exposure did not

Table 6 The effect of TC (42 °C for 6 h; posthatch day 28) during 4 time terminals (at the beginning and after 1, 3 and 5 h of TC) on relative mRNA levels of Atrogin-1, cKM, avUCP, DIO3 and DIO2 in the Muscle of broiler chicks subjected to different TM protocols at ED 12–18 ($n = 5$)

Parameters	Groups	Treatments				
		Control	TM ₁	TM ₂	TM ₃	TM ₄
Atrogin-1	TN	1.00 ± 0.05 ^{aw}	0.20 ± 0.01 ^{bw}	0.26 ± 0.05 ^{bw}	0.28 ± 0.08 ^{bw}	0.30 ± 0.09 ^{bw}
	TC ₁	10.65 ± 0.02 ^{ax}	5.60 ± 1.06 ^{bx}	6.40 ± 1.14 ^{bx}	5.52 ± 1.04 ^{bx}	6.61 ± 1.05 ^{bx}
	TC ₃	20.13 ± 0.82 ^{ay}	10.99 ± 1.50 ^{by}	9.52 ± 1.08 ^{by}	9.11 ± 1.88 ^{by}	9.66 ± 1.44 ^{by}
	TC ₅	28.30 ± 5.40 ^{az}	16.02 ± 1.10 ^{bz}	15.21 ± 1.34 ^{bz}	16.11 ± 1.40 ^{bz}	15.96 ± 1.21 ^{bz}
cKM	TN	1.00 ± 0.04 ^{aw}	1.29 ± 0.03 ^{aw}	1.25 ± 0.03 ^{aw}	1.25 ± 0.03 ^{aw}	1.36 ± 0.02 ^{aw}
	TC ₁	1.60 ± 0.52 ^{ax}	1.33 ± 0.33 ^{aw}	1.95 ± 0.81 ^{ax}	1.95 ± 0.88 ^{ax}	1.66 ± 0.42 ^{aw}
	TC ₃	1.96 ± 0.81 ^{ax}	1.56 ± 0.43 ^{aw}	1.55 ± 0.32 ^{aw}	1.72 ± 0.66 ^{aw}	1.53 ± 0.51 ^{aw}
	TC ₅	1.89 ± 0.84 ^{ax}	1.04 ± 0.22 ^{aw}	1.37 ± 0.63 ^{aw}	1.44 ± 0.32 ^{aw}	1.45 ± 0.40 ^{aw}
avUCP	TN	1.00 ± 0.32 ^{aw}	0.97 ± 0.71 ^{aw}	1.34 ± 0.24 ^{aw}	1.43 ± 0.40 ^{aw}	1.39 ± 0.19 ^{aw}
	TC ₁	1.30 ± 0.21 ^{aw}	1.18 ± 0.31 ^{aw}	1.20 ± 0.11 ^{aw}	1.70 ± 0.51 ^{aw}	1.05 ± 0.21 ^{aw}
	TC ₃	1.87 ± 0.60 ^{ax}	1.30 ± 0.29 ^{aw}	1.08 ± 0.33 ^{aw}	1.37 ± 0.23 ^{aw}	1.33 ± 0.21 ^{aw}
	TC ₅	1.63 ± 0.40 ^{aw}	1.10 ± 0.24 ^{aw}	1.54 ± 0.45 ^{aw}	1.09 ± 0.21 ^{aw}	1.79 ± 0.71 ^{aw}
DIO3	TN	2.00 ± 0.01 ^{aw}	2.36 ± 0.01 ^{bw}	2.69 ± 0.01 ^{bw}	2.63 ± 0.03 ^{bw}	2.78 ± 0.03 ^{bw}
	TC ₁	3.43 ± 0.07 ^{ax}	4.43 ± 0.02 ^{bx}	4.25 ± 0.08 ^{bx}	4.27 ± 0.03 ^{bx}	4.29 ± 0.08 ^{bx}
	TC ₃	3.42 ± 0.06 ^{ax}	4.37 ± 0.10 ^{bx}	4.34 ± 0.03 ^{bx}	4.32 ± 0.05 ^{bx}	4.31 ± 0.02 ^{bx}
	TC ₅	3.56 ± 0.06 ^{ax}	4.32 ± 0.03 ^{bx}	4.38 ± 0.07 ^{bx}	4.34 ± 0.01 ^{bx}	4.37 ± 0.08 ^{bx}
DIO2	TN	4.88 ± 0.01 ^{aw}	2.27 ± 0.01 ^{bw}	2.27 ± 0.03 ^{bw}	2.26 ± 0.02 ^{bw}	2.25 ± 0.03 ^{bw}
	TC ₁	3.17 ± 0.03 ^{ax}	1.87 ± 0.08 ^{bx}	1.90 ± 0.03 ^{bx}	1.89 ± 0.01 ^{bx}	1.13 ± 0.03 ^{bx}
	TC ₃	3.25 ± 0.02 ^{ax}	1.90 ± 0.02 ^{bx}	1.13 ± 0.08 ^{bx}	1.32 ± 0.09 ^{bx}	1.34 ± 0.04 ^{bx}
	TC ₅	3.15 ± 0.03 ^{ax}	1.86 ± 0.01 ^{bx}	1.32 ± 0.03 ^{bx}	1.34 ± 0.04 ^{bx}	1.38 ± 0.04 ^{bx}

^{a-w}Within the same row, means ± SD with different superscripts differ significantly ($P < 0.05$)

^{w-z}Within the same gene, means ± SD with different superscripts differ significantly ($P < 0.05$)

significantly change the enzyme activities in the chicken's serum. This is supported by previous finding reported that, the activities of CK and AST were not influenced even by acute heat exposure [24]. The current findings suggest that virtually no cellular damage, resulting in leakage of intracellular enzymes into the blood, took place. Therefore, the thermotolerance acquisition induced by TM was safe to the birds' tissues.

Publications concerning the effect of high ambient temperature on serum electrolytes status in chickens are contradicting. Although, non-significant changes of all examined electrolytes have been observed in plasma of TM and subsequent TC at posthatch day 28 chicks in the current study, Khone et al. [25] reported increased blood potassium level in heat stress birds whereas, Ait-Boulahsen et al. [26] noted otherwise. Lin et al. [12] reported that, chloride level was increased and potassium level was decreased by high temperature in birds. McDaniel et al. [27, 28] reported a significant decrease of calcium and phosphorus levels in heat stressed birds. Furthermore, Zulkifli et al. [29] reported that serum levels of sodium and chloride were significantly

higher and serum potassium were significantly lower 14 days after heat exposure in broiler chickens.

DIO2 acts as an outer-ring 5'-deiodinase by removing iodine from the 5' outer-ring site to convert T₄ to the more active form T₃, while DIO3 acts as an inner-ring deiodinase 5-deiodinase to remove iodine from the inner-ring of T₄ and T₃ and convert these hormones to inactive forms including reverse triiodothyronine (rT₃) and diiodothyronine (T₂) [15]. The current data indicated that, TM induced a significant down-regulation of DIO2 and significant up-regulation of gene expression of DIO3 in muscle of TM chicks compared to control. Furthermore, TC at posthatch day 28 induced up-regulation of DIO3 and down-regulation of DIO2 gene expression in TM chicks and in control birds however, this effect was higher in TM birds compare to control. The lower DIO2 and higher DIO3 expression in TM birds is consistent with the decrease in plasma T₃ concentration reported in the current study and also with decrease of plasma T₃ reported previously in birds and mammals [30, 31] and fast growing chickens [15]. The persistence of increase of DIO3 and decrease of DIO2

expression in TM birds than control during TC might explain why plasma T₃ concentrations in TM birds were lower than that of control during TC. Nevertheless, TM decreased O₂ consumption of embryos, indicating a potentially lower metabolic rate in these animals [5, 32]. The current study showed that, mRNA expression of DIO3 significantly increased while that of DIO2 significantly decreased in muscle of TM birds. These findings suggest that TM has a long-term overall negative effect on thyroid hormone metabolism in muscle. However, the physiological impact of such changes remains to be determined, since the activity of deiodinase enzymes is not necessarily correlated with their mRNA expression.

Atrogin-1 an E3 ubiquitin ligase also referred to as MAFbx (muscle atrophy F-box), plays a vital role in muscle atrophy [33, 34]. Atrogin-1 expressed only in skeletal muscle. Its expression increased in catabolic conditions that result in muscle atrophy [34, 35]. The current results indicated that, TM induced a significant down-regulation of Atrogin-1 compared to the control. Moreover, TC at posthatch day 28 induced up-regulation of gene expression of this gene in muscle of TM and control chicks. However, the up-regulation of Atrogin-1 expression in muscle of TM birds was lower than that of control. These findings indicated that, all tested TM protocols decrease the effect of muscle degeneration and this is consistent with non-significant changes that observed in enzymes activities of CK. In response to TC, Atrogin-1 was up regulated and this dynamic profile is consistent with previous reports of muscle injury response, whereby the expression of atrogin-1 was concomitant with atrophy and recovery [36]. Atrogin-1 regulated by FOXO transcription factors, via nuclear localization of FOXO in response to various stimuli that inhibit PI3K/Akt (protein Kinase B) signaling and subsequently drive atrophy [37, 38]. The mechanism of down-regulation of Atrogin-1 by TM could be furtherly investigated and studying the phosphorylation of Akt/FOXO axis might be of great benefits. As reported above, the increment of plasma total proteins and albumin concentrations together with down-regulation of Atrogin-1 in muscle of TM birds are two mechanisms that perhaps induced by TM as a sort of protection of muscle mass against possible atrophy induced by TC. The current study demonstrated that TM and subsequent TC did not affect the gene expression of cKM and avUCP in muscles of all investigated chicks. The current findings regarding the cKM are in consistent with the non-significant changes that observed in serum CK enzyme activities of all TC chicks. In addition, the down-regulation of Atrogin-1 and elevation of total proteins confirmed these findings. The non-significant changes of avUCP in muscle of all TC birds might give an indication that, the action

taken by the body of birds subjected to TM was enough to counter act the thermal challenges.

In conclusion, the present study indicated that, TM improved thermotolerance acquisition by decreasing each of basal metabolic rate and muscle atrophy. The decrease in basal metabolic rate induced via reduction of T₃ concentration in plasma with up and down regulation of DIO3 and DIO2, respectively. However, the decrease in muscle atrophy conducted by stimulation of protein biosynthesis and down-regulation of Atrogin-1 gene expression.

Conclusion

In conclusion, our findings strongly indicated that, TM improved thermotolerance acquisition by decreasing basal metabolic rate and muscle injury during thermal stress. Basal metabolic rate decreased via reduction of plasma T₃ concentration with up and down regulation of expression of DIO3 and DIO2, respectively in muscles. Muscle injury protected by stimulation of protein biosynthesis and down-regulation of Atrogin-1 expression.

Abbreviations

TM: Thermal manipulation; TC: Thermal challenge; TN: Thermal-neutral naïve; RH: Relative humidity; ED: Embryonic day; avUCP: Avian uncoupling protein; DIO3: Deiodinase, iodothyronine, type III; DIO2: Deiodinase, iodothyronine, type II; AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; CK: Creatine kinase; CGT: Gamma-glutamyl transferase; T3: 3,5,3-triiodothyronine; T4: Thyroxine; rT3: Reverse triiodothyronine; T2: Diiodothyronine; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; cKM: Creatine kinase muscle.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MA and SE are equally contributed in the study. MA carried out the experimental design and participated in hatching management, TM and heat challenge, sequence alignment, RNA isolation, RT-PCR and drafted the manuscript. SE carried out the biochemical analysis and performed the statistical analysis. SE participated in design of the study and coordination, hatching management, TM and heat challenge, real time RT-PCR, drafted the manuscript and approved the final manuscript. RA and TA participated in RNA isolation, RT-PCR and drafted the manuscript. AD and SA participated in hatching management, TM and heat challenge, sequence alignment, RNA isolation, RT-PCR. All authors read and approved the final manuscript.

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