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Effect of thermal manipulation during embryogenesis on gene expression of myogenic regulatory factors pre and post hatch in broilers

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ABSTRACT

A total of 1,440 fertile eggs were divided randomly and equally into 5 treatment groups; control (no TM) and 4 treatment groups: TM_1, TM_2, TM_3, and TM_4 that were thermally subjected to 39°C for 18 h with 65% relative humidity daily, during embryonic days ED 7–11, ED 11–15, ED 15–18 and ED 7–18, respectively. Out of TM conditions that were investigated, TM_1 resulted in a significant improvement in expression of myogenic factor-5 (Myf5), myoblast determination protein (MyoD) and myogenin (MyoG) in pre and post hatch broiler muscle when compared to the control. This study aimed to determine the optimum time for thermal manipulation during embryogenesis that may result in improvement in expression of myogenic regulatory factors (MRFs): Myf5, MyoD, MyoG and myostatin (MSTN) in broiler muscle. Thus, the results of this research may provide a means of improvement of myogenesis of pectoral and thigh muscles of broilers proceeding from early embryogenesis (ED7) through post-hatch day.

Keywords: Broiler, Myoblast determination protein, Myogenic factor-5, Myogenin, Myostatin, Thermal manipulation

Development of skeletal muscle proceeds from early embryogenesis through adulthood (Piestun *et al.* 2009). This development depends on number and size of muscle cells and the rate of protein synthesis and/or degradation (Braun and Gautel 2011, Mukund and Subramaniam 2020). Skeletal muscle myoblasts can proliferate; thereby generating a larger population of myoblasts, or it can irreversibly differentiate and form a myocyte (Piestun *et al*. 2009, Almada and Wagers 2016).

Previous researchers had indicated that post-natal myofiber growth in avian species is considered to occur at early growth phase, and it is characterized by a high level of satellite cell mitotic activity (Mozdziak *et al*. 2000, Yin *et al*. 2013). Myoblasts proliferation and myocytes differentiation are directly regulated by signal pathways, which regulate the number, size and strength of myocytes (Olson 1992, Stockdale 1992, Piestun *et al*. 2009). Furthermore, molecular pathways (mitogenic growth) and extracellular myogenic regulatory factors such as Myf5, MyoD, MyoG and MSTN were considered very important for muscle protein synthesis and degradation rates (Hernandez *et al*. 2017). Upon satellite cell activation, the muscle regulatory factors Myf5 and MyoD are expressed in embryo followed by MyoG expression as the cells enter differentiation (Hernandez *et al.* 2017, Evano and Tajbakhsh 2018, de las Heras-Saldana *et al.* 2019). Whereas MSTN expression inhibits satellite cell proliferation and differentiation by maintaining them in quiescent state (down regulation of MyoD) (McCroskery *et al.* 2003, Garikipati and Rodgers 2012).

Satellite cells can also be activated in skeletal muscles under stress conditions (Evano and Tajbakhsh 2018). However, environmental heat stress and/or excessive metabolic heat production can cause muscle damage (Clarkson and Sayers 1999). Previously, Tazawa *et al.* (2004) reported that deviations from optimum incubation temperatures, ranging from 37 to 37.5°C, might affect embryo size, organ and skeletal growth, metabolic rate, physiological development and hatching success. It has been stated that incubation at higher temperatures accelerated embryonic growth (Yalcin and Siegel 2003). Therefore, this study was to determine the optimum age of the embryo for thermal manipulation during embryogenesis that may improve mRNA gene expression of Myf5, MyoD and MyoG in both the pectoral and thigh muscles of Ross 708 broilers.

MATERIALS AND METHODS

Pre-egg incubation management: All experimental procedures including experimental incubation and hatching management conditions were approved by King Faisal University's Animal Care and Use Committee (KFU-ACUC), session (5) date 27/4/2017. A total of 1,700 Ross 708 broiler fresh eggs were procured from a 36-week-old broiler flock (from Al-Ahsa, Saudi Arabia) and stored at

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24°C for 12 h in a separate room free of dust and any source of contamination. The broken as well as abnormally small (58 g) and large eggs (65 g) were excluded before the first day of incubation. After discarding these eggs, 1550 number of eggs with uniform weight were taken for experiment.

Egg incubation and thermal manipulation management: The 1,550 eggs with uniform weight were pre-heated in semi-commercial incubators (type OVA-Easy 380 Advance Series II, Brinsea, Sandford, UK; each incubator has 384 hens eggs capacity) at 27°C for 8 h. Further, all eggs were incubated at 37.8℃ with 56% relative humidity (RH). The eggs in all incubators were turned through 45° every hour up to ED18. At embryonic day 7, egg candling was performed to exclude any dead embryos and infertile eggs, ending up with 1,440 fertile eggs. These fertile eggs were distributed randomly into five treatment groups (288 eggs/ incubator); the first group remained incubated at 37.8°C with 56% RH normal temperature (control group). Whereas TM_1, TM_2, TM_3 and TM_4 were thermally subjected to 39°C for 18 h with 65% RH daily during ED7–11, ED11–15, ED15–18 and ED7–18. The incubation temperature and RH were raised from day 7 to 11 (TM_1) and 11-15d (TM_2), 15-18d (TM_3) and 7-18d (TM_4).

Hatching management: From ED18-ED20, fertile eggs of all groups were incubated at 36.6°C and 70% RH. Till ED 21 temperature was lowered to 36.1°C. Upon hatching and full feather drying (approximately 2 h post hatch); chickens (no sex separation) were recorded and transferred to the Agricultural and Veterinary Research and Training Station at King Faisal University where the field study was conducted. Water and feed (ARASCO, Riyadh, Saudi Arabia) were supplied *ad lib*. to the chicks, and they were kept for brooding at an initial house temperature of 31 ± 1°C, which was reduced by an average of 0.2–0.3°C per day to achieve a final house temperature of 22 ± 1°C by day 24 post-hatch. The light system design was 24 h of light in the first 3 days, 20 h of light and 4 h of darkness per day from day 3 to day 7, then 16 h of light and 8 h of darkness per day up to day 35.

Sampling management: Pectoral and thigh muscle samples from embryos of all five groups were taken at the end of ED11, ED15, ED18 and 35d post hatch. The samples were subjected to RNA isolation (75 embryos, n=5) and further gene expression studies were conducted for MyoF-5, MyoD, MyoG and MyoS.

RNA isolation and semi-quantitative real time RT-PCR analysis: Myf5, MyoD, MyoG and MSTN mRNA expression levels at embryonic day 11, 15 and 18 and post-hatch day 35 were analysed and quantified using a semi-quantitative real-time RT-PCR. The pectoral and thigh muscles were homogenized by Bead Ruptor (24 Bead Mill Homogenizer, OMNI, USA) and total RNA was extracted using the PureZOL™ RNA isolation method (BIO-RAD, Catalog #732-6890, Hercules, CA, USA). DNA was removed using a DNase I kit (Ambion).

The purity and concentration of RNA was estimated by measuring the ratio of absorbances (260/280) using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (Bio-Tek, USA). The RNA (2 µg) was reverse transcribed to cDNA in a reaction mixture using an iScriptc DNA synthesis kit (BIO-RAD, Catalog #170-8890, Hercules, CA, USA).

A semi-quantitative Real-Time PCR analysis (BIO-RAD, Hercules, CA, USA) was performed using the ssoAdvanced™ SYBR Green Supermix kit (BIO-RAD, Catalog #170-5270, Hercules, CA, USA). A total of 20 µl reaction mixture was prepared using 10 µl of the master mix, 2 µl of the forward primer pm/µl, 2 µl of the reverse primer pm/µl (Supplementary Table 1), 2 µl of cDNA from the sample, and 4 µl of nuclease-free water. PCR conditions consisted of an initial denaturation step of 95°C for 1 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec and 72°C for 10 sec, with a final melting temperature at 95°C for 20 sec. Samples were run in triplicates. GAPDH and Actin 1 were kept as housekeeping genes.

Statistical analyses: The original data were arranged using Excel 2007 software (Microsoft Corporation, Redmond, WA, USA). The data for Myf5, MyoD, MyoG and MSTN mRNA gene expressions was expressed as means±SE. The relative quantitative expression results were calculated using comparative ct-($2^{-\Delta\Delta Ct}$) method acording to Livak and Schmittgen (2001). A two-way ANOVA followed by an all-pairs Bonferroni test were applied to compare the different parameters in each treatment group using IBM SPSS Statistics 20 software (IBM, Chicago, USA). Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

In TM_1 and TM_4 thermal manipulation from ED7 to ED11 resulted in a highly significant increase ($p<0.05$) in the expression of Myf5, MyoD and MyoG in both the pectoral and thigh muscles when compared to the control (Tables 1, 2 and 3). It was observed that relative fold change expression of Myf5 gene in both thigh and pectoral muscle tissue samples increased compared to control and other treatment groups. The change in expression level was not significant.

The relative fold change expression of MyoD in pectoral tissue of TM_2 group decreased significantly ($p<0.05$) whereas the expression of the same in thigh muscle was not significantly different from control group. The change in the relative expression of MyoG gene when compared between TM_2 and control was also not significant (Table 3, $p<0.05$). The expression of MSTN gene was not observed in Control and as well as in all treatment samples at ED11 and ED15.

In TM_3 group, thermal manipulation from ED15–ED18 resulted in downregulation ($p<0.05$) of relative expression of Myf5 and MyoG genes in both the muscle tissues when compared to Control (Tables 1 and 3)

In ED18, thermal manipulation of TM_3 from ED15 to ED18 showed a significant reduction of Myf5 and MyoG mRNA expressions in both pectoral and thigh muscles when compared to those of the control (Tables 1 and 3) at

Table 1. Relative normalized expression of mRNA levels of Myf5 in pectoral and thigh muscles at embryonic days 11, 15 and 18

Myf5	ED11		ED15			ED18		
	Expression (fold)			Expression (fold)			Expression (fold)	
	Pectoral	Thigh		Pectoral	Thigh		Pectoral	Thigh
Control*	1.0±0.7a	1.0±0.4a	Control*	1.0±0.2a	1.0±0.1a	Control*	1.0±0.3a	1.0±0.3a
TM_1	5.29±0.4b	2.58±0.3b	TM_1	----	----	TM_1	0.55±0.1a	0.53±0.06a
TM_2	----	----	TM_2	1.07±0.2a	0.93±0.2a	TM_2	0.93±0.2a	0.71±0.1a
TM_3	----	----	TM_3	----	----	TM_3	0.48±0.03b	0.46±0.08b
TM_4	----	----	TM_4	----	----	TM_4	0.37±0.03b	0.58±0. 1a

Control, 37.8°C; TM_1, Thermal manipulation from ED7-11 at 39°C for 18 h; TM_2, Thermal manipulation from ED11-15 at 39°C for 18 h; TM_3, Thermal manipulation from ED15-18 at 39°C for 18 h; TM_4, Thermal manipulation from ED7-18 at 39°C for 18 h.

a–c, Within the same day, means ± SD with different superscripts differ significantly ($p<0.05$). *, Compared to the control of each embryonic day ($p<0.05$).

($p<0.05$). However, mRNA expression of MyoD was found significantly higher in TM_1, TM_3 and TM_4 when compared to the control. The highest expression was observed in TM_1 group ($p<0.05$) followed by TM_3 and TM_4 in muscles of both the tissues (Table 2). Gene expression of MSTN was observed to be greater in TM_3 group compared to Control ($p<0.05$); whereas its expression in TM_1 was not detected in muscle but even though expressed in thigh muscle, its expression was not significantly different from control (Supplementary Table 2).

Furthermore, comparative gene study was also performed on the relative normalized expression of Myf5, MyoD, MyoG and MSTN mRNA levels in both pectoral and thigh muscles collected at ED11, ED15 and ED18. The results revealed that the gene expression of Myf5, MyoD and MyoG were upregulated during early embryogenesis (ED7-ED11) and was downregulated at ED15 up to ED18 (Supplementary Fig. 1).

The effect of TM during embryogenesis on Myf5, MyoD, MyoG, and MSTN mRNA expressions at post-hatch day35 are given in Figs. 1 and 2. In the pectoral muscle of the post-hatch chicks at day 35, embryonic thermal manipulation did not cause significant difference ($p<0.05$) of Myf5 mRNA expression in TM_2, TM_3 and TM_4 when compared to the control ($p<0.05$), while it was significantly higher ($p<0.05$) in TM_1 when compared to the control (Fig. 1). The difference in Myf5 gene expression of the thigh muscle due to the same treatment was not significant when compared to control ($p<0.05$, Fig. 2). The expression of MyoD and

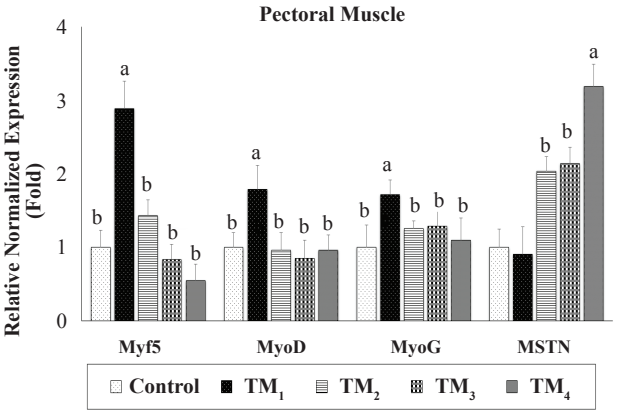

Fig. 1. Effect of thermal manipulation (TM) during embryogenesis on the mRNA level of Myf5, MyoD, MyoG and MSTN in pectoral muscle at post-hatch day 35. Means with different superscripts differ significantly ($p<0.05$).

MyoG genes was highest ($p<0.05$) in the pectoral muscles of 35d chicks of TM_1 group when compared to rest of the groups (Fig. 1). In thigh muscle, expression of the same genes was significantly greater ($p<0.05$) in all the treatment groups when compared to control (Fig. 2). The expression of MSTN gene in pectoral muscle was observed to be highest ($p<0.05$) in TM_4 followed by TM_2 and TM_3 when compared to control and TM_1 (Fig. 1). Similar results were observed for thigh muscle of TM_4 but its expression was less ($p<0.05$) in other groups when compared to control (Fig. 2).

Table 2. Relative normalized expression of mRNA levels of MyoD in pectoral and thigh muscles at embryonic days 11, 15 and 18

MyoD	ED11		ED15			ED18		
	Expression (fold)			Expression (fold)			Expression (fold)	
	Pectoral	Thigh		Pectoral	Thigh		Pectoral	Thigh
Control*	1.0±0.9a	1.0±0.7a	Control*	1.0±0.1a	1.0±0.08a	Control*	1.0±0.4a	1.0±0.3a
TM_1	6.88±0.8b	5.62±1.3b	TM_1	----	----	TM_1	5.30±0.5b	4.69±0.3b
TM_2	----	----	TM_2	0.28±0.01b	1.10±0.05a	TM_2	1.03±0.8a	1.08±0.1a
TM_3	----	----	TM_3	----	----	TM_3	3.80±0.9d	3.3±0.6d
TM_4	----	----	TM_4	----	----	TM_4	2.05±0.4c	2.21±0.1c

Control, 37.8°C; TM_1, Thermal manipulation from ED7-11 at 39°C for 18 h; TM_2, Thermal manipulation from ED11-15 at 39°C for 18 h; TM_3, Thermal manipulation from ED15-18 at 39°C for 18 h; TM_4, Thermal manipulation from ED7-18 at 39°C for 18 h.

a–c, Within the same day, means ± SD with different superscripts differ significantly ($p<0.05$). *, Compared to the control of each embryonic day ($p<0.05$).

Table 3. Relative normalized expression of mRNA levels of MyoG in pectoral and thigh muscles at embryonic days 11, 15 and 18

MyoG	ED11		ED15			ED18		
	Expression (fold)		Expression (fold)			Expression (fold)		
	Pectoral	Thigh		Pectoral	Thigh		Pectoral	Thigh
Control*	1.0±0.5a	1.0±0.3a	Control*	1.0±0.1a	1.0±0.2a	Control*	1.0±0.4a	1.0±0.3a
TM_1	4.31±0.7b	2.48±0.3b	TM_1	----	----	TM_1	0.89±0.1a	0.37±0.05b
TM_2	----	----	TM_2	1.40±0.1a	0.78±0.2a	TM_2	1.82±0.8b	0.96±0.1c
TM_3	----	----	TM_3	----	----	TM_3	0.36±0.06c	0.47±0.1b
TM_4	----	----	TM_4	----	----	TM_4	0.25±0.02c	0.41±0.1b

Control, 37.8°C; TM_1, Thermal manipulation from ED7-11 at 39°C for 18 h; TM_2, Thermal manipulation from ED11-15 at 39°C for 18 h; TM_3, Thermal manipulation from ED15-18 at 39°C for 18 h; TM_4, Thermal manipulation from ED7-18 at 39°C for 18 h.

a–c, Within the same day, means ± SD with different superscripts differ significantly ($p<0.05$).*, Compared to the control of each embryonic day ($p<0.05$).

In the present study, the results showed that thermal manipulation at 39°C for 18 h, at early age ED7-11, prolongs stimulatory effect on muscular mRNA gene expression of myogenic regulatory factors Myf5, MyoD, MyoG as compared to the control. This is in accordance with Halevy *et al*. (2001) who reported that mild heat exposure at an early age (post-hatch day 3) results in the acceleration of myogenesis mediated by specific local myogenic regulatory factors. Similarly, it has been reported that TM (39.5°C at ED12-18 for 3 and 6 h) resulted in the enhancement of cell differentiation as indicated by expression levels of myogenic regulatory factors (Piestun *et al.* 2009). On the other hand, Gabriel *et al*. (2003) suggested that myogenic proliferation and differentiation events are compromised by variation in environmental temperature during avian embryogenesis. They observed that that the chicken embryos that were subjected to TM at 44°C after ED4 for 1 h had delayed myofiber formation.

The significant increase in gene expression of myogenic regulatory factors Myf5, MyoD and MyoG at 39ºC in TM_1 in the present study indicates that small increase in incubation temperature changes the expression level of the genes positively instead of reduction in the expression, as mentioned by Gabriel *et al.* (2003). It seems that early TM at (ED7-11) has long effect on the expression of myogenic regulation factors at later age post hatch 35d.

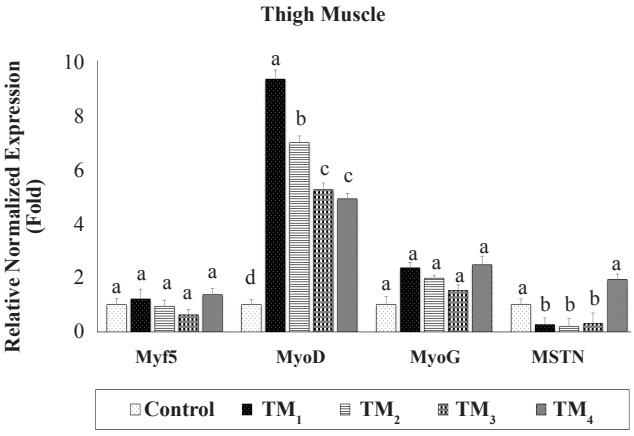

Fig. 2. Effect of thermal manipulation (TM) during embryogenesis on the mRNA level of Myf5, MyoD, MyoG and MSTN in thigh muscle at post-hatch day 35. Means with different superscripts differ significantly ($p<0.05$).

In ross chickens, strong MSTN mRNA expression was seen during the blastoderm stage of embryos and remained high till embryonic day 2 (Kocamis *et al.* 1999). On the contrary, myostatin expression could not be detected in ross chickens during early (ED7-11) and middle (ED11-15) embryogenesis. This is in agreement with Kocamis *et al*. (2000) who reported that MSTN expression could not be detected in cob chicken embryos at early age, indicating absence of inhibitory function of MSTN on development/ growth of both pectoral and thigh muscle of the embryos. Therefore, any external stimuli such as thermal manipulation may enhance growth by stimulation of other growth factors as well as activation of myogenic regulatory factors.

On the other hand, the detection of MSTN expression was observed in late embryogenesis (ED15-18) especially in TM_3 compared to the control. Thus, myogenic proliferation and differentiation maybe inhibited by MSTN and maintains with down–regulation of myogenic activation and regulatory factors (Halevy *et al*. 2006). It seems that TM during middle or late embryogenesis has no strong effect on the expression of myogenic activation and regulation factors at later age on post-hatch days 35 and on MSTN expression, causing decrease in growth rate. Thus, myogenic regulatory factors Myf5, MyoD, MyoG and MSTN may be the major determinants of myogenesis that maintain overall post-natal pectoral and thigh muscle mass. The results of present study indicate that application of thermal manipulation at 39°C for 18 h dailly during ED7-11 is crucial for increasing the expression of myogenic factors. It can be concluded that early thermal manipulation during embryonic day 7 to 11 is the best and safe for thermal manipulated protocols that may be applied for commercial broiler embryos to express myogenic genes which may improve and may enhance broilers' muscle development and share in meat consumption demands.

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