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Thermal Manipulation during Broiler Chicken Embryogenesis Modulates the Splenic Cytokines' mRNA Expression

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Abstract

The increased incubation temperature has several impacts on the physiology and development of broiler chicken embryos. However, the impact of these conditions on embryonic immunity is unclear. The aim of this study is to evaluate the effect of intermittent thermal manipulation during embryogenesis (TM) on the mRNA expression of cytokines in the spleen of chicken embryos. In this study, the IL-4, IL-6, IL-8, IL-15, IL-16, IL-17, IL-18, IFN-γ, IFN-β, IFN-α, TNF-α and IL-1β genes are evaluated. The eggs of the TM group were subjected to thermal manipulation at 39^{o}C and 65 % relative humidity for eighteen hours/day during embryonic days (ED) 10-18, whereas the eggs of the control group were kept at 37.8^{o}C and 56 % RH throughout the incubation period. On ED 18, the spleen was collected from the embryos in order to evaluate the mRNA levels of cytokines by relative quantitation real time RT-PCR. On the day of hatching, the hatchability rate, body weight, and cloacal temperature (T^{c}) of the hatched chicks were recorded. TM significantly increased the mRNA expression of IL-6, IL-8, IL-4, IL15, IL-16, IL-17, IL-18, IFN-γ, IFN-β, IFN-α, TNF-α and IL-1β in the spleen of broiler chicken embryos on ED eighteen. However, TM did not significantly affect the hatchability rate, T^{c} and the body weight of chicks on the day of hatching. In conclusion, results of the present study suggest that TM modulates the cytokine expression in broiler embryos, but did not lead to significant impacts on the hatchability rate and hatchling body weights, and cloacal temperatures.

Keywords: Broilers, Thermal manipulation, Interleukins, Interferons, Cytokines

1. Introduction

The incubation temperature is an essential factor for a normal development of broiler chicken embryos (*Gallus gallus domesticus*) (Nakage *et al*. 2003, Lourens *et al*. 2005, Lourens *et al.* 2006, Wineland *et al*. 2006, Brand *et al*. 2007, Yalçin *et al*. 2007). Eggs incubated at temperatures higher than the standard conditions have severe effects on the physiology and development of broiler chicken embryos (French 1997). It was found that increased incubation temperatures have deleterious effects on the immune organs and immune system (Thaxton *et al*. 1968, Heller *et al*. 1979, Mashaly *et al*. 2004, Oznurlu *et al*. 2010).

On the other hand, intermittent increased incubation temperature, also called thermal manipulation (TM), was suggested to enhance post-hatch heat tolerance in broiler chickens (Al-Zghoul *et al*. 2013, Loyau *et al*. 2014, Al-Zghoul *et al*. 2015, Al-Zghoul *et al*. 2015, Al-Zghoul *et al*. 2015, Morita *et al*. 2016, Al-Rukibat *et al*. 2017). Furthermore, in pekin ducklings, TM during periods after the incubation day ten led to beneficial effects on the immune system during embryogenesis and during post-hatch lipopolysaccharide-challenge (Shanmugasundaram *et al*. 2018, Shanmugasundaram *et al.* 2019). TM led to increased IL-6, IFN-γ and IL-10 cytokines, and to increased MHCI and MHCII gene expression in pekin duck embryos (Shanmugasundaram *et al*. 2018). However, Liu *et al*. (2013) reported that increased incubation temperature during the middle stage of pekin ducks embryogenesis led to repressive effects on immunity and the development of immune organs.

Cytokines are extracellular, small, signaling proteins that have a critical role in immunity, both during the development of immune system and in the cases of immune response to pathogens or to certain stimulus (Giansanti *et al*. 2006, Kaiser 2010, Davison *et al*. 2011). Cytokines could be secreted by every cell type in vertebrates (Kaiser 2010, Davison *et al*. 2011). They were classified depending on their function, the cells that secret them, or on the cells that they act upon (Giansanti *et al*. 2006). Cytokines include interleukins (IL), tumor necrosis factors (TNF), interferons (IFN), chemokines, transforming growth factor beta family (TGF-β) and colony stimulating factors (CSF) (Davison *et al.* 2011). In birds, TNF-α, IL-1β, IL-18, IL-17, IL-6, IL-16 and IL-8 are known to act as pro-inflammatory cytokines. Chicken IL-15 promotes T-cells proliferation, and IFN-γ is a signal for the cell mediated immune response, while IL-4 activates the antibody-mediated immunity. Chicken type I –interferons (IFN-α and IFN-β) possess antiviral activity

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(Kaiser and Stäheli 2014). Abdul-Careem *et al*. (2007) reported that cytokines have a function in the maturation and shaping of spleen in chicken embryos.

In broiler chickens, the effect of TM on mRNA expression of proinflammatory cytokines during post hatch heat stress exposure had been evaluated (Al-Zghoul *et al.* 2019), however, the impact of high incubation temperatures on the embryonic development and immune status of broiler chicken embryos still needs further investigations. Therefore, the aim of this study is to evaluate the influence of intermittent thermal manipulation during embryogenesis (TM) on the mRNA levels of the cytokines (signaling molecules that are important in immune response and have roles in embryonic development) in the spleen of broiler embryos. The evaluated cytokines were: IL-4, 6, 8, 15, 16, 17, 18, IFN-γ, IFN-α, IFN-β, IL-1β and TNF-α.

2. Materials and Methods

2.1. Ethical Statement

All experimental procedures that are followed in this study were approved by the Jordan University of Science and technology Animal Care and Use Committee (JUST-ACUC).

2.2. Incubation Management and Sampling

A total of six-hundred fertile eggs of the Indian River broiler breed with a uniform weight were obtained from an Indian River breeder (Irbid, Jordan). The abnormal and broken eggs were excluded, and the approved eggs were incubated in commercial Type-I HS-SF incubators (Masalles, Barcelona, Spain). The eggs were subdivided into two treatment groups: the control group and the thermal manipulation (TM) group. The eggs of the control group were maintained at 37.8 °C and 56 % relative humidity (RH) throughout the embryogenesis period, while those of the TM group were subjected to 39°C and 65 % RH for eighteen hours/day during days ten to eighteen of embryogenesis. On day seven of incubation, the eggs were examined by candling and the infertile eggs, and the eggs containing dead embryos were removed.

On embryonic day (ED) eighteen, six eggs were randomly selected from each group, and the spleen samples were collected for the purpose of evaluating the mRNA expression of cytokines by real time RT-PCR analysis. On hatching day, the number of hatched chicks and their cloacal temperatures (T^c) and body weights were recorded.

2.3. Total RNA Extraction and Reverse Transcription

Splenic total RNA was isolated using Direct-Zol™ RNA MiniPrep (Zymo Research, Irvine, USA) with TRI Reagent® (Zymo Research, Irvine, USA) according to the manufacturer's procedures. RNA concentrations were determined using XS2 Spectrophotometer (BioTek Instruments, Inc., USA). 2 µg total RNA from each sample was used for the cDNA synthesis (reverse transcription) using a Power cDNA Synthesis Kit (Intron Biotechnology، Kyungki-Do, Korea).

2.4. Real-Time PCR and mRNA Relative-Quantitation

QuantiFast SYBR® Green PCR Kit (Qiagen corp., CA, USA) was used on a Rotor-Gene Q MDx5 plex instrument (Qiagen corp., CA, USA). Briefly, the 20 µL reaction mix was prepared from 10 µL of master mix, 1.2 µL forward primer (12 pmol), 1.2 µL reverse primer (12 pmol), 1 µL cDNA from the sample, and 6.6 µL of nuclease-free water. The PCR cycles employed the following parameters: 95°C for five minutes; forty cycles of 95°C for ten seconds followed by thirty seconds at 55°C; and 72°C for ten seconds with final melting at 95°C for twenty seconds. The fluorescence emission detection was during the extension step. The fold changes in the gene expression were normalized to the 28S ribosomal RNA, which was utilized in this study as an internal control. The single target amplification specificity was checked using the melting curve. The fold changes in gene expression were calculated automatically. Table 1 presents the primers' sequences that were used in the real time PCR analysis.

2.5. Statistical Analysis

All Statistical analyses were conducted using the IBM SPSS statistics 23 software (IBM software, Chicago, USA). The data of cloacal temperature, body weight, and relative mRNA levels of IL-1β, TNF-α, IL-6, IL-8, IFN-α, IFN-β, IFN-γ, IL-4, IL-15, IL-16, IL-17 and IL-18 were expressed as means ± SD. An independent t-test was used to compare the difference between the controls vs. the TM treatment groups. The hatchability was assessed using the Chi-square statistical test. Parametric differences were considered statistically significant when $P < 0.05$.

Table 1. Primer sequences of genes used in the real time RT-PCR analysis.

The gene	Forward	Reverse
IL-6	GCGAGAACAGCATGGAGATG	GTAGGTCTGAAAGGCGAACAG
IL-4	GAGAGCATCCGGATAGTGAATG	TGTGGAGGCTTTGCATAAGAG
IL-8	CTGCGGTGCCAGTGCATTA	AGCACACCTCTCTTCCATCC
IL-15	GTGGTCAGACGTTCTGAAAGAT	CAGGTTCCTGGCATTCTATATCC
IL-16	GGAACAAAGCAGCCCAGTTC	GGCTGTGGTGTGCACCTGTA
IL-17	CTCCGATCCCTTATTCTCCTC	AAGCGGTTGTGGTCCTCAT
IL-18	AGGTGAAATCTGGCAGTGGAAT	TGAAGGCGCGGTGGTTT
IFN-γ	CAAGTCAAAGCCGCACATC	CGCTGGATTCTCAAGTCGTT
IFN-α	ATGCCACCTTCTCTCACGAC	AGGCGCTGTAATCGTTGTCT
IFN-β	CCTCAACCAGATCCAGCATTA	TAGTTGTTGTGCCGTAGGAAG
IL-1β	GGGCATCAAGGGCTACAA	CTGTCCAGGCGGTAGAAGA
TNF-α	GACAGCCTATGCCAACAAGTA	GAATTAAGCAACAGCCAGCTATG
28S rRNA	CCTGAATCCCGAGGTTAACTATT	GAGGTGCGGCTTATCATCTATC

3. Results

3.1. Effect of Intermittent TM on The Splenic mRNA Levels of Cytokines in Broiler Chicken Embryos

The effect of intermittent TM at 39°C for 18 h/day during EDs 10-18 on the splenic mRNA expression of cytokines in the broiler embryos is shown in Figure 1 (A-L). Intermittent TM significantly increased the mRNA expression of IL-6, IL-8, IL-4, IL15, IL-16, IL-17, IL-18, IFN-γ, IFN-β, IFN-α, TNF-α and IL-1β in the spleen of broiler chicken embryos on ED 18 (*P*-value< 0.05).

3.2. Effect of Intermittent TM on Hatchability Rate, Hatchling Tc and Hatchling Body Weight of Broiler Chicks

Figure 2 (A-D) presents the effect of intermittent TM at 39°C for 18 h/day during EDs 10-18 on the number of hatched chicks, hatchability, cloacal temperature, and the body weight on the day of hatch. No significant difference was observed in the hatchability rate, hatchling T^c, and hatchling body weight between the TM and control groups (*P*-value > 0.05).

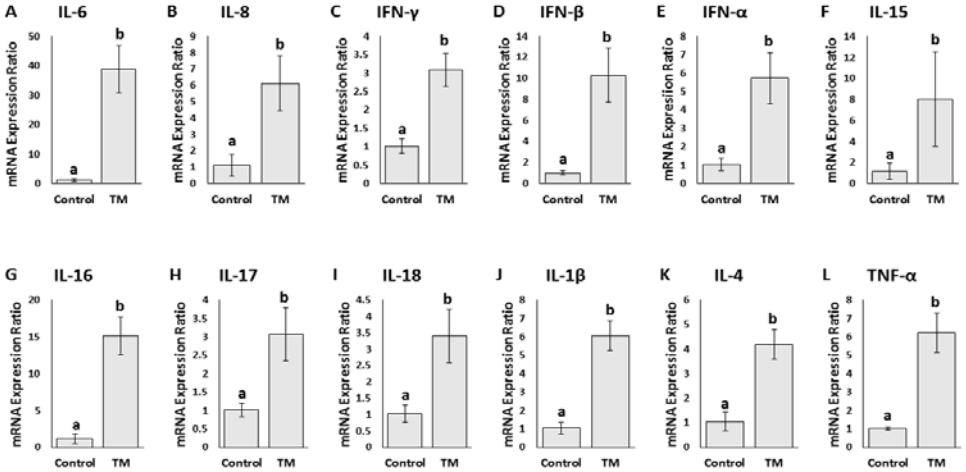

Figure 1. Effect of intermittent TM on the mRNA levels of cytokines, (A) IL-6, (B) IL-8, (C) IFN-γ, (D) IFN-β, (E) IFN-α, (F) IL-15, (G) IL-16, (H) IL-17, (I) IL-18, (J) IL-1β, (K) IL-4, (L) TNF-α, in the spleen of broiler chicken embryos on embryonic day eighteen .

a-b means ± SD with different superscripts differ significantly ($p < 0.05$).

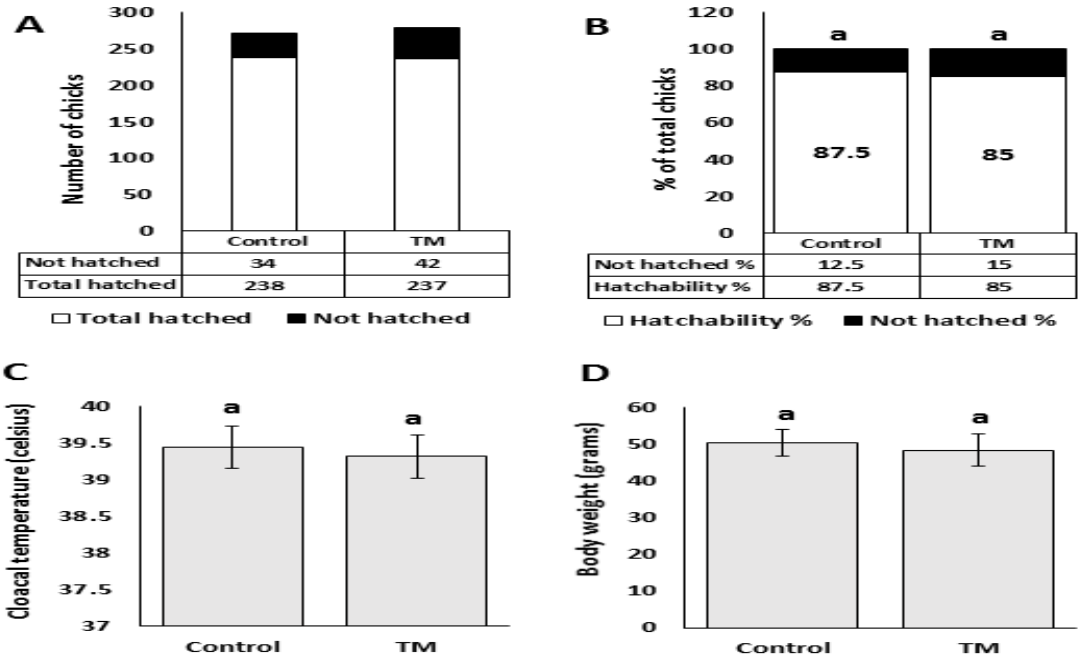

Figure 2: Effect of intermittent TM on the number of hatched chicks (A), the hatchability rate (B), hatchling T^c (C), and hatchling body weights (D) of broiler chicks. $^{a-b}$ values with different superscripts differ significantly.

4. Discussion

The incubation conditions of broiler chickens, especially the incubation temperature, had been proven to affect embryonic development, leading to significant impacts on hatched chicks' quality and post-hatch physiology and performance (Decuypere and Michels 1992, Lourens *et al*. 2005, Lourens *et al*. 2007). The aim of the present study is to evaluate the effect of TM at 39°C and 65 % RH for 18 h/day during incubation days 10 to 18 on the splenic mRNA levels of cytokines (IFN-α, IFN-β,

IFN-γ, IL-4, IL-8, IL-15, IL-16, IL-17 and IL-18) in broiler chicken embryos, as well as on the hatchability, hatchling body weights, and cloacal temperatures.

Cytokines are small proteins that have a function in signaling and immunoregulation. Their major function is during the immune response against pathogens, however, they are also important in the regeneration and healing of injured tissues (Wigley and Kaiser 2003, Eming *et al*. 2009).

In this study, TM increased the IFN-α, IFN-β, IFN-γ, IL-4 and IL-15 mRNA levels in the spleen of broiler embryos. IFNs are mainly upregulated in response to viral infections (Wigley and Kaiser 2003, Giansanti *et al*. 2006), and IL-15 has a function in lymphocytes proliferation, intestinal epithelium growth, as well as stimulating cytokines' expression (Wigley and Kaiser 2003).The role of interferons and IL-15 in the response to heat stress is not clear and no previous studies were found in the literature describing IFN-α, IFN-β and IL-15 expression during heat stress in broilers. In humans, the IFN-α level was reported to be elevated during a heat stroke (Leon 2007). Previously, IFN-γ expression was found to be increased in chickens exposed to heat stress (Song *et al*. 2017, Xie *et al*. 2017). However, Ohtsu *et al*. (2015) had shown that heat stress decreased the IFN-γ expression in broiler chickens. In Pekin duck embryos, TM led to increased IFN-γ expression (Shanmugasundaram *et al.* 2018). IFN-γ is mainly upregulated during cell-mediated immune response through Th1 cells (Wigley and Kaiser 2003, Giansanti *et al*. 2006). In contrast, IL-4 is a Th2 cytokine that plays a role in the humoral (antibody) immune response (Giansanti *et al*. 2006). Ohtsu, *et al*. (2015) had found that broilers exposed to heat stress had upregulated the IL-4 expression. The immune response is functionally polarized into either cell-mediated or into the antibody-mediated response (Mosmann *et al.* 1986, Degen *et al*. 2005). Cytokines were reported to be expressed in minute amounts during standard embryonic conditions (Abdul-Careem *et al.* 2007); thus, the current results suggest that TM chicks were under stress due to the non-standard incubation condition. Previously, heat stress was reported to disturb the normal levels of cytokines and the Th1 - Th2 polarization signals in broiler chickens (Ohtsu *et al*. 2015).

In the current study, TM increased the splenic mRNA levels of the IL-1β, TNF-α, IL-6, IL-8, IL-16, IL-17 and IL-18 in broiler embryos.IL-1β, TNF-α, IL-6, IL-8, IL-16, IL-17 and IL-18 are pro-inflammatory cytokines, that have a major function in native immunity and the activation of acute phase response (Wigley and Kaiser 2003, Giansanti *et al.* 2006). Pro-inflammatory cytokines play a critical role in the regeneration and repair of damaged and stressed tissues, especially IL-8 and IL-6 (Rennekampff *et al*. 2000, Streetz *et al*. 2000, Bosch *et al*. 2002, Wigley and Kaiser 2003, Eming *et al*. 2007, Crouser *et al*. 2009, Eming *et al*. 2009, McFarland-Mancini *et al*. 2010, Welc *et al*. 2013, Cheng *et al*. 2015, Phillips *et al*. 2015). Previously, heat stress was found to increase the expression of IL-1β, IL-6, IL-8 and TNF-α in broiler chickens and mammals, and to reduce IL-8 plasma level in chickens (Leon 2007, Heled *et al*. 2013, Varasteh *et al.* 2015, Lan *et al*. 2016, Al-Zghoul *et al*. 2019). Recently, it has been found that chickens' IL-6 functions as a heat-shock gene, since its transcription is induced by HSF3 during heat stress (Prakasam *et al*. 2013). In Pekin duck embryos, TM was found to increase the IL-6 expression (Shanmugasundaram *et al*. 2018). Moreover, it was reported that the IL-18 expression was not changed during heat stress in broiler chickens (Ohtsu *et al*. 2015); however, its level was modulated in mammals (Heled *et al.* 2013). Furthermore, artificial stress induced by corticosterone administration resulted in increased IL-1β, IL-6, IL-8 and IL-18 expression in chickens (Shini and Kaiser 2009, Shini *et al*. 2010). To the authors' knowledge, no previous studies could be found in the literature about the impact of heat stress on the expression of IL-16 and IL-17. Since stress conditions induce proinflammatory cytokines' expression, the present results could be described as the stress response in the broiler embryos.

In the current study, TM did not affect the hatchability rate of broiler chicks. This finding is consistent with two previous studies (Lourens *et al.* 2005, Zaboli *et al*. 2016); however, TM at 39.5^{o}C for 12 h/day during ED 7-16 was reported to decrease the hatchability rate, and TM at 39.5^{o}C for 3 h/day during ED 8-10 and at 38.8^{o}C for 18 h/d during ED 10-18 was found to increase the hatchability rate (Collin *et al*. 2007, Piestun *et al.* 2008, Piestun *et al.* 2008, Al-Zhgoul *et al*. 2013). These contradicting results may be attributed to the strain type, age factors, or incubation conditions (relative humidity and temperature).

The present study shows that TM did not change the hatchling weights and cloacal temperatures. The findings concerning hatchling weights in the previous studies were contradictory; different studies had shown that TM increased, decreased, or did not affect the hatchling weights (Hulet *et al*. 2007, Yalçin *et al*. 2007, Narinç *et al.* 2016, Zaboli *et al.* 2016, Al-Rukibat *et al*. 2017). The present results of the hatchling T^{c} are consistent with two previous studies (Soren *et al.* 2012, Al-Rukibat *et al.* 2017); however, other studies had shown that TM decreased (Collin *et al*. 2005, Collin *et al.* 2007, Al-Zghoul *et al*. 2013, Piestun *et al.* 2013), or increased the hatchling T^{c} in broiler chicks (Al-Zghoul *et al*. 2015). The present findings suggest that although TM increased the cytokines mRNA expression, it did not lead to deleterious impacts on the hatchability rate, weight, and T^{c} of the hatched chicks. The increased cytokines' expression in the thermally- manipulated chicks might be attributed to the fact that TM stimulated an immune response in the embryos, thus, leading to an improved immune response during post-hatch life. Previously, it was found that TM increased the cytokines' expression in Pekin duck embryos, and led to an enhanced post-hatch immune response to a lipopolysaccharide injection (Shanmugasundaram *et al*. 2018, Shanmugasundaram *et al.* 2019). Further studies are required to evaluate the effect of TM on post-hatch immune challenges. On the other hand, the modulation of cytokines' expression during embryogenesis might affect the development of embryos and differentiation of different types of tissues, since cytokines are important players in the differentiation of embryonic stem cells (Chadwick *et al*. 2003, Kristensen *et al.* 2005, Zhou *et al*. 2010). However, this impact on differentiation might be beneficial for the post-hatch performance, as shown in several studies, or might have deleterious effects on the development and post-hatch

health status (Loyau *et al.* 2014, Morita *et al*. 2016, Narinç *et al*. 2016, Al-Zghoul *et al.* 2019).

5. Conclusion

In conclusion, the results of the present study suggest that intermittent thermal manipulation modulates the cytokines' expression in broiler embryos, but does not lead to significant impacts on the hatchability rate, hatchling body weights, and cloacal temperatures. Further studies are required to evaluate the impacts of thermal manipulation on the immune challenges during post-hatch life.

Conflict of Interest

The authors declare that they have no conflict of interest.

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