

mRNA Expressions of Methylation Related Enzymes and Duration of Thermal Conditioning in Chicks

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DNA methylation regulates gene expression by modifying the nucleosome structure of DNA, without altering the gene sequence. It has been reported that DNA methylation reactions are catalyzed by several enzymes. In chickens, thermal conditioning treatment affects the central DNA methylation levels. The purpose of this study was to clarify the changes in DNA methylation and demethylation factors during thermal conditioning in the hypothalamus of 3-day-old chicks. Male chicks (3-days old) were exposed to $40 \pm 0.5^\circ\text{C}$ as a thermal conditioning treatment for 1, 2, 6, 9, or 12 h. The control chicks were kept in a thermoneutral zone ($30 \pm 0.2^\circ\text{C}$). After thermal conditioning, the mRNA levels of DNA methyltransferase (DNMT)-1, -3a, -3b, and ten-eleven translocation (TET)-1, -2, and -3 in the hypothalamus were measured by q-PCR. The mRNA levels of DNMT-3a and TET-1 were increased by thermal conditioning. Moreover, the expression level of TET-1 increased with the loading time of the thermal conditioning. The gene expressions of DNMT-1, DNMT-3b, TET-2, and TET-3 were not affected by thermal conditioning. Since DNMT-3a is a catalyst for *de-novo* DNA methylation and TET-1 catalyzes the oxidation of methylated cytosine, it is suggested that the thermal conditioning increased the activation of DNA methylation and demethylation factors, which occur in the hypothalamus of neonatal chicks.

Key words: chicks, DNA methylation, DNA methyl transferase, ten-eleven translocation, thermal conditioning

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Introduction

DNA methylation and demethylation are known epigenetic mechanisms of reorganization. It has been reported that there is a negative correlation between methylation levels of cytosine-phosphate-guanine (CpG) islands and their gene expression (Li *et al.*, 2011). DNA methylation is the addition of a methyl group present in the body to cytosine, one of the bases in a gene (Moore *et al.*, 2013). This reaction is mediated by several enzymes. DNA methyltransferases (DNMTs) have the ability to attach a methyl group to cytosine (Goll and Bestor, 2005; Smith and Meissner, 2013).

DNMTs have several subtypes, each with different roles in DNA methylation. DNMT-1 acts as a catalyst in the methylation maintenance reaction during DNA replication (Bird and Wolffe, 1999; Song *et al.*, 2011). The other DNA methylation reaction, *de novo* DNA methylation, is catalyzed by DNMT-3a and -3b (Hsieh, 1999). In contrast, the DNA demethylation reaction, which is important in gene expression regulation as DNA methylation, is mediated by the ten-eleven translocation (TET) family of enzymes (Pastor *et al.*, 2013).

Chickens, like other animals and plants, undergo epigenetic reorganization, and DNA methylation is especially important during embryogenesis (Li *et al.*, 2016). In addition, early thermal conditioning has been reported to alter DNA methylation levels in the hypothalamus of chickens (Yossifoff *et al.*, 2008; Cramer *et al.*, 2019; Rosenberg *et al.*, 2020). Thermal conditioning is a type of stress loading treatment, in which heat loading is applied during the neonatal period and incubation period to improve thermotolerance after growth (Yahav and McMurtry, 2001; Yahav *et al.*, 2004). However, the detailed kinetics of DNA methylation and demethylation mechanisms during thermal condition-

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ing are unclear.

The aim of this study was to elucidate the DNA methylation and demethylation phenomena caused by thermal conditioning. The mRNA expression of DNA methylation-related enzymes, such as DNMTs and TETs, is associated with DNA methylation and demethylation mechanisms (Takeshima *et al.*, 2020). In zebrafish, exposure to environmental stress affects DNMT gene expression and DNA methylation levels (Aluru *et al.*, 2015). In chickens, there is an association between DNA methylation levels and DNMT gene expression during embryogenesis (Li *et al.*, 2016), and it has been suggested that nutrition stress affects the expression of DNA methylation-related genes and alters DNA methylation levels in chickens (Kang *et al.*, 2017). Some researchers have reported that these DNA methylation-related genes are expressed in the hypothalamus of chickens and are associated with DNA methylation (Yu *et al.*, 2008). Thus, we investigated the changes in gene expression levels of DNMTs and demethylase TETs in the hypothalamus during thermal conditioning in the neonatal period.

Materials and Methods

Birds were handled in accordance with the regulations of the Animal Experiment Committee of Hiroshima University (authorization No. C19-15) and complied with Law No. 105 and Notification No. 6 of the Japanese government.

Animals

Day-old male Chunky chicks were used in this study (Fukuda Hatchery, Okayama, Japan). They were kept in a climate-controlled room with continuous lighting and $30 \pm 0.2^\circ\text{C}$. Chicks were housed in polypropylene boxes ($36 \times 40 \times 30$ cm) with sawdust litter at a population density of 6 chicks per box during the experimental period. They were provided with free access to a commercial starter diet (Nichiwa Sangyo Co. Ltd., Kobe, Japan) and water until the end of the experiment.

Experimental Design

At day 3, the chicks were distributed into six groups ($n=8$ per group). The average body weight of each group was as uniform as possible. Thereafter, the chicks in the treatment groups were exposed to a high-temperature environment ($40 \pm 0.5^\circ\text{C}$; Ouchi *et al.*, 2020) for 1, 2, 6, 9, or 12 h. The size

of the chamber used for exposure was $90 \times 90 \times 115$ cm. The control chicks were left in the thermoneutral zone ($30 \pm 0.2^\circ\text{C}$). Immediately after the treatment, all groups of chicks were anesthetized with isoflurane (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and decapitated for tissue sample collection. Diencephalic tissues were collected from all groups of chicks and stored at -80°C until total RNA isolation.

RNA Isolation and Real Time RT-PCR

Total RNA was isolated from harvested diencephalic tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and purity of the isolated total RNA were measured using a spectrophotometer (NanoDrop ONE: Thermo Scientific, Inc.) at the 260/280 nm absorbance ratio. Total RNA was then treated with DNase (Ambion, Austin, TX, USA). cDNA was synthesized from DNase-treated total RNA using the Prime Script RT reagent kit (TaKaRa Bio Inc., Shiga, Japan). Gene expression was evaluated using a light cycler system (Light Cycler Nano: Roche Applied Science, IN, USA). The primer sequences are shown in Table 1. The PCR protocol was as follows: denaturation phase at 95°C for 5 min followed by 45 cycles at 95°C for 10 s and 60°C for 20 s. The total volume of PCR was $20 \mu\text{L}$, containing $2 \mu\text{L}$ of cDNA, $1.5 \mu\text{L}$ of each $0.2 \mu\text{M}$ primer, $10 \mu\text{L}$ of 2 FastStart Essential DNA Green Master (Roche Life Science, Basel, Switzerland), and $5 \mu\text{L}$ of PCR grade water. Ribosomal protein S17 (RPS17) was used as the geometrical mean of the internal control, and the $2^{-\Delta\Delta\text{Ct}}$ method was used to normalize the data.

Statistical Analysis

The data were analyzed using the commercially available package, StatView (Version 5, SAS Institute, Cary, USA, 1998). Data were evaluated by one-way analysis of variance (ANOVA) for thermal conditioning treatment. When significant effects were detected, a post-hoc test was performed using the Tukey-Kramer test for comparison with the control group. Statistical significance was set at $P < 0.05$. All data are expressed as mean \pm SEM.

Table 1. Prime sequences for real time PCR

Gene name	Forward (5' → 3')	Reverse (5' → 3')	Accession no.
RPS17	AAGCTGCAGGAGGAGGAGAGG	GGTTGGACAGGCTGCCGAAGT	NM_204217
DNMT-1	CTCAAATCTTCCAAGGGGA	AAACACGGGTTGTCATCATA	NM_206952
DNMT-3a	GGGTTYGTTYGGTTGATTTG	GGGACACCGCTGATCGTTTAAA	NM_001024832
DNMT-3b	TGCAAATGTGGACTGCAGAG	TGCAGCTTGAGGGTTTTTGG	NM_001024828.3
TET-1	GGCACTACATCAACCTCATC	CTGTGCTTTCCCTCAACAA	XM_025151680.1
TET-2	TTTTGCATGAGTCTGTCAGC	TGTTCTCACAGCCAAAGTGC	NM_001277794.1
TET-3	TGCAGGAGAACCTCAACCATAC	TGCCTGGTGATGGTTTTG	XM_015297468

RPS17, ribosomal protein S17; DNMT-1, DNA methyltransferase 1; DNMT-3a, DNA methyltransferase -3a; DNMT-3b, DNA methyltransferase 3b; TET-1, ten-eleven translocation 1; TET-2, ten-eleven translocation 2; TET-3, ten-eleven translocation 3.

Results

Effect of Length of Heat Exposure on Gene Expression of DNMTs in Chicks

Fig. 1 shows the gene expression of DNMTs in 0 (control), 1, 2, 6, 9, and 12 h of thermal conditioning. The expression level of DNMT-1 was not affected by thermal conditioning (Fig. 1A) while the mRNA level of DNMT-3a was upregulated by thermal conditioning (Fig. 1B). However, there was no significant difference in the gene expression of DNMT-3a in the hypothalamus between the processing times of thermal conditioning. Meanwhile, the expression level of DNMT-3b in the hypothalamus did not differ between the groups (Fig. 1C).

Effect of Length of Heat Exposure on Gene Expression of TETs in Chicks

The effect of the processing length of thermal conditioning on the gene expression of TETs in chicks is shown in Fig. 2. The gene expression of TET-1 increased with the processing time of thermal conditioning treatment (Fig. 2A). The expression levels of TET-1 in the 6, 9, and 12 h treatment groups was almost double that of the control group. On the other hand, there were no differences in the mRNA levels of TET-2 and TET-3 between the groups.

Discussion

The present study showed changes in gene expression of DNA methylation-related genes in the hypothalamus after thermal conditioning treatment. The gene expression of methyltransferases (DNMT-1, DNMT-3a, and DNMT-3b) and demethylation-related proteins (TET-1, TET-2, and TET-3) were investigated. The mRNA level of DNMT-3a was increased, while the levels of DNMT-1 and -3b were not changed by thermal conditioning treatment (Fig. 1). The TET-1 expression level increased with the processing time of the thermal conditioning treatment, but there were no differences between groups in gene expression of TET-2 and TET-3 (Fig. 2).

DNA methylation and demethylation are known epigenetic mechanisms of reorganization. It controls translation by binding a methyl group to CpG islands in the genome promoter region (Bernstein *et al.*, 2007; Miranda and Jones, 2007; Triantaphyllopoulos *et al.*, 2016). DNMTs play important roles in CpG methylation and transfer methyl groups from S-adenosyl methionine (SAM) to CpG binding sites (Anderson *et al.*, 2012; Crider *et al.*, 2012; Baardman *et al.*, 2015; Hervouet *et al.*, 2018). Inheritance methylation, which maintains methylation states during replication, is catalyzed by DNMT-1. The enzyme has a high affinity for both methylated cytosine (5-methyl cytosine) and SAM as a methyl group donor (Bacolla *et al.*, 1999; Fatemi *et al.*, 2001). In this study, the gene expression of DNMT-1 in the hypothalamus was not affected by thermal conditioning (Fig. 1A). Therefore, regardless of the duration of thermal conditioning, DNA methylation status might be maintained under normal conditions. *De-novo* methylation is catalyzed by DNMT-3a and -3b. DNMT-3a has an affinity for un-

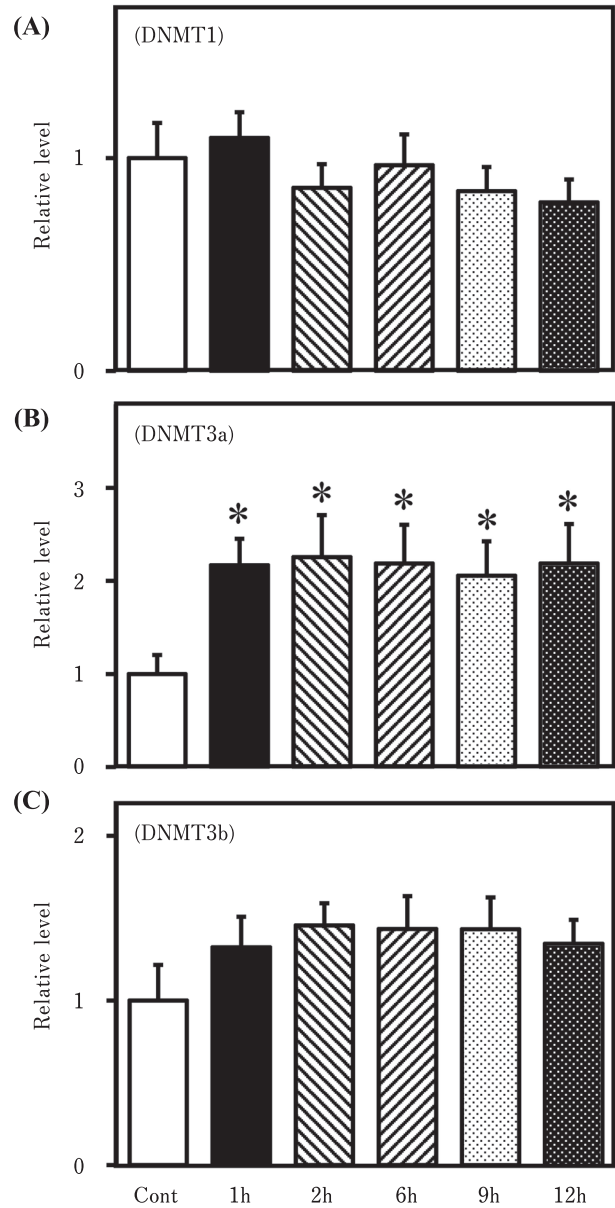


Fig. 1. **The gene expression levels of DNMT-1 (A), DNMT-3a (B) and, DNMT-3b (C) in 1, 2, 6, 9 and 12 h of thermal conditioning treated chicks and control chicks.** Data were expressed as means \pm SEM. Asterisks refer to the level of statistical significance, compared with control (*, $P < 0.05$). The number of chicks in each group was $n = 8$. Cont: control, DNMT-1: DNA methyl transferase 1, DNMT-3a: DNA methyl transferase 3a, DNMT-3b: DNA methyl transferase 3b.

methylated cytosine, whereas DNMT-3b acts on both 5-methyl cytosine and unmethylated cytosine (Okano *et al.*, 1999; Hervouet *et al.*, 2018). It has been reported that thermal conditioning treatment at an early age alters DNA methylation levels in the central nervous system (Yossifoff *et al.*, 2008; Cramer *et al.*, 2019). In addition, Yossifoff *et al.*

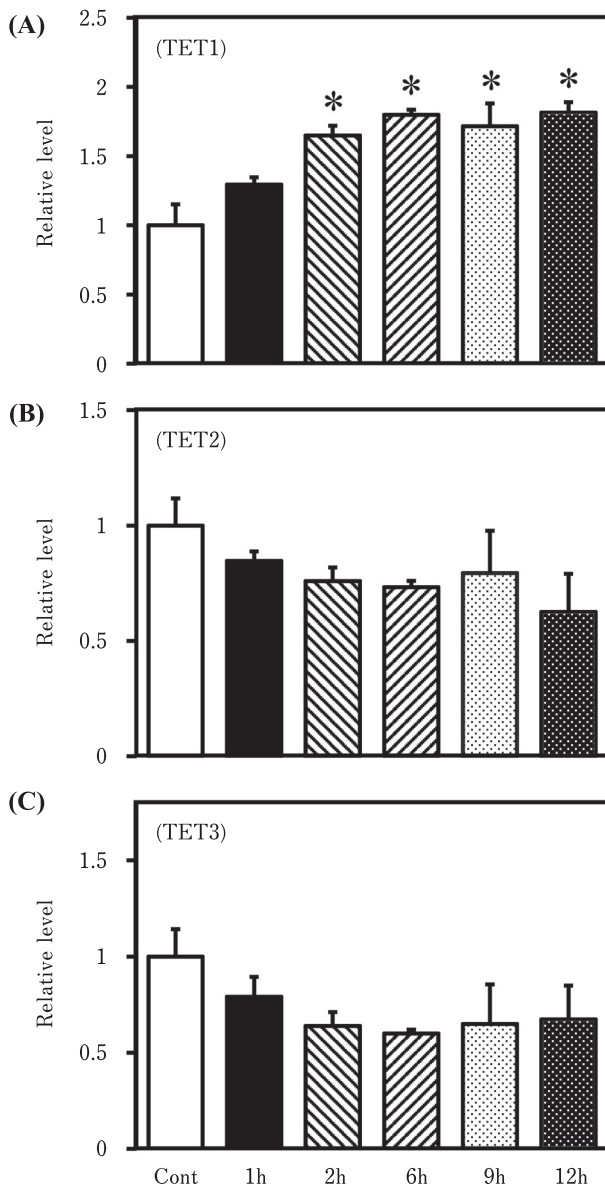


Fig. 2. The gene expression levels of TET-1 (A), TET-2 (B) and, TET-3 (C) in 1, 2, 6, 9, 12 h of thermal conditioning treated chicks and control chicks. Data were expressed as means \pm SEM. Asterisks refer to the level of statistical significance, compared with control (*, $P < 0.05$). The number of chicks in each group was $n=8$. Cont: control, TET-1: ten-eleven translocation 1, TET-2: ten-eleven translocation 2, TET-3: ten-eleven translocation 3.

(2008) revealed that the gene expression level of DNMT-3a in the hypothalamus was increased by thermal conditioning. In this study, similar to a previous report, an increase in DNMT-3a gene expression level due to thermal conditioning was confirmed. This result supports the theory that thermal conditioning significantly alters *de novo* DNA methylation in the central nervous system. However, it is still unclear which genes are methylated by thermal conditioning and the

association between methylation modification and acquired thermotolerance. Thus, further studies are needed for an extensive methylation analysis.

5-methyl cytosine is not a completely stable DNA modification. Fig. 3 shows the DNA demethylation pathway. There is a process of demethylation called passive demethylation because of the lack of maintenance of 5-methyl cytosine methylation during DNA replication (Valinluck and Sowers, 2007). In addition, TET family proteins are known to cause demethylation of 5-methyl cytosine by oxidizing 5-methyl cytosine to 5-hydroxy methyl cytosine, 5-formylcytosine, and 5-carboxycytosine (Tahiliani *et al.*, 2009; Guo *et al.*, 2011; Ito *et al.*, 2010; Ito *et al.*, 2011; Wu and Zhang, 2017). Three subtypes of TET (TET-1, TET-2, and TET-3) catalyze the conversion of 5-methyl cytosine to 5-hydroxy methyl cytosine (Kriaucionis and Heintz, 2009; Tahiliani *et al.*, 2009; Ito *et al.*, 2010). Previous studies have indicated that 5-hydroxy methyl cytosine is not recognized by the methylation-maintaining enzyme DNMT-1, and it is converted to cytosine during replication. This demethylation mechanism is similar to the passive demethylation of 5-methyl cytosine. Similar to mammals, TET family proteins are expressed in chickens and are involved in DNA demethylation (Okuzaki *et al.*, 2017). In this study, the gene expression of TET-1 in the hypothalamus increased with increasing duration of thermal conditioning (Fig. 2A). Therefore, it seemed that thermal conditioning advanced the conversion from 5-methyl cytosine to 5-hydroxy methyl cytosine. The 5-hydroxy methyl cytosine, which is increased by TET-1, is converted to cytosine by the passive demethylation pathway (Fig. 3). It is therefore suggested that thermal conditioning highly demethylates genes in the hypothalamus.

The present study showed that thermal conditioning methylates and demethylates genes in the central nervous system. It has been reported that thermal conditioning changes gene expression in the hypothalamus of chickens, as well as the DNA methylation levels of genes such as corticotropin-releasing hormone and brain-derived neurotrophic factor (Yossifoff *et al.*, 2008; Tanizawa *et al.*, 2014; Cramer *et al.*, 2019; Ouchi *et al.*, 2020, in press). These genes are important for stress response and thermoregulation in chickens (Siegel, 1980; Mujahid and Furuse, 2008; Nobel *et al.*, 2011; You *et al.*, 2020). However, it is unclear which gene's epigenetic reorganization is associated with the acquisition of thermotolerance due to thermal conditioning. In addition, there are few reports of thermal conditioning-induced DNA demethylation, suggesting that further research is needed.

Conflicts of Interest

The authors declare no conflict of interest.

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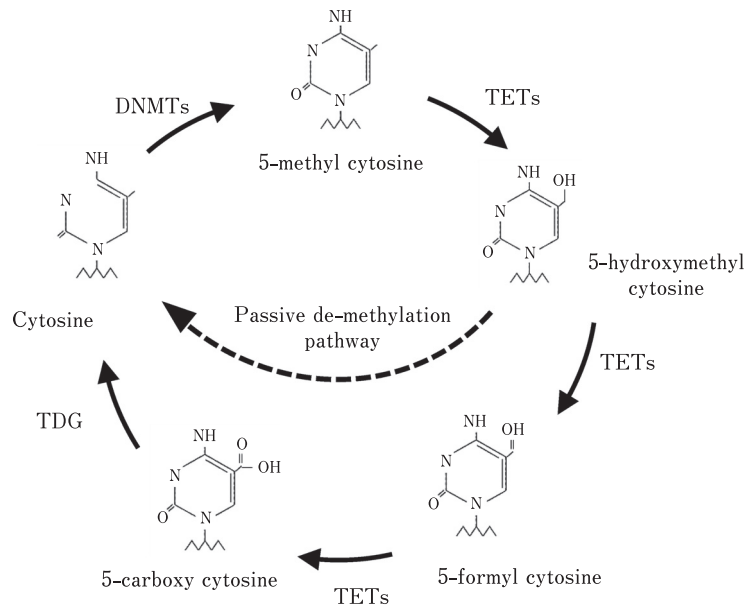


Fig. 3. **Schematic chart of DNA methylation and demethylation cycle.** DNMTs: DNA methyl transferases, TET: ten-eleven translocases, TDG: thymine DNA glycosylase.

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