

Effects of Thermal Conditioning and Folic Acid on Methylation of the BDNF Promoter Region in Chicks

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This study aimed to investigate the effects of thermal conditioning and folic acid on the methylation levels of the avian brain-derived neurotrophic factor (BDNF) promoter region at the M3 and M9 positions in the early life of broiler chicks. In Experiment 1, male broiler chicks (day 3 of life) were orally injected with methyl cellulose solution with or without folic acid (25 mg). The chicks in the heat-treatment groups were immediately exposed to a high ambient temperature ($40 \pm 0.5^\circ\text{C}$) for 12 h, while chicks in the non-heat treatment groups were left in the thermoneutral zone ($30 \pm 0.5^\circ\text{C}$). The groups were as follows: 1) no thermal conditioning group without folic acid (control), 2) thermal conditioning group without folic acid, 3) no thermal conditioning group with folic acid, and 4) thermal conditioning group with folic acid. In Experiment 2, treatments were similar to those in Experiment 1, except for the usage of female chicks. After the treatments, the methylation levels of the BDNF promoter in chicks were determined using semiquantitative PCR. There were no significant differences between groups in the levels of methylation at the M3 position in both males and females as a result of thermal conditioning and folic acid treatment. Interestingly, significant effects of thermal conditioning and folic acid treatment on methylation at the M9 position were found. BDNF methylation levels at M9 significantly decreased following thermal conditioning, while folic acid suppressed demethylation in both male and female chicks. These data suggest that folic acid and thermal conditioning affects DNA methylation patterns in the central nervous system of chicks, regardless of sex.

Key words: brain-derived neurotrophic factor, chicks, DNA methylation, folic acid, thermal conditioning

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Introduction

Early life experiences affect the structural and functional development of the nervous system in animals, leading to the development of physiological abilities. Several studies provide evidence indicating that epigenetic modifications of the genome exert effects later in life (Sweatt, 2009; Szyf, 2009). DNA methylation is an epigenetic modification that causes gene expression or phenotypic changes without changing the primary DNA sequence (Berry *et al.*, 2010;

Crider *et al.*, 2012).

Early thermal conditioning to improve the ability of chickens to survive heat stress involves the exposure of young chicks to a high ambient temperature for half a day or a whole day. This increases heat tolerance, weight gain, and mortality (Yahav and Hurwitz, 1996; Yahav and McMurtry, 2001). These changes have been related to DNA methylation or histone modification (Yossifoff *et al.*, 2008; Kislouk and Meiri, 2009). As for a candidate of the epidemic episode, Yossifoff *et al.* (2008) suggested that the acquisition of heat tolerance through thermal conditioning occurred through the methylation of the brain-derived neurotrophic factor (BDNF) promoter region in the hypothalamus of chicks. The methylation levels in some cytosine-guanine dinucleotide (CpG) sites of the promoter region were analyzed, and significant changes at positions M1, M3, and M9 were found after heat exposure of Cobb male chicks (Yossifoff *et al.*, 2008). Several reports (e.g., Tanizawa *et al.*, 2014; Ouchi *et al.*, 2020) have revealed the acquisition of heat tolerance by thermal conditioning using various

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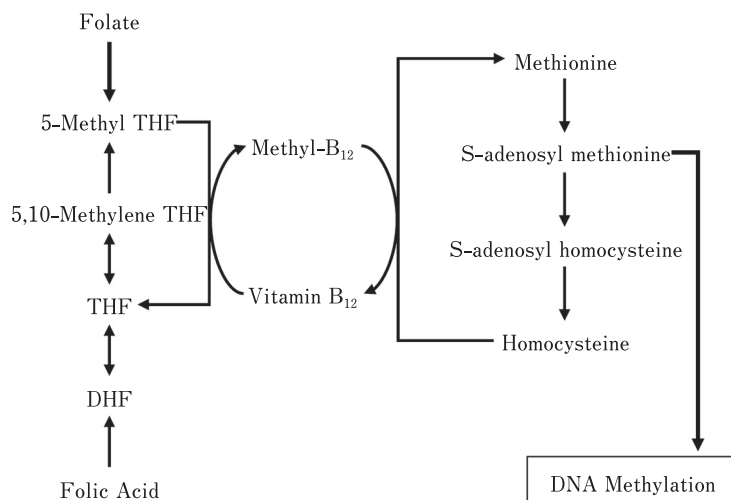


Fig. 1. Schematic chart of the relationship between folic acid and DNA methylation. DHF: dihydrofolate; THF: tetrahydrofolate

breeds or lines of chickens; however, there have been few investigations regarding line or sex differences in the methylation levels of the BDNF promoter region. Information regarding these differences should help understand the mechanism underlying the acquisition of thermotolerance by thermal conditioning and enable the utilization of thermal conditioning in poultry production.

It is necessary to accelerate thermal conditioning-induced heat tolerance in order to improve poultry production, such as through nutritional supplementation. Methylation of DNA requires a methyl donor. Folic acid is an important source of methyl moieties, which are used to synthesize S-adenosyl methionine, the methyl donor for DNA methylation (Fig. 1). There are several studies on the interactions between folate and folic acid status and DNA methylation (Crider *et al.*, 2012). However, there is no information about the effect of folic acid on the methylation of the BDNF promoter region in chicks.

This study aimed to investigate the effects of early thermal conditioning and folic acid treatment on the methylation levels of the avian BDNF promoter regions M3 and M9 in Chunky chicks. In addition, we compared the effects of sex on methylation levels after thermal conditioning in the chicks.

Materials and Methods

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

Animals

Day-old male and female broiler chicks (Chunky: Ross 308) were obtained from local hatcheries (Fukuda Hatchery, Okayama, Japan). The chicks were maintained in a room with 24-h lighting, at a temperature of $30 \pm 0.2^\circ\text{C}$, in poly-

propylene boxes with sawdust litter ($36 \times 40 \times 30$ cm), and at a population density of six chicks per box during the experimental period. The chicks were given free access to a commercial starter diet (Nichiwa Sangyo Co. Ltd., Kobe, Japan) and water until the end of the experiment.

Preparations of Drugs

Folic acid (pteroylglutamic acid) was obtained from Wako Pure Chemical Industries (Osaka, Japan) and suspended in a 0.25% methyl cellulose solution. The suspension was settled on the stirrer with the heater kept at $38 \pm 0.5^\circ\text{C}$ during treatment administration.

Experimental Design

In Experiment 1, male chicks (3 days old) were distributed into four groups ($n=8$ per group) based on their body weight, so that the average body weight was similar in all groups. The groups were as follows: 1) no thermal conditioning group without folic acid (control), 2) thermal conditioning group without folic acid, 3) no thermal conditioning group with folic acid, and 4) thermal conditioning group with folic acid. Chicks were orally administered 0.1 mL of a 0.25% methyl cellulose solution with or without folic acid (50 mg/kg) using a syringe with a silicone tube. The dose of folic acid was determined based on a previous report (Gao *et al.*, 2017), and the average body weight. Immediately after folic acid administration, the chicks in the treatment group were exposed to a high ambient temperature ($40 \pm 0.5^\circ\text{C}$; inner size of heat chamber: $90 \times 90 \times 115$ cm; Ouchi *et al.*, 2020) for 12 h, and those in the control group were kept in the thermoneutral zone ($30 \pm 0.5^\circ\text{C}$). The duration of heat exposure (12 h) was based on the report by Yossifoff *et al.* (2008). In Experiment 2, female chicks (3 days old) grouped similarly to those in Experiment 1 were orally administered folic acid and transferred to the heat chamber for 12 h ($n=8$ per group). After these treatments, chicks in all groups were anesthetized with isoflurane (FUJIFILM Wako Pure Chemi-

cal Corporation, Osaka, Japan) and decapitated. Immediately after decapitation, the diencephalic tissues of the chicks were removed and stored at -80°C till DNA extraction was performed.

DNA Methylation Analysis by Bisulfite Modification

DNA methylation analysis was performed for the BDNF promoter region at positions M3 and M9, and a schematic chart is presented in Fig. 2. DNA was extracted from diencephalic tissues using a commercial DNA isolation kit (Takara Bio Inc., Shiga, Japan), and purified DNA was measured using a spectrophotometer (NanoDrop ND-2000c, Thermo Scientific, Inc.) at 260 nm. The purity of the DNA was also analyzed by measuring absorbance at 260 and 280 nm. Bisulfite analysis of BDNF promoter methylation was performed using the MethylEasy Xceed Rapid DNA Bisulfite Modification Kit (Human Genetic Signatures Pty. Ltd., New South Wales, Australia), according to the manufacturer's instructions. The methylation levels of the BDNF promoter were determined by semiquantitative PCR. The primers used for methylation-specific PCR are presented in Table 1.

Statistical Analysis

Data were analyzed using the commercially available package, StatView (Version 5, SAS Institute, Cary, USA, 1998); two-way ANOVA was used to analyze the effects of thermal conditioning and folate treatment. When effects were found to be significant, a post-hoc test was performed using the Tukey-Kramer test. Statistical significance was considered at $P < 0.05$. All data are expressed as mean \pm standard error of the mean (SEM).

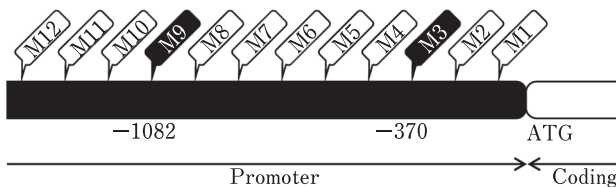


Fig. 2. Schematic diagram of the CpG sites upstream of the translational start site (ATG) of the BDNF coding region. White characters with black backgrounds show the sites analyzed for methylation level.

Results

DNA Methylation by Thermal Conditioning and Oral Administration of Folic Acid in Male Chicks

Figure 3 shows the methylation levels of the avian BDNF promoter region at the M3 and M9 positions in male chicks. There were no significant differences in methylation levels at M3 among groups (left panel; $P > 0.05$). At M9, the main effects of treatments and interaction between thermal conditioning and folic acid treatment were significant (right panel; $P < 0.05$), and the methylation level in the thermal conditioned group without folic acid was significantly lower than those in the other groups.

DNA Methylation by Thermal Conditioning and Oral Administration of Folic Acid in Female Chicks

The methylation levels of the avian BDNF promoter region at the M3 and M9 positions in female chicks are shown in Fig. 4. Although no significant changes in the BDNF methylation levels at M3 in chicks after thermal conditioning and folic acid treatment were detected (left panel; $P > 0.05$), there were significant effects of both treatments and interaction on the levels of BDNF methylation at M9 (right panel; $P < 0.05$). Similar to male chicks, thermal conditioning without folic acid decreased the methylation level of the BDNF promoter region in female chicks.

Discussion

In this study, we measured the methylation levels of the BDNF promoter region in the central nervous system of chicks subjected to thermal conditioning and orally administered folic acid. We also compared the effects of these treatments between males and females. There were no significant differences between groups in the level of methylation at the M3 position in both males and females, and no effects of thermal conditioning and folic acid were found. Interestingly, there were significant differences between groups in response to thermal conditioning, folic acid treatment, and the interaction between thermal conditioning and folic acid treatment at the M9 position. Heat exposure significantly decreased BDNF methylation levels at M9, while folic acid treatment suppressed demethylation in both male and female chicks.

Table 1. Primer sequences for methylation-specific PCR

CpG site	F/R	Sequence (5' -3')
M3		
Methylated	F	GTGAAACGTTGTGTGTTAAATAG
Unmethylated	F	GTTGAAATGTTGTGTGTTAAATAG
M + U	R	AAATCAAATACTACATAAACTCCT
M9		
Methylated	F	GTGTTGGTAGGAATGACGTTTTG
Unmethylated	F	GTGTTGGTAGGAATGATGTTTTG
M + U	R	AACACCAACTAACAACATCAATAAA

F, forward primer; R, reverse primer; M + U, methylated and unmethylated

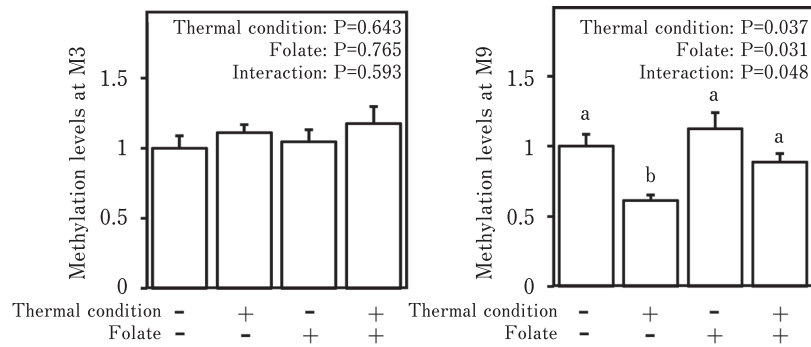


Fig. 3. **Methylation levels at the M3 (left panel) and M9 (right panel) positions of the avian BDNF promoter region in 3-day-old male chicks.** Data are expressed as mean \pm SEM. Means with different letters are significantly different at $P < 0.05$.

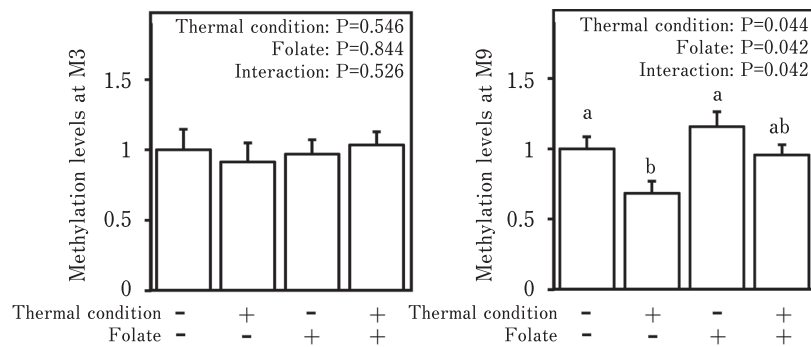


Fig. 4. **Methylation levels at the M3 (left panel) or M9 (right panel) position of the avian BDNF promoter region in 3-day-old female chicks.** Data are expressed as mean \pm SEM. Means with different letters are significantly different at $P < 0.05$.

An epigenetic change is a change in the gene expression or cell phenotype that is inherited by progeny cells without any change in the DNA sequence; epigenetic changes include histone modifications and DNA methylation (Kelly *et al.*, 2010; Meissner, 2010; Portela and Esteller, 2010). DNA methylation, the focus of this study, regulates gene expression through the binding of methyl groups to the CpG islands of the genome (Moore *et al.*, 2013). In chickens, DNA methylation levels of the BDNF promoter regions are changed by thermal conditioning at an early age (Yossifoff *et al.*, 2008). Here, the methylation level of the M3 position of the BDNF promoter region (Fig. 2) was not affected by thermal conditioning; however, thermal conditioning downregulated the methylation level of the M9 position of the BDNF promoter region (Fig. 2, 3, and 4). In a previous report, thermal conditioning of Cobb chicken upregulated the DNA methylation level of M3 and downregulated that of M9 (Yossifoff *et al.*, 2008). The results of this study did not match those of this previous report. In this study, Chunky (Ross 308) chicks were used as the experimental model. The results of the present study suggested that differences in

DNA methylation patterns and susceptibility to DNA methylation depend on the line of chickens. In humans, DNA methylation patterns differ between Caucasians, African Americans, and Hispanics (Adkins *et al.*, 2011; Zhang *et al.*, 2011).

The effects of environmental factors, such as immune response and thermoregulation (Cryan and Wolf, 2003; Kelein and Flanagan, 2016), differ among genders, similar to DNA methylation. In humans, the effects of prenatal fasting on DNA methylation levels in metabolism-related genes, such as the leptin and insulin growth factor 2 receptor genes, vary between males and females (Tobi *et al.*, 2009). In addition, the effects of environmental factors on DNA methylation differ between males and females in mammals, including humans (Davegårdh *et al.*, 2019; Maschietto *et al.*, 2017; Kippler *et al.*, 2013; Gallou-Kabani *et al.*, 2010; Liu *et al.*, 2010). In this study, changes in the DNA methylation levels of the BDNF promoter region due to thermal conditioning did not differ between male and female chicks (Fig. 3 and 4). DNA methylation is affected by gender; however, some genes were affected, while others were not (Tobi *et al.*,

2009). Global DNA methylation patterns may differ among genders in chickens. Thermal conditioning did not cause changes in the methylation patterns of the BDNF promoter region among genders.

Folic acid acts as a methyl group donor in the process of DNA methylation (Fig. 1). Dietary folate is metabolized to 5-methyltetrahydrofolate (5-methyl THF) and is converted to tetrahydrofolate (THF) in the gut and liver, where methyl groups are simultaneously released to the methionine synthesis reaction (Fig. 1). S-adenosyl methionine (SAM) is a metabolite of methionine that is converted to S-adenosyl-homocysteine (SAH). In this conversion, methyl groups are released from SAM and are used for DNA methylation (Fig. 1). A relationship has been found between folate and global DNA methylation (Cravo *et al.*, 1994, 1998; Pufulete *et al.*, 2005). In general, demethylation has been confirmed in the tumor tissues and blood of patients with cancer. Dietary and supplementary folate have been reported to suppress the demethylation of tumor-related genes (Levine *et al.*, 2003; Christensen *et al.*, 2010; Wallace *et al.*, 2010; Kim *et al.*, 2011); thus, folate affects, not only the global DNA methylation patterns but also the level of DNA methylation in specific genes. In this study, the methylation level at M3 of BDNF was not affected by folate; however, demethylation was found at M9 due to the attenuation of the effect of thermal conditioning by orally administered folate (Fig. 3 and 4). Folate has been used in patients with cancer and suppresses DNA demethylation (Wallace *et al.*, 2010; Kim *et al.*, 2011); these findings are consistent with those of the present study. DNA methylation occurs through two mechanisms: *de novo* methylation, which involves the methylation of unmethylated genes, and inheritance methylation, which involves the maintenance of methylation during DNA replication. DNA methyltransferases (DNMTs) bind a methyl group to the cytosine in the base of SAM (Hervouet *et al.*, 2018). However, the mechanism of DNMT action during thermal conditioning in chicks remains unclear. Future studies on this topic are needed.

It has been suggested that the methylation of the BDNF promoter region is involved in heat tolerance induction by thermal conditioning in chicks (Yossifoff *et al.*, 2008). BDNF plays a pivotal role in neuronal development and neurogenesis (Poo, 2001; Lee *et al.*, 2002; Scharfman *et al.*, 2005) and in feeding regulation and energy metabolism in animals (Nakagawa *et al.*, 2000; Xu *et al.*, 2003). Therefore, thermotolerance in thermally-conditioned chickens can be attributed to the epidemic regulation of BDNF gene transcription. However, the present results regarding changes in DNA methylation in response to thermal conditioning and folic acid treatment did not coincide with the findings of Yossifoff *et al.* (2008). It is unlikely that the methylation levels of the avian BDNF promoter region are critical for the acquisition of thermotolerance by early thermal conditioning in chicks. Further study of epigenetic changes related to thermal conditioning is necessary to elucidate thermotolerance acquisition in chicks.

In conclusion, our findings indicate that the oral admini-

stration of folic acid partially affected DNA methylation patterns in the central nervous system of chicks, regardless of sex. Additionally, our findings suggest that changes in BDNF promoter methylation at M3 may differ depending on the genetic line of chicks.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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