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Flavangenol regulates gene expression of HSPs, anti-apoptotic and anti-oxidative factors to protect primary chick brain cells exposed to high temperature

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ABSTRACT

Heat-stress exposure increased the expression of heat-shock proteins (HSPs), B-cell lymphoma 2 (BCL-2) and anti-oxidative enzymes to maintain normal cellular function by attenuating the oxidative reaction and apoptosis. Reducing the stress response or enhancing anti-stress capability is an important goal in animal production. Our previous study indicated a protective role of flavangenol, a pine bark extract, in chicks after three hours of high-temperature exposure. However, the cellular mechanism of flavangenol was not clarified *ex vivo*. In the current study, we investigated the effect of flavangenol on cellular apoptosis and oxidation in heat-stressed treated chick brain cells (mixed neurons and glia cells). The primary brain cells were isolated from the diencephalon of 14-day-old chicks and cultured at 41.5 °C (to mimic the body temperature of young chicks), and were treated with flavangenol from day 3 of isolation to day 8. Cells were kept bathed in the cell culture dish under a high temperature (HT: 45 °C, 20 or 60 min) on day 8 and were then collected for analysis of cell viability as well as for HSP and other related gene expression. Flavangenol treatment significantly increased cell viability and BCL-2 mRNA expression, and attenuated HSP-70 and BCL-2-associated X protein mRNA expression. Moreover, flavangenol treatment elevated the mRNA expression of glutathione peroxidase in the HT group, which indicates that cellular anti-oxidative ability was strengthened by flavangenol. In conclusion, flavangenol may play a protective role in cells damaged or killed by heat stress by increasing cellular anti-oxidative pathways.

1. Introduction

Heat stress is a serious problem for poultry because it is challenging for birds to maintain their body temperature when exposed to a high ambient temperature (HT) due to the absence of sweat glands (Ensminger et al., 1990) and their high metabolic rate (Geraert et al., 1993). HT has an enormous impact on the induction of cytotoxicity through, for example, increasing cell death and cellular reactive oxygen species (ROS) (Fulda et al., 2010; Akbarian et al., 2016). A cellular defense system that protects cells from various environmental stressors, including heat stress, is required for cell survival and to maintain normal physiology (Drew, 2012; Surai, 2016).

Heat stress causes cellular protein to misfold and aggregate, and upregulates a group of molecular chaperones called heat-shock proteins (HSPs) (Nollen and Morimoto, 2002; Wallace et al., 2015). HSP-70 and -90, usually used as a stress marker, are the widely studied groups of HSP families due to their rapidly enhanced gene expression following exposure to heat stress (Wang et al., 2013). During heat exposure, HSPs are increased in cells to prevent cellular protein degradation (Kiang and Tsokos, 1998). To produce HSPs, heat-shock factors (HSFs), the specific transcription factors of HSPs, are essential. Among these, HSF-1 and -3 are necessary for HSP production in avians (Morimoto, 1998; Akerfelt et al., 2010). Both HSFs and HSPs exist in all organisms as a biological defense system for supporting the survival of organisms against environmental stress (Kiang and Tsokos, 1998).

Heat stress could also induce cell death and oxidative stress. It has been reported that heat stress induces early apoptosis through activation of caspase-9 and -3 in the mitochondria in human umbilical vein endothelial cells (Gu et al., 2014). Under such a condition, a family of proteins, the B-cell lymphoma 2 (BCL-2) family, is involved in

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Table 1
Primers used for real-time PCR.

Gene	Accession no.	Sequences 5′–3′(forward / reverse)	Annealing Temperature (°C)	Product Size (bp)
HSP-70	AY143691.1	5′−GGGAGGACTTTGACAACCGA-3′/ 5′-CAAAGCGTGCACGAGTGATG-3′	60	219
HSP-90	NM_001109785	5′-GAAGACTCCCAGAACCGCAA-3′/ 5′-ACCTGGTCCTTTGTCTCACC-3′	60	155
HSF-1	L06098.1	5′-TGAAGCATGAGAACGAGGCG-3′/ 5′-CGGCTGTATTTCGGCATGGA-3′	60	199
HSF-3	L06126.1	5′-GTGTGACAGAGTGCCACAATA-3′/ 5′-ACAGCTGCAGGAGACTATGC-3′	62	215
Caspase-3	NM_204725.1	5′-TTCCACCGAGATACCGGACT-3′/ 5′-AAACTGCTTCGCTTGCTGTG-3′	62	179
BCL-2	NM_205339.2	5′−GATGTGCGTCGAGAGCGTCAA-3′/ 5′-GTGCAGGTGCCGGTTCAGGT-3′	64	91
Bax	XM_422067.4	5′- ACAGTTCTGCCTGGTGTCCTTG -3′/ 5′- TGTGTTTAAGGCACTGAACGGA-3′	60	183
GFAP	XM_418091.6	5′- CGGGCAGGATGGATTTCTCTT-3′/ 5′- TCGATGTAACTGGCGAAGCG-3′	60	115
MAP-2	XM_025152487.1	5′- CCTGCTGACCAACTGTTTGC-3′/ 5′- CCCCCATGCAGGGATTTTCT-3′	60	141
Nestin	NM_205033.2	5′- CAACGAGCCTACATTGCTAA-3′/ 5′- CTCATCTGGGAACTCACATTC-3′	60	289
iNOS	NM_204961.1	5′-CAAGAGATGGACAAGGGCCA-3′/ 5′-GGCCAATAAGAATGCACGGC-3′	60	150
SOD	NM_205064.1	5′-TTACCGGCTTGTCTGATGGA-3′/ 5′-ATGCAGTGTGGTCCGGTAAG-3′	60	229
GPX	NM_001277853.1	5′-TGTTCGAGAAGTGCGAGGTG-3′/ 5′-TTGATGGTCTCGAAGTGGCG-3′	64	229
RP-II	NM_001006448.1	5′-CGACGGTTTGATTGCACCTG-3′/ 5′-CAATGCCAGTCTCGCTAGTTC-3′	64	161

Primers were designed with Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) for heat-shock proteins (HSP-70 and −90), heat-shock factors (HSF-1 and −3), caspase-3, B-cell lymphoma 2 (BCL-2), BCL-2-associated X protein (Bax), glial fibrillary acidic protein (GFAP), microtubule-associated protein-2 (MAP-2), nestin, inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD), glutathione peroxidase (GPx) and RNA polymerase II (RP-II).

maintaining normal tissue homeostasis by regulating apoptosis in the cells (Robertson et al., 1997). The BCL-2 family consists of members that either promote or inhibit apoptosis, and among these, BCL-2-associated X protein (Bax) was reported to promote apoptosis by inducing the release of cytochrome c and then activated caspase-9 and -3 (Robertson et al., 1997). On the other hand, BCL-2 was found to repress apoptotic cell death (Robertson et al., 1997). On the other hand, heat stress increases cellular ROS, such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical as well as peroxynitrite ($ONOO^-$). ROS, a pro-oxidant, is generated by the oxygen from respiration and nitric oxide (NO), which is produced by nitric oxide synthase (NOS) from L-arginine (Valko et al., 2007). Oxidative stress occurs when there is an imbalance between the pro-oxidants and the antioxidants induced by various stressors, including heat stress (Fulda et al., 2010). Normally there is a state of equilibrium between pro-oxidant species and anti-oxidant defense mechanisms, such as the enzymatic defense factors, including superoxide dismutase (SOD) and glutathione peroxidase (GPX) (Suzuki and Mittler, 2006; Valko et al., 2007). With these cellular defense mechanisms, cells could survive for a certain period of time during intense heat stress. However, these cellular protective mechanisms would not be sufficient under prolonged heat stress. Supplementation of the antioxidants is also a well-known practice for ameliorating the negative impacts of heat stress or oxidative stress (Brambilla et al., 2008; Tawfeek et al., 2014). It has been reported that polyphenols extracted from the tamarind seed coat can reduce both heat and oxidative stress in broilers (Aengwanich and Suttajit, 2010). Other antioxidants, such as pycnogenol, grape seed extract and resveratrol, also showed neuroprotective effects against either oxidative stress or apoptosis (Peng et al., 2002; Abdou and Wahby, 2016; Zhao et al., 2017). Flavangenol, a French maritime pine bark extract, is one of the effective polyphenol-rich antioxidants that has been used as a supplement as well as a skin-care substance (Yoshida et al., 2011; Furumura et al., 2012). It has also been reported that flavangenol has the capacity to protect against hepatotoxicity, inhibit lipid peroxidation and increase cellular anti-oxidative ability (Shimada et al., 2009; Yoshida et al., 2011; Ko et al., 2014). Previously, we found that chronic oral administration of flavangenol attenuated HSP gene expression, and demonstrated its protective function in central and peripheral tissues in heat-exposed neonatal chicks (Yang et al., 2016). However, the mechanism by which flavangenol protects the cellular state and the reason HSPs declined as a result of flavangenol treatment are still not clear. In the present study, we conducted experiments using primary chick brain cells to clarify the beneficial effects of flavangenol on HSP expression during heat exposure. We also examined whether flavangenol has a protective effect in cells and how it influences anti-apoptotic and anti-oxidative factor gene expression. Finally, glial fibrillary acidic protein (GFAP), a specific marker of glial cells, and microtubule-associated protein-2 (MAP-2) as well as nestin, the makers of neuron were further examined to investigate which type of brain cells were influenced by flavangenol.

2. Materials and methods

2.1. Animals

Day-old male layer chicks (Julia) (*Gallus gallus domesticus*) were purchased from a local hatchery (Murata hatchery, Fukuoka, Japan). The chicks were housed in groups of 20 in metal cages (floor space: 36 cm × 50 cm; height: 30 cm) at a constant temperature of 30 ± 1 °C and under continuous light until they were 14 days old. Food [Commercial starter diet (Adjust diets); Toyohashi Feed and Mills Co. Ltd., Aichi, Japan] and water were available *ad libitum*. This study was performed in accordance with the guidelines for animal experiments in the Faculty of Agriculture of Kyushu University and Law No. 105 and Notification No. 6 of the Japanese government.

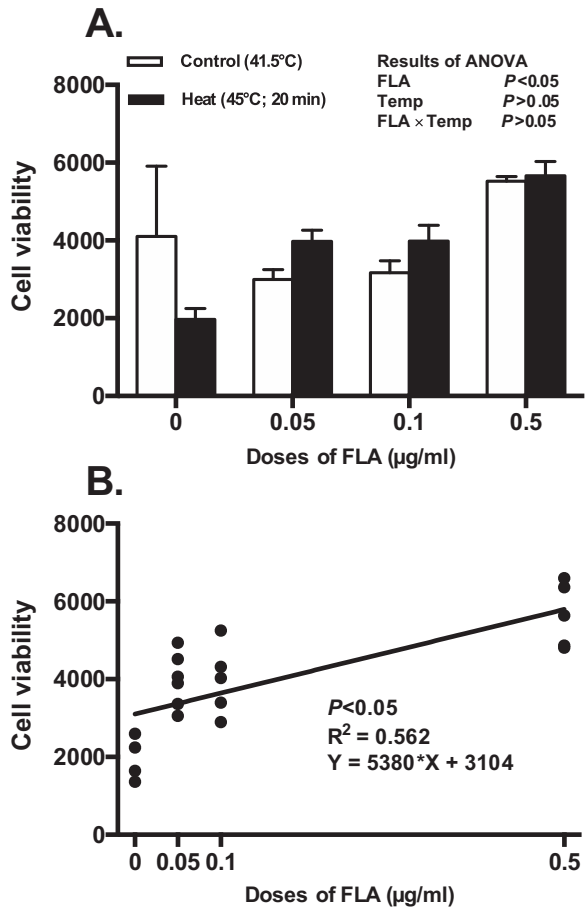

Fig. 1. (A) Cell viability of chick brain cells in a control thermoneutral temperature (CT; 41.5 ± 1 °C) or a high temperature (HT; 45 ± 1 °C) for 20 min where the cells were treated with flavangenol at 0, 0.05, 0.1 and 0.5 μg/ml every day. Values are mean ± SEM of the 4–6 samples in each group. (B) Correlation between dose of flavangenol and cell viability in HT groups. FLA: flavangenol, Temp: temperature.

2.2. Cell culture

Cell culture was performed using modified methods from previous studies (Todd et al., 2013; Kumar and Mallick, 2016). In brief, primary brain cells (mixed neurons and glia cells) were isolated from the diencephalon of chicks. The diencephalon was removed from the chicks (at 14 days old) and immediately transferred to an ice-cold Hibernate-E complete medium (Hibernate-E with 2% B-27 plus supplement and 0.5 mM GulutaMAX; Gibco, USA). Then the diencephalon was cut into small pieces with surgical scissors and incubated with papain (Worthington, USA) at 37 °C for 30 min. Then the solution containing brain tissue fragments was filtered and washed in the Hibernate-E complete medium twice. Finally, the cells were plated at a density of 1×10^5 cm^{-2} in 6-well plates in a Neurobasal complete medium (Neurobasal with 2% B-27 plus supplement and 0.5 mM GulutaMAX; Gibco, USA). All the plates were coated with 0.05 mg/ml poly-D-lysine (Sigma, USA) overnight before being used. The cells were maintained under a control thermoneutral temperature (CT: 41.5 °C) in a humidified 5% CO_2 incubator and the culture medium was changed every two days. The experiment was conducted at a cell density of over 85%. The reason for selecting the incubation temperature at 41.5 °C was to mimic the physiological body temperature of young chicks.

2.3. Experimental design

In Experiment 1, cells were treated with flavangenol (0, 0.05, 0.1 and 0.5 μg/ml) from day 3. The flavangenol® powder (including mainly 3.06% catechin, 0.24% epicatechin, 0.13% ferulic acid, 2.9% procyanidin B1, 1.3% procyanidin B3 and total polyphenol 75.0% (Folin-Denis) as per Toyo Shinyaku Co., Ltd. Saga, Japan) was dissolved in sterile double-distilled water (DDW) and added into the culture medium until the final concentration was obtained. On day 8, thermal heat stress was applied by immersing the culture plates into a water bath at 45 °C for 60 min. After undergoing the heat-stress, cells were harvested immediately for the extraction of total RNA and the synthesis of cDNA. All the cDNA samples were stored at −30 °C until their gene expression was analyzed by real-time PCR. In Experiment 2, cells were treated with flavangenol (0 and 0.5 μg/ml) on day 3 and heat stress (20 min) was applied by the same method described in Experiment 1. After undergoing heat stress, the cells were collected immediately for the same purpose as in Experiment 1.

2.4. Cell viability

The effects of heat stress and flavangenol on cell viability were determined by using cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan). The cells prepared in Section 2.2 were placed at a density of 5×10^3 cells/well in 96-well plates 48 h before undergoing heat stress. Following their incubation, they were treated with flavangenol (0, 0.05, 0.1 and 0.5 μg/ml) from day 3 to the day of the experiment. On the day of the experiment, the cells were further divided into control and heat-stress (45 °C, 20 min) groups. After the latter group had undergone heat stress, 10 μl CCK-8 solution was added into each well. Then the cells were further incubated at 41.5 °C in a humidified incubator for 240 min. After the 240-min incubation, the cellular fluorescence was immediately recorded at 450 nm with a plate reader (Wallac 1420 ARVOmx, PerkinElmer, Japan). The number of cells was calculated following the protocol using a standard curve.

2.5. Isolation of total RNA and quantitative real-time PCR

Total RNA was isolated from the chick brain cells by the use of RNAiso Plus (Takara, Shiga, Japan), as described in the manufacturer's protocol. A reverse transcription reaction was performed using the PrimerScript™ RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan) according to the manufacturer's protocol, using 1 μg of total RNA. All primers were tested by routine PCR (TaKaRa PCR Thermal Cycler Dice®, Takara, Shiga, Japan) and gel electrophoresis before real-time PCR was carried out. To quantify the expression of target genes, real-time PCR was conducted using Startagene MX3000P (Agilent Technologies, Tokyo, Japan) with a denaturation step at 95 °C for 30 s, 40 cycles of amplification at 95 °C for 15 s, and a primer-specific temperature for 30 s. The primer sequences are listed in Table 1. The mRNA level was calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). The calculated levels were normalized using mRNA levels of RNA polymerase II (RP-II). A dissociation curve was determined for each sample to confirm the specificity of the PCR conditions.

2.6. Nitrogen oxides assay

Since the phenol red contained in the Neurobasal medium could distort the final results as described on the protocol, Neurobasal medium without phenol red (Gibco, USA) was used in the experiment involving the analysis of the nitrogen oxides (NOx). The experimental design was the same as that described in Experiment 1. After 60 min of exposure to HT, the cells were given a recovery time of 360 min at a temperature of 41.5 °C to make sure that enough NOx was released from the cells. The recovery time was determined on the basis of a report that described the half-life of nitrate in the plasma as being 5–6 h (Zhang, 2017). Then the culture mediums were quickly collected into plastic tubes and centrifuged at 1000*g* for 15 min, and the supernatant was collected into new tubes. NOx analysis was conducted using a

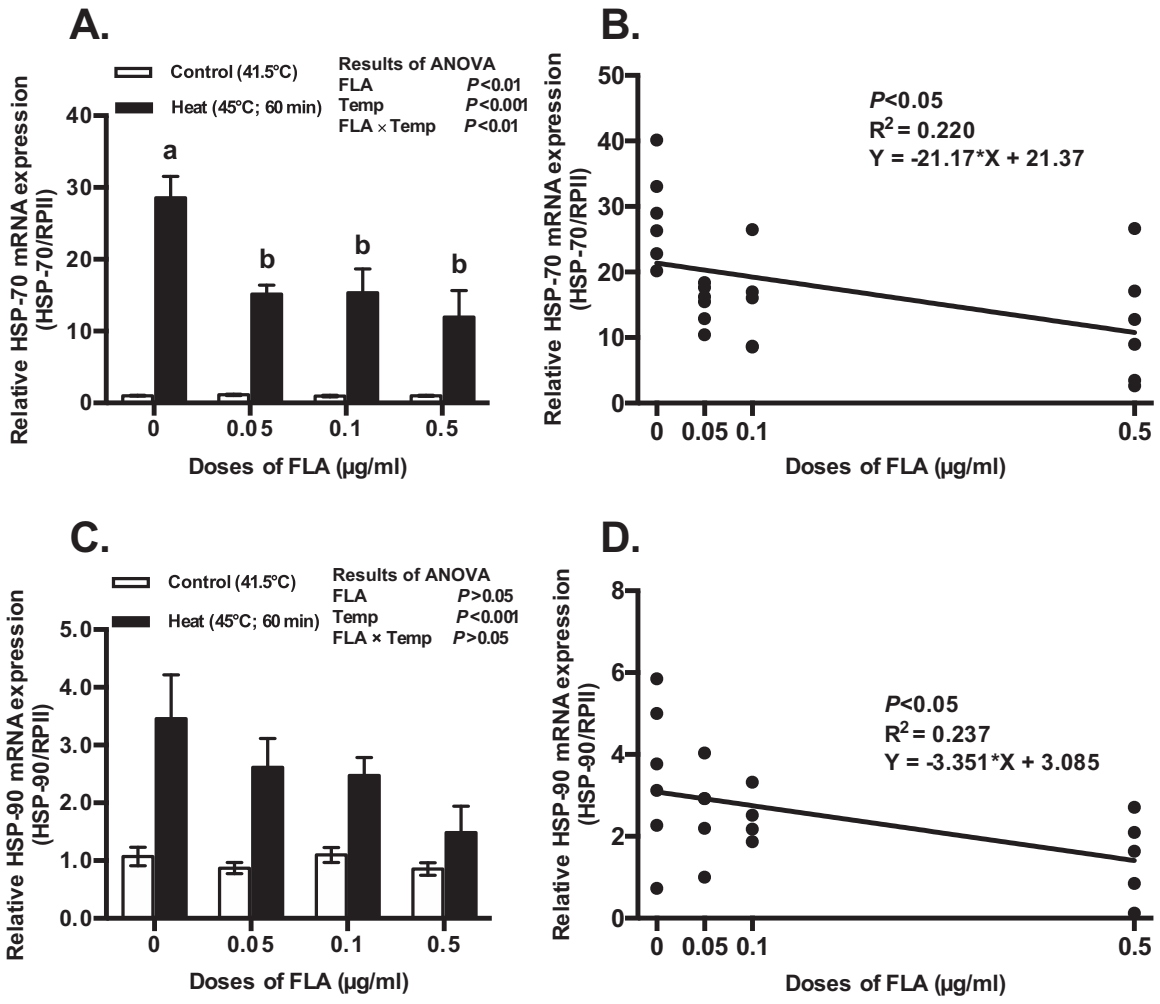

Fig. 2. Gene expression of cellular HSP-70 (A) and HSP-90 (C) exposed to a control thermoneutral temperature (CT; 41.5 ± 1 °C) or a high temperature (HT; 45 ± 1 °C) for 60 min where cells were treated with flavangenol at 0, 0.05, 0.1 and 0.5 μg/ml every day. Values are mean ± SEM of the 4–6 samples in each group. Correlation between dose of flavangenol and HSP gene expression in HT groups is shown in B and D. FLA: flavangenol, Temp: temperature.

commercial kit (Dojindo, Kumamoto, Japan), as described in the manufacturer's protocol, based on the reaction with 2, 3-diaminonaphthalene (DAN). The fluorescence of DAN was detected at 450–465 nm with a plate reader (Wallac 1420 ARVOmx, PerkinElmer, Japan). The NOx concentrations were calculated following the protocols with a standard curve.

2.7. Immunocytochemistry

In Experiment 2, cells were cultured on coverslips with a diameter of 18 mm from day 1 to the day of the experiment. Treatment with flavangenol (0 and 0.5 μg/ml) and the application of heat stress (45 °C, 20 min) were carried out using the same procedures described above. Cell immunocytochemistry was carried out in a form that was modified from that previously described (Steel et al., 2012; Kumar and Mallick, 2016). After undergoing heat stress, cells were washed in cold PBS and fixed in 4% paraformaldehyde on ice for 20 min, and were then washed in cold PBS and incubated in 1% BSA/PBS with 0.25% Triton X-100 at room temperature for 30 min of blocking. The immunocytochemistry of HSP-70 was conducted to determine its expression pattern and localization in the primary brain cells. In brief, after blocking, the cells were incubated for 2 h with a specific anti-mouse HSP-70 primary antibody (1:2000 dilution, Abcam, ab2787, USA). After being washed with PBS, the cells were incubated for 60 min with a goat anti-mouse IgG-Cy3 labeled secondary antibody (1:1000 dilution, Invitrogen, USA). Then the coverslips were air-dried in the dark and mounted on a glass slide using mounting solution with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen, USA). Images were captured using the following microscopy system: Leica DM16000B-AFC with a Leica DFC365FX monochrome digital camera with a 20 x lens and LAS AF 3.1.0 controller software.

2.8. Statistical analysis

Data were analyzed by factorial one-way or two-way analysis of variance (ANOVA) with respect to HT and flavangenol treatments, using StatView Version 5.0. A Tukey-Kramer test was performed as a means separation test to compare the data for each treatment when the interaction was found to be significant. Correlation and regression analyses were performed using Prism 6 (GraphPad Software, LA Jolla, CA). Statements of significance were based on $P < 0.05$. Data were expressed as means ± S.E.M.

3. Results

3.1. Cell viability following flavangenol treatment for 20 min under HT and CT

The effects of heat stress and flavangenol on cell viability are shown in Fig. 1. Although 45 °C heat stress for 20 min did not affect cell

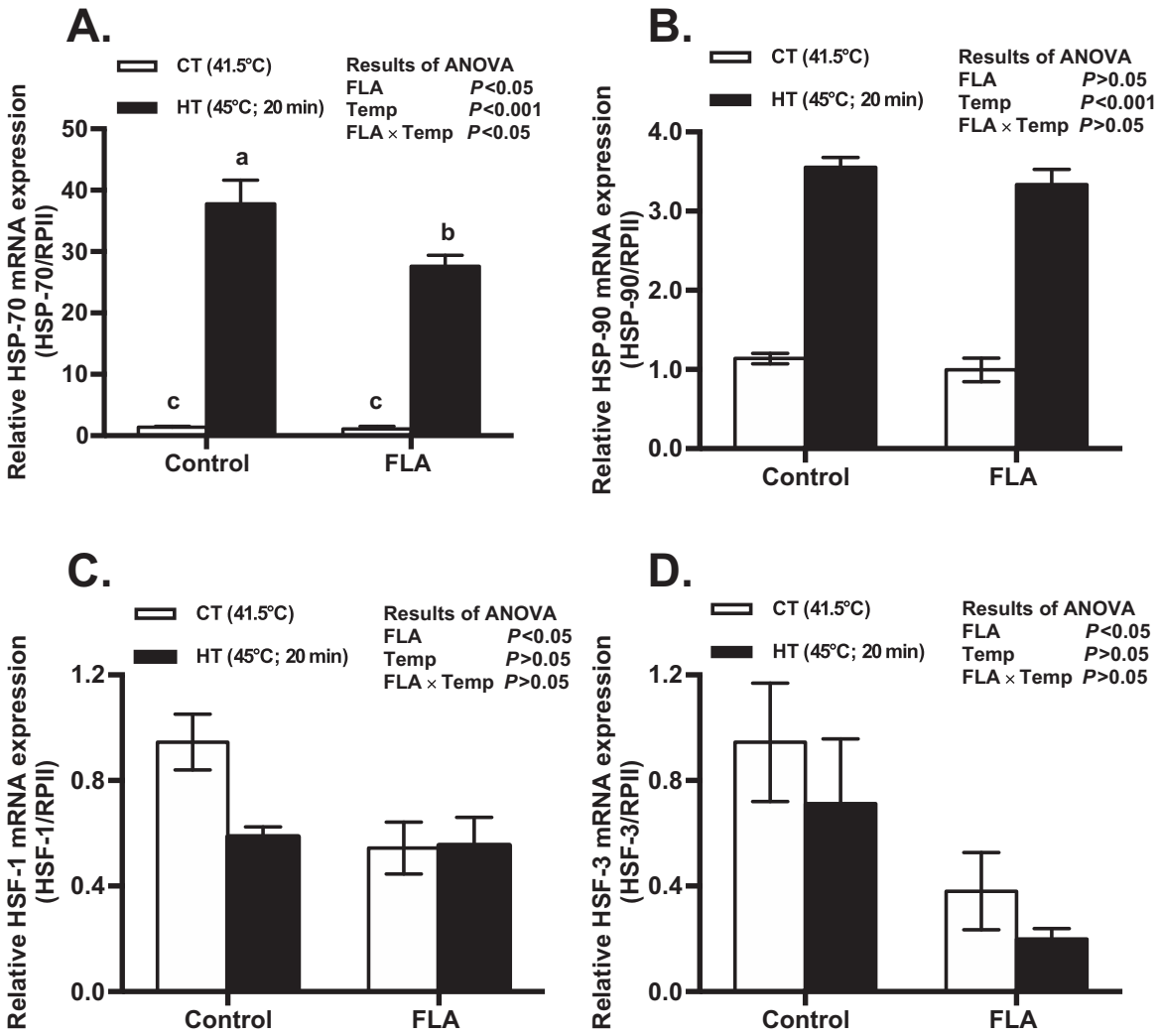

Fig. 3. Gene expression of cellular HSP-70 (A), HSP-90 (B), HSF-1 (C) and HSF-3 (D) exposed to a control thermoneutral temperature (CT; 41.5 ± 1 °C) or a high temperature (HT; 45 ± 1 °C) for 20 min where cells were treated with flavangenol at 0 and 0.5 μg/ml every day. Values are mean ± SEM of the 4–6 samples in each group. FLA: flavangenol, Temp: temperature.

viability, flavangenol-treated cells showed significantly increased cell viability (Fig. 1 A). A significant ($P < 0.05$) positive correlation between cell viability and flavangenol dose in the HT groups was also found (Fig. 1 B).

3.2. HSP-70 and -90 mRNA expression in flavangenol-treated cells following 60 min exposure to heat stress

The gene expression of both HSP-70 and -90 was significantly ($P < 0.05$) increased in the chick brain cells after 60 min exposure to HT. Furthermore, HSP-70 and -90 mRNA expression decreased in a dose-dependent fashion in the brain cells (Fig. 2 A, C). Significant ($P < 0.05$) negative correlations between mRNA expression and flavangenol doses in the HT groups were found for both HSP-70 and -90 (Fig. 2 B, D), indicating that the gradually increasing doses of flavangenol in the current study were effective for a linear reduction in the expression of HSPs.

3.3. Cellular gene expression of HSP-70 and -90, as well as of HSF-1 and -3, in flavangenol-treated cells following 20 min exposure to heat stress

Gene expression of both HSP-70 and -90 was significantly ($P < 0.05$) increased after exposure to HT for 20 min (Fig. 3 A and B). Compared with the control group, the mRNA expression of HSP-70 was also found to be significantly ($P < 0.05$) lower in the HT-treated flavangenol group (Fig. 2 A). However, no significant change was found between the flavangenol groups in terms of HSP-90 expression (Fig. 3 B). It was also found that flavangenol significantly ($P < 0.05$) reduced the mRNA expression of HSF-1 and HSF-3 under both CT and HT (Fig. 3 C and D).

3.4. Cellular gene expression of caspase-3, BCL-2 and Bax in flavangenol-treated cells following 20 min exposure to heat stress

Heat stress significantly ($P < 0.05$) increased caspase-3 gene expression after 20 min of exposure to HT, which indicated that the apoptosis had progressed under HT (Fig. 4A). However, there was no significant influence of flavangenol on caspase-3 gene expression. Interestingly, BCL-2 gene expression was significantly increased in the flavangenol-treated groups (Fig. 4B). Bax gene expression was also found to be increased significantly ($P < 0.05$) after exposure to HT (Fig. 4C). Importantly, lower gene expression of Bax was found in the HT-treated flavangenol group compared to the control HT group (Fig. 4C).

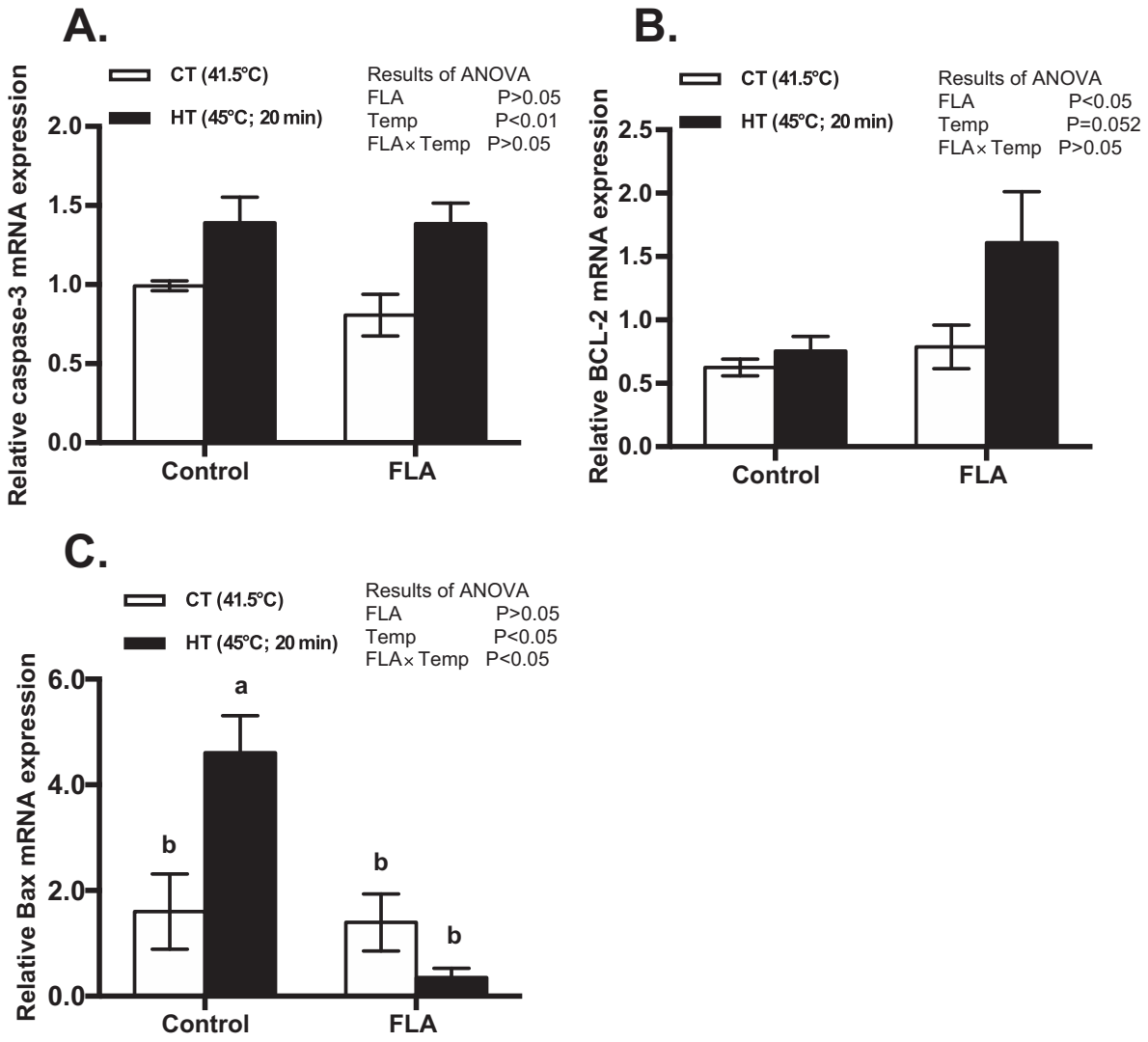

Fig. 4. Gene expression of cellular caspase-3 (A), BCL-2 (B) and Bax (C) exposed to a control thermoneutral temperature (CT; 41.5 ± 1 °C) or a high temperature (HT; 45 ± 1 °C) for 20 min where cells were treated with flavangenol at 0 and 0.5 μg/ml every day. Values are mean ± SEM of the 4–6 samples in each group. FLA: flavangenol, Temp: temperature.

3.5. Cellular SOD, GPx and iNOS, gene expression as well as NOx concentration in flavangenol-treated cells following exposure to heat stress

Heat stress significantly ($P < 0.05$) increased SOD as well as GPx gene expression following 20 min of exposure to HT (Fig. 5A and B). However, no significant change was found in SOD gene expression as a result of the effect of flavangenol. Importantly, a significant ($P < 0.05$) interaction between temperature and flavangenol treatment was found in the GPx mRNA expression, indicating that flavangenol treatment increased GPx expression under HT, while this effect was not apparent under CT. On the other hand, flavangenol reduced iNOS gene expression after 20 min of exposure to HT (Fig. 5C). Furthermore, NOx concentration (NO_2/NO_3) was also reduced by flavangenol after 60 min of exposure to HT following 6 h recovery time (Fig. 5D). A significant ($P < 0.05$) interaction between temperature and flavangenol was found in the NOx concentrations, which suggested that flavangenol could reduce NOx concentrations at 0.1 and 0.5 μg/ml under HT compared with the control group.

3.6. Cellular GFAP, MAP-2 and nestin gene expression in flavangenol-treated cells following 20 min exposure to heat stress

No significant changes were found either HT or flavangenol treatment in the mRNA expression of GFAP, MAP-s and nestin (Fig. 6A-C). However, a significant ($P < 0.05$) interaction between temperature and flavangenol was found in the GFAP mRNA expression, which suggested that flavangenol can reduce GFAP mRNA expression under HT compared with the control HT group.

3.7. Cellular localization of HSP-70 in flavangenol-treated cells

Immunocytochemistry revealed that HSP-70 mainly displayed a diffuse cytoplasmic, but not nuclear, distribution under CT (Fig. 7A). Heat stress for 20 min caused an increased expression of HSP-70 (Fig. 7B). It was also found that HSP-70 was distributed not only in the cytoplasm but also in the nucleus (Fig. 7B), which is in agreement with previous reports (Welch and Feramisco, 1984; Ellis et al., 2000; Steel et al., 2012), and suggested that dynamic changes take place in its localization as a chaperone-dependent protection measure. Interestingly, reduced HSP-70 expression occurred when cells were treated with flavangenol, in comparison with the HT group (Fig. 7E). HSP-70

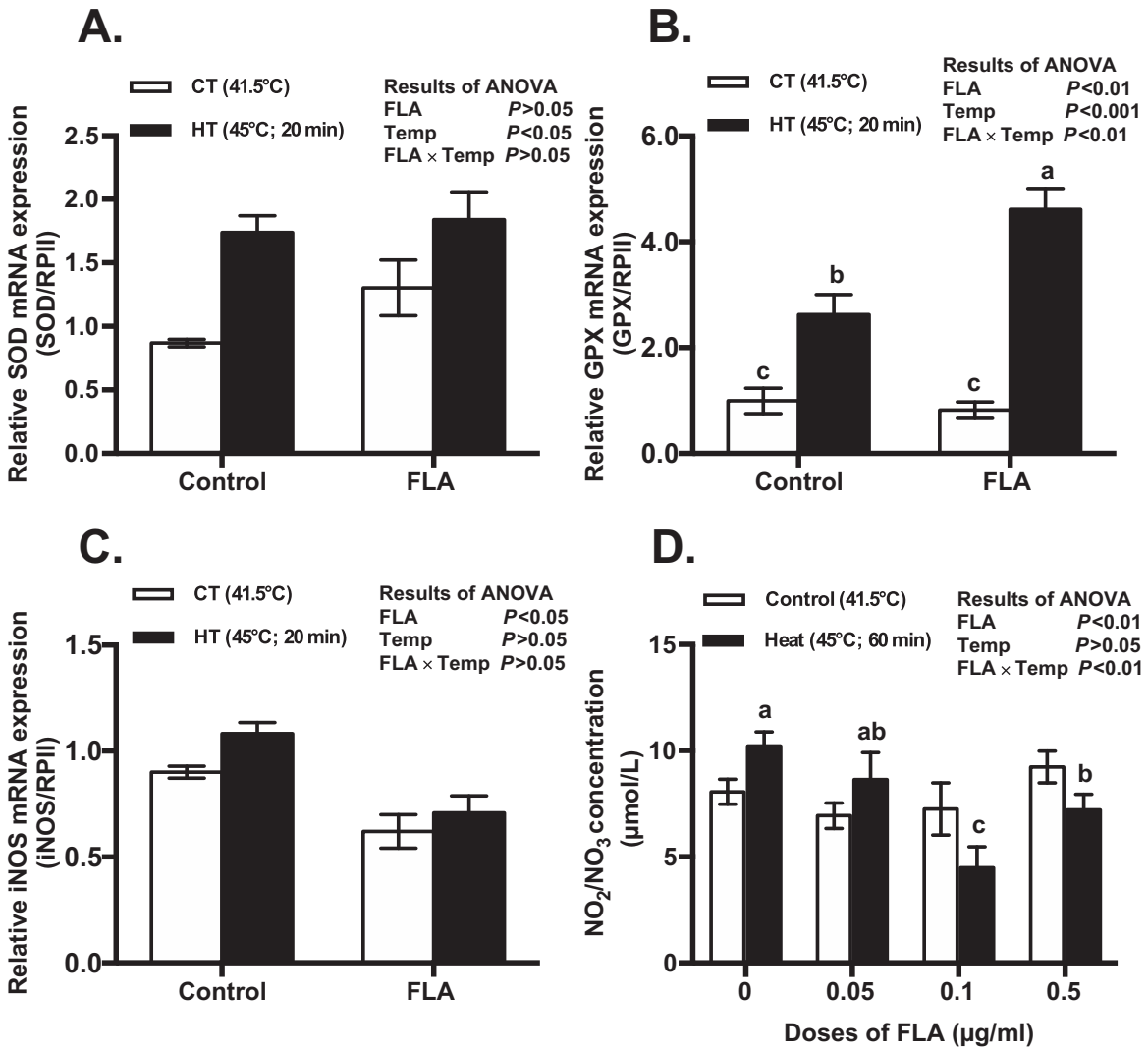

Fig. 5. Gene expression of cellular SOD (A), GPX (B) and iNOS (C) exposed to a control thermoneutral temperature (CT; 41.5 ± 1 °C) or a high temperature (HT; 45 ± 1 °C) for 20 min where cells were treated with flavangenol at 0 and 0.5 μg/ml every day. NOx concentration (D) of culture medium exposed to a control thermoneutral temperature (CT; 41.5 ± 1 °C) or a high ambient temperature (HT; 45 ± 1 °C) for 60 min where cells were treated with flavangenol at 0, 0.05, 0.1 and 0.5 μg/ml every day. Values are mean ± SEM of the 4–6 cells in each group. FLA: flavangenol, Temp: temperature.

expression had almost disappeared after 120 min of HT through protein degradation (Fig. 7C, D, F, G).

4. Discussion

In the current study, we conducted *ex vivo* experiments to investigate whether flavangenol could ameliorate the effects of heat stress in cells, including its effects on HSPs and anti-apoptotic and anti-oxidative factors, by regulating the cellular defense system. The results showed that flavangenol attenuated the gene expression of HSP-70, HSFs and iNOS and also upregulated BCL-2 and GPX gene expression.

Cell viability is an important measure of live, metabolically active cells *ex vivo* and it has been widely used to determine the effects of various stressors or drugs on normal functions of cells and on their metabolism (Butler et al., 2013). Gu et al. (2014) reported that heat stress (45 °C, 120 min) significantly reduced cell viability in human umbilical vein endothelial cells. In the current study, however, cell viability was not affected by heat stress, which indicated that the duration of our experimental HT (45 °C, 20 min) was not sufficient to induce cell death. This may be partly, but not entirely, explained by the difference in physiological body temperature between mammalian and avian species, it being greatly higher in the latter. Importantly, cell viability in groups with a low and a medium concentration of flavangenol was not influenced; however, a high concentration of flavangenol increased cell viability under both CT and HT. Yang et al. (2014) found that pycnogenol, another type of pine bark extract, showed reduced cell viability when the concentration exceeded 10 μg/ml in cancer cells. The present results demonstrated that the dose of flavangenol (0.05, 0.1 and 0.5 μg/ml) did not show cytotoxicity and that the highest dose was suitable for cell growth. The increased cell viability that was due to the high dose of flavangenol suggests that flavangenol may have a cellular protective function, preventing cell death under HT.

HSP has been reported to be increased by various environmental stressors, including heat stress, as a cellular defense mechanism. In the current study, experimental HT for 60 min increased the expression of HSP-70 and -90 mRNA in cultured chicken primary brain cells. These results indicated that our experimental HT acted on the cells to activate the cellular defense mechanism against heat-stress-induced damage as HSP gene expression increased rapidly after heat stress, as reported elsewhere (Stetler et al., 2010; Yang et al., 2016). Notably, the expression of HSP mRNA following treatment with flavangenol declined in a dose-dependent fashion in the chick brain cells after 60 min of exposure to HT. These results were in agreement with our previous *in vivo* studies involving chicks (Yang et al., 2016). Gorman et al. (1999)

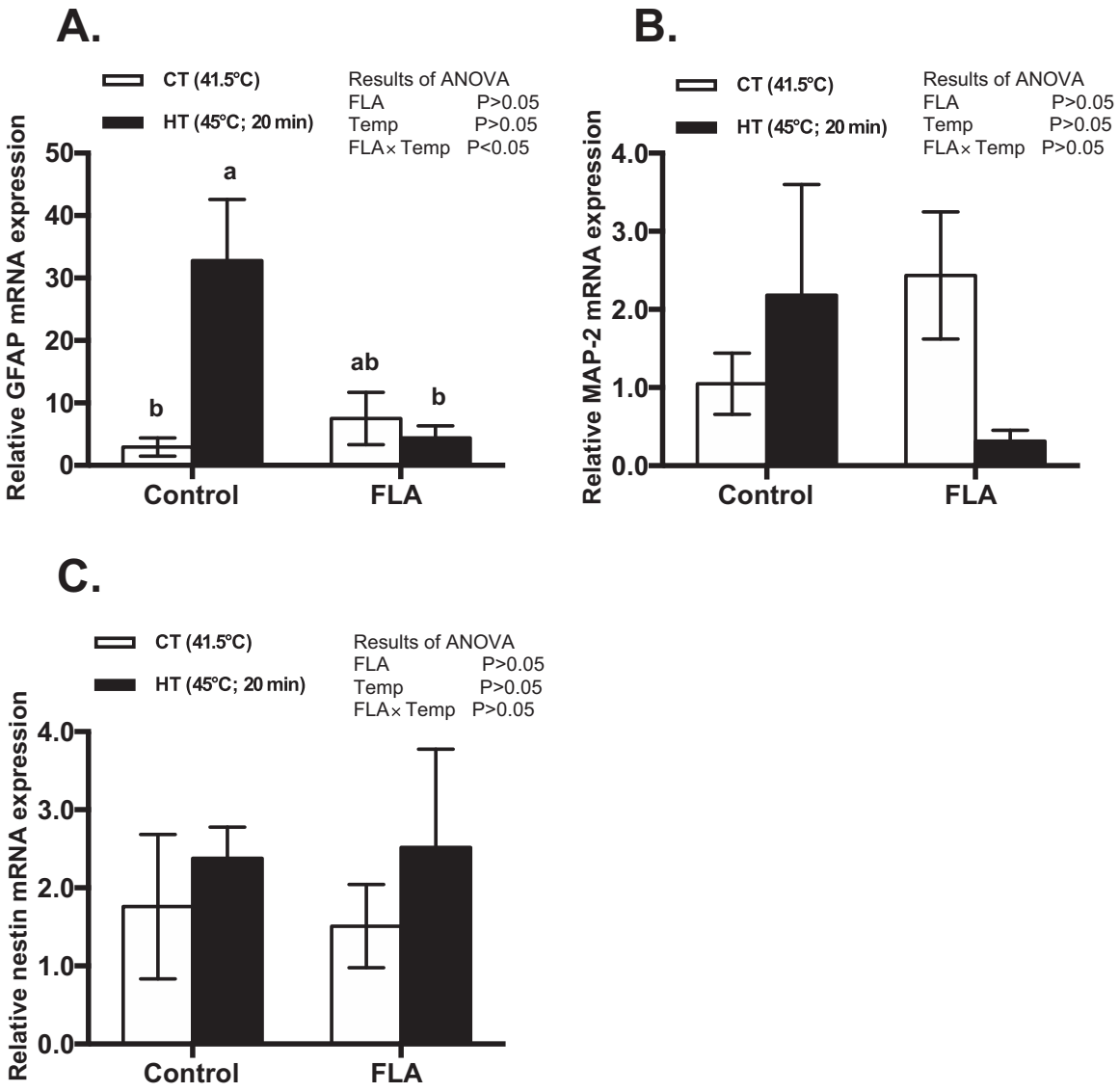

Fig. 6. Gene expression of cellular GFAP (A), MAP-2 (B) and nestin (C) exposed to a control thermoneutral temperature (CT; 41.5 ± 1 °C) or a high temperature (HT; 45 ± 1 °C) for 20 min where cells were treated with flavangenol at 0 and 0.5 μg/ml every day. Values are mean ± SEM of the 4–6 samples in each group. FLA: flavangenol, Temp: temperature.

found that in the presence of the antioxidant molecules pyrolidine dithiocarbamate or 1, 10-phenanthroline, the induction of HSP-72 and -27 was significantly reduced after heat stress (42 °C, 60 min) in HL-60 cells. It was also found that HSP-70 gene expression in the heart and liver of broilers was reduced by grape seed extract and vitamin C supplementation during chronic heat stress (Hajati et al., 2015). Yun et al. (2012) also suggested that Sprague Dawley rats supplied with vitamin C showed reduced HSP-70 mRNA expression in the liver tissue. Therefore, it might be that the antioxidants reduce HSP expression in the cells, probably as a means of protecting normal cellular functions against heat-stress damage.

We further found that gene expression of both HSP-70 and -90 was also increased by 20 min of exposure to HT. These results indicated that 20 min of 45 °C heat exposure was a sufficient stressor to induce HSP in cells. Importantly, the current results showed that the flavangenol group had a lower HSP-70 gene expression compared with the control group under HT. This result further proved that the function of flavangenol could be to reduce cellular susceptibility to heat stress. However, HSP-90 did not change as a result of the flavangenol treatment after 20 min of exposure to HT, but decreased following the 60-min treatment, suggesting a combined effect of the HSP family on different adverse conditions (Fig. 2 C). Both HSF-1 and -3 were also attenuated by flavangenol after 20 min of exposure to HT. HSFs are essential molecules for organisms in terms of protecting them against acute stress, and they are also known as the transcriptional regulator of the HSP genes (Akerfelt et al., 2010). HSF-1 is ubiquitous, whereas HSF-3 has been suggested as an avian-specific transcription factor for HSP (Morimoto, 1998). Therefore, the reduced level of HSP-70 and -90 following treatment with flavangenol after 60 min of HT may have been a result of the attenuated HSFs. All of these results further confirmed that flavangenol-treated cells showed a lower gene expression of stress markers under heat stress, which strongly suggests that flavangenol affords cellular protective functions.

Inducible HSP-70 is expressed in both the cytoplasm and the nucleus before heat stress and is rapidly translocated from the cytoplasm to the nucleus during heat shock, as reported elsewhere (Welch and Feramisco, 1984; Ellis et al., 2000; Steel et al., 2012). In the current study, heat shock increased the HSP-70 protein level and also changed the distribution of cellular HSP-70. These phenomena were more aggregated in the nucleus under HT, while these aggregations were not

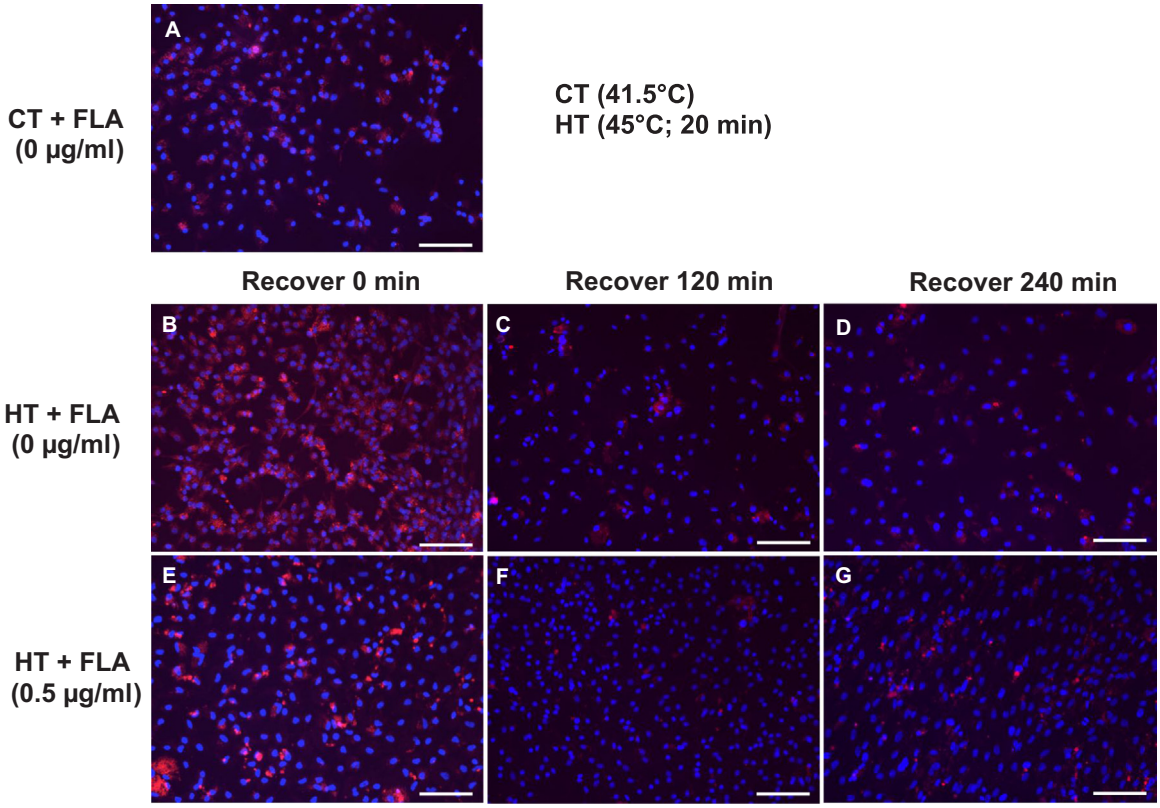

Fig. 7. HSP-70 staining of chicken brain cells exposed to a control thermoneutral temperature (CT; 41.5 ± 1 °C; A) or a high temperature (HT; 45 ± 1 °C) for 20 min where the cells in the HT group were treated with flavangenol at 0 and 0.5 μg/ml every day. After exposure to HT, cells were returned to 41.5 ± 1 °C for recovery. Cells were harvested after recovery for 0, 120 and 240 min (B to G). HSP-70 (red) and DAPI (blue) were detected by immunofluorescence. Scale bars represent 100 μm. FLA: flavangenol. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

observed after 120 min. The differences in the localization of HSP-70 during and after HT in the current results indicated normal heat-shock responses in the chick brain cells. Interestingly, flavangenol-treated cells that were subjected to heat shock showed a similar level of expression of HSP-70 to the CT groups, which provides clear evidence that flavangenol reduces HSP expression.

To clarify whether flavangenol affects the anti-apoptotic and anti-oxidative defense system under HT, the expression of apoptotic and oxidative marker genes was analyzed in the current study. It has been reported that heat stress enhanced caspase-3 activity in cultured rat glial cells (Li et al., 2018). Li et al. (2016) also reported that heat stress increased caspase-3 and Bax gene expression and induced apoptosis in bovine granulosa cells. The present experimental HT (45 °C, 20 min) increased caspase-3 gene expression and Bax gene expression was also increased in the control HT group. These results indicated that the apoptosis was enhanced inside the cells due to HT. Importantly, flavangenol increased the BCL-2 and decreased Bax gene expression. BCL-2, an anti-apoptotic protein, and Bax, a pro-apoptotic protein, have been shown to play an important role in regulating apoptosis (Fulda et al., 2010). Gu et al. (2014) reported that overexpressing BCL-2 in human umbilical vein endothelial cells significantly attenuated heat-stress-induced apoptosis. Zhao et al. (2017) also reported that resveratrol, an effective antioxidant, declined Bax which was induced by the spinal cord injury in rats. However, caspase-3, the downstream factor of apoptosis pathway was not changed by flavangenol, which indicated that flavangenol may not directly affect the apoptosis. Therefore, our results indicated that the used dose of flavangenol up-regulated BCL-2 and down-regulated Bax gene expression under HT, but these changes did not affect caspase-3. Therefore, we investigated other possibilities as discussed below.

Heat stress has been reported to be a vital environmental stressor, stimulating oxidative stress (Martindale and Holbrook, 2002; Chowdhury et al., 2014; Slimen et al., 2014), while endogenous SOD and GPX expression can be enhanced to minimize the oxidative damage (Zelko et al., 2002; Valko et al., 2007). In the present study, HT was found to significantly increase SOD and GPX gene expression, which showed evidence of causing oxidative damage inside the cells. Even though flavangenol did not affect SOD gene expression, GPX gene expression was significantly increased by treatment with flavangenol under HT. Heat stress would increase the mitochondrial $O_2^{\bullet-}$ level, which is the precursor of most ROS. $O_2^{\bullet-}$ can dismutate to H_2O_2 through a reaction catalyzed by SOD (Slimen et al., 2014). H_2O_2 is most efficiently scavenged by the enzyme GPX with glutathione (GSH) as the electron donor (Valko et al., 2007). Our results indicated that flavangenol may be able to enhance the cellular anti-oxidative ability and scavenge the toxic H_2O_2 against heat stress by increasing the GPX. It was also reported that cellular BCL-2 overexpressing cells have a higher level of total cellular GSH as protection against ROS-induced apoptosis (Mirkovic et al., 1997; Fulda et al., 2010). Therefore, the enhanced gene expression of BCL-2 through treatment with flavangenol may also have contributed to increasing the level of GPX under HT in the current study. On the other hand, flavangenol also possessed the ability to reduce iNOS gene expression as well as the NOx level. It is known that NOS generates NO from L-arginine and that NO combined with $O_2^{\bullet-}$ will generate $ONOO^-$, which is a strong oxidant that can oxidize protein and initiate lipid peroxidation (Nordgren and Fransen, 2014; Tomanek, 2015). Therefore, these results further suggested that flavangenol could suppress cellular oxidative damage by reducing the toxic NOx.

GFAP is an important protein expressed in astrocytes in the central nervous system and plays crucial roles in the development and differentiation of astrocytes (Fukasawa et al., 2011). It was reported that the expression of GFAP increased when the glial cells were injured (Sharma

et al., 1992; Callaghan and Sriram, 2005). Sharma et al. (1992) also reported the expression of GFAP was induced in the rat brain after 4 h of heat exposure. Therefore GFAP was often used as a biomarker of neurotoxicity (Callaghan and Sriram, 2005). In the current study, our experimental HT (45 °C, 20 min) increased GFAP mRNA expression in chicken brain cells, which indicated the neurotoxicity occurred in the brain cells by heat exposure. Importantly, flavangenol attenuated GFAP mRNA expression under HT which provided a strong evidence that flavangenol may have the beneficial role to protect astrocytes against heat stress- induced neurotoxicity. However, the neuron marker MAP-2 and nestin were not changed by HT or flavangenol, which indicated these parameters were not influenced by our experimental HT or flavangenol treatment.

In summary, we demonstrated that flavangenol could protect chick brain cells from heat stress by increasing both cell viability and BCL-2 and GPX gene expression and by inhibiting Bax, iNOS, NOx and GFAP expression. Reduced expression of HSFs and HSPs in the cells indicated that the cellular state had been improved by flavangenol under HT. The present finding suggests that the mechanism through which flavangenol provides a cellular protective function is involved in enhancing the cellular anti-oxidative ability.

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Disclosure summary

The authors of this manuscript have nothing to declare.

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