Original Article

Reducing the Effects of Oxidative and Nitrosative Stress on Viability of the Broiler Blood Cells when Exposed to High Ambient Temperatures by Using Tannic Acid

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Abstract

The study aimed to investigate the effect of tannic acid (TA) on total antioxidant power (FRAP), malondialdehyde (MDA), hydrogen peroxide (H2O2), nitric oxide (NO), and the viability of broiler blood cells (BBC) exposed to high ambient temperatures. The BBC from a broiler chicken was exposed to temperatures ranging from 41.5°C to 46°C and incubated with TA at concentrations of 0 (the positive control group, TPCG), 5, 10, 15, and 20 µg/mL. Results showed that MDA, H2O2, and NO levels of the control group (TCG) which BBC was maintained at 41.5°C were significantly lower than TPCG. The viability of TPCG was lower than TCG. TA reduced H2O2 and NO levels and increased BBC viability, especially at the optimal concentration of 10µg/mL. These findings indicate that TA has the potential antioxidant and cytoprotective effects on BBC under heat stress conditions.

Keywords: Broiler blood cells (BBC), tannic acid, oxidative stress, nitrosative stress, viability

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Introduction

The poultry industry faces challenges due to climate change-induced high ambient temperatures, negatively affecting broiler growth, health, and quality (Choi and Kim, 2020; Goel, 2021). Heat stress in chickens leads to over production of reactive oxygen species (ROS), causing oxidative stress, cell damage, and even death (Choi and Kim, 2020; Pan *et al*., 2021; Wen *et al*., 2021). Generally, ROS are small reactive molecules that play critical roles in the regulation of various cell functions, chemical and biological processes. In many chemical and biological processes, they are usually formed as molecules, ions, or radicals, such as hydrogen peroxide (H_2O_2). ROS participate in many important processes and react with various target molecules (Vitiello *et al*., 2021). Oxidative stress occurs when there is an imbalance between pro-oxidants and antioxidants (Pazzini *et al*., 2015; Fratta Pasini and Cominacini, 2022). The excess of free radicals, such as ROS, induces harmful effects such as lipid peroxidation, damage to deoxyribonucleic acid (DNA), proteins, and enzymes etc. (Pazzini *et al*., 2015; Juan *et al*., 2021). Nitrosative stress refers to the joint biochemical reactions of nitric oxide (NO) and superoxide when an oxygen metabolism disorder occurs in the body. The peroxynitrite anion produced during this process can nitrate several biomolecules, such as proteins, lipids, and DNA, which further induces cell death (Wang *et al*., 2021). When broiler blood cells (BBC) were exposed to high ambient temperature, they were under oxidative and nitrosative stress (Wandee *et al*., 2022; Srinontong *et al*., 2023).

Tannic acid (TA), a plant polyphenol with antioxidant properties, is considered a potential solution to mitigate these effects (Gulcin *et al*., 2010; Aengwanich and Boonsorn, 2017; Chen *et al*., 2022). Due to the antioxidant activity of TA which could replace the synthetic antioxidants (Al-Hijazeen *et al*., 2016), dietary TA supplementation could be applied as a biological antioxidant for poultry nutrition in hot climatic conditions (Hidayat *et al*., 2021). However, TA effects on farm animals can vary from positive to toxic. Higher TA doses induce a stress condition in chickens (Ramaha *et al*., 2020). This was consistent with the report of Abed *et al*. (2013) who found that TA has both, antioxidant and oxidant potency. Feeding TA decreased body weight gain and feed intake, on the other hand, it increased feed conversion ratio (FCR) of broilers under high temperature (Hidayat *et al*., 2021). However, Aengwanich and Boonsorn (2017) found that TA extracted from cassava leaves had no effect on productive performance and gut ecology of broilers. In the case of blood cells, Ramaha *et al*. (2020) reported that immunosuppressive effects of chicken fed 25 or 30 g TA/kg diet included extremely decreased numbers of white blood cells, and serum IgM and IgY levels were decreased in a dose-dependent manner. Also, Abed *et al*. (2013) found that TA could stimulates eryptosis, a suicidal death characterized by cell shrinkage and cell membrane scrambling with the appearance of phosphatidylserine at the erythrocyte surface. Conversely, Huyut *et al*. (2016) reported that TA could decrease the sensitivity to oxidation of erythrocytes.

Our previous study found that when the BBC were exposed to high ambient temperatures, the BBC were under heat stress, and would then progress into apoptosis. This property could allow use of BBC as an *in vitro* model of broiler cells response to high ambient temperature due to broilers having low heat tolerance. In addition, we also found that when BBC were exposed to the ambient temperature at 46°C, superoxide, malondialdehyde (MDA), and NO increased.

However, information on the optimal level of TA to prevent oxidative and nitrosative stress in broilers under heat stress is limited. This study aimed to explore the effect of TA on total antioxidant power (FRAP), MDA, H_2O_2, NO, and the viability of the BBC under high ambient temperatures.

Materials and Methods

This study was approved by the Institution's Ethics Committee on Animal Experimentation of Mahasarakham University (license number: IACUC-MSU-11/2022).

Animal: This experiment was adapted from the method which was developed by Aengwanich and Wandee (2022). The experiment was conducted in February 2022 at the laboratory, Faculty of Veterinary Sciences, Mahasarakham University, Maha Sarakham, Thailand. One healthy male Cobb broiler, 28 days of age, was obtained from a commercial poultry farm in Mahasarakham province. The broilers were raised in open housing of 8x24 m, with an ambient temperature ranging from 26°C to 35°C. The bird was fed *ad libitum* with commercial feed with continuous light and water supplies (Note: Broiler was used as a blood donor. The BBC in this study were cells that were collected from the broiler directly and comparable to the cultured cell lines used for conventional *in vitro* experiments).

Treatments and Experimental design: The design of the experiment was completely randomized (CRD), consisting of 6 treatments. In the first group, BBC were maintained at 41.5°C throughout the experimental period (the control group, TCG). In the second to the sixth groups, BBC were diluted with TA at concentrations of 0 (the positive control group, TPCG), 5, 10, 15 and 20 µg/mL of phosphate buffered saline (PBS), pH 7.4 (Sigma), and maintained at ambient temperatures increasing from 41.5°C to 46°C, each treatment (group) consisting of 3 replications. (Note: Prior to the start of the experiment, we conducted a preliminary study to determine the optimal level of TA for the BBC, found that the level that did not cause damage to the BBC was in the range of 0-20 µg/mL)

1 ml of blood sample was collected from the jugular vein of the broiler and placed in a tube containing heparin (BD vacutainer®, sodium heparin tube). The heparinised broiler blood was then added to PBS, pH 7.4 and centrifuged (Hettich Rotina 380R, Andreas Hettich GmbH & Co. KG, Germany) at 2500 rpm (769x*g*) for 5 min. The supernatant was discarded. This process was performed twice. TA (Rankem™) solution at concentrations of 5, 10, 15 and 20 µg/mL of PBS pH

7.4 was prepared. The test tubes were divided into 6 groups. Each group comprised 3 test tubes. TCG and TPCG, blood were diluted in 1:200 PBS, pH 7.4. In the other 4 groups, broiler blood was diluted with TA at concentrations of 5, 10, 15 and 20 µg/mL of PBS, pH 7.4, each concentration with the same ratio of dilution as TCG and TPCG. The diluted blood samples were placed in 2 water baths (Memmert, Memmert GmbH + Co. KG, Germany). In the first water bath, the temperature was maintained at 41.5°C throughout the experimental period (TCG) for 3 h. In the second water bath, TPCG and groups diluted with different levels of TA were conditioned at 41.5°C before starting the experiment after 30 min when the temperature was then continuously increased from 42°C to 46°C. At 42°C to 46°C; BBC were maintained to each temperature for 30 min per temperature, total 3 h. At the end of the incubation period, all test tubes were removed from the water bath. Then FRAP, MDA, NO and H_2O_2 in the supernatant, and viability of blood cells were assayed from sedimented blood.

Determination of Indicators: Supernatant FRAP was measured using the ferric reducing ability of plasma assay. Briefly, the procedure was as follows. 300 mM sodium acetate (Ajax Finechem Pty. Ltd.) buffer (pH 3.6), 40 mM HCl (QRëC™), 10 mM TPTZ (2,4,6-tri[2-pyridyl]-s-triazine) (Aldrich), and 20 mM $FeCl_3.6H_2O$ (PanReac AppliChem) were prepared. 10mL of 300 mM acetate buffer, pH 3.6, 1 mL of 10 mM TPTZ, 1 mL of 20 mM $FeCl_3.6H_2O$ were mixed as a working FRAP reagent (freshly prepared). 20 µL of supernatant was mixed with 180 µL working FRAP reagent in 96 well plates (Thermo Scientific) and incubated at room temperature for 5 min. The optical density at 595 nm was measured by Tecan Infinite® 200 Microplate Reader (Tecan Trading AG, Männedorf, Switzerland). $FeSO_4.7H_2O$ (Ajax Finechem Pty. Ltd.) was used as the standard (Aengwanich and Wandee, 2021).

MDA in the supernatant was investigated using the thiobarbituric acid reactive substance assay (TBARS assay). Briefly, the procedure was as follows. 0.01 mL of sample was assayed by the addition of 3 mL (0.05 $molL^{-1}$) of HCl (QRëC™) and 1 mL (0.67%) of thiobarbituric acid (Fluka). Cocktails were heated for 30 min at 100°C, cooled with running tap water, and then 4 mL of n-butyl alcohol (QRëC™) was added. The mixture was shaken in a vortex mixer and centrifuged (Hettich Rotina 380R, Andreas Hettich GmbH & Co. KG, Germany) at 3000 rpm (1008×*g*) for 10 min. The optical density at 450 nm was measured by Tecan Infinite® 200 Microplate Reader (Tecan Trading AG, Männedorf,Switzerland). 1,1,3,3-tetramethoxypropane (Aldrich) at 0-50 µmol/L was used as the standard (Aengwanich and Wandee, 2022).

The method used for measuring NO were as follows. Griess's reagent was prepared composed of sulfanilamide (DC Fine Chemicals) 1% (w/v) and naphtyl ethylene diamin dihydrochloride (PanReac AppliChem) 0.1% (w/v), and phosphoric acid (QRëC™) 1.25% (v/v) by combining all three constituents in a bottle. Distilled water was added to a final volume of 100 mL, and thoroughly shaken. Equal proportions of Griess's reagent and supernatant were then mixed and let stand for 15 min. The optical density at 540 nm was measured by Tecan Infinite® 200 Microplate Reader (Tecan Trading AG, Männedorf, Switzerland). Sodium nitrite was used as the standard (Aengwanich and Wandee, 2022).

Measurement of H_2O_2 was performed according to the method of Orprayoon *et al*. (2020). Briefly, the procedure was as follows, 1 mL of 2.25 mM $FeSO_4.7H_2O$ (Ajax Finechem Pty. Ltd.) was placed into the test tube. Then 1 mL of supernatant was added and left to react for 5 min after which, 1 mL of 4 mM norfloxacin (Sigma-Aldrich) was added and the mixture was left to react for 3 min. The optical density at 440 nm was measured by Tecan Infinite® 200 Microplate Reader (Tecan Trading AG, Männedorf, Switzerland). H_2O_2 (Vidhyasom Co. Ltd.) was used as the standard.

The viability of BBC was examined as follows: MTT formazan (Sigma) was dissolved in acetone (Merck) at 5 mg/ mL and then were filtrated using syringe filters (Minisart filter 0.2 µm, Sartorius, Germany). Then 1 mL of diluted blood sample from a test tube was placed in a microtube, and centrifuged (Hettich Rotina 380R, Andreas Hettich GmbH & Co. KG, Germany) at 2500 rpm (769 x *g*) for 5 min and 900 µL of supernatant was discarded. Then the rest of the diluted blood was transferred (100µL) to a well plate (Thermo Scientific) and was incubated at 41.5 °C for 75 min, 95 µL of supernatant was discarded and 150 µL of DMSO (Loba Chemie. Pvd. Ltd) added. The optical density at 540 nm was measured by Tecan Infinite® 200 Microplate Reader (Tecan Trading AG, Männedorf, Switzerland). The percentage of viability was calculated as the following formula: % cell viability = (OD of the treatment group /OD of TCG) X 100.

Statistical analysis: The statistical analysis model of the present study was as follows:

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

Where: Y_{ij} is the j^{th} observation of the i^{th} treatment
μ is overall mean
τ_i is the treatment effect of the i^{th} treatment
ε_{ij} is the random error

The normal distribution of data was tested. Data were analysed using analysis of variance (Proc ANOVA). Means were separated by Duncan's multiple range tests (SAS® studio). The level of significance was identified at $p < 0.05$

Results

As shown in the (Figs. 1-5), FRAP of TCG was significantly lower than TPCG ($p < 0.05$). At 46 °C, FRAP of BBC that were diluted with TA significantly increased ($p < 0.05$) when the concentration of TA increased (Fig. 1).

MDA of TCG was significantly lower than TPCG ($p < 0.05$). MDA of BBC that were diluted with TA at the concentration of 15 µg/mL was significantly higher than the values found with of TCG and BBC that were diluted with TA at the concentration of 5 µg/mL ($p < 0.05$) (Fig. 2).

H_2O_2 level of TCG was significantly lower than TPCG ($p < 0.05$). At 41.5°C to 46°C, H_2O_2 of TPCG was significantly higher than all groups that were diluted with TA ($p < 0.05$). At 41.5°C to 46°C, H_2O_2 of BBC that diluted with TA at the concentration of 15 µg/mL was significantly higher than those of other BBC that were diluted with TA, and H_2O_2 of BBC that diluted with TA at the concentration of 20 µg/mL was significantly lower than other groups ($p < 0.05$) (Fig. 3).

The NO level of TCG was significantly lower than TPCG ($p < 0.05$). NO of TPCG was significantly higher than BBC that were diluted with TA ($p < 0.05$), except for BBC that had been diluted with TA at the concentration of 15 µg/mL (Fig. 4).

Viability of TPCG was significantly lower than TCG ($p < 0.05$). Viability of TPCG was significantly lower than other BBC that were diluted with TA ($p < 0.05$). Viability of BBC that were diluted with TA at the concentration of 10 µg/mL was significantly higher than other BBC that diluted with TA ($p < 0.05$) (Fig. 5).

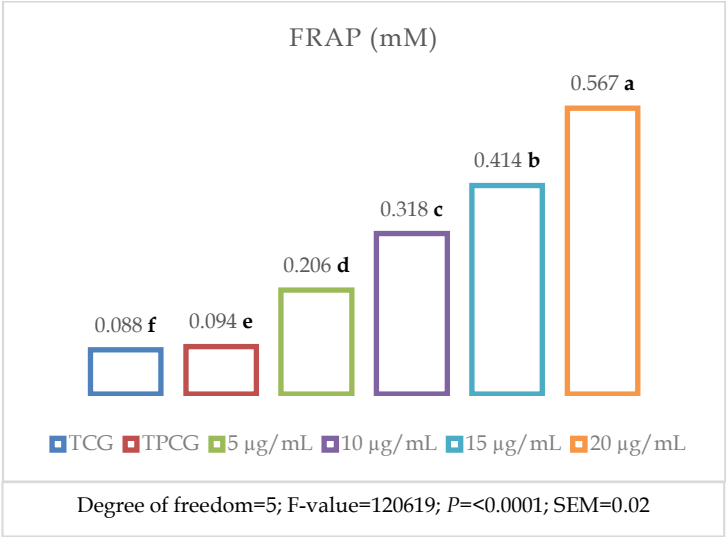

Figure 1 Total antioxidant power (FRAP) in the supernatant of the control group (TCG), the positive control group (TPCG), and BBC at 41.5°C to 46°C that were diluted with tannic acid (TA) at concentrations of 5, 10, 15 and 20 µg/mL, respectively. Means with different letters are significantly different among treatments ($p < 0.05$), p =*P*-value, and SEM= standard error of the mean.

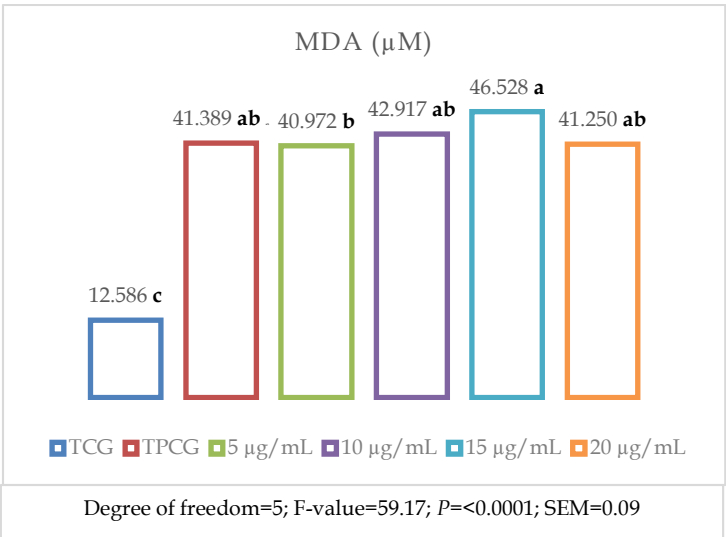

Figure 2 Malondialdehyde (MDA) in the supernatant of the control group (TCG), the positive control group (TPCG), and BBC at 41.5°C to 46°C that were diluted with tannic acid (TA) at concentrations of 5, 10, 15 and 20 µg/mL, respectively. Means with different letters are significantly different among treatments ($p < 0.05$), p =*P*-value, and SEM= standard error of the mean.

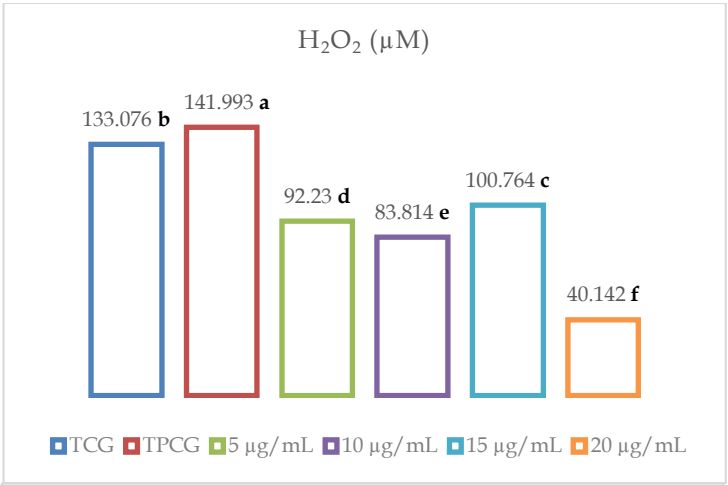

Figure 3 Hydrogen peroxide (H_2O_2) in the supernatant of the control group (TCG), the positive control group (TPCG), and BBC at 41.5°C to 46°C that were diluted with tannic acid (TA) at concentrations of 5, 10, 15 and 20 µg/mL, respectively. Means with different letters are significantly different among treatments ($p < 0.05$), *p* =*P*-value, and SEM= standard error of the mean.

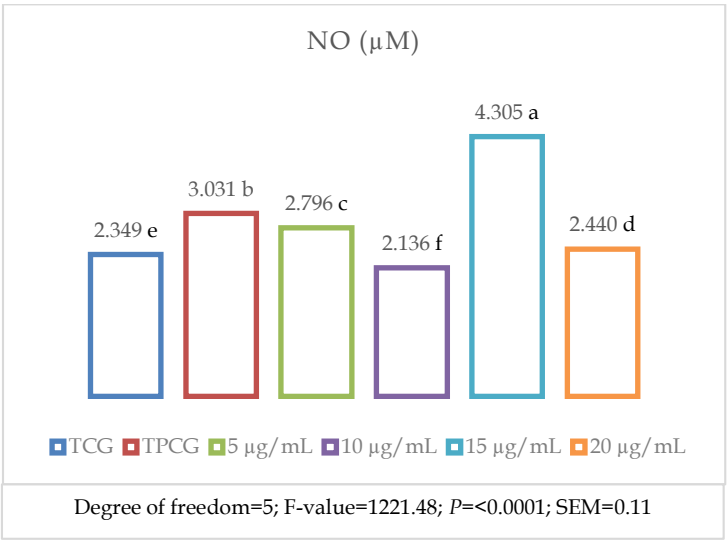

Figure 4 Nitric oxide (NO) in the supernatant of the control group (TCG), the positive control group (TPCG), and BBC at 41.5°C to 46°C that were diluted with tannic acid (TA) at concentrations of 5, 10, 15 and 20 µg/mL, respectively. Means with different letters are significantly different among treatments ($p < 0.05$), *p* =*P*-value, and SEM= standard error of the mean.

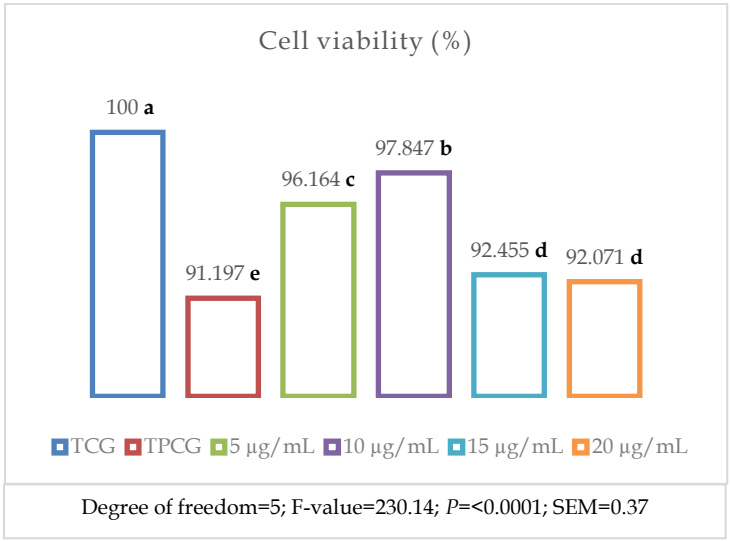

Figure 5 Viability of the control group (TCG), the positive control group (TPCG), and BBC at 41.5°C to 46°C that were diluted with tannic acid (TA) at concentrations of 5, 10, 15 and 20 µg/mL, respectively. Means with different letters are significantly different among treatments ($p < 0.05$), p =*P*-value, and SEM= standard error of the mean.

Discussion

This trial sought to find a way to reduce heat stress by an *in vitro* study using BBC as a model. Although there have been trials that show the use of TA for reducing stress and oxidative stress in broilers under the exposure to high ambient temperature, some aspects remain unstudied. This study was designed to fulfill the gap of research to explain the efficacy of TA in BBC that were under heat stress, oxidative and nitrosative stress. Due to it has been reported that TA could reduce oxidative stress through the NF-κB/Nrf2 pathway (Jina *et al*., 2020). In addition, a polyphenol substance (including TA) could also reducing nitrosative stress through inhibition of the inducible nitric oxide synthase (iNOS) enzyme, which is a key enzyme in NO synthesis mechanism (Luduvico *et al*., 2020).

It was found that the ambient temperature in TCG may be due to the normal body temperature of the chickens in the range of 41.0-42°C (Wilson *et al*., 1989), which is the normal body temperature of the broiler, but in TPCG, BBC were exposed to the ambient temperature ranging from 41.5°C to 46°C . Therefore, there may be an increase in FRAP levels due to the BBC membrane becoming damaged when under oxidative stress (Aengwanich and Wandee, 2021) and releasing some antioxidants from the cells, resulting in an increase in the FRAP of the BBC in TPCG. Furthermore, at 41.5°C to 46°C, FRAP of BBC that had been diluted with TA increased when the concentration of TA increased. One reason for the FRAP measurements in this experiment was measurement of the level of total antioxidant capacity in the supernatant to assess whether the BBC diluent contained the required levels of TA in the supernatant.

It is well known that primary targets of free radicals are cellular lipids (Maurya *et al*., 2021). Lipid peroxidation often occurs in response to oxidative stress, where ROS cause the oxidation of lipids containing carbon-carbon double bonds in membrane lipid bilayers (Mas-Bargues *et al*., 2021). MDA is one of the products of lipid peroxidation. Generally, this substance is measured by a reliable method which is known as one of the most popular methods for determining the status of oxidative stress (Maurya *et al*., 2021; Mohammadi *et al*., 2021). In the present study, MDA of TCG was lower than TPCG. The result of this experiment was similar to the study of Wandee *et al*. (2022) who found that when BBC were exposed to the ambient temperature at 46°C, MDA increased. In addition, MDA of BBC that were diluted with TA at the concentration of 15 µg/mL was higher than those of TCG and BBC that were diluted with TA at the concentration of 5 µg/mL. This result was similitude to the study of Xue *et al*. (2020) who found that TA levels may induce increased MDA formation.

H_2O_2 is the most abundant ROS subspecies in cells (Herb and Schramm, 2021). H_2O_2 is a ROS that is not a free radical, and is less reactive than free radicals (Herb *et al*., 2021; Shields *et al*., 2021). Generally, H_2O_2 is an endogenous ROS produced by mitochondria through the dismutation of superoxide by mitochondrial superoxide dismutase (Jia and Sieburth, 2021). The overproduction of H_2O_2 can cause extensive oxidative stress responses, which, in turn, lead to mitochondrial dysfunction, cell damage, and death (Jeong *et al*., 2021). In the present study, H_2O_2 of TCG was lower than TPCG. The results of this study are consistent with the study by Srinontong *et al*. (2023) who found that H_2O_2 of BBC at 46°C increases when compared with at 41.5°C. Moreover, H_2O_2 of TPCG was higher than other BBC groups that diluted with TA. This result shows that TA could reduce H_2O_2 level such as TA at the concentration of 20 µg/mL could reduce H_2O_2 levels of BBC, which was lower than other groups. The result of this study was similar to those of Gulcin *et al*. (2010) who found that TA had an effective H_2O_2 scavenging activity.

NO is endogenously generated by numerous cells throughout the body. L-Arginine, molecular oxygen, NADPH and tetrahydrobiopterin (BH_4) are important

substrates or co-factors that lead to generation of NO (Carlström *et al*., 2021). NO is implicated in several cellular functions, including proliferation, differentiation, and even in protection against oxidative stress. On the other hand, excessive production of NO could induce apoptotic cell death (Akaraphutiporn *et al*., 2021). In this study, NO of TCG was lower than in TPCG. This finding was similar to that of Wandee *et al*. (2022) who found that when maintained BBC at 46°C, NO of BBC increased. In addition, Vinoth *et al*. (2016) also found that when chicks were exposed to high environmental temperature, this condition caused over production of NO. Besides, NO of TPCG was higher than other BBC that were diluted with TA, except the BBC that was diluted at the concentration of 15 µg/mL. This result showed that TA could decrease NO production of BBC when they were at high ambient temperature. However, the NO of BBC maintained at 46°C that had been diluted with TA at the concentration of 15 µg/mL was higher than TPCG and other TA diluted groups. This result was similar to that found in the study by Guo *et al*. (2021), which observed that some levels of TA supplementation caused increase in the NO production.

Ibtisham *et al*. (2018) found that when chicken embryonic fibroblast cells were maintained at 40-44°C, their viability increased. However, in the present study, the viability of BBC that were exposed to temperatures ranging from 41.5 °C to 46°C was lower than that of TCG. This result was consistent with the report of Aengwanich and Wandee (2022) who found that, from an ambient temperature of 44°C onwards, apoptosis of broiler blood cells increased with elevated ambient temperature. In addition, Ibtisham *et al*. (2018) reported that the cause of cell death at high ambient temperature resulted from oxidative stress. In this study, the viability of TPCG was lower than other groups which had been diluted TA at 46°C. The results of this study are consistent with a study by Srinontong *et al*. (2023) which found that when the ambient temperature rose to 46°C, the viability of BBC decreased. This result showed that TA could increase viability of BBC under heat stress. Moreover, the result of this experiment was in line with a study by Perumal *et al*. (2019) who found that TA had antioxidant activity and reduced the death of human embryonic kidney (Hek-293) cells. In addition, viability of BBC that had been diluted with TA at the concentration of 10 µg/mL was higher than other BBC groups that were diluted with TA. The results indicated that TA at 10 µg/mL was an optimal TA level, as TA at this level increased viability of BBC higher than TPCG and other BBC groups that diluted with TA.

In conclusion, when BBC were maintained at high ambient temperature at 41°C to 46°C, they were under heat stress and oxidative and nitrosative stress. It was found that at those ambient temperatures, the levels of MDA, H2O2 and NO increased. On the other hand, the viability of BBC decreased. When TA was added into the diluent of BBC. This substance could reduce oxidative and nitrosative stress, resulting in MDA, H2O2 and NO becoming decreased, and BBC viability increasing. The optimum level of TA to reduce oxidative and nitrosative stress and increase viability of BBC when exposed to high ambient temperature was found to be 10µg/mL.

The study suggests that TA can be an effective solution to combat the negative effects of heat stress on broilers. Further research is needed to explore TA's application in the poultry industry and its potential implications for broiler health and welfare. However, this study was an in vitro study. Practical application in broiler, it is necessary to take into account the level suitable for broilers at different stages of life, bioavailability, and the level of TA in the bloodstream is consistent with the study results. This requires further study at the farm level.

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