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Reduced expression of HSP

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Research Article

Reduced Expression of Heat Shock Protein (HSP) 70 Gene by Ascorbic Acid Supplementation in Broiler Chickens Exposed to Transportation Stress to Maintain the Quality of Meat and Hematological Parameters

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Abstract

Background: Transportation process causes broilers suffer from stress and affects its meat quality. **Objective:** This study aims to determine the effect of transportation treatment and ascorbic acid supplementation on HSP 70 gene expressions, hematological status and meat quality of broiler chickens. **Methodology:** The study was designed in a completely randomized design using a unidirectional pattern with 50 female broilers of Lohmann MB 202 aged 5 weeks. The broilers were equally divided into 5 treatments. Treatment 1 was broilers with no ascorbic acid supplementation and transportation treatment as a control 1. Treatment 2 was broilers with no ascorbic acid supplementation but transportation treatment applied (a control 2). Treatment 3 was broilers supplemented with ascorbic acid at a dose of 360 mg L^{-1} in drinking water for 2 h before being transported. Treatment 4 was broilers supplemented with ascorbic acid at a dose of 360 mg L^{-1} in drinking water for 2 h immediately after being transported. Treatment 5 was broilers with transportation treatment and were supplemented with ascorbic acid at a dose of 360 mg L^{-1} of drinking water for 2 h before and after being transported. The transportation treatment was imposed for 5 h starting from 10:00 am-14:00 pm. Rectal temperatures of all experimental broilers were measured. For birds in T4 and T5, their rectal temperatures were measured again after they were supplemented with ascorbic acid after transportation. Next, blood samples were used to test hematology variables. These were followed by expression measurement of HSP 70 genes and meat quality. All data were tabulated and analyzed with variant analysis and LSMEAN. **Results:** Transportation treatment was found to increase HSP gene expression, rectal temperature, heterophil percentage, H/L ratio, pH of the meat and water holding capacity of the meat. The transportation treatment also reduced total erythrocytes, hemoglobin, hematocrit value, lymphocytes and monocytes. Supplementation of ascorbic acid for 2 h just before the broilers were transported and for 2 h immediately after they were transported decreased HSP 70 gene expression, rectal temperature, pH of the meat and water holding capacity of the meat. The supplementation of ascorbic acid also increased total erythrocytes, hemoglobin, hematocrit, total leukocytes, lymphocytes, heterophils and the ratio of H/L. **Conclusion:** Supplementation of ascorbic acid 2 h before broilers are transported reduces broiler's stress and maintains their meat quality.

Key words: Transportation, broiler, HSP 70, hematological, meat quality

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Poultry production has the highest contribution for national meat supply. In 2013, the national production of poultry meat was 1788.9 t, consisting of 82.72% of broiler meat, 16.1% of domestic poultry meat, 3.9% of culling laying chicken and 1.7% of duck meat[1]. The success of the high supply of poultry meat is partly due to the availability of high-quality breeds and the availability of complete technology packages. However, there is one factor that sometimes goes unnoticed in the poultry production industry. The factor is the transportation of chicken from growers to slaughter-houses. The transportation factor is actually a short process in the poultry industry. Nonetheless, the activity often causes significant losses. This happens because during the transportation, chickens are in the discomfort environment which will be a potential stressor for the poultry. Losses of transported chickens could happen due to the high number of deaths during the transportation process and the declining of meat quality due to the effect of stress during the transportation.

There are some factors triggering the emergence of haulage stress i.e., the catching process, the loading and unloading of the poultry into and out of the truck, the handling process when the poultries are transferred from the cage to the box, the density of poultry in the box, social disruption, limitation of motion, heat radiation, wind, noise and vibration[2-8]. All these stressors affect the physiological responses simultaneously, resulting in acute stress experienced by the poultries.

Stress could affect the balance of biochemical reactions, especially the formation of weak bonds such as enzymes and the denaturation of proteins including enzymes in the body of the chickens. As a result, enzymes cannot work properly, leading to disruption of biochemical reaction in the body, including homeostasis disruption[9-11]. When this disruption is happened, the body would attempt to return the homeostasis state to its original condition before the stress. When the attempt fails, the body would use genetic pathways, for instance, by activating HSP 70 genes[7,8,12,13].

The HSP 70 genes can be activated and deactivated in accordance with the metabolic signal received from internal factors, such as hormones and external factors, such as nutrition[14]. Some essential and non-essential bioactive components of feeds affect a number of cellular processes associated with health and disease prevention, including carcinogen metabolism, hormonal balance, cell signaling, cell cycle control, apoptosis and angiogenesis. Components of bioactive food often modify processes in the body simultaneously. Therefore, the issue of stress happened during the poultry transport could be addressed using nutrigenomic approach[15]. This could be done, for example, by supplementing antioxidant vitamin to the DNA. The supplementation could affect HSP 70 genes protecting proteins that are sensitive to stress. This means the effects of stress can be reduced and before the poultries are slaughtered, they are in a good condition which will produce a high quality of meat[16,17].

Ascorbic acid is the example of vitamin that belongs toantioxidant group. Ascorbic acid could slow down the oxidation reaction of substances that get oxidized easily, although the concentration of the substances is low[18,19]. Under a normal condition, chicken can synthesize ascorbic acid in the body. However, when chickens are under stress, the requirement of ascorbic acid will increase which could be obtained from feed or drink[4,5,20]. Ascorbic acid plays an important role in the thyroid activities, as dopamine β-hydroxylase co-substrate in norepinephrine formation, so that the chickens could stimulate the heart rate and dilatation of the peripheral vessels to lower body temperature[4,5,21]. A study of effectiveness of the use of ascorbic acid as an antioxidant to reduce the influence of the transportation stress has been conducted[4,5,22,23]. However, similar study which focuses on gene expression of HSP 70 as a major indicator of stress of chicken andits association with the quality of the meat is limited. Therefore, the aim of this study was to determine the meat quality of broilers chickens exposed to transportation stress shortly after slaughtering.

MATERIALS AND METHODS

Research location: Rearing of broilers and transportation stress test were conducted in the Teaching Farm Laboratory of Faculty of Animal Science, University of Mataram, Lingsar Village, Lingsar District, West Lombok. Measurements of hematology parameters were conducted in the Immune-biology Laboratory of Faculty of Science, University of Mataram. Test of HSP 70 gene expression was conducted in the laboratory of the Cultivation Research, Sekotong West Lombok. Analysis of the meat quality was conducted in the Laboratory of Animal Products Processing Technology of Faculty of Animal Science, University of Mataram.

Experimental design: This study was a completely randomized design with 5 treatments using a unidirectional pattern with 50 female broilers of Lohmann MB 202 aged 5 weeks. This means that each treatment group had 10 chickens. The chickens were divided into 5 treatments.

Treatment 1 (T1) was the broiler chickens without ascorbic acid supplementation and without transportation treatment (control 1). Treatment 2 (T2) was the broiler chickens with transportation treatment without ascorbic acid supplementation (control 2). Treatment 3 (T3) was the broiler chickens with transportation treatment and ascorbic acid supplementation at a dose of 360 mg L^{-1} of drinking water for 2 h just before being transported. Treatment 4 (T4) was the broiler chickens with transportation treatment and with ascorbic acid supplementation at a dose of 360 mg L^{-1} of drinking water for 2 h shortly after transportation. Treatment 5 (T5) was the broiler chickens with transportation treatment and with ascorbic acid supplementation at a dose of 360 mg L^{-1} of drinking water for 2 h before and after being transported.

Transportation treatment: All of the chickens used in the study were identified using a wing band. The chickens were put into the transportation box after which they were taken around from 10.00 am to 02.00 pm using an open vehicle. For the experimental broiler chickens in treatments 2-5, their body temperatures were measured directly after being transported. Blood of chickens from T2 and T3 were sampled to measure the hematology parameters. After blood sampling, chickens from treatment 2 and 1 were immediately slaughtered to obtain the liver of the chickens (for gene expression of HSP 70 parameter) and breast meat for measurement of the meat quality. Chickens in the treatment 4 and 5 after rectal temperature measurements were rested for 2 h and were given ascorbic acid supplementation. This was followed by the rectal temperature measurement for the second time and blood sampling and slaughtering process.

Blood samples were taken from the wing venous using 1 cc insulin syringe. After that, the sample was inserted into a tube containing 5 mL EDTA (ethylene diamine tetra acetic acid). The livers were taken using sterile equipment and put into a 1.5 mL tube containing 500 mL of RNA (Ribonucleic acid). Both samples were stored at a temperature of -80°C for analysis of total RNA.

Measurements

Body temperature (rectal temperature): In T1, the measurement of rectal temperature was conducted when other treatment groups were transported. In T2 and T3, rectal temperatures were measured shortly after the chickens were transported. For T4 and T5, the measurement of rectal temperature was done twice, shortly after they were transported and 2 h later (after supplementation of ascorbic acid). These works were performed by inserting a thermometer into the cloaca.

Hematology parameters: Hematology parameters measured were (1) The total number of erythrocytes, (2) Hemoglobin levels, (3) The value of hematocrit, (4) The total number of leukocytes and (5) The differential count of leukocytes (percentage of heterophils, eosinophil, basophils, monocytes and lymphocytes) and the ratio of H/L.

Erythrocyte: Measurement of erythrocyte was conducted using counting chambers method[24]. Twenty milliliters of blood containing EDTA was put into a 4000 mL of Hayem solution using a micropipette. The mixture of the solution was then rinsed and mixed evenly. The solution was then incubated for two minutes. Next, the solution was put into Improved Neubauer counting chamber. The number of erythrocytes could be counted on five areas of erythrocyte box with 40x magnification of objective lenses. The number of erythrocytes was determined by multiplying the number of erythrocytes with 10000 mm^3.

Hemoglobin: The level of hemoglobin was measured using spectrophotometer method[24]. Twenty milliliters of blood containing EDTA were inserted using a micropipette. Thereafter, the blood sample was mixed with Drabkin solution. The solution was then rinsed, mixed until smooth and incubated for 3 min. The absorbance was read at a wavelength of 540 nm using a spectrophotometer (UV-visible). Hemoglobin level was calculated by multiplying the absorbance by a factor (g dL^{-1}).

Hematocrit: The measurement of hematocrit value was done using micro-hematocrit methods[24]. Blood sample was inserted into the micro-hematocrit tube. The bottom part of the tube was closed using wax. The tube was put in a hematocrit centrifuge (Hettich) then centrifuged at 15,000 rpm for 5 min. The percentage of blood was read using a hematocrit measuring instrument.

Leukocytes: The total number of leukocyte was calculated using the counting chamber method[24]. Three hundred and eighty milliliters of Turk's solution was inserted using a micropipette into a glass tube. After that, 20 mL of blood containing EDTA was mixed with the Turk's solution using a micropipette. The solution was then rinsed, thoroughly mixed and then incubated for 2 min. The mixture of the

solution was put into the counting chamber of Improved Neubauer. Leukocyte was counted in four areas using 10x magnification of objective lenses. The total number of the white cell was determined by multiplying the number of leukocytes results count with 50 mm^3.

Differential count of leukocyte: The differential count of leukocyte was calculated using the Rapid method[24]. Five milliliters of blood sample was dripped onto the end of the glass object using a micropipette until the blood stick and spread to the edge of the glass slider. The blood was removed with an inclination of 35°. After that, the preparation was dried and fixed with methanol. The next step was the preparation was colored using eosin (color 1) for 20-30 sec. The preparation was then colored for the second time for 15-30 sec. The preparation was subsequently rinsed with running water until the smear clean and dried. The preparation was read under a microscope with the help of emersion oil. The percentage of each differential leukocyte cell was calculated with the help of a hand counter.

Expression of HSP 70 gene

Total RNA extraction: Total RNA extraction was analysed using a prochedure conducted by Tamzil *et al.*[6] as follow: "Each liver tissue was sampled to find total RNA. Extraction of total RNA was performed using total RNA Mini Kit 50 preps produced by Gene Aid. Twenty five milligrams of liver samples was dissolved with 400 mL RB buffer and 4 mL mercaptoethanol in a 1.5 mL tube. Next, the sample was incubated for 3 min at a room temperature. After that, the samples were moved to filter column and centrifuged at 1000×g for 30 sec. Colum filter pair was disposed andto sediment component, 400 mL of 70% ethanol was added and vortexed until homogeneous. The sample was moved to RB column and was centrifuged at 14000×g for 1 min. After the sedimentation was disposed, RB column was transferred to a 2 mL new tube and WI buffer was added after that. This step was followed by centrifugation at 14000×g for 30 sec. After the sediment was disposed, 600 mL of wash buffer was added then it was centrifuged at 14000×g for 30 sec. After the sediment was disposed, it was centrifuged at 14000×g for 2 min and the column was transferred into 1.5 mL tube. After that, 50 mL of RNase-free water sample was added and it was kept for 2 min followed by centrifugation at 14000×g for 1 min. The RNA was then stored for approximately 45 min. The process was continued by a reverse transcriptase reaction".

Reverse transcriptase: Reverse transcriptase was analysed using a prochedure conducted by Tamzil *et al.*[6] as follow: "Synthesis of single-stranded DNA was performed using total RNA via reverse transcriptase reaction, using Transcription Synthetic First Strand cDNA Kit. Five milliliters sample of total RNA, 1 mL oligo (dT) and 15 mL H_2O were put into a 0.2 mL tube. The solution was heated at 65°C for 10 min and then immediately was moved into an ice bath. Six milliliters of 5X buffer, 0.5 mL of inhibitor RNase, 0.5 mL of dNTP and 0.5 mL of reverse transcriptase were added into the sample solution. The solution mix was then incubated at 55°C for 30 min and 85°C for 5 min. After the process finished, cDNA was ready for use".

Real-time quantitative PCR (RT-PCR): Testing was performed using real-time PCR quantitative using DNA samples obtained from the reverse transcriptase reaction of tRNA genes HSP 70. Primer used was the primer of GADPH (housekeeping): F-5'GTGTTATCATCTCAGCTCCCTCAG-3', R-5'GGTCATAAGACC CTCCACAATG-3' and HSP70: F-5'GACAAGAGTACAGGGAAG GAGAAC-3', R-5'CTGGTCACTGATC TTTCCCTTCAG-3'[25] with a length of the product was 222 bp PCR.

Meat quality

Meat pH: Measurement of this variable was performed using a method summarized by Soeparno[26]. Ten grams of broiler breast meat was put into a 100 mL beaker. Ten milliliters of distilled water was added next. After that the mixture was stirred and kept for 2 h at a temperature of 5°C. The solution was then equilibrated at a temperature of 23°C. The measurement of pH was performed using a digital pH meter.

Water holding capacity: Measurement of this variable was performed using a method summarized by Soeparno[26]. Three hundred milligrams (0.3 g) of chicken meat was placed on a glass plate. The sample was then covered with filter paper. On the top of filter paper another glass plate was put. This system was then pressed using a load weighing 35 kg for 5 min. After that, the pressed sample was drawn on a transparent plastic. Wet area outside the sample was measured using graph paper (cm^2). Water holding capacity (mg) was determined using the following equation:

$$H_2O\ (mg) = \frac{\text{Wet area } (cm^2)}{0.0948} \times 8.0$$

$$\text{Water holding capacity} = \text{Total water percentage } (\%) - \frac{H_2O\ (mg) \times 100\%}{300\ mg}$$

Cooking loss: Measurement of cooking loss was performed using a method summarized by Soeparno[26]. Five grams of sample (x) was put into a plastic bag. It was then cooked for 1 h at a temperature of 80°C. After that, the sample was taken out from the plastic bag and then separated from its broth. Next, the sample was swabbed using tissue without being pressed. Lastly the sample was weighed (Y). Cooking loss was determined using equation:

$$\frac{X - Y}{x} \times 100\%$$

Tenderness: Measurement of tenderness was performed using a method summarized by Soeparno[26]. Sample of broiler meat of 1 cm^3 was put into the base of a penetrometer. Needle of the device was arranged so the sample could meet with the end of the needle. As a result, the needle was in 0 position. A load weighing 50 g (a) was freed at the same time with a stopwatch for 10 sec. The depth of the needle was shown by penetrometer scale (b). Tenderness of the meat was determined using:

$$\frac{b}{a} / t\,(mm\ dt)$$

Statistical analysis: The collected data were tabulated and analyzed using analysis of variance and LS-MEANS using the GLM procedures SAS software[27]. The differences between the means of groups were identified by Tukey's test with the significant levels of $p<0.05$ and $p<0.01$.

RESULTS AND DISCUSSION

The effects of transportation treatment and ascorbic acid supplementation in drinking water on HSP 70 gene expression and rectal temperature are presented in Table 1. Transportation treatment increased HSP 70 gene expression and rectal temperature ($p<0.01$). On the other hand, ascorbic acid supplementation could reduce HSP 70 gene expression and rectal temperature ($p<0.05$) after the transportation treatment. This result implied that during transportation the chickens indeed experienced stress. Some studies showed that during the transportation process the chickens experienced stress due to the process of catching, loading and unloading into and out of the truck, the handling process when the chickens were transferred from cage to the box, the density of the chickens in the box, social disorder, motion limitation, heat radiation, wind, noise and vibration. All of

Table 1: Effect of transportation and supplementation of ascorbic acid in drinking water on expression of HSP 70 gene, cloacal temperature, hematological status and meat quality of broiler chickens

	Treatment					
Parameters	1	2	3	4	5	p-value
Expression of HSP 70 gene (relative mRNA level)	0.819±2.576^{c}	11.418±2.822^{a}	5.088±2.231^{ab}	1.270±2.822^{c}	2.368±2.576^{c}	<0.0004
Cloacal temperature 1 (°C)	41.287±0.245^{c}	43.167±0.283^{a}	42.400±0.262^{ab}	41.814±0.262^{c}	41.950±0.283^{c}	<0.0001
Cloacal temperature 2 (°C)*	-	-	-	40.385±0.2748^{a}	40.933±0.295	
Hematological status						
Erythrocytes (×10^{6} mm^{-3})	3.116±0.269^{a}	2.500±0.249^{b}	2.950±0.269^{a}	2.957±0.249^{a}	2.825±0.233^{a}	<0.0001
Hemoglobin (%)	1.000±1.162^{a}	0.600±0.300^{b}	1.000±0.439^{a}	0.875±0.411^{a}	1.000±1.162^{a}	<0.9448
Hematocrit values (%)	63.975±3.555^{a}	47.486±3.800^{b}	60.467±4.105^{a}	61.828±3.800^{a}	50.400±4.105^{b}	<0.0001
Leukocytes (×10^{3} mm^{-3})	3.984±0.210^{a}	3.216±0.257^{b}	3.539±0.361^{a}	3.728±0.256^{a}	3.699±0.292^{a}	<0.0001
Leukocyte differentiation						
Lymphocytes (%)	55.000±7.97^{a}	25.000±7.97^{b}	47.333±2.06^{a}	42.250±2.820^{a}	48.286±3.01^{a}	<0.0001
Heterophils (%)	21.875±2.39^{c}	29.166±2.76^{b}	21.428±2.56^{c}	28.571±2.560^{b}	23.833±2.76^{c}	<0.0001
Eosinophil (%)	9.000±9.720	8.286±3.674	11.760±2.510	8.125±3.437	8.000±9.720	<0.0732
Basophils (%)	6.000±1.423	6.857±0.538	6.267±0.367	6.875±0.503	4.000±1.422	<0.0871
Monocytes (%)	5.400±0.713	4.514±0.270	4.653±0.184	5.025±0.252	6.600±0.713	<0.1792
H/L ratio	0.398±0.086^{c}	1.166±0.099^{a}	0.453±0.092^{c}	0.676±0.092^{b}	0.493±0.099^{c}	<0.0001
Meat quality						
pH	5.650±0.063^{a}	6.270±0.063^{b}	5.633±0.073^{a}	5.633±0.073^{a}	5.567±0.073^{a}	<0.0501
Cooking loss	2.212±0.099	1.316±0.114	2.414±0.106	2.486±0.106	1.850±0.115	<0.2472
Water holding capacity	14.666±1.30^{c}	20.125±1.12^{a}	14.000±1.20^{c}	13.571±1.203^{c}	14.000±1.30^{c}	<0.0501
Tenderness	2.500±0.191	2.667±0.220	2.286±0.204	2.428±0.204	3.000±0.221	<0.0792

$^{a-c}$Means in the same row without common superscript differ at $p<0.05$, treatment 1: Experimental chickens without transportation treatment and ascorbic acid supplementation, treatment 2: Experimental chickens with transportation treatment but without ascorbic acid supplementation, treatment 3: Experimental chickens with transportation treatment and supplementation of ascorbic acid at a dose of 48 mg L^{-1} in drinking water for 2 h before being transported, treatment 4: Experimental chickens with transportation treatment and supplementation of ascorbic acid at a dose of 360 mg L^{-1} in drinking water for 2 h after being transported, treatment 5: Experimental chickens with transportation treatment and supplementation of ascorbic acid at a dose of 360 mg L^{-1} in drinking water for 2 h before and after being transported, *Rectal temperature measured after 2 h of transportation

these factors affected physiological conditions the experimental chickens simultaneously, leading to acute stress[2,5,8]. When the transportation test was performed, the ambient temperature was around 29.9-36.4°C which could be classified as stress temperature for the chickens, since for the adult broilers the comfortable temperature is 26-27°C[28]. Under stress conditions, the chickens will attempt to restore homeostasis of the body to the condition before the stress. When the stress continues and the body cannot handle it, HSP genes, will be activated, including HSP 70 genes which only function under the stress condition[29]. The HSP 70 genes are very sensitive to environmental changes[30]. This was the reason why in this study, HSP 70 gene expression of chickens belonged to T1 (control 1) was lower ($p<0.01$) compared to chickens belonged to T2 (control 2). The increase in the expression of HSP 70 gene as the effect of transportation treatment means that the transportation process indeed cause the chickens to suffer stress[8,30]. Another study showed that HSP 70 gene expression of chickens reared under heat stress condition was higher than that of reared in normal condition[12]. Study of HSP gene expression in CG sites of ducks experiencing stress is higher than those without stress[31]. Ewing *et al.*[9] defined that stress is a condition of living things that cause increase of temperature or other stressors coming from both external and internal body of the livestock. Morberg[10] defined stress as any biological process that could threat and disrupt homeostasis of animals and every stressor causes a negative impact on animal welfare.

Data presented in Table 1 provides information that supplementation of 360 mg L^{-1} of ascorbic acid in drinking water could reduce gene expression of HSP 70 after being transferred. Under normal conditions, exogenous ascorbic acid supplementation is not required, because it can be synthesized internally by the chicken. However, when the chickens are under stress, the need for the ascorbic acid increases, meaning an extra ascorbic acid is needed to be supplemented[32,33]. Physiologically, ascorbic acid is required in hydroxylation reactions on the nervous system and the adrenal medulla[16]. In addition, ascorbic acid plays a role in thyroid activities, such as a co-substrate of dopamine β-hydroxylase in the formation of norepinephrine to increase the ability of chickens to dispose of body heat to stimulate the heart rate and dilating the peripheral vessels[21]. Ascorbic acid plays a role in increasing the antioxidant activity of vitamin E, which happens by converting tocopheroxy radical into the active form of vitamin E, so the danger of heat stress can be avoided[20]. This is the reason why ascorbic acid supplementation in this study could reduce HSP 70 gene expression in broiler chickens, which is the main indicator of chickens suffering from stress after the transport.

Chickens from T4 and T5 gave the same HSP 70 gene expression ($p>0.05$) but gave lower expression compared to chickens from treatment 3 ($p<0.01$). The low expressions of HSP 70 gen in chickens in T4 and T5 indicated the lower stress condition due to the rest period for 2 h after the transportation treatment. The HSP 70 genes can act as savior for living creatures in an emergency situation[34]. When the homeostasis of the body is back to normal, the expression of HSP 70 genes will slowly decrease and then go back to dormant state as they were prior to the stress[29]. This is what causes HSP 70 gene expressions of chickens from T4 and T5 are lower than that of from T3. Nonetheless, all three methods of supplementation of ascorbic acid tested gave the same result as the expression of HSP 70 with that of chickens from T1 ($p>0.05$). However, this result was lower than the level of the expression of HSP 70 from group of chickens in T2 ($p<0.01$). Thus, it means that supplementation of ascorbic acid at a dose of 360 mg L^{-1} in drinking water can minimize the effect of transportation stress.

Data of the effects of treatment on rectal temperature (Table 1) showed that transportation increased rectal temperature ($p<0.01$) and supplementation of ascorbic acid was able to reduce the increased rectal temperature ($p<0.01$). The in creased rectal temperature as the impact of the transportation treatment causes the chickens to suffer stress. During the transportation treatment, the chickens were exposed to high temperature and the situation was worsened by other stressors. This combination simultaneously affects the physiological conditions of the experimental chickens[2,3,8]. The temperature during the transportation was unfavorable for the chickens (29.9-36.4°C), while the comfortable temperature for broilers are around 26-27°C[28]. As a consequence, the chickens experienced difficulty in releasing the excess of body heat to the environment, leading to the increased body temperature[6,8,35]. Normal body temperature of chickens ranges between 40.5-41.5°C[36]. When they are exposed to high temperatures, the body temperature will rise. This was shown from a study in which laying chicken, kampong chicken and Arab chicken were exposed to 40°C for 0.5-1.5 h. The result showed that the body temperature rose to 44.99°C[6,8].

Data presented in Table 1 showed that the experimental chickens in T3, T4 and T5 had the same body temperature ($p>0.05$) with chickens in T1, but lower compared to those in T2 ($p<0.01$). This means that the treatment of ascorbic acid supplementation of 360 mg L^{-1} in drinking water can help the broiler to avoid transport stress. This condition indicates that the physiological functions of ascorbic acid in the activity of the thyroid, which is a co-substrate of dopamine to stimulate the heart rate and dilating the peripheral is normal, meaning the body temperature of chicken receiving supplementation

of ascorbic acid is lower than the body temperature of chicken that do not get ascorbic acid supplementation. The mean body temperature of the chickens of T1 was 41.287±0.245°C, whereas in the T2, the mean increased to 43.167±0.283°C. Nonetheless, the average body temperature of the chickens in the group of chickens receiving ascorbic acid supplementation decreased to around 41°C (normal temperature)[8,35].

When the second measurement was performed, rectal temperatures (after 2 h of transportation treatment) of the chickens in T4 and T5 were normal. The normal temperature happened because the body conditions went back to normal due to the influence of the break.

The effect of the treatment on the total number of erythrocytes, hemoglobin, hematocrit, total number of leukocytes and leukocyte differentiation are presented in Table 1. It could be seen that the transportation treatment lowered total of erythrocytes, hemoglobin, hematocrit and the total number of leukocytes ($p<0.01$). Supplementation of ascorbic acid could increase the total erythrocytes, hemoglobin, hematocrit and total leukocyte of the broiler chickens experiencing acute stress due to transportation ($p<0.01$).

The level of erythrocytes, hemoglobin and hematocrit values of the experimental chickens decreased because the transportation caused stress on the chickens. The stress experienced by the chickens affects the synthesis, stability and activities of enzyme, as well as some biochemical compounds in the body[6-8,29]. Another effect of the stress is its interference on the performance of the erythropoietin hormone, which is responsible for the formation of erythrocytes, triggering the emergence of erythropoiesis[37], as well as a decrease of hematocrit value[7,8,38]. The decreasing levels of hemoglobin triggered by stress have been reported by Tamzil *et al*.[7] and Tamzil[8]. The studies report that kampong chickens, arab chickens and laying chickens suffering from heat stress experienced the decrease in Hb level after being exposed to heat stress at 40°C for 0.5 up to 1.5 h. Erythropoiesis process in some of species of poultry has also been widely reported in some studies, such as by Tamzil *et al*.[7], Tamzil[8], Altan *et al*.[35] and Coles[38]. The studies stated that there is a strong relationship between the numbers of erythrocytes with a hematocrit va The decrease in hematocrit value partly is caused by the damage of erythrocytes, decreased production of erythrocytes or decrease in the number or size of erythrocytes.

Table 1 also shows that the levels of erythrocytes, hemoglobin and hematocrit of experimental chickens in T3, T4 and T5 are the same as those in T1 ($p>0.05$), but higher than those in T2. The reason for this is that ascorbic acid supplementation can protect the experi ntal chickens from the effect of the transportation stress. Ascorbic acid plays a role in thyroid activity as a co-substrate of dopamine β-hydroxylase in the formation of norepinephrine, to stimulate heart rate and peripheral vessel dilatation to allow body heat dissipation into the environment[4,5,21]. As a consequence, body temperature is not too high, which in turn does not affect the erythropoietin hormone, resulting in h levels of erythrocytes, hemoglobin and hematocrit values in the group of chickens that are subjected to transportation stress and supplemented with ascorbic acid are similar to those of experimental chickens without transportation treatment and ascorbic acid supplementation. This result proves that ascorbic acid is a good antioxidant to counteract free radicals when poultry are suffering stress[20].

Table 1 shows that the chickens in T2 experienced the decrease in total leukocyte and lymphocyte percentage, but the percentage of heterophil and the ratio H/L ($p<0.01$) increased. Nonetheless, thepercentage of eosinophil, basophils and monocytes in the experimental chickens in T group ($p>0.05$) did not change. It can also be seen that the effect of ascorbic acid supplementation on total leukocyte, lymphocyte percentage, heterophil and the ratio H/L experimental chickens in T3, T4 and T5 are the same as those of T1 (control 1) ($p>0.05$).

The changes of the number of leukoc , lymphocytes percentage, heterophil and the value of the H/L ratio is caused by the influence of stress arising from transportation process[2-5,7,8,39]. Chickens suffering stress are characterized by the change in differentiation of blood leukocytes (lymphocytes, heterophil, basophils, monocytes and the H/L ratio)[7,8,35,39]. The changes happen because chickens experiencing stress cause the increase of glucocorticoid hormone level, especially corticosterone hormone[6,8,40]. A study regarding the emergence of stress caused by feed deficiency in poultry showed the increase levels of the corticosterone hormone and the H/L ratio[40]. Heat stress tested on kampong chickens, Arab chickens and broilers showed the increases levels of the corticosterone hormone and the H/L ratio[6,8,41]. This is the reason why the increased levels of the corticosterone hormone and the H/L ratio in poultry or N/L in humans can be used as a stress indicator[40]. The increased of corticosterone hormone in the blood can obstruct the performance of the immune system in the body such as lymphocyte proliferation, immunoglobulin production, production of cytokines, cytotoxicity and anti-inflammatory agents[42].

Data of the transportation treatment effects and supplementation of ascorbic acid on meat quality are presented in Table 1. Transportation treatment increased pH and water holding capacity ($p<0.05$) but did not affect cooking loss value and tenderness ($p>0.05$). Supplementation of ascorbic acid lowered pH and water holding capacity of broiler meat experiencing stress caused by transport treatment but did not affect tenderness and water holding capacity.

Data of effects of transportation treatment and ascorbic acid supplementation on the pH of the meat, cooking loss, water holding capacity and tenderness of broiler meat are given in Table 1. It can be seen that transportation treatment caused the increases in pH value, water holding capacity and tenderness of meat ($p<0.05$), but it did not affect cooking loss values ($p>0.05$). On the contrary, ascorbic acid supplementation lowered the pH value and water holding capacity and increased cooking loss values of chickens which had experienced stress due to transportation treatment ($p<0.05$).

The increases pH values and water holding capacity of meat are related to the stress caused by the transportation treatment of the experimental chickens[2,3]. Chickens suffering from stress just before slaughtered produce meat with a lower quality[41,43,44]. When stress occurs, glycolytic enzyme activity increases within the chicken body, leading to the increase of glycolysis and ATP hydrolysis[45]. After slaughtering, muscle glycogens undergo glycolysis process enzymatically and produce lactic acids that trigger changes in the pH value of the meat[46]. Lactic acid accumulation will stop after the depletion of glycogen reserve. Lack of muscle glycogen reserves causes limited anaerobic glycolysis process and limited lactic acid accumulation, causing small decrease in pH value[47]. Chickens suffering from stress before being slaughtered have excessive glycogen accumulation and lactic acid in their bodies, resulting in meat having high pH value. This leads to the increase in water holding capacity of meat, as well as a decrease in the shelf life of meat[48,49]. Meat having high pH value is associated with a lower shelf life, because meat having high pH is a favorable environment for bacteria to grow[41,49].

When the data in Table 1 is considered carefully, it can be seen that experimental chickens in T3, T4 and T5 were able to bring back the pH of meat back to normal, which was 5.6 (similar to the pH of meat in T1), while the pH of meat in experimental chickens in T2 was 6.270. This means that supplementation of ascorbic acid at 360 mg L^{-1} in drinking water for 2 h before and after the transportation can reduce the increase in pH and water holding capacity[48,49]. These cases can be used as an indicator to predict that ascorbic acid supplementation could prolong the shelf life of broiler meat[41,49].

From data in Table 1, it can be seen that the experimental chickens in T3, T4 and T5 were able to bring back the pH of the meat back to normal. The controls experimental chickens (T1) had lower pH of the meat. This means that the supplementation of ascorbic acid of 360 mg L^{-1} in drinking water for 2 h before and after transport can reduce the increase in pH value, water holding capacity, tenderness of meat[48,49] and can extend the shelf life of meat[40,49].

There are three different systems of ascorbic acid supplementation used in this study. It was found that supplementation of ascorbic acid of 360 mg L^{-1} in drinking water for 2 h before the transportation treatment or 2 h shortly after the transportation treatment was an applicable system, while the supplementation of ascorbic acid for 2 h before the transport and followed by 2 h shortly after the transport was less-applicable.

CONCLUSION

It can be concluded that the transportation treatment causes the chickens to suffer from stress characterized by the increased of HSP 70 gene expressions. The HSP 70 gene expression can be reduced using ascorbic acid supplementation of 360 mg L^{-1} in drinking water in 2 h shortly before the transportation or for 2 h shortly after the transportation. The supplementation treatment could prevent the decrease quality of chicken meat because of the stress caused by the transportation process.

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