Hematological Response of Chickens with Different Heat Shock Protein 70 Genotypes to Acute Heat Stress

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Abstract: Hematological responses in chicken with different HSP 70 genotypes to acute heat stress were studied using 28 kampong chickens, 24 Arabic chickens and 4 commercial chickens. The experimental chickens were selected randomly from a group of chickens with HSP 70 genotypes identified and were exposed to ambient temperature (40°C) for 0.5, 1.0 and 1.5 h. Results showed that erythrocyte, hematocrit and leukocyte in all chicken lines decreased in response to acute heat stress with the highest decrease in commercial chickens, followed by Arabic and kampong chickens. Regardless of acute heat stress exposure, there was no significant difference in erythrocyte, hemoglobin, leukocyte, heterophil, eosinophil, basophil, lymphocyte, monocyte and heterophil/lymphocyte ratio in all chicken lines studied. Arabic and commercial chickens had lower hematocrit as compared to kampong chickens. However, acute heat stress increased the percentage of heterophil, basophil, lymphocyte and heterophil/lymphocyte ratio without affecting eosinophil and monocyte. It was found that there was no interaction between lines of chicken and acute heat-stress exposure on the hematological parameters measured. The lowest percentage of lymphocytes was found in chickens with DD HSP 70 genotype while the highest was found in chickens with AD genotype. The results indicated that there was a relationship of heat resistance or tolerance to lymphocyte expression. Chickens that were the most tolerant to acute heat stress had the highest lymphocyte percentage (AD genotype) whereas those that were the least tolerant had the lowest lymphocyte percentage (DD genotype).

Key words: HSP 70 gene, acute heat stress, kampong chicken, Arabic chicken, hematology

INTRODUCTION
Kampong chicken is Indonesian native chicken which is raised for meat and egg production locally. This local chicken line survive well in traditional management system and has been a significant contribution to food security in rural areas. The weakness of kampong chicken is its slow growth rate and low egg production potential (Nishida et al., 1980; Iskandar et al., 2000). To meet the needs and requirement of chicken meat and eggs consumption in Indonesia, Arabic and commercial chicken (broiler and layer) were introduced. The three lines of chicken have different development and domestication backgrounds and have different physiological capacities to respond to various breeding environments. Kampong chicken is a descendant of the red jungle fowl (Gallus gallus) which is domesticated in South East Asia including Indonesia (Fumihito et al., 1996; Sartika and Iskandar, 2007; Sulandari et al., 2007a,b). Arabic chicken is a local layer hen coming from overseas and is a descendant of braekel kriel silver and braekel kriel gold chickens (European local chicken) (Sulanidari et al., 2007a,b; Sartika and Iskandar, 2007), where as commercial chicken (broiler and layer) is a layer type developed in sub-tropical and cold climate regions.

Chicken is warm-blooded animal (homeothermic) with body temperature ranges between 40.5-41.5°C (Etches et al., 2008). A characteristic of poultry is it does not have sweat glands and almost all parts of its body are covered by feathers. These biological characteristics make poultry raised in warm areas susceptible to heat stress (Cooper and Washburn, 1998; Lin et al., 2005; Al-Fatafah and Abu-Dieyeh, 2007; Al-Ghamdi, 2008; Al-Aqil and Zulkifli, 2009; Zulkifli et al., 2009; Ajakaiye et al., 2010). Stress in poultry can decrease erythrocyte count (Toghyani et al., 2006), hemoglobin concentration (Hilman et al., 2000; Puvadolpirod and Thaxton, 2000), hematocrit (Coles, 1982; Altan et al., 2000; Zulkifli et al., 2009), percentages of leukocytes (Nathan et al., 1976; Mashaly et al., 2004) and leukocyte differentiation (Altan et al., 2000).

When poultry is stress, homeostasis zone in the body is disturbed and the control and integrating systems of the body attempt to restore the conditions to the
homeostasis zone. Some of the activities that the poultry does to restore the homeostasis zone are panting, reducing feed intake and increasing water consumption (Tamzil et al., 2013b), sprinkling its body with dust and regulating body metabolism to maintain a constant body temperature. When stress cannot be overcome physiologically, genetic method will be used by activating HSP genes including HSP 70 working only under stress conditions. HSP 70 genes contained several polymorphic sites that can be used as chicken’s marker that are more tolerant at high temperatures (Mazzi et al., 2003; Zhen et al., 2006; Gaviol et al., 2008; Tamzil et al., 2013a). Tamzil et al. (2013a) successfully mapped the HSP 70 genotypes in kampong, Arabic and commercial chickens and showed that there were such seven genotypes in kampong chicken (AA, AB, AC, CC, AD, DD and BC genotypes), six genotypes in Arabic chickens (AA, AB, AC, CC, AD and BC genotypes) and one genotype in commercial chicken (DD genotype). Genotype mapping indicate that heat tolerant or resistant parameters of chickens to heat stress are related to the genotype of the chickens (Tamzil et al., 2013b). The present study evaluated the interaction between lines of chickens and genotype of HP 70 and heat tolerance of chickens to heat stress with focus on the hematological dynamics and cellular immune systems.

MATERIALS AND METHODS

Birds: Twenty eight kampong, twenty four Arabic and four commercial chickens (laying hen type) were randomly selected from groups which HSP 70 genotypes had been identified by using Polymerase Chain Reaction (PCR)-Single Strand Conformation Polymorphism (SSCP).

Heat stress exposure: This study was designed in a completely randomized design with a 2x4 factorial arrangement. The first factor was chicken line which consisted of 2 levels i.e., kampong chicken and Arabic chicken. The second factor was the duration of acute heat stress exposure (40°C ambient temperature) which consisted of 4 levels i.e., 0 (as a control without heat stress), 0.5, 1.0 and 1.5 h. Twenty eight kampong chickens of genotypes AA, AB, AC, CC, AD, DD and BC and twenty four Arabic chickens of genotypes AA, AB, AC, CC, AD and BC and four commercial chickens of genotype DD were used. One bird of each HSP 70 genotype from kampong, Arabic and commercial chickens was assigned as a control (without exposure to any heat stress) and the others were exposed to acute heat stress test on 40°C for 0.5, 1.0 and 1.5 h in a chamber. The chamber was square shaped, from wooden board and in 33x33x75 cm³. The chamber was also equipped with heater, thermostat, blower, digital thermometer, ventilation, feed and water spot. At the base of the chamber, a divider wire was placed and aluminum foil was placed on top of it to collect the manure. Commercial chicken was not included in this design due to only one genotype was found (DD and no replicates). Acute heat stress test was done alternately. Two hours prior to heat stress, chickens were fasted but water was available ad libitum.

Blood parameter measurements: After heat-stress challenge test was conducted, blood samples were taken through brachial vein using a 1 cc insulin syringe then it was put into 5 mL EDTA tube. The sample from each chicken was used for measurement of erythrocytes, hemoglobin, hematocrit, leukocytes and leukocyte differentiation.

Erythrocytes: The number of erythrocyte was counted using count room method (Kolmer et al., 1959). Twenty microliter of blood in EDTA was put into 4000 μL Hayem solution using micropipette, then it was rinsed and mixed until the solution was homogen, after that it was incubated for two minutes. The result was put into Improved Neubauer count room. The number of erythrocytes was counted on five field boxes with 40 times magnification of objective lens. The number of erythrocytes was determined by multiplying the result of counted erythrocytes with 10000 (mm³).

Hemoglobin: The percentage of hemoglobin was measured by using Spectrophotometer method (Kolmer et al., 1959). Twenty microliters of blood in EDTA was put into Drabkin solution using micropipette and then the solution was homogenized and incubated for 3 min. Absorbance of the solution was read at the wave length of 540 nm using a spectro photometer (UV-Visible). Hemoglobin value was calculated by multiplying the absorbance with hemoglobin factor (g/dL).

Hematocrit values: Hematocrit value was measured by using microhematocrit method according to method of Kolmer et al. (1959). Blood sample was drawn into microhematocrit tube and the bottom part of the tube was blocked by paraffin. The tube was put in a hematocrit centrifuge (Hettick) and was centrifuged at 15,000 rpm for five minutes. The percentage of blood cell was read using hematocrit measurement tools.

Leukocyte: The effect of acute heat stress on leukocyte concentration in various HSP 70 genotypes was measured by using counting chamber method (Kolmer et al., 1959). Three hundred and eighty microliters of Turk solution was put into a tube glass using micropipette. Twenty microliters of blood sample in EDTA was added to the Turk solution using micropipette. After that, the reaction mixture was rinsed and mixed until it was homogen and the solution was incubated for two minutes. After incubation, the solution
was poured into counting chamber of Improved Neubauer. Concentration of leukocytes was determined in four areas using 10 times magnification objective of lens. Concentration of leukocyte was determined by multiplying the number of counted leukocyte results with 50 (mm$^3$).

**Leukocyte differentiation:** The value of leukocyte differentiation was calculated by using the Rapid methods (Kolmer et al., 1959). Five microliters of blood using a micropipette was dropped at the end of the glass object and the drop was allowed to adhere and spread on the edge of the glass slider. Blood was spreaded in the object glass with 35°C tilt. The preparation was dried and fixated with methanol. The next step was staining by dipping the preparation in eosin for 20-30 sec. After that it was moved and dipped in the second staining for 15-30 sec. Then it was rinsed by using running water and then dried. The preparation was read under a microscope using emersion oil. The number of each leukocyte cell was counted.

**Data analysis:** The effects of chicken lines and acute heat stress on all observed variables were analyzed using analysis of variance and when there was a significant difference, further test was done using least square mean. The effect of heat stress in commercial chicken and the effect of genotype HSP 70 against all commercial chickens kept at 33°C at the age of 21 days were widely studied. Acute heat stress in poultry decreases the number of erythrocyte (Puvadolpirod and Thaxton, 2000; Toghyani et al., 2006) and hemoglobin (Hilman et al., 2000). Concentration of erythrocyte in commercial chickens kept at 33°C at the age of 21 days is $2.31\times10^6$ (Toghyani et al., 2006). Artificial stress using ACTH treatment decreases the level of erythrocyte from $2.01\pm0.08\times10^5$ to $1.92\pm0.05\times10^5$ cell/mm$^3$ (Puvadolpirod and Thaxton, 2000).

The data in Table 1 also provided information that the acute heat stress exposure for only 0.5 hour did not affect the value of hematocrit and leukocytes (p>0.05), but when the heat stress exposure was extended to 1 h it decreased hematocrit and leukocytes values significantly. The data indicated that the 0.5 h of exposure to heat stress was still on the alarm phase. However, when heat stress exposure was extended to 1 and 1.5 h, stress status increased to resistant phase or even to exhaustion phase (dead). The decrease of hematocrit value was caused by the high ambient temperature exposure that lead to the decreased number of erythrocytes (erythropoiesis) which in turn affected the hematocrit value. The decrease of hematocrit value might be due to the damage of erythrocytes, the decrease of erythrocyte production or the decrease of erythrocytes number and size (Coles, 1982; Hilman et al., 2000; Altan et al., 2000) or due to the increase in water consumption during the exposure to heat stress (Tamzil et al., 2013b) that eventually diluted blood cell concentrations, included leukocyte. The decrease number of leukocytes during acute exposure to heat stress was caused by the increased concentrations of glucocorticoid hormones in plasma.
The low value of $r$ indicated that the increased expression of HSP 70 gave a small contribution to the decrease of hemoglobin levels, while the increasing level of the corticosterone hormone was negatively correlated with hemoglobin concentration with $r = -0.9$. It meant that the increase in corticosterone hormone concentration in the blood would be followed by a decrease in hemoglobin concentration.

Acute heat stress increased the expression of HSP 70 and the levels of the corticosterone hormone (Tamzil et al., 2013b) but decreased erythrocytes concentration. HSP 70 expression and corticosterone hormone levels were negatively correlated with hematocrit value ($r = -0.3$ and $-0.8$, respectively). This meant that the increased expression of HSP 70 gave small effect on the decrease of hematocrit values but the increased levels of the corticosterone hormone would be followed by a decrease in hematocrit value. On the other hand, erythrocyte concentration in blood were positively correlated with hematocrit values ($r = 0.8$). This meant that the decrease in erythrocyte concentration would be followed by a decrease in hematocrit value.

Effect of acute heat stress in kampong and Arabic chicken on leukocyte differentiation (heterophil, eosinophil, basophil, lymphocytes and monocytes percentage) and ratio of H/L were presented in Table 2. It could be seen that there was no interaction between lines of chicken and percentage of heterophil, basophil, lymphocytes, monocytes and ratio of H/L (p>0.05). Kampong and Arabic chickens had the same levels of percentage of leukocyte differentiation (p>0.05). Acute heat-stress exposure increased the percentage of heterophil, basophils and the ratio of H/L and decreased the percentage of lymphocytes and monocytes (p<0.01) but did not affect the concentration of eosinophil (p>0.05).

The data in Table 2 showed that kampong and Arabic chickens had relatively the same leukocyte differentiation values, but when it compared to the leukocyte differentiation of commercial chicken, the percentages of heterophil, basophil and the ratio of H/L in commercial chicken were relatively higher and the percentages of
HSP 70 and the percentage of lymphocytes was found. However, a negative correlation between the expression and heterophil percentage and ratio H/L was found, with $r = 0.5$ and $r = 0.6$, respectively. During acute heat stress was assumed to contribute to these phenomena. Positive correlations between HSP 70 expression and heterophil percentage and ratio H/L were found, with $r = 0.5$ and $r = 0.6$, respectively. However, a negative correlation between the expression of HSP 70 and the percentage of lymphocytes was found ($r = 0.5$). It meant that the increase in HSP 70 expression would result in an increase in heterophil percentage and ratio of H/L and a decrease in lymphocytes percentage in the blood. When $r$ values between corticosterone hormone level and leukocyte components and $r$ value between HSP 70 expression and leukocyte components were observed carefully, it appeared that corticosterone hormone levels in the blood had higher contribution to the percentage of leukocyte components as compared to HSP 70 expression. Davis et al. (2008) suggested that stressed poultries higher corticosterone hormone percentage and ratio of H/L. This is the reason why the ratio of H/L can be used as an indicator of stress and higher glucocorticoid level in the blood (Davis et al., 2008). Corticosteroids can inhibit the immune system functions in the body, including the proliferation of lymphocytes, immunoglobulin production, cytokine production, cytotoxicity and anti-inflammatory agents (Munck et al., 1984).

The effect of HSP 70 genotype on the value of erythrocytes, hemoglobin, hematocrit, leukocytes and leukocyte differentiation were presented in Table 3. It can be seen that the percentage of blood erythrocytes in 7 HSP 70 genotypes studied, erythrocyte value was around 2.354-2.540x10^6/mm³. The highest erythrocyte concentration was found in BC genotype, followed by AB, DD, AC, CC, AA and AD genotypes. The data in Table 3 also showed that all HSP 70 genotypes in this study had relatively the same hemoglobin concentrations, even though there was a tendency that the hemoglobin concentration was the highest in chickens with AA genotype and the lowest was in chicken with DD genotype. Table 3 also showed that each chicken HSP 70 genotype had different hematocrit values. The highest hematocrit percentage was found in BC genotype followed by AA, AC, AD, AB, DD genotypes and the lowest was found in CC genotype. The data in Table 3 also indicated that each HSP 70 genotype had different levels of leukocytes. The highest value of leukocytes was found in AD genotype, followed by AC, AB, BC, AA, CC genotypes and the lowest was in DD genotype.

| Table 2: Value of leukocyte differentiation in kampong and Arabic chickens exposed to acute heat stress |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Treatment                                    | Heterophil (%)                               | Eosinophil (%)                               | Basophil (%)                                  | Lymphocytes (%)                              | Monocytes (%)                                 | H/L ratio                                     |
| Chicken lines (CL)                           | 13.33±0.35                                  | 0.17±0.14                                    | 0.52±0.42                                    | 85.99±0.35                                   | 1.00±0.00                                    | 0.16±0.04                                    |
| Arabic chicken                              | 13.58±0.33                                  | 0.25±0.21                                    | 0.42±0.35                                    | 85.67±0.35                                   | 0.08±0.17                                    | 0.16±0.05                                    |
| Acute heat stress (AHS)                      | Control                                      | 9.35±0.49                                    | 0.10±0.14                                    | 0.00±0.00                                    | 90.18±0.52                                   | 0.36±0.20                                    | 0.10±0.07                                    |
|                                            | 0.5 h                                         | 12.24±0.45                                  | 0.25±0.17                                    | 0.38±0.20                                    | 87.21±0.48                                   | 0.00±0.00                                    | 0.14±0.06                                    |
|                                            | 1 h                                           | 14.75±0.46                                  | 0.33±0.33                                    | 0.58±0.12                                    | 84.33±0.50                                   | 0.00±0.00                                    | 0.17±0.06                                    |
|                                            | 1.5 h                                          | 17.48±0.45                                  | 0.15±0.02                                    | 0.92±0.12                                    | 81.58±0.48                                   | 0.00±0.00                                    | 0.21±0.06                                    |
| Effect                                      | Chicken line (CL)                            | **                                          | NS                                           | NS                                           | NS                                           | NS                                           |
|                                            | Acute heat stress (AHS)                       | **                                          | NS                                           | **                                          | NS                                           | NS                                           |
|                                            | CLxAHS                                        | **                                          | NS                                           | NS                                           | NS                                           | NS                                           |

**: Highly significant (p<0.01), NS: Not significant (p>0.05)
The data in Table 3 also showed that the percentage of heterophil in this study was around 11.62-16.25%. The highest percentage was found in chickens with DD genotype followed by CC, AB, BC, AA, AC genotypes and the lowest percentage was found in AD genotype. It meant that HSP 70 genotypes that most susceptible to heat stress was the DD genotype and on the other hand AD genotype was a genotype candidate of HSP 70 chickens that was relatively resistant to the dangers of heat stress. From the data in Table 3 it was clear that the highest percentage of basophils was found in chickens with DD HSP 70 genotype, followed by AA, AC, CC and BC (with the same percentage) AD genotype and the lowest percentage of basophils was found in chickens with AB genotype. It was also clear that HSP 70 genotype containing the highest percentage of lymphocytes was AD genotype and the lowest percentage was found in chickens with DD genotype. These data provided information that HSP 70 genotype that was most vulnerable to the dangers of heat stress was the DD genotype while AD was the one that was the most resistant. Thus AD genotype was a candidate of HSP 70 genotypes that was resistant to heat stress.

Data on HSP 70 chicken genotype and monocyte percentage in Table 3 also showed that HSP 70 chicken genotype affects the percentage of monocytes in the blood. AC genotype had the highest percentage of blood monocytes followed by AA and DD genotypes with the same percentage of monocytes, whereas others genotypes did not have monocytes. Heterophil and lymphocyte ratio in various HSP 70 genotypes showed that H/L ratio were different in different HSP 70 genotypes. The highest value of H/L ratio was found in chicken with DD HSP 70 genotype while the lowest was found in chickens with AD genotype. These data provided information that the DD HSP 70 genotype was the most susceptible to heat stress, while the most heat-resistant genotype was the AD genotype. Thus it can be concluded that AD genotype is a candidate of HSP 70 genotypes chickens that is resistant to heat stress.

**Conclusion:** It is concluded that there was a relationship between chicken lines and HSP 70 genotypes of chicken to the level of heat resistance. Kampong and Arabic chickens have better heat resistance than commercial chickens. AD HSP 70 genotype is a heat-resistant genotype candidate while DD genotype is the opposite, is a heat intolerant genotype.

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