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Adaptation to oxidative stress and impact of chronic oxidative stress on immunity in heat-stressed broilers

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ABSTRACT

1. Glutathione peroxidase activity and serum malondialdehyde of heat-stressed broilers were increased in the early period of heat exposure, and then these parameters decreased.
2. The lesion scores of bursa of Fabricius in heat-stressed broilers were increased and decreased in accordance with the activity of glutathione peroxidase and serum malondialdehyde.
3. High environmental temperature had not affected relative bursa of Fabricius weight and NDV-HI titer of heat-stressed broilers.
4. We concluded that heat-stressed broilers could adapt to oxidative stress, and environmental temperature set at 38±2 °C had not affected humoral immunity.

1. Introduction

Homeostasis is constantly challenged by intrinsic and extrinsic stressors (Lin et al., 2006). Heat stress is of major concern for poultry, especially in the hot regions of the world because of the resulting poor growth performance, immunosuppression and high mortality rate (Mujahid et al., 2005, 2007a). When the temperature exceeds 30 °C, signs of heat stress are likely to appear (Yardibi and Turkay, 2008). Biochemical and physiological events associated with hyperthermia can potentially promote reactive oxygen species formation (Flanagan et al., 1998; Mujahid et al., 2007a). Mujahid et al. (2006) reported that acute heat stress resulted in increased levels of reactive oxygen species in mitochondria. Excessive levels of reactive oxygen species result in the disturbance of balance between the oxidation and antioxidant defense systems, causing lipid peroxidation (LPO), oxidative damages to proteins and DNA (Lin et al., 2006) and biological molecules (Ando et al., 1997).The important line of defense against reactive oxygen species is a system of antioxidant enzymes including glutathione peroxidase, superoxide dismutase and catalase (Milinkovic-Tur et al., 2007). Mahmoud and Edens (2003) found that after broilers were exposed to heat stress glutathione peroxidase activity increased. Lipid bilayers of cell membrane are the common biological target of reactive oxygen species. Malondialdehyde (MDA) is the principal product of polyunsaturated fatty acid peroxidation. Mujahid et al. (2007b) reported that plasma malondialdehyde of heat-stressed chickens significantly increase more than control chickens.

High environmental temperatures affect the development of a specific immune response in chickens. Thaxton et al. (1968) were the first to demonstrate that high environmental temperatures affect the development of specific immunity. When chicks were exposed to environmental temperatures ranging from 32.2 to 43.0 °C for short intermittent periods of constant high environmental temperature or cyclic high environmental temperature, the resulting antibody response to sheep red blood cell was reduced significantly (Bartlett and Smith, 2003). Moreover, Aengwanich (2008) reported that after broilers were under heat stress, bursa of Fabricius shrunk. The cortex area of the bursa increased while the medulla area decreased. Moreover, the number of lymphocytes in both the cortex and medulla of the bursa in broilers under heat stress decreased.

All of the aforementioned documents indicate that when chickens were exposed to high environmental temperatures, they were under heat and oxidative stress. These conditions impaired the immune system. Knowledge about the adaptation to chronic oxidative stress and the impact of chronic oxidative stress from heat stress to the humoral immune system in broilers has not

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been reported. Therefore, the objective of this experiment was to study the adaptation to oxidative stress and the impacts of chronic oxidative stress from prolonged high heat exposure to the humoral immune system in broilers. Results from this study would provide fundamental knowledge for using antioxidants to reduce oxidative stress and improve immunity in poultry production in the tropical regions.

2. Materials and methods

This research was performed under the care and use of experimental animals committee of Mahasarakham University.

2.1. Birds

One hundred broilers, 21 days of age, infectious diseases-free were obtained from a commercial farm near Mahasarakham University and transferred to the laboratory of the Faculty of Veterinary Medicine and Animal Science, Mahasarakham University. The experiment was performed during April–July, 2007. Experiments began after a 7-day adaptation period. The chickens were fed a standard ration *ad libitum* with continuous light and water supplies.

2.2. Experimental design

Factorial in CRD was the design of this experiment. Broilers were separated into two groups; group 1, fifty broilers were maintained in the environmental temperature at $26\pm2\,^{\circ}C$, and group 2, fifty broilers were maintained in the environmental temperature at $38\pm2\,^{\circ}C$. Each group of broilers were divided into 5 subgroups (ten broilers/subgroup) for determining glutathione peroxidase activity, malondialdehyde, NDV-HI titer, relative bursa weight and lesion scores of bursa of Fabricius on days 1, 4, 7, 11 and 21 of the experimental period.

2.3. Blood sampling

Broilers were restrained manually and 3.0 ml of blood sample was collected from the cervical vein using a 3-ml syringe, 23-gauge needle 1.5 in. in length then placed in a microtube with EDTA for determining hematological values. The samples were cooled to approximately $4\,^{\circ}C$ (Ritchie et al., 1994), using icepacks and transferred to the laboratory within 2 h after blood collection.

3. Parameters measurement

3.1. Glutathione peroxidase activity

Glutathione peroxidase activities were assayed by a modification of a procedure previously described by Koller et al. (1984). 50 μl of EDTA blood was diluted with 50 μl of isotonic saline. 400 μl of double distilled water was added and the samples were freeze-thawed at $-70\,^{\circ}C$ (15 min) to hemolyze the red blood cells. After thawing, 0.5 ml of double strength Drabkins reagent (0.0016 M KCN, 0.0012 M $K_3Fe(CN)6$, 0.0238 M $NaHCO_3$) was added, and the blood–reagent mixture was kept on ice until its addition to the reagent-buffer pool. The reagent-buffer pool was freshly prepared and a total of 2.8 ml was used for each sample. Each 2.8 ml contained 2.58 ml of 0.5 M phosphate buffer, (pH 7), 0.10 ml of 0.0084 M NADPH, 0.10 ml of 0.15 M glutathione reduced (GSH) liquid, 0.01 ml of glutathione reductase (GR) (used as supplied) and 0.01 ml of 1.125 M NaN_3. All reagents were kept on ice during the analysis. The assay was performed by adding 0.1 ml of the blood–Drabkins mixture to 2.8 ml of the reagent-buffer pool and allowed to come to room temperature for a minimum of 5 min. The reaction was initiated by adding 0.1 ml of 0.002 M H_2O_2. The change in absorbance was measured at 340 nm for 4 min, and the first 2 min of nonlinear NADPH consumption was disregarded. To determine the nonenzymatic oxidation of GSH, a blank was prepared with the reagent-buffer pool, using 0.1 ml of double distilled water to replace the hemolysate. The glutathione peroxidase activity is reported as mUnits/mg hemoglobin (mU/mg Hgb).

$$\text{Activity (units/mg Hgb)} = \frac{*A \text{ sample} - *A \text{ blank} \times 0.00176.22 \times 10^3}{5 \times 10^{-6}}$$

3.2. Lipid peroxidation

Lipid peroxidation of heat-stressed broilers determined malondialdehyde, a secondary breakdown product of lipid peroxidation. The content of thiobarbituric acid (TBA) reactive MDA products in serum was determined by a modification of a procedure previously described by Gur et al. (2003). Briefly, 0.01 ml of the sample was assayed by the addition of 3 ml of 0.05 mol/L of HCl and 1 ml of 0.67% thiobarbituric acid. Cocktails were heated for 30 min at $100\,^{\circ}C$, cooled with running tap water, and then added with 4 ml of n-butyl alcohol (15:85;V/V), shaking by vortex mixer and centrifuging at 3000 rpm for 10 min. The absorbance at 532 nm was compared with that of 1,1,3,3 tetramethoxypropane standard.

3.3. ND HI titer

Newcastle disease (ND) HI titer was determined by a procedure in the following information: a 2-fold serial dilution of serum was made in a 96-well, V-shaped bottom microlitre plate containing 25 μl of buffer of pH 7.2–7.4, and 25 μl of serum in all wells. Twenty five microlitres of ND virus antigen was added to all the wells except for the last row (the controls). Serum dilutions ranged from 1:2 to 1:2048. The antigen serum mixture was incubated for 10 min at $37\,^{\circ}C$. Fifty microlitres of a 0.5% erythrocyte suspension was then added to each well and the wells were re-incubated for 30 min. A positive serum, a negative serum, erythrocytes and antigens were also included as controls. The highest dilution of serum causing complete inhibition of erythrocyte agglutination was considered as the end point. The geometric mean titer was expressed as the reciprocal log2 values of the highest dilution that displayed anti-ND-HI.

3.4. Relative bursa weight and lesion scores of bursa of Farbricius

On days 1, 4, 7, 11 and 21 of experimental period, the body weight of broilers maintained in 2 conditions were determined and they were killed. Bursa of each broiler was collected and weighed. The relative bursa weight of broilers that were maintained in 2 controlled room temperatures on days 1, 4, 7, 11 and 21 was calculated. Lesion scores of bursa of Fabricius were performed. Briefly, bursa was fixed in 10% buffered formalin, then sectioned, and stained with hematoxylin and eosin (H&E) for microscopic examination (Luna, 1968). The lesion scores were judged in the following information: *Score* 0 (100%): normal finding; *Score* 1 (80%): some follicles in the bursa shrunk. Space between the follicle septum was found; *Score* 2 (60%): each lobule in the bursa shrunk more than *Score* 1, and each follicle was separated from the others. The space between follicles within the bursa was larger than the *Score* 1; *Score* 3 (40%): each follicle in the bursa shrunk more than *Score* 2. Each follicle in the bursa was

completely separated from the others and the space between follicles within the bursa was larger than the *Score* 2 (Aengwanich, 2008).

3.5. Statistic analysis

Data were analyzed by using the ANOVA procedure of Statistical Analysis System. Means were separated by Duncan's multiple range tests (Duncan, 1955). The level of significance was determined at $P< 0.05$.

4. Results

After broilers were maintained in two conditions: condition 1, broilers were maintained in a controlled room temperature at 26 ± 2 °C. Condition 2, broilers were maintained in a controlled room temperature at 38 ± 2 °C. On days 1, 4, 7, 11 and 21 of the experimental period, glutathione peroxidase activity, malondialdehyde in serum, relative bursal weight, lesion scores of bursa of Fabricius and NDV-HI titer were investigated. On days 1, 4, 7 and 11 of the experimental period, glutathione peroxidase activity of broilers maintained at 38 ± 2 °C was significantly higher than that of broilers at 26 ± 2 °C ($P<0.05$) (Fig. 1). Glutathione peroxidase activity of broilers were significantly decreased on day 4 ($P<0.05$) and during day 4 through day 21 of the experimental period, glutathione peroxidase activity was not significantly different (Table 1). On days 1, 4, 7 and 11 of the experimental period, the malondialdehyde of broilers maintained at 38 ± 2 °C was significantly higher than that of broilers maintained at 26 ± 2 °C ($P<0.05$) (Fig. 2). The malondialdehyde of broilers were significantly decreased on day 7 ($P<0.05$), and on days 7 and 11, MDA of broilers were significantly higher than on day 21 of the experimental period ($P<0.05$) (Table 1). Relative bursa weight of broilers on days 1, 4, 7, 14 and 21 of the experimental period, and both conditions, was not significantly different ($P>0.05$) (Table 1). On days 4, 7, 11 and 21 of the experimental period, lesion scores of bursa of Fabricius in broilers maintained at 38 ± 2 °C were significantly higher than that of broilers maintained at 26 ± 2 °C ($P<0.05$) (Fig. 3). Lesion scores of bursa of Fabricius in broilers were significantly increased on day 4 ($P<0.05$), stable during days 7 and 11, and then were significantly decreased on day 21 ($P<0.05$) of the experimental period (Table 1). On days 7 and 11 of the experimental period, NDV-HI titer of broilers were significantly lower than on day 21 of ($P<0.05$) of the experimental period (Table 1).

5. Discussion

During days 1–11 of the experimental peroid, glutathione peroxidase activity of the broilers maintained in a high environmental temperature increased higher than that of broilers maintained at thermoneutral. The glutathione peroxidase activity was highest on day 1 of heat exposure, and then decreased on days 4, 7, 11 and 21 of the experimental period. This phenomenon was similar to the report of Mahmoud and Edens (2003), Milinkovic-Tur et al. (2007) and Yardibi and Turkay (2008), they found that when broilers were exposed to high environmental temperature, their glutathione peroxidase activities increased. After broilers were exposed to high environmental temperature, the malondialdehyde level in the serum of broilers increased to the highest level on day 1, but was not different with day 4, and then later decreased to days 7 and 11, and the malondialdehyde level was lowest on day 21, respectively. Therefore, the malondialdehyde level of heat-stressed broilers was in accordance with the pattern of glutathione peroxidase activity. Several reports showed data about the adaptation of animals to stressors such as Moberg and Mench (2000) found that when animals were subjected to repeated stress in the first few days after exposure, they usually show an increased response and then later the response decreased. Moreover, Aengwanich (2007) found that when chickens were exposed to high heat the heterophil/lymphocyte ratio (stress parameter of chicken) was increased on day 7, and later decreased on days 14 and 21 of the experimental period. Therefore, we concluded that heat-stressed broilers could adapt to high environment temperatures and oxidative stress.

Reactive oxygen species have been demonstrated to be mediators of cellular injury (Flanagan et al., 1998). Several studies have suggested that exposure to heat results in oxidative stress, which in turn can lead to cytotoxicity (Mujahid et al., 2005). Moreover, Mujahid et al. (2005) reported that heat stress increase oxygen radical, possibly by the disruption of the electron transport assemblies of the membrane. The high level of reactive oxygen species causes cellular damage (Beckman and Ames, 1998). Bursa of Fabricius is an important ovallike shaped gland which locates at the proctodaeal region of the cloaca in birds (Whittow, 2000). This organ is known to be a primary lymphoid organ in birds where immunologically competent cells are produced, while it is also a secondary or peripheral lymphoid organ which produces antibodies (Tsuji and Miyoshi, 2001). In this study, the lesion scores of bursa of Fabricius in heat-stressed broilers were increased on day 4, the highest increased on days 7 and 14, and then decreased to day 21 of the experimental period,

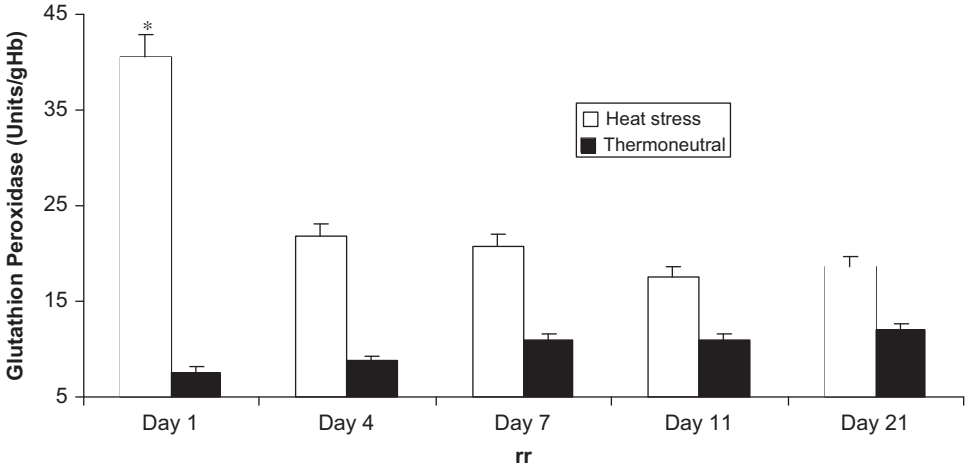

Fig. 1. Glutathione peroxidase activity of boilers were maintained at 26 ± 2 °C and 38 ± 2 °C on days 1, 4, 7, 11 and 21 of the experimental period.

Table 1
Effect of high environmental temperature on glutathione peroxidase activity, malondialdehyde level, relative bursa weight, lesion scores of bursa weight and HI titer of Newcastle disease virus in broilers under chronic heat stress.

Parameters	Days of experimental period					SEM	Temperature		SEM	*p*-value		
	Day 1	Day 4	Day 7	Day 11	Day 21		H	TN		T	P	T X P
Glutathione peroxidase (units/gHb)	24.050[a]	15.310[b]	16.130[b]	14.210[b]	15.300[b]	0.800	23.830[A]	10.170[B]	2.020	**	**	**
Malondialdehyde (μM)	237.630[a]	232.080[a]	155.530[b]	124.310[b]	85.450[c]	11.310	249.340[A]	84.660[B]	28.270	**	**	**
Relative bursa weight (mg/BW)	0.001	0.001	0.001	0.001	0.050	0.020	0.020	0.001	0.050	NS	NS	NS
Lesion scores of bursa	0.100[c]	0.800[b]	1.300[a]	1.350[a]	1.000[b]	0.070	1.720[A]	0.100[B]	0.180	**	**	**
HI titer	2.830[a,b]	2.860[a,b]	3.470[a]	3.120[a]	1.880[b]	0.320	3.090	2.580	0.790	NS	**	NS

H = Heat stress temperature, TN = Thermoneutral, P = Experimental period, T = Environmental temperature, HI titer = Hemagglutination inhibition titer of Newcastle disease virus (NDV-virus).

[a–c] Within row, mean with no common superscript differ significantly ($p<0.05$).
[A–B] Within row, mean with no common superscript differ significantly ($p<0.01$).
** Significantly different at ($p<0.01$).

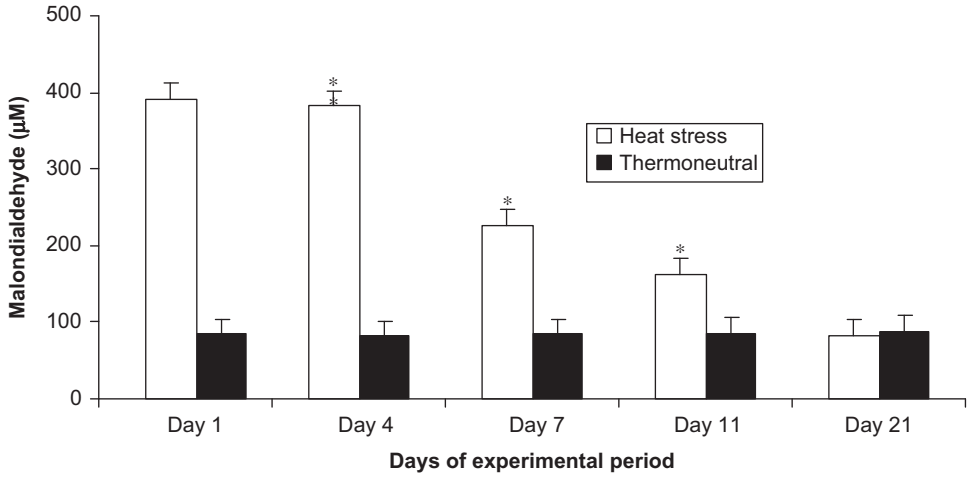

Fig. 2. Malondialdehyde levels of boilers were maintained at 26±2 °C and 38±2 °C on days 1, 4, 7, 11 and 21 of the experimental period.

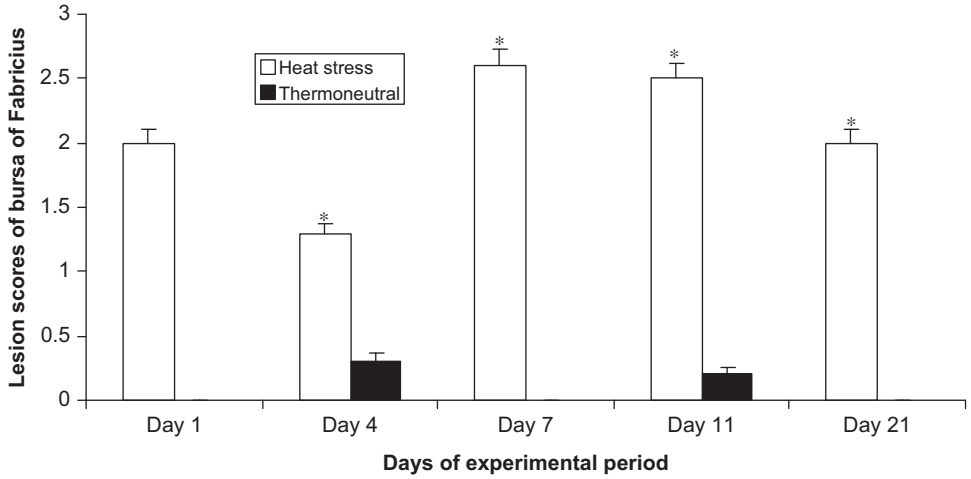

Fig. 3. Pathological lesion scores of boilers were maintained at 26±2 °C and 38±2 °C on days 1, 4, 7, 11 and 21 of the experimental period.

respectively. This result was in accordance with the report of Anwar et al. (2004) and Aengwanich (2008), they found that bursa of Fabricius of heat-stressed broilers were atrophied when examined under histopathological observation after broilers had prolonged exposure to high environmental temperature. The lesion scores pattern of bursa of Fabricius was in accordance with the pattern of glutathione peroxidase activity and malondialdehyde levels. This phenomenon indicated that the abnormality of bursa of Fabricius might occur from the oxidative damage occurring during the period they were under heat stress.

Anwar et al. (2004) reported when broilers were under heat stress, the relative weight of major immunological organs such as spleen, thymus and bursa of Fabricius of them decreased. This was in accordance with the report of Bartlett and Smith (2003), they found that relative bursa weight of heat-stressed broiler decreased. Whereas, in this study, the relative bursal weight and

NDV-HI titer of broilers throughout the experimental period were not different between heat stress temperature and thermoneutral. This result was different from the report of Bartlett and Smith (2003), they found that when chicks were exposed to environmental temperature ranging from 32.2 to 43.0 °C for short intermittent period of constant high environmental temperature or cyclic high environmental temperature, the resulting antibody response to sheep red blood cell was reduced significantly. Besides, Mashaly et al. (2004) reported that high environmental temperature inhibits antibody production in laying hen. This phenomenon indicated that even if the environmental temperature set at 38 ± 2 °C induced broilers to heat stress, oxidative stress, and caused damage of bursa tissue, but did not affect the humoral immunity of this bird.

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References

Aengwanich, W., 2007. Comparative ability to tolerate heat between Thai indigenous, Thai indigenous crossbred and broilers chickens by using heterophil/lymphocyte ratio. Pak. J. Biol. Sci. 10, 1840–1844.

Aengwanich, W., 2008. Pathological changes and effect of ascorbic acid on lesion scores of bursa of fabricius in broilers under chronic heat stress. Res. J. Vet. Sci. 1, 62–66.

Ando, M., Katagiri, K., Yamamoto, S., Wakamatsu, K., Kawahara, I., Asanuma, S., Usuda, M., Sasaki, K., 1997. Age-related effects of heat stress on productive enzymes for peroxides and microsomal monooxygenase in rat liver. Environ. Health Perspect. 105, 726–733.

Anwar, B., Khan, S.A., Aslam, A., Maqbool, A., Khan, K.A., 2004. Effects of ascorbic acid and acetylsalicylic acid supplementation on the performance of broiler chicks exposed to heat stress. Pak. Vet. J. 24, 109–111.

Beckman, K.B., Ames, B.N., 1998. The free radical theory of ageing matures. Physiol. Rev. 78, 547–581.

Bartlett, J.R., Smith, M.O., 2003. Effects of different levels of zinc on the performance and immunocompetence of broilers under heat stress. Poult. Sci. 82, 1580–1588.

Duncan, D.B., 1955. Multiple ranges and multiple *F*-test. Biometrics 11, 1–42.

Flanagan, S.W., Moseley, P.L., Buettner, G.R., 1998. Increased flux of free radicals in cells subjected to hyperthermia: detection by electron paramagnetic resonances spin trapping. FEBS Lett. 431, 285–286.

Gur, E.S., Erdinc, A., Serdar, Z., Gur, H., 2003. Influence of acute exercise on oxidative stress in chronic smokers. J. Sports Sci. Med. 2, 98–105.

Koller, L.D., South, P.J., Exon, J.H., Whitbeck, G.A., Maas, J., 1984. Comparison of selenium levels and glutathione peroxidase activity in bovine whole blood. Can. J. Comp. Med. 48, 431–433.

Lin, H., Decuypere, E., Buyse, J., 2006. Acute heat stress induces oxidative stress in broiler chickens. Comp. Biochem. Physiol. Part A 144, 11–17.

Luna, L.T., 1968. Manual of Histologic Staining Methods of the Armed Force Institute of Pathology. McGraw-Hill Book Company, New York.

Mahmoud, K.Z., Edens, F.W., 2003. Influence of selenium source on age-related and mild heat stress-related changes of blood and liver glutathione redox cycle in broiler chickens (*Gallus domesticus*). Comp. Biochem. Physiol. Part B 136, 921–934.

Mashaly, M.M., Hendricks, G.L., Kalama, M.A., Gehad, A.E., Abbas, A.O., Patterson, P.H., 2004. Effect of heat stress on production parameters and immune responses of commercial laying hens. Poult. Sci. 83, 889–894.

Milinkovic-Tur, S., Stojevic, Z., Pirsljin, J., Zdelar-Tuk, M., Poljicak-Milas, N., Ljubic, B.B., Gradinski-Vrbanac, B., 2007. Effect of fasting and refeeding on the antioxidant system in cockerels and pullets. Acta Vet. Hung. 55, 181–189.

Moberg, G.P., Mench, J.A., 2000. The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. CAB International, New York.

Mujahid, A., Yoshiki, Y., Akiba, Y., Toyomizu, M., 2005. Superoxide radical production in chicken skeletal muscle induced by acute heat stress. Poult. Sci. 84, 307–314.

Mujahid, A., Sato, K., Akiba, Y., Toyomizu, M., 2006. Acute heat stress stimulates mitochondrial superoxide production in broiler skeletal muscle, possibly via downregulation of uncoupling protein content. Poult. Sci. 85, 1259–1265.

Mujahid, A., Pumford, N.R., Bottje, W., Nakagawa, K., Miyazawa, T., Akiba, Y., Toyomizu, M., 2007a. Mitochondrial oxidative damage in chicken skeletal muscle induced by acute heat stress. J. Poult. Sci. 44, 439–445.

Mujahid, A., Akiba, Y., Toyomizu, M., 2007b. Acute heat stress induces oxidative stress and decreases adaptation in young white leghorn cockerels by down-regulation. Poult. Sci. 86, 364–371.

Ritchie, B.W., Harrison, G.J., Harrison, R.L., 1994. Avian Medicine: Principle and Application. Wingers Publishing, Inc., Florida.

Thaxton, P., Sadly, C.R., Glick, B., 1968. Immune response of chicken following heat exposure or injection with ACTH. Poult. Sci. 47, 264–266.

Tsuji, T., Miyoshi, M., 2001. A scaning and transmission electron microscopic study of the lymphoreticular framework in the chicken Fabricius' bursa. Med. Bull. Fukuoka Univ. 28, 63–75.

Whittow, G.C., 2000. Sturkie's Avian Physiology. Academic Press, Florida.

Yardibi, H., Turkay, G., 2008. The effects of vitamin E on the antioxidant system, egg production and egg quality in heat stressed laying hen. Turk. J. Vet. Anim. Sci. 32, 319–325.