

Effects of Early Age Feed Restriction and Dietary Ascorbic Acid on Heterophil/Lymphocyte and Tonic Immobility Reactions of Transported Broiler Chickens

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ABSTRACT : Broiler chickens were subjected to (i) pre-treatment for 24 h with 1,200 ppm L-ascorbic acid in their drinking water on day 42 (AA), (ii) 60% feed restriction on days 4, 5 and 6 (FR), (iii) combination of AA and FR (AAFR), or (iv) neither treatment (control). On day 43, the birds were crated and transported in an open truck for either 60 min or 120 min. Heterophil/lymphocyte ratios (HLR) were determined prior to (T_0) and 20 h following the 60 min (T_{60}) and 120 min (T_{120}) journeys. Prior to transportation, all treatment groups had similar HLR. Following T_{60} , the AAFR chicks had smaller increase in HLR than their control, AA and FR counterparts. The AA, FR and AAFR birds exhibited lower HLR than controls following T_{120} . The means HLR of AA, FR and AAFR did not differ significantly. Irrespective of journey time, AA and AAFR birds had shorter tonic immobility duration than their FR and control counterparts. The tonic immobility duration of all birds increased with journey duration. (*Asian-Aust. J. Anim. Sci.* 2003, Vol 16, No. 10 : 1545-1549)

Key Words : Feed Restriction, Ascorbic Acid, Heterophil/Lymphocyte Ratios, Tonic Immobility Duration, Transportation, Broiler Chickens

INTRODUCTION

Pre-slaughter handling, crating and transport of broiler chickens have been associated with exposure to numerous potential stressors including, handling by humans, feed withdrawal, noise, vibration, thermal extremes, social disruption, crowding, and restriction of movement (Nicol and Scott, 1990). There is a wealth of literature indicating that pre-slaughter process can result in physiological and behavioural changes indicative of acute stress (Freeman et al., 1984; Zulkifli et al., 2000a; 2001) and extreme fearfulness (Cashman et al., 1989; Zulkifli et al., 2000a; 2001). Work in our laboratory showed that dietary supplementation of ascorbic acid (AA) (Zulkifli et al., 2000a; 2001), and regular visual contact with humans (Zulkifli et al., 2002a) reduced duration of tonic immobility (TI) and heterophil/lymphocyte ratio (HLR) in broiler chickens subjected to the traumatic experience of pre-slaughter process.

There is the question of whether the birds' ability to cope with stress attributed to pre-slaughter handling, crating and transport can be altered. Exposure to a stressor during the neonatal stage has the potential to modify many facets of a bird's physiology and behaviour later in life. Previous studies showed that stressful experiences during the neonatal stage may perturb homeostasis, but there can be long-term benefits in improving ability to cope with stress later in life (Gross and Siegel, 1993; Zulkifli and Siegel,

1995). For example, exposure of 5-day-old broiler chicks to elevated temperature improved survivability in otherwise lethal heat treatment at the age of 42 day (Arjona et al., 1988; Yahav and Hurwitz, 1996). An animal does not always have to be preconditioned to the same stressor for habituation to take place. Work in our laboratory indicated that early age feed restriction enhanced the ability of chickens to cope with high ambient temperatures (Zulkifli et al., 1994a, b; 2000b) and subsequent feed deprivation (Zulkifli et al., 1995) as juveniles compared to those fed *ad libitum* throughout the experiment. Therefore, it is reasonable to hypothesise that subjecting chicks to early age feed restriction can dampen physiological stress responses to pre-slaughter process. The purpose of the present study was to evaluate whether early age feed restriction and its combination with dietary ascorbic acid supplementation has any influence on HLR and duration of TI when broiler chickens are subjected to handling, crating and road transporting. In this study, HLR was used as an index of stress. The reliability of HLR as a biological index of stress in avian species is well documented (Maxwell, 1993). Furthermore, a strong relationship between ascorbic acid treatment and HLR response has been documented (Gross, 1988a). There are numerous reports that TI is a robust measure of bird's fearfulness (Jones, 1986). The substantial evidence for the positive relationship between antecedent fear state and the duration of TI has been extensively reviewed (Jones, 1986).

MATERIALS AND METHODS

A total of 400 day-old male broiler chicks (Avian) were

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Table 1. Mean (\pm SEM) heterophil/lymphocyte ratios when the treatment x journey duration interaction was significant

Treatment	Journey duration		
	0 min	60 min	120 min
AA	0.39 \pm 0.03 ^z	0.52 \pm 0.03 ^{by}	0.60 \pm 0.03 ^{bx}
FR	0.36 \pm 0.02 ^z	0.50 \pm 0.07 ^{by}	0.60 \pm 0.03 ^{bx}
AAFR	0.33 \pm 0.02 ^z	0.43 \pm 0.02 ^{cy}	0.59 \pm 0.04 ^{bx}
Control	0.37 \pm 0.04 ^y	0.65 \pm 0.07 ^{ax}	0.71 \pm 0.01 ^{ax}

^{a-c} Means within a column-subgroup with differing letters differ significantly ($p \leq 0.05$). ^{x-z} Means within a row-subgroup with differing letters differ significantly ($p \leq 0.05$). AA=pre-treatment for 24 h with 1,200 ppm L-ascorbic acid in their drinking water on day 42.

FR=60% feed restriction on days 4, 5 and 6. AAFR=combination of AA and FR. Control=neither treatment.

purchased from a local hatchery. Forty chicks were housed in each of 12 floor pens with deep litter of wood shavings in a conventional open-sided house. All chicks were wing banded, vaccinated against Newcastle disease and fed on standard broiler starter and finisher. Feed and water were available *ad libitum* and the chicks were supplied with 24 h of light daily. The equal number of chicks was subjected to the following 4 treatments: (i) 60% feed restriction on d 4, 5 and 6 (FR), (ii) pre-treatment for 24 h with 1,200 ppm L-ascorbic acid (F. Hoffman-La Roche, Kuala Lumpur, Malaysia) in the drinking water on d 42 (AA), (iii) 60% feed restriction on d 4, 5 and 6, and pre-treatment for 24 h with 1,200 ppm L-ascorbic acid in the drinking water on d 42 (AAFR), and (iv) *ad libitum* feeding and untreated drinking water (control). The feed restriction was 60% of the previous day's intake of the control group. The dosage of ascorbic acid used in the present study was based on documented findings that inclusion of 1,200 ppm ascorbic acid in the drinking water for 24 h is effective in reducing stress and fear responses of handled and transported broiler chickens (Zulkifli et al., 2000c; 2001). Plastic bottle drinkers with a capacity of 4 L were used (10 birds per drinker). To maintain the stability of crystalline acid ascorbic in solution, the drinkers of AA and AAFR birds were replenished every 4 h with fresh AA solution. In order to avoid the confounding effect of periodic disturbance to the birds, the drinkers of the FR and control chicks were also replenished every 4 h. At 43 d of age (08:00 h), all the birds were individually removed from cages, spray painted on the back (to facilitate identification of different treatment groups), carried by the legs in an inverted manner and placed in plastic crates (0.80 \times 0.60 \times 0.31 m) at 10 birds to each crate. The birds were randomly crated, irrespective of treatment group and home cage. The crates were loaded in an open truck and equal number of birds were transported for either 60 min or 120 min with an average speed of 70 km/h. The journey covered highways, roads with heavy traffic and traffic lights. At the time of transporting, the ambient temperature and relative humidity was about 32°C and 80%, respectively. Prior to transportation (T_0), and

following the 60 min (T_{60}) and 120 min (T_{120}) journeys, the crates of birds were unloaded, and 20 birds from each treatment group were randomly chosen and tested individually for duration of TI in a separate room (no visual contact with other birds). Birds were carried by both legs in an inverted manner to the room. A modification of procedure described by Benoff and Siegel (1976) was used. The TI was induced as soon as the birds were carried to the separate room by gently restraining them on their right side by the legs and wings for 15 s. The experimenter then retreated approximately 1 m and remained within the sight of the bird but made no unnecessary noise or movement. Direct eye contact between the observer and the bird was avoided as it may prolong TI duration (Jones, 1986). A stopwatch was started to record latencies until the bird righted itself. If the bird righted in less than 10 s, it was captured again and restraining procedure was repeated. If the TI was not induced after three attempts the duration of TI was considered as 0 s. The maximum duration of TI allowed was 600 s (Zulkifli et al., 2000c; 2001). The number of inductions required to attain TI was recorded. Approximately five birds could be tested simultaneously.

Both birds that were used and not used for TI measurement were returned to their home cages. Prior to and 20 h following transportation, blood samples (0.3 mL) were obtained from 10 birds (those that were not used for TI measurement) per treatment-journey subgroup for heterophil and lymphocyte counts. Blood samples were collected in tubes containing EDTA as anticoagulant. Blood smears were prepared using May-Grunwald-Giemsa stain and heterophil and lymphocyte counted to a total of 60 cells (Gross and Siegel, 1983). Gross (1990) indicated that HLR response to a short-duration stress peaked after 20 h.

Data obtained were subjected to two-way ANOVA using SAS software (1982) with treatment, journey duration and their interaction as the main effects. When interactions between main effects were significant, comparisons were made within each experimental variable. Differences between means were determined using Duncan's multiple range test. Statistical significance was declared at $p \leq 0.05$.

RESULTS

Analyses of variance to examine the effects of treatment and journey duration on HLR data showed a significant interaction between the main effects (Table 1). At T_0 , HLR was not significantly affected by the treatment group. Following T_{60} , the AAFR birds had lower HLR than their FR, AA and control counterparts. The control chicks exhibited higher HLR response than those of FR and AA. However, the mean HLR of FR, AA and AAFR birds was not significantly different after being transported for T_{120} . Similarly, the HLR of the control chicks was higher than the

Table 2. Mean (\pm SEM) duration of tonic immobility and number of inductions needed to induce tonic immobility by treatment and journey duration

Treatment	Duration of tonic immobility (s)	Inductions (no)
AA	155 \pm 14.7 ^b	2.00 \pm 0.15
FR	207 \pm 17.7 ^a	1.53 \pm 0.13
AAFR	155 \pm 14.1 ^b	1.53 \pm 0.11
Control	203 \pm 19.9 ^a	1.37 \pm 0.10
Journey duration		
0 min	122 \pm 9.09 ^c	1.65 \pm 0.12
60 min	185 \pm 13.03 ^b	1.68 \pm 0.12
120 min	232 \pm 16.36 ^a	1.50 \pm 0.10

^{a-c} Means within a column-subgroup with differing letters differ significantly ($p < 0.05$). AA=pre-treatment for 24 h with 1,200 ppm L-ascorbic acid in their drinking water on day 42. FR=60% feed restriction on days 4, 5 and 6. AAFR=combination of AA and FR. Control=neither treatment.

other three groups. Irrespective of treatment, HLR increased with journey duration.

There was no significant treatment \times journey duration interaction for TI duration. Irrespective of journey duration, subjecting chicks to AA and AAFR significantly reduced TI duration as compared to those of FR and control groups (Table 2). The means TI durations of AA and AAFR did not differ significantly. The TI durations of all the birds were positively related to journey duration. The longest TI duration was obtained following the 120 min journey. Neither treatment nor journey duration had a significant effect on number of inductions to induce TI.

DISCUSSION

As expected, irrespective of treatment group, there was an elevation in HLR following the 60 min and 120 min journeys. Transportation of poultry is known to elevate HLR (Zulkifli et al., 2000a; 2001) and plasma corticosterone concentration (Freeman et al., 1984) indicative of stress response. The importance of journey duration in eliciting stress reaction was noted in the present study. A higher HLR was obtained after longer journeys. Similarly, Freeman et al. (1984) noted an increase in plasma corticosterone with journey time. This is expected because longer journey would result in a higher risk of exposure to noxious and stressful stimuli.

There is considerable debate over which aspect of pre-slaughter process is the most traumatic for the chickens. Swarbrick (1986) reported that loading and unloading were more stressful than the journey itself. On the other hand, broilers that were crated and transported for 40 min had higher plasma levels of corticosterone than those that were subjected to simulated catching or handling procedures (Duncan, 1989). Similarly, Zulkifli et al. (2001) reported that birds transported for 40 min were more "stressed" than

those that were crated and left stationary in the laboratory, as measured by HLR. The positive relationship noted here between journey duration and HLR suggests that transportation per se can be stressful to broiler chickens. Although during transit birds may be exposed to an array of potential stressors, high ambient temperature is a major factor in the elicitation of physiological stress responses (Mitchell and Kettlewell, 1998). The high relative humidity in the tropics may exacerbate the heat stress problem in broilers during transportation.

The present findings are consistent with those of Saterlee et al. (1989), and Zulkifli et al. (2000a; 2001) that AA supplementation is beneficial in ameliorating physiological stress response in birds subjected to the traumatic events of catching, crating and transportation. Irrespective of journey duration, AA birds had lower HLR than their control counterparts. Previous studies (Zulkifli et al., 1994a,b; 1995; 2000b) indicated that early age feed restriction can alter the ability of birds to cope with high ambient temperatures and feed deprivation at market age. The present findings suggest that the FR birds had smaller increases in HLR following transportation as compared to controls. Although FR may alleviate the detrimental effects of heat stress (Zulkifli et al., 1994a,b; 2000b) and starvation (Zulkifli et al., 1995) during transit, transported birds are also exposed to other potential stressors including acceleration, vibration, motion, impacts, social disruption and noise. It is not clear whether the reduced HLR in the transported FR birds was attributed to lower stress response to high temperature and feed deprivation or to other stressors during transit as well. However, because both FR and AA birds had similar HLR following transportation, and dietary ascorbic acid supplementation can dampen stress response to handling and crating (Saterlee et al., 1989; 1994; Zulkifli et al., 2000a; 2001), there is a possibility that the stress-ameliorating effect of FR is not confined to high temperature and starvation only.

The stress-ameliorating effect of FR could be associated with an increase in the expression of heat shock protein 70 which may have considerable impact on tolerance to both thermal and non-thermal stresses (Zulkifli et al., 2002b). Sapolsky (1992) postulated that stresses early in life may increase the concentration of hippocampal corticosteroid receptors which enhance negative feedback sensitivity and decrease numbers of glucocorticoid-binding globulin like receptors in the pituitary.

As measured by HLR, it appears that both AA and FR birds were less "stressed" following the road transportation. Thus, it can be concluded that both AA and FR procedures are equally effective in dampening stress responses. However, FR has its advantages over AA because the former is more cost effective and practical. There is evidence that AAFR can further improve the ability of birds

to cope with stress during transit as compared to AA and FR alone. However, this superiority was only noted following the 60-min journey and not thereafter.

As expected TI was significantly prolonged by transportation. Cashman et al. (1989) indicated that harvesting, loading and unloading may have contributed to the overall fear following transit, because exposure to human beings, social disruption and crating are all capable of inducing fear in the domestic fowl. The present findings and those of Cashman et al. (1989) suggest that journey time and subsequent TI reaction are strongly and positively related. Thus, it appears that stimuli during transit per se and not just loading and unloading procedures, are vital in augmenting underlying fearfulness.

The shorter TI duration in AA birds observed in the current study is in agreement with previous reports (Saterlee et al., 1993; 1994; Jones et al., 1996; Zulkifli et al., 2000a; 2001). Saterlee et al. (1993) indicated that supplementation of ascorbic acid attenuated fear reactions in Japanese quail that were subjected either to cooping procedure (stressed by capture, inescapable exposure to novelty, a reduction of floor space and ambient temperature, deprivation of food and water and limited transportation) or remained undisturbed.

Although FR reduced HLR response to transportation, as measured by TI duration, it appears that FR failed to attenuate fear reactions before and after transportation. The TI durations of FR birds were not significantly different from controls but longer than those of AA. Thus, it appears that the association between HLR and TI duration is negative. This fact is consistent with that reported by Campo and Redondo (1996), who reported that hens laying pink eggs were less fearful and more stressful than control hens, as indicated by the TI reaction and the HLR, respectively. Similarly, Zulkifli et al. (2000a) reported that although rough inverted handling prolonged TI duration as compared to gentle upright handling in broilers, both procedures resulted similar HLR response. On the contrary, Beuving et al. (1989) found that White Leghorns showing long TI had higher HLR than those showing short TI. Mauldin et al. (1979) showed that birds from the less fearful line had a significantly larger number of lymphocytes, although they were smaller in diameter. In discussing the relationship between fear and stress responses, Campo and Redondo (1996) indicated that although both fear and stress reactions are not synonymous, they are closely linked and the interpretation of the above findings is equivocal.

Unlike physiological stress reaction, the AAFR combination did not further attenuate underlying fearfulness in broiler chickens. Because FR had no significant effect on TI reaction, it is expected that the TI durations of AAFR and AA birds were similar.

In conclusion, these results suggest that AA and AAFR

alleviated both fear and physiological stress reactions to transportation in broiler chickens and thus have important implications for the welfare of poultry. Although the present findings suggest that birds can be treated with ascorbic acid 24 h prior to anticipated traumatic event, it is not always possible to predict frightening events. Hence, continuous treatment with ascorbic acid may be necessary. However, optimum dosage and cost analyses are some of the aspects that require further investigation before any recommendations can be made. Although the FR treatment had negligible effect on underlying fearfulness, it offers a feasible method for dampening physiological stress response to transportation in broiler chickens.

REFERENCES

- Arjona, A. A., D. M. Denbow and W. D. Weaver. 1988. Effects of heat stress early in life on mortality of broilers exposed to high environmental temperatures prior to marketing. *Poult. Sci.* 67:226-231.
- Benoff, F. H. and P. B. Siegel. 1976. Genetic analyses of tonic immobility in young Japanese quail (*Coturnix coturnix japonica*). *Anim. Learn. Behav.* 4:160-162.
- Beuving, G., R. B. Jones and H. J. Blokhuis. 1989. Adrenocortical and heterophil/lymphocyte responses to challenge in hens showing short or long tonic immobility reactions. *Br. Poult. Sci.* 30:175-184.
- Campo, J. L. and A. Redondo. 1996. Tonic immobility reaction and heterophil to lymphocyte ratio in hens from three Spanish breeds laying pink eggshells. *Poult. Sci.* 72:2193-2199.
- Cashman, P. J., C. J. Nicol and R. B. Jones. 1989. Effects of transportation on the tonic immobility fear reactions of broilers. *Br. Poult. Sci.* 30:211-221.
- Freeman, B. M., P. J. Kettlewell, A. C. C. Manning and P. S. Berry. 1984. Stress of transportation in broilers. *Vet. Rec.* 114:286-287.
- Gross, W. B. and H. S. Siegel. 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. *Avi. Dis.* 27:972-979.
- Gross, W. B. and P. B. Siegel. 1993. General principles of stress and welfare. In: (Ed. T. Grandin), *Livestock, Handling and Transport*, CAB International, Wallingford, UK, pp. 21-34.
- Gross, W. B. 1990. Effect of exposure to a short-duration sound and the stress response of chickens. *Avi. Dis.* 34:759-761.
- Jones, R. B. 1986. The tonic immobility reaction of the domestic fowl: a review. *Wild's. Poult. Sci. J.* 42(1):82-96.
- Jones, R. B., D. G. Saterlee, J. Moreau and D. Waddington. 1996. Vitamin C supplementation and fear-reduction in Japanese quail: short term cumulative effects. *Br. Poult. Sci.* 37:33-42.
- Mauldin, J. M., P. B. Siegel and W. B. Gross. 1979. Interfacing genetics, behavior and husbandry in White Leghorns presented with *E. coli* challenge. *Appl. Anim. Ethol.* 5:347-359.
- Mitchell, M. A. and P. J. Kettlewell. 1998. Physiological stress and welfare of broiler chickens in transit: solutions not problems! *Poult. Sci.* 1803-1814.
- Nicol, C. J. and G. B. Scott. 1990. Pre-slaughter handling and transport of broiler chickens. *Appl. Anim. Behav. Sci.* 28:57-

73.

- Sapolsky, R. M. 1992. *Stress, the Aging Brain and the Mechanism of Neuron Death*. MIT Press, Cambridge, MA.
- SAS Institute, 1982. *SAS® User's Guide: Statistics*. (Ed. A. Ray). SAS Institute Inc., Cary, NC.
- Saterlee, D. G., I. Aguilera-Quintana, B. J. Munn and B. A. Krautman. 1989. Vitamin C amelioration of the adrenal stress response in broiler chickens being prepared for slaughter. *Comp. Biochem. Physiol.* 94A:569-574.
- Saterlee, D. G., R. B. Jones and F. H. Ryder. 1993. Effects of vitamin C supplementation on the adrenocortical and tonic immobility fear-reactions of Japanese quail genetically selected for high corticosterone response to stress. *Appl. Anim. Behav. Sci.* 35:347-357.
- Saterlee, D. G., R. B. Jones and F. H. Ryder. 1994. Effects of ascorbyl-2-polyphosphate on adrenocortical activation and fear-related behaviour in chickens. *Poult. Sci.* 73:194-201.
- Swarbrick, O. 1986. The welfare during transport of broilers, old hens and replacement pullets. In: *Welfare of Animals in Transit*. British Veterinary Association Animal Welfare Foundation, London, UK. pp. 82-97.
- Yahav, S. and S. Hurtwitz. 1996. Induction of thermotolerance in male broiler chickens by temperature conditioning at an early age. *Poult. Sci.* 75:402-406.
- Zulkifli, I. and P. B. Siegel. Is there a positive side to stress? *Wld's Poult. Sci. J.* 51:63-76.
- Zulkifli, I. M., T. Che Norma, C. H. Chong and T. C. Loh. 2000. Heterophil/lymphocyte and tonic immobility reactions to pre-slaughter handling in broiler chickens treated with ascorbic acid. *Poult. Sci.* 79:402-406.
- Zulkifli, I., E. A. Dunnington, W. B. Gross and P. B. Siegel. 1994a. Food restriction early or later in life and its effect on adaptability, disease resistance, and immunocompetence of heat stressed dwarf and nondwarf chickens. *Br. Poult. Sci.* 35:203-214.
- Zulkifli, I., E. A. Dunnington, W. B. Gross and P. B. Siegel. 1994b. Inhibition of adrenal steroidogenesis, food restriction and acclimation to high ambient temperatures in chickens. *Br. Poult. Sci.* 35:417-426.
- Zulkifli, I., H. S. Siegel, M. M. Mashaly, E. A. Dunnington and P. B. Siegel. 1995. Inhibition of adrenal steroidogenesis, neonatal feed restriction and pituitary-adrenal axis response to subsequent fasting in chickens. *Gen. Comp. Endocrinol.* 97:49-56.
- Zulkifli, I., J. Gilbert, P. K. Liew and J. Ginsos. 2002a. The effects of regular visual contact on tonic immobility, heterophil/lymphocyte ratio, antibody and growth responses in broiler chickens. *Appl. Anim. Behav. Sci.* 79:103-112.
- Zulkifli, I., M. T. Che Norma, C. H. Chong and T. C. Loh. 2001. The effects of crating and road transportation on stress and fear responses of broiler chickens treated with ascorbic acid in the hot, humid tropics. *Arch. Geflügelk.* 65(1):33-37.
- Zulkifli, I., M. T. Che Norma, D. A. Israf and A. R. Omar. 2000b. The effect of early age feed restriction on subsequent response to high environmental temperatures in female broiler chickens. *Poult. Sci.* 79:1401-1407.
- Zulkifli, I., M. T. Che Norma, D. A. Israf and A. R. Omar. 2002b. The effect of early age feed restriction on heat shock protein 70 response in heat-stressed female broiler chickens. *Br. Poult. Sci.* 43:141-145.