Effect of Stocking Density and Supplemental Ascorbic Acid on Growth, Organ Weight, Mixed Function Oxidase in Hepatic Microsomes, Lipid Metabolism and Plasma Corticosterone in Male Broiler Chicks

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Abstract The experiment was conducted with male broiler chicks to determine if stocking density and/or dietary ascorbic acid (AA) affect growth, organ weight, lipid metabolism, mixed function oxidase (MFO) in hepatic microsomes and plasma corticosterone concentration. Chicks (7 days of age) were housed in cages at 217, 325 and 625 cm² per chick. The chicks in each stocking density were given a diet supplemented with or without 0.2% AA for 14 days. The stocking density and/or supplemented AA did not affect body weight gain and feed efficiency for 14 days. Body weight gain, however, tended to decrease with the increased stocking density in chicks fed the diet without AA. The thymus weight was significantly decreased by the density of 217 cm²/chick regardless of being supplemented with AA. The plasma cholesterol and lipid concentrations decreased with the increased stocking density and the supplemented AA, but the carcass fat content did not differ between treatments. The supplemental AA significantly decreased plasma corticosterone concentration. The components of MFO, cytochrome b5 and NADH-cytochrome b5 reductase, decreased with the increase in stocking density. The supplemental AA increased the contents of cytochromes P-450 and b5, but decreased the activity of NADPH-cytochrome c reductase. The plasma corticosterone concentration correlated negatively with the content of cytochrome b5 and the activity of NADH-cytochrome b5 reductase per 100 g of body weight. These results show that stocking density and/or AA administration altered lipid metabolism, the components of the hepatic microsomal MFO and the plasma corticosterone concentration under the condition that they did not influence growth and feed utilization. The supplemental AA is a modifier of MFO even in chicks kept under nutritionally proper conditions. In addition, the effect of ascorbic acid on lipid metabolism and the plasma corticosterone appeared to be associated with the changes in the some components of the MFO.

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Animals reared in stressful conditions resulted in the reduction of growth, feed utilization, and immunoresponse²⁵⁾. Stocking density is a stressor and affects growth, reproductivity and plasma corticosterone concentration in hens¹¹⁾ and broilers³²⁾. Plasma corticosterone concentration is a candidate physiological indicator of animals subjected to stressor (s)^{15, 17, 24)}. Administration of glucocorticoids to chicks increased body fat deposition and decreased body protein retention^{4, 5)}, and immunity^{16, 19, 30)}. Moreover, AKIBA *et al.*²⁾ showed a significant positive correlation between plasma corticosterone concentration and abdominal fat weight in chicks kept in a proper nutritional and environmental condition.

Hepatic microsomal mixed function oxidase (MFO) is responsible for metabolizing steroids in chicks as well as mammals⁴¹⁾. TAKAHASHI and JENSEN⁴²⁾ postulated that the MFO in hepatic microsomes was possibly activated by ascorbic acid (AA). Furthermore, PARDUE and THAXTON³⁰⁾ noted that supplemental AA ameliorated the immunosuppression associated with exogenous cortisol. Supplemental AA also improved heat resistance and mortality associated with elevated environmental temperature^{30, 31)} and increased egg production and the hatching of fertile eggs in broiler breeders³³⁾. Thus, AA would be an anti-stress nutrient though it is not an essential nutrient in chicks kept in proper nutritional and environmental conditions. It, therefore, appears that improvement in growth, nutrient utilization, and immunoresponse by supplemental AA in chicks subjected to stressful conditions could be, in part, associated with an activation of the MFO in hepatic microsomes. This experiment was undertaken to determine if the administration of AA and stocking density would affect growth, lipid metabolism, activity of MFO, plasma corticosterone and immunocompetent organs in chicks.

Materials and Methods

Male broiler chicks (7 days of age) were housed in wire floor cages and given a 24 hour photoperiod. The chicks were allotted randomly to twelve pens of 2 each, and six pens of 4 or 6 each to achieve stocking density of 650, 325, 217 cm² per chick. Half of the pens in each stocking density were provided a basal diet (Table 1). The remainder were fed a diet containing 0.2% AA. Feed and water were given *ad libitum*. At 21 days of age, the chicks and remaining feed were weighed. Six chicks from each stocking density were randomly selected and placed in a box and taken to an adjoining place where blood was taken from a heart. The chicks were bled for less than 3 min and plasma was separated by the centrifuge at 1,500 × g for 15 min and stored until analysis. After bleeding, the chicks were killed and organ weight was determined. Carcasses and plasma were kept at -20°C. For determination of mixed function oxidase in hepatic microsomes, half of the liver were placed in 0.1 M Tris-HCl buffer (pH 7.40) at 0°C and replaced in ice-cold 0.25 M sucrose in the buffer, minced and soaked over 10 min. The liver was homogenized in the

Table 1. Composition of basal diet

Ingredients	(%)
Corn	58.86
Soyben meal	30.00
Soy protein	3.00
Soyben oil	4.00
Calcium phosphate, Dibasic	1.72
Calcium carbonate	1.26
Sodium chrolide	0.32
DL-Methionine	0.25
Vitamin mixture ¹⁾	0.40
Mineral mixture ¹⁾	0.42
Calculated analysis	
Metabolizable energy (kcal/kg)	3150
Crude protein (%)	21.5

¹⁾ See AKIBA and MATSUMOTO (1978)

buffer with Ultradispaser (Yamato Co. Ltd., Japan) for 15 seconds at 0°C and then centrifuged at $10,000 \times g$ for 10 min twice. To the supernatant we added calcium chloride (final concentration, 8 mM) stirred it for 10 min at 4°C, and spun it at $20,000 \times g$ for 40 min at 4°C. The pellet was resuspended in the buffer with 0.15% KCl (pH 7.4) and spun again under the same conditions. The microsomal pellets were suspended in 20% glycerol in 0.1M Tris-HCl buffer and used for assay within 3 days. Cytochromes P-450 and b5 were measured by the method of OMURA and SATO²⁹⁾. NADPH-cytochrome c and NADH-cytochrome b5 reductases were measured by the methods of PHILLIPS and LANDON³⁵⁾, and TAKESUE and OMURA⁴⁴⁾, respectively. Plasma cholesterol and lipids were determined by the methods of ZURKOWSKI⁴⁷⁾ and BRAGDON⁸⁾, respectively. Plasma corticosterone was extracted by the method of SCOTT and DIXON³⁷⁾ and determined using a reverse phase high performance liquid

chromatography¹⁴⁾. Carcasses were grounded by a meat chopper and a portion of the ground carcasses were extracted by the method of FOLCH *et al.*¹³⁾ and then weighed. Data were analyzed by the 2×3 factorial model with interaction using the General Linear Models Procedure of Statistical Analysis system³⁹⁾.

Results

Table 2 shows the effect of supplemental AA and/or stocking density on body weight gain, feed efficiency, and weights of the liver, spleen, bursa Fabricius and thymus per unit of 100 g body weight in chicks. Body weight gain and feed efficiency did not significantly differ between treatments. The higher stocking density, however, resulted in a tendency toward increasing body weight gain in chicks fed the basal diet. The supplemental AA did not affect body weight gain. The higher stocking density reduced relative liver weight regardless of supplement with AA. The weights of the

Table 2. Effect of supplemental ascorbic acid (AA) and/or stocking density on weight of liver, spleen, bursa Fabricius and thymus per 100 g body weight in chicks

AA (%) Density (cm ² /chicks)	0			0.2		
	625	325	217	625	325	217
Body weight gain (g/14 days)	487±16	493±19	529±3	501±27	496±8	501±18
Feed efficiency	0.64±0.03	0.67±0.04	0.62±0.03	0.64±0.03	0.64±0.03	0.61±0.04
Liver (g/100 g BW)	2.43±0.14	2.25±0.05	2.25±0.09	2.36±0.07 ^a	2.20±0.07 ^{ab}	2.08±0.10 ^b
Spleen (mg/100 g BW)	95±6	102±11	90±9	99±9	113±7	89±15
Bursa (mg/100 g BW)	362±63	328±17	315±16	339±38	358±11	377±61
Thymus (mg/100 g BW)	658±60	650±51	546±36	696±70	656±76	553±25

Probability>F

Significance of effects	d.f.	Body weight gain	Feed efficiency	Weight of			
				liver	spleen	bursa	thymus
AA	1	NS	NS	NS	NS	NS	NS
Density	2	NS	NS	*	NS	NS	*
AA×Density	2	NS	NS	NS	NS	NS	NS

1. Mean±SE with 12 observations.

2. Mean±SE with 6 observations in 217cm² per chick and 3 observations in 325 and 625 cm² per chick, respectively.

3. Mean±SE with 6 observations.

a,b; Significant difference within the same AA level ($P<0.05$).

Initial body weight was 160 ± 5 g. *, $P<0.05$. **, $P<0.01$. NS, not significant.

bursa and spleen did not significantly differ between treatments. Bursa weight, however, tended to be decreased with the higher stocking density in chicks fed the basal diet. Thymus weight significantly decreased with the increasing stocking density regardless of supplement with AA.

Table 3 shows effect of supplemental AA and/or stocking density on plasma concentrations of lipids, cholesterol and corticosterone, and carcass fat content in chicks. Plasma lipid concentration was significantly decreased by the supplemented AA. The increased stocking density and supplemental AA significantly reduced the plasma cholesterol concentration. The reducing effect of the supplemented AA on the plasma cholesterol concentration appeared to be effective in chicks kept at the higher stocking density. The stocking density and AA significantly affected plasma corticosterone concentration. The highest values of the plasma corticosterone concentration were observed in chicks kept at the density of 217 cm² per chick and the lowest were observed in chicks kept at the density of 325 cm² per chick regardless

of supplement with AA. The supplemented AA significantly reduced the plasma corticosterone concentration. A significant interaction between the stocking density and AA was found for the plasma corticosterone. Carcass fat content was in the range of about 12 to 13.5% and did not differ among the treatments.

Table 4 shows the effect of supplemental AA and/or stocking density on contents of cytochromes P-450 and b 5, activities of NADPH-cytochrome c reductase and NADH-cytochrome b 5 reductase in hepatic microsomes of chicks. The content of cytochrome b 5 significantly decreased with the increase in the stocking density. There was a tendency toward decreasing the content of cytochrome P-450 associated with the increased stocking density. Supplemental AA significantly increased the contents of cytochromes P-450 and b 5. The activity of NADPH-cytochrome c reductase was significantly decreased by the supplemental AA, but not affected by the changes in stocking density. Significantly decreased activity of NADH-cytochrome b 5 reductase was observed in

Table 3. Effect of supplemental ascorbic acid (AA) and/or stocking density on plasma concentration of lipids, cholesterol, and corticosterone and carcass fat content in chicks

AA (%) Density (cm ² /chick)	0			0.2		
	625	325	217	625	325	217
Plasma						
lipid (mg/100mg)	464±15	462±21	446±19	414±20	394±14#	391±23
cholesterol (mg/100ml)	136±6 ^a	127±9 ^a	106±7 ^b	112±3 ^{a#}	121±4 ^a	108±5 ^b
corticosterone (ng/ml)	9.2±1.4 ^b	7.8±1.3 ^b	42.3±11.5 ^a	4.4±0.8#	3.1±0.6#	5.2±1.0#
Carcass fat (%)	12.1±0.9	13.3±0.5	12.2±0.4	12.5±0.7	12.1±0.6	12.5±0.7
Probability>F						
	d.f.	Lipids	Cholesterol	Corticosterone	Carcass fat	
Significance of effects						
AA	1	**	*	**	NS	
Density	2	NS	**	**	NS	
AA×Density	2	NS	*	**	NS	

1. Means±SE with 6 observations. *, P<0.05. **, P<0.01. NS, not significant.

a, b; Significant difference within the same AA level (P<0.05).

#; Significant difference within the same stocking density (P<0.05).

Stocking Density and Ascorbic Acid

Table 4. Effect of supplemental ascorbic acid (AA) and/or stocking density on content of cytochrome P-450 and cytochrome b5, and activity of NADPH-cytochrome c reductase and NADH-cytochrome b5 reductase in hepatic microsomes per unit of 100 g body weight of chicks

AA (%)	0			0.2		
Stocking density(cm ² /chicks)	625	325	217	625	325	217
Cytochrome P-450 ²	6.3±0.9	5.9±0.6	5.2±1.1	7.7±0.9*#	7.8±0.6*#	5.6±0.4 ^b
Cytochrome b5 ²	10.3±0.7 ^a	8.7±0.6 ^{ab}	7.4±1.1 ^b	11.6±1.2#	12.2±0.6#	10.0±0.6
NADPH-cytochrome c reductase ³	831±74	775±40	707±68	496±29#	542±35#	520±23#
NADH-cytochrome b5 reductase ⁴	12.1±0.7 ^a	10.7±0.7 ^a	7.8±0.6 ^b	9.6±0.6#	9.2±0.5	8.9±0.5
Probability>F						
d.f.	Cytochrome P-450	Cytochrome b5	NADPH-cytochrome c	NADH-cytochrome b5		
Significance of effects						
AA 1	*	**	**		NS	
Density 2	NS	*	NS		**	
AA×Density 2	NS	NS	NS		*	

1. Mean±SE with 6 observations.

2. nmol/100 g body weight.

3. nmol/min/100 g body weight.

4. μmol/min/100 g body weight.

a,b; Significant difference within the same AA level (P<0.05).

#; Significant difference within the same stocking density (P<0.05).

*, P<0.05. **, P<0.01. NS, not significant.

Table 5. Correlation coefficients among the components of MFO and Plasma lipids, cholesterol and corticosterone and carcass fat

	Cytochrome P-450 (nmol/100 g BW)	Cytochrome b5 (nmol/100 g BW)	NADPH-cytochrome c reductase (nmol/min/100 g BW)	NADH-cytochrome b5 (μmol/min/100 g BW)
Plasma lipids (mg/100 ml)	-0.142	-0.244	0.508**	0.402*
Plasma cholesterol (mg/100 ml)	-0.126	-0.030	0.345*	0.505**
Plasma corticosterone (ng/100 ml)	-0.270	-0.406*	0.192	-0.382*
Carcass fat (%)	-0.053	-0.173	0.198	0.069

*, P<0.05. **, P<0.01.

chicks kept at the higher stocking density. The supplemental AA tended to decrease the activity of NADH-cytochrome b5 reductase. There was a significant interaction between the stocking density and supplemental AA for NADH-cytochrome b5 reductase.

Table 5 shows correlation coefficients between the components of MFO per 100 g body weight and plasma concentrations of lipids, cholesterol

and corticosterone and carcass fat content. Plasma lipids and cholesterol concentrations were, significantly, positively correlated with hepatic microsomal NADPH-cytochrome c reductase and NADH-cytochrome b5 reductase per 100 g of body weight. The plasma corticosterone concentration was, significantly, negatively correlated with hepatic microsomal cytochrome b5 content and the activity of

NADH-cytochrome b 5 reductase per 100 g body weight. No significant correlation was found between the components of MFO and carcass fat content.

Discussion

In the present experiment, the stocking density of 217, 325 or 625 cm² per chick did not significantly affect body weight gain and feed efficiency from 1 to 3 weeks of age: this is consistent with the result of PESTI and HOWARTH³⁴⁾ although the deleterious effects of the high stocking density on the growth of chicks has been reported by some investigators^{7, 20)}. PESTI and HOWARTH³⁴⁾ and DAFWANG *et al.*¹²⁾ observed that the stocking density of about 100-140 cm² per chick decreased body weight gain and feed efficiency as compared to that of over 200 cm² per chick. These results indicate that less than 150 cm² per chick of stocking density, at least, should be required in order to induce growth depression by changes in stocking density at this age. It seems that chicks might reduce body weight gain if they are kept at the 217 cm² per chick for long periods because higher corticosterone concentration was observed at this density. Our experiment also reconfirmed the results of the experiments that dietary AA did not stimulate consistently the growth of chicks fed nutritionally adequate diets and maintained under "nonstressed" conditions^{9, 26)}.

The thymus weight significantly decreased in chicks subjected to 217 cm² per chick, and corticosterone concentration in the plasma was higher at this stocking density. The results possibly support the hypothesis that the stocking density affected immunocompetence, since it has been known that the plasma corticosterone was increased under certain stressful condition, and that administration of corticosterone reduced immunity and immunocompetent organ weight^{16, 18, 30)}. On the other hand, DAFWANG *et al.*¹²⁾ suggested that the stocking density did not appear to affect

antibody titer to sheep red blood cell (SRBC) and spleen weight. Thus, the effect of the stocking density on the immunocompetence of chicken has remained obscure. The supplemental AA served as an anti-immunosuppressive agent in chicks administrated with cortisol at pharmacological level³⁰⁾. McCORKLE *et al.*²⁴⁾ showed that the AA supplementation in chicks kept at "nonstressed" condition did not affect antibody production to a T-lymphocyte dependent antigen such as SRBC. It is recognized that the thymus is a maturation center in which stem cells differentiate into T-lymphocytes³²⁾. Combined with the results of McCORKLE and the present study that supplemental AA did not affect weights of immunocompetent organs, the supplemental AA would not affect, at least, T-lymphocyte dependent immunity under "nonstressed" conditions.

The present experiment first demonstrated that the stocking density and supplemental AA affected the components of the MFO. However, it is not surprising that the stocking density affected MFO activity since it has been established that many hormones including glucocorticoids modified, directly or indirectly, the MFO activity in hepatic microsomes of mammals which were unable to synthesize AA²³⁾. It is known that dietary AA affects MFO activity in mammals. AA deficiency in guinea pigs, which essentially require AA for the normal growth and the normal metabolism of nutrients, reduced the MFO activity in hepatic microsomes⁴⁶⁾. It has also been shown that ingestion of xenobiotics would increase the requirement of ascorbic acid several-fold for inducing the maximum activity of drug-metabolizing enzymes in mammals²²⁾. The mode of action of AA to increase hepatic microsomal MFO of mammals has been considered as follows: a) inhibition of cytochrome degradation and/or promotion of cytochrome synthesis, b) maintenance of phospholipid structure in endoplasmic reticulum, and c) protection of occurrence of lipid peroxida-

tion⁴⁶⁾. On the other hand, the cause of changes in the MFO activity of chicks fed a diet containing AA is not clearly explainable since chicks used in the present study were not treated with any particular xenobiotics and are capable of synthesizing AA. Another question is why components of the MFO changed in opposite directions to each other due to AA feeding.

A higher corticosterone may be a factor for increasing the fat deposition in broiler chicks^{2,4,5)}. The results of our experiment showed that the body fat content did not change even when the plasma corticosterone concentration was changed by the stocking density and/or supplemental AA. The different results between the earlier works and the present experiment may be caused by the experimental periods. When dietary and environmental conditions change, hormones will respond firstly, and, thereafter, nutrient utilization or retention may change. The concept was approved by the results of the time course studies with chicks^{3,43)} of the changes in dietary composition, the plasma estrogen concentration and liver lipid deposition. The plasma corticosterone concentration was significantly reduced by feeding a diet with 0.2% AA in this experiment with broiler chicks, being in good accordance with the results of the experiment with laying hens treated with or without 150 ppm AA²⁸⁾. According to SCHMELING and NOCKELS³⁶⁾, the corticosterone synthesis would be inhibited by feeding AA in mature hens: In the hens fed AA, the pool size of the plasma corticosterone was smaller and its excretion rate was slower than the hens which received no AA. The MFO in hepatic microsomes is known to play an important role in steroid metabolism and an activation of the MFO in hepatic microsomes would be related to an increasing production of water soluble products from steroids⁴¹⁾. The present experiment showed that supplemental AA significantly changed the activity of the MFO in hepatic micro-

somes, and a significant negative correlation was observed between the plasma corticosterone concentration, and the content of cytochrome b 5 and the activity of NADPH-cytochrome b 5 reductase. A possible explanation for the reduction of the plasma corticosterone concentration by supplemental AA would be that an increase in the MFO activity in hepatic microsomes accelerated the rate of hydroxylation of a corticosterone. Although this would be supported by the *in vitro* results that drug-activated MFO produced more hydroxylated metabolites of corticosterone than an inactivated MFO¹⁰⁾, further study is required to define our concept.

The reducing effect of supplemental AA on blood cholesterol has been demonstrated in rats and rabbits given a high cholesterol diet³⁸⁾ and chicks treated with propylthiouracil⁴⁰⁾. This study reconfirmed the effect of supplemental AA on the plasma cholesterol concentration in broiler chicks even when they were fed a nutritionally adequate diet. On the other hand, supplemental AA did not affect the plasma cholesterol concentration in hens²⁷⁾. Different responses between the results of laying hens and broiler chicks are not explained at this time. In studies of liver homogenate and *in situ* liver perfusions, cholesterol synthesis from mevalonate was significantly depressed in the AA-deficient guinea pigs when compared to the control⁴⁵⁾. MFO in the liver plays an important role in the 7- α -hydroxylation of cholesterol during the conversion of bile acids to cholesterol. The AA-deficient guinea pigs reduced the activity of this rate-limiting step in cholesterol metabolism⁵¹⁾, leading to an accumulation of cholesterol in tissues and blood. As described above, chickens are able to synthesize AA but guinea pigs are not. However, metabolic demand of AA in chickens probably increases when they receive particular stress^{30,31,33)}. The present experiment has shown that the AA requirement for maintaining normal lipid and steroid metabolism and, the

MFO function may exceed, at least in part, amounts of AA biosynthesis in chicken under the condition of the present experiment. However, we have not demonstrated whether the responses of cholesterol metabolism and MFO activity toward AA in chicks are similar as are those observed in guinea pigs.

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雄ブロイラーの成長、臓器重量、脂質代謝、肝臓 ミクロソーム薬物代謝酵素活性および血漿 コルチコステロン濃度に対する飼育密度と アスコルビン酸添加の影響

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雄ブロイラーの成長、臓器重量、脂質代謝、肝臓ミクロソーム薬物代謝酵素活性および血漿コルチコステロン濃度に対する飼育密度とアスコルビン酸添加の影響を検討した。7日齢の雄ブロイラーを217, 325, 625 cm²/鶏の飼育密度で14日間飼育し、それぞれの飼育密度の半数の鶏には0.2%アスコルビン酸添加飼料を給与した。増体、飼料効率には、飼育密度およびアスコルビン酸添加の有意な影響は認められなかったが、アスコルビン酸無添加区では、飼育密度の増加にともない増体が低下する傾向がみられた。胸腺重量は、アスコルビン酸添加の有無にかかわらず高飼育密度条件下で低下した。血漿中コレステロールおよび脂質濃度は、飼育密度の増加およびアスコルビン酸添加により低下した。血漿コルチコステロン濃度も、アスコルビン酸添加により低下した。体脂肪含量は、飼育密度およびアスコルビン酸添加の影響を受けなかった。肝臓薬物代謝酵素を構成しているチトクロム-b5量およびNADPH-チトクロムc還元酵素活性は、飼育密度の増加にともない低下した。アスコルビン酸添加は、チトクロム-P450量およびチトクロムb5量を増加させたが、NADPH-チトクロムc還元酵素活性を低下させた。血漿コルチコステロン濃度と肝臓薬物代謝酵素の構成成分のチトクロムb5量およびNADH-チトクロムb5還元酵素活性の間には負の相関が認められた。これらの結果は、成長に影響を及ぼさない条件下の飼育密度およびアスコルビン酸添加が、雄ブロイラーの脂質代謝、肝臓薬物代謝酵素活性および血漿コルチコステロン濃度に影響を及ぼしていることを示している。アスコルビン酸添加が、肝臓ミクロソーム薬物代謝酵素活性、特にチトクロムb5量を修飾することも示された。また、脂質代謝の変化と肝臓薬物代謝酵素活性は、調節が直接的か間接的かは不明であるが、両者とも同時に制御されていることが示唆された。

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