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Effects of Squid Ink on Growth Performance, Antioxidant Functions and Immunity in Growing Broiler Chickens*

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ABSTRACT : This study was conducted to explore the effects of squid ink on growth performance, immune functions and antioxidant ability of broiler chickens during a period of six weeks. Either sex Arbor Acres broilers were equally allotted to 4 groups with 3 replicates of 20 chickens each. Broilers diets for the 4 test groups were prepared separately with starter and finisher phases. Control chickens were fed with basal diet and birds of group Exp 2, Exp 4 and Exp 6 were fed with the basal diet supplemented with 2%, 4% and 6% of squid ink, respectively. Broilers were sacrificed to investigate antioxidant parameters of sera, indices of thymus, spleen and bursa of fabricius and spleen lymphocyte proliferation, as well as growth performance on the 21th and 42th day. The results revealed that, i) squid ink promoted growth performance of broilers during days 22 to 42 and the whole trial period ($p<0.05$ or $p<0.01$); ii) squid ink elevated relative weight of the three immune organs during the starter phase and spleen lymphocyte proliferation throughout the experiment ($p<0.05$); iii) squid ink increased SOD activity and decreased MDA level in sera from broilers during the whole period ($p<0.05$). The above results suggest that squid ink could improve growth performance, antioxidant ability and immune functions of growing broiler chickens and be employed in the development of feed additives for animals. (**Key Words :** Squid Ink, Antioxidant Ability, Immune Function, Growth Performance, Broilers)

INTRODUCTION

Squid ink produced at the end of the cuttlefish maturation process is a suspension of melanin granules in a viscous colorless medium. Ink gland cells of the digestive tract in the mantle cavity degenerate and shed their contents into the ink sac, acting as a reservoir of the exhausted material. Ejection of dark ink from the sac is a defensive means cuttlefish employed to avoid dangers and risks.

Background researches showed that squid ink is an intermixture, besides large amounts of melanin, the ink contains proteins, lipids, glycosaminoglycans and various minerals, etc. The main components are melanin and protein-polysaccharides complex. Double-layer structural melanin granule consists of inner high density melanin and external low density organic compound.

It is well documented that squid ink is a multifunctional marine bioactive-material, it not only promotes thromboxane and kills cancer cells, it also possesses of leukocyte-number elevating, anti-oxidant, anti-radiation, anti-retrovirus and anti-bacterial properties (Zhong et al., 2009). In recent years, we discovered that squid ink could ameliorate chemotherapeutic injury induced by cyclophosphamide in model animals; mice or rats (Wang et al., 2009, 2010b; Liu et al., 2009; Zhong et al., 2009). To the present, although pharmacological roles of squid ink have been studied by researchers for many years, this marine material is still discarded. A correct understanding and utilization of squid ink may lead to its reuse and reduce the waste of a marine resource.

In our previous papers, we reported the chemotherapeutic protective effects of squid ink against cyclophosphamide damage in rats and mice, but another finding is still unreported. As a byproduct of the study, we found that squid ink extract could slightly improve growth performance of rats or mice (data not show). This raised the possibility that the discarded ink could act as a growth promoter and be used as a animal food additive. To inquire

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into the nutritious effects of squid ink, we investigated the effects of squid ink on growth performance, antioxidant ability in serum and immunity of growing broiler chickens. This study aimed to provide a basic theory for the use of squid ink in animal feed.

MATERIALS AND METHODS

Preparation of squid ink

Fresh cuttlefish were purchased from a fishmonger and rapidly transferred to the laboratory where they were dissected, the ink was collected and diluted with the basic broiler diet to the appropriate ratios before administration to animals.

Animals and experimental design

Day-old healthy Arbor Acres broilers were purchased from a commercial hatchery, either sex chickens were equally allotted to four test groups, one control group and three treatment groups, with three replicates of twenty birds each reared in stainless steel battery brooders. The room temperature was maintained at 33±1°C up to seven days old and then gradually reduced to 26±1°C by 21 days of age after which, animals were maintained at room temperature. Uniform management and vaccination schedules were followed for all the birds.

Experimental diets

Experimental diets were formulated separately for starter phase (day 0 to 21) and finisher phase (day 22 to 42). Control group animals were fed with basal diet shown in Table 1. Dietary treatments for three experimental groups were formulated with the basal diet which in addition contained a percentage of squid ink. Groups Exp 2, Exp 4 and Exp 6 were formulated to contain an additional 2%, 4% and 6% squid ink respectively; the ink was in lieu of the components in the basal diet.

Traits measured

Ten chickens from each group were randomly sampled at day 21 and 42. The selected broilers were weighed and feed intake (FI) was recorded to calculate body weight gain (BWG) and feed conversion ratio (FCR). The birds were sacrificed, blood was collected to prepare serum to measure antioxidant ability. Thymus, spleen and bursa of fabricius were weighed separately to calculate organ indices expressed as a percentage of body weight, in addition, the spleen was collected for the lymphocyte proliferation assay.

Antioxidant parameters (total SOD activity and MDA content) in sera were measured with detection kits developed by Nanjing Jiancheng Bioengineering Institute from China according to the manufacturer's protocol.

Lymphocyte proliferation assay was performed according to the following process. Spleen was homogenated in 6 ml Hank's solution and centrifugated at a speed of 1,000 rpm for 3 min. The harvested supernatant was centrifugated at speed of 1,000 rpm for 5 min, and then the cells at the bottom of test tube were washed with Hank's solution three times, 1,000 rpm for 5 min every time. Cells were suspended in RP1640 medium and diluted to 1×10^7 cells/ml suspension and placed in 96-well plates. The contents of each experimental well contained 100 μl cell suspension and 30 μl ConA (100 μg/ml) while the control well contained 100 μl cell suspension, 30 μl RP1640 medium and 5% FBS per well. There were three replicates for each sample. After incubation in a humidified atmosphere (37°C, 5% CO_2) for 48 h, 20 μl MTT (5 mg/ml) was added to each well. The microplates were vibrated for 5 min on a vibrator and incubated for another 4 h in a humidified atmosphere. 100 μl SDS (10%) solution dissolved in 0.01 mol/L HCl solution was added into each well to stop color reaction. After 10 min, the plates were read at 570 nm by a ELISA plate reader.

Table 1. Composition of basal diet

Items	Starter (d 1-21)	Finisher (d 22-42)
Ingredients (%)		
Corn	56.20	59.60
Soybean meal	25.30	21.30
Wheat middling	6.00	6.00
Fish meal	2.50	1.00
Cottonseed meal	2.00	3.00
Rapeseed meal	2.30	2.50
Calcium hydrophosphate	1.30	1.30
Limestone	1.50	1.50
Sodium chloride	0.30	0.30
Soybean oil	1.60	2.50
Premix	1.00	1.00
Nutrients levels		
ME (MJ/kg)	12.01	12.30
CP (%)	20	18
Ca (%)	1.0	0.9
Available P (%)	0.45	0.40
Met (%)	0.46	0.36

The premix provided following per kg of diet. Day 1-21: Mn 66 mg, Zn 44 mg, Cu 9 mg, Fe 50 mg, I 0.4 mg, Se 0.3 mg, V_A 8,000 IU, V_{D3} 1,000 IU, V_E 30 IU, V_{K3} 1 mg, V_{B1} 2 mg, V_{B2} 5.5 mg, D-calcium pantothenate 12 mg, V_{B5} 50 mg, V_{B6} 2.5 mg, V_{B12} 0.6 mg. Day 22-42: Mn 66 mg, Zn 44 mg, Cu 9 mg, Fe 50 mg, I 0.4 mg, Se 0.3 mg, V_A 7,000 IU, V_{D3} 875 IU, V_E 20 IU, V_{K3} 1 mg, V_{B1} 2 mg, V_{B2} 4.5 mg, D-calcium pantothenate 12 mg, V_{B5} 50 mg, V_{B6} 2.5 mg, V_{B12} 0.6 mg.

Statistical analysis

Experimental data were analyzed by ANOVA using the SAS statistical software (SAS Institute, 1999). Results were

Table 2. Effects of squid ink on performance of broiler chickens

Items	Con	Exp 2	Exp 4	Exp 6
Day 1-21				
Average daily weight gain (g)	31.07±1.71	31.98±1.26	33.65±2.01	33.72±2.20
Average daily feed intake (g)	50.95±3.04	51.49±2.83	57.21±4.87	56.65±4.90
Feed conversion ratio	1.64±0.06	1.61±0.05	1.70±0.07	1.68±0.06
Day 22-42				
Average daily weight gain (g)	72.12±2.93^{Bb}	80.66±3.05^{bc}	84.24±3.03^{Ac}	85.28±3.64^{ac}
Average daily feed intake (g)	156.50±5.61^{B}	179.87±4.03^{Aa}	192.91±3.99^{Ab}	188.47±4.62^{Aab}
Feed conversion ratio	2.17±0.07	2.23±0.05	2.29±0.04	2.21±0.08
Day 1-42				
Average daily weight gain (g)	48.62±2.33^{b}	54.64±2.49^{bc}	57.51±2.06^{ac}	56.98±2.43^{ac}
Average daily feed intake (g)	98.21±3.55^{Bb}	115.29±3.46^{ac}	120.20±4.29^{Ac}	121.94±4.70^{Ac}
Feed conversion ratio	2.02±0.03	2.11±0.06	2.09±0.05	2.14±0.05

Dietary treatments were as follows. Con: basal diet; Exp 2: basal diet and 2% of squid ink; Exp 4: basal diet and 4% of squid ink; Exp 6: basal diet and 6% of squid ink. In the same row, values with different lowercase superscripts mean significant difference ($p<0.05$), those with different capital letters are extremely different ($p<0.01$).

expressed as the means and standard errors. Differences were separated by Duncan's multiple range test. Significance was considered at $p<0.05$ or $p<0.01$.

RESULTS

Effects of squid ink on growth performance of broilers

In Table 2, during the starter phase no differences were observed in BWG, FI and FCR among all groups, but in the finisher phase and the whole trial period, broilers treated with different levels of squid ink supplement showed an increased body weight gain compared with the control group. Except for group Exp 2, BWG in the other two experimental groups were significantly higher than those in the control group ($p<0.05$ or $p<0.01$). The FI in the three treated groups were greater than that in control birds ($p<0.05$ or $p<0.01$). However, no difference was observed in FCR among the four groups throughout the experiment. Overall, birds diets supplemented with squid ink increased broiler BWG and FI during days 22 to 42.

Effects of squid ink on immune functions of broilers

The indices of thymus, spleen and bursa of fabricius of the experimental chickens, as well as spleen lymphocyte proliferation assay, were investigated to explore the effects of squid ink on immune functions of growing Arbor Acres broilers. The results are showed in Table 3. It can be seen that during days 1 to 21, squid ink significantly elevated indices of the three immune organs and promoted spleen lymphocyte proliferation ($p<0.05$) when stimulated by Con A. During the finisher phase, although spleen lymphocyte proliferation was at the same level ($p<0.05$) as the starter phase, the marine bioactive mixture did not induce a similar effect on indices of the organs ($p>0.05$). In addition, the

Table 3. Effects of squid ink on immune functions of broiler chickens

Items	Con	Exp 2	Exp 4	Exp 6
Day 21				
Thymus index (mg/g)	4.16±0.14^{b}	4.66±0.13^{a}	4.61±0.16^{a}	4.71±0.16^{a}
Spleen index (mg/g)	0.68±0.07^{b}	0.79±0.08^{ab}	0.93±0.07^{a}	0.96±0.09^{a}
Bursa of fabricius index (mg/g)	2.19±0.23^{b}	2.46±0.20^{ab}	2.93±0.19^{a}	2.84±0.25^{ab}
Spleen lymphocyte proliferation (A_{570nm})	0.34±0.04^{b}	0.46±0.03^{a}	0.52±0.06^{a}	0.55±0.08^{a}
Day 42				
Thymus index (mg/g)	2.83±0.17	2.96±0.15	2.99±0.12	3.01±0.13
Spleen index (mg/g)	2.15±0.19	2.23±0.16	2.31±0.13	2.29±0.21
Bursa of fabricius index (mg/g)	1.84±0.16	2.11±0.23	2.47±0.31	2.22±0.26
Spleen lymphocyte proliferation (A_{570nm})	0.42±0.06^{b}	0.60±0.04^{a}	0.67±0.07^{a}	0.68±0.08^{a}

Dietary treatments were as follows. Con: basal diet; Exp 2: basal diet and 2% of squid ink; Exp 4: basal diet and 4% of squid ink; Exp 6: basal diet and 6% of squid ink. In the same row, values with different lowercase superscripts mean significant difference ($p<0.05$).

results showed that the relative organ weight of the three immune organs differed among the three treatment groups. The index of thymus increased ($p<0.05$) in response to three doses of squid ink, whereas the spleen was insensitive to low-dose of squid ink and bursa of fabricius was only sensitive to a mid-dose of squid ink.

Effects of squid ink on antioxidant functions of broilers

Results in Figure 1 show that the antioxidant activity of squid ink on broiler chickens. During the whole trial period, the marine bioactive material markedly elevated total SOD activity in serum from chickens ($p<0.05$), though the low-dose of squid ink did not induce significant changes ($p>0.05$). Meanwhile, as an important antioxidant ability parameter, MDA level in sera from the high dose of squid ink group was higher than that in control birds ($p<0.05$) on day 21. During days 22 to 42, contents of the products of lipid peroxidation in sera from treatment chickens were all higher than that in untreated broilers ($p<0.05$). In addition, both SOD activity and MDA levels were not different among the three treatment groups.

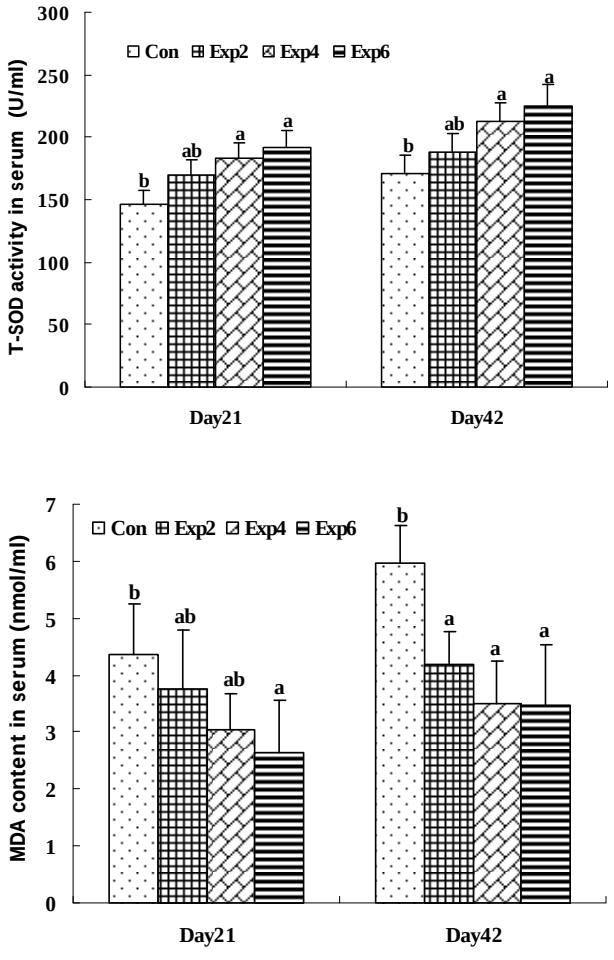

Figure 1. Effects of squid ink on serum antioxidant ability of broiler chickens. Dietary treatments were as follows. Con: basal diet; Exp 2: basal diet and 2% of squid ink; Exp 4: basal diet and 4% of squid ink; Exp 6: basal diet and 6% of squid ink. In the same day, values with different lowercase superscripts mean significant difference ($p<0.05$).

DISCUSSION

Squid ink is a mixture containing melanin, protein, carbohydrate and lipid. It is reported that protein constitutes approximately 10.08% of squid ink from *Sepialla maindroni*. Additionally, the dark natural material contains nine fatty acids (approximately 1.34%) of which 43.4% is unsaturated fatty acid and 56.6% is saturated fatty acid plus sixteen amino acids of which aspartic acid is the richest. Many mineral elements are important components of the ink, of which calcium is the most common element (Takaya et al., 1994b; Chen et al., 2000; Zhen et al., 2002). In our previous unpublished study, we showed that the dark ink employed in this study contained approximately protein (6.33%), melanin (15.41%), saccharides (3.15%), lipid (0.19%) and water (74.92%).

Superoxide dismutase, a member of the first line of defense in the antioxidant system of living cells, works at preventing the production of free radicals. Superfluous free radicals may damage protein and nucleic acids, and induces lipid peroxidation to produce large amounts of MDA that injures cells and results in disequilibrium of the internal environment and diseases. Consequently, SOD activity and MDA levels can reflect the antioxidant ability of body. In recent years, the antioxidant ability of squid ink was discovered. Background researches showed that melanin of squid ink, like superoxide dismutase, can catalyze O^{2-} to H_2O_2, and thus avoid the free radical chain reaction triggered by O^{2-} (Chen et al., 2007). Zhang et al. (2003) reported that squid ink elevated SOD activity in the liver and kidney of mice in a dose-dependent manner. Our previous studies showed that squid ink upregulated SOD activities decreased by cyclophosphamide in kidney, spleen, heart, lung and brain of mice and downregulated MDA contents in these organs (Liu et al., 2009; Wang et al., 2009, 2010b; Zhong et al., 2009). Our subsequent investigations confirmed that the function was related to polysaccharides present in squid ink (Wang, 2010a). In this paper, squid ink increased SOD activity and decreased MDA levels in growing broilers sera in a dose-dependent manner. Consequently, squid ink improved the antioxidant ability of chickens. The results further confirmed antioxidant ability of squid ink.

It is documented that squid ink could improve immune functions, such as inducing the activity of natural killer cells (He et al., 2002) and lymphokine activated killer cells (Wang et al., 2001), activating macrophages (Takaya et al., 1994a), inducing production of many cytokines (Lü et al.,

1999; He et al., 1999; Xie and He, 2001) and promoting immune adhesive capacity of erythrocytes (Liu et al., 1996). Thymus, spleen and bursa of fabricius are the main immune organs of chickens. Thymus and bursa of fabricius are central immune organs where lymphocytes are produced and differentiate. Fowl B lymphocytes mature in the bursa of Fabricius and T lymphocytes mature in thymus while the spleen is a peripheral immune organ for immune response. Liveweight increases of immune organs results from cell growth and proliferation and the weight gain of immune organs indicates the promotion of the immune function of an animal. Our previous research found that squid ink increased the lymphocyte proliferation index and antioxidant ability of mouse spleens (Zhong et al., 2009). Therefore, in the present study we hypothesized that squid ink could enhance immune function of broilers. As expected, squid ink promoted the relative weight of thymus, spleen and bursa of fabricius in chickens during days 1 to 21, as well as spleen lymphocyte proliferation throughout experiment. The results indicated that squid ink could accelerate early development of the immune organs and immune response.

Also, the findings of the study revealed that body weight gain and food intake were affected by feeding the basal diets containing squid ink to broilers. These positive effects were likely to be related the rich nutritious ingredients of squid ink. However, it should be mentioned here that the significant growth-promoting effect was observed in the finisher phase and whole trial period. These two parameters were not affected by the experimental diets during the starter phase. It is further observed that an additional dose of squid ink did not bring about uniform outcome. Consequently, on a basis of an overall consideration, the mid-dose (4% of the diet) of squid ink may be a more appropriate level of feed additive for chickens than either the low or high dose of marine material.

REFERENCES

Chen, S. G., C. H. Xue, Y. Xue, Z. J. Li, X. Gao and Q. Ma. 2007. Studies on the free radical scavenging activities of melanin from squid ink. Chin. J. Mar. Drugs. 26(1):24-27.

Chen, X. E. 2000. Study on nutritional composition of *Sepialla maindroni* ink. J. Zhejiang Ocean Univ. 19(4):324-326.

He, S., G. L. Xie and M. C. He. 2002. Studies of sepia influence on NK cells activity and effect on inhibition tumor in mice. Chin. J. Mar. Drugs. 21(5):37-39.

He, S., G. L. Xie, F. Z. Liu and C. L. Lü. 1999. The study about IL-2 activity induced by sepia in mice. Chin. J. Mar. Drugs. 18(1):11-12.

Liu, C. Y., Q. Teng, X. Y. Zhou and R. L. Tan. 1996. Effects of sepia on erythrocyte's adhesion to tumor cells. Chin. J. Mar. Drugs. 15(3):14-16.

Liu, H. Z., G. Wang, Y. Z. Guo, J. Q. Pan, Y. Huang, J. P. Zhong and K. Li. 2009. Protective effect of squid ink on cyclophosphamide-induced oxidative injury of kidney in mice. Chin. J. Nephrol. 25(10):804-805.

Lü, C. L., S. He, B. X. Liu, L. X. Na, J. Z. Yan and G. Fu. 1999. The immuno-stimulating activations of squid ink and its extracts. Chin. J. Mar. Drugs. 18(2):32-34.

Takaya, Y., H. Uchisawa, H. Matsue, B. Okuzaki, F. Narumi, J. Sasaki and K. Ishida. 1994a. An investigation of the antitumor peptidoglycan fraction from aquid ink. Biol. Pharm. Bull. 17(6):846-849.

Takaya, Y., H. Uchisawa, K. Hanamatsu, F. Narumi, B. Okuzaki and H. Matsue. 1994b. Novel fucose-rich glycosaminoglycans from squid ink bearing repeating unit of trisaccharide structure (-6GalNAc alpha 1-3GlcA beta 1-3Fuc alpha 1-)n. Biochem. Biophys. Res. Commun. 198(2):560-567.

Wang, G. 2010a. Study of protective effects sepia ink polysaccharides on chemotherapy. M.D. Thesis, Guangdong Ocean University, Zhanjiang, China.

Wang, G., H. Z. Liu, J. L. Wu, Q. W. Zeng, Y. P. Chen, C. L. Yang and J. P. Zhong. 2010b. Study of sepia ink extract on protection from oxidative damage of cardiac muscle and brain tissue in mice. Chin. JMAP. 27(2):95-98.

Wang, G., Y. Z. Guo, S. B. Guan, J. P. Zhong, J. Q. Pan, Y. Huang and H. Z. Liu. 2009. Protective effect of sepia ink extract on cyclophosphamide-induced pulmonary fibrosis in mice. Chin. J. Mar. Drugs. 28(1):36-40.

Wang, Q., C. L. Lü, C. Lü, J. Liu, Y. M. Cao and J. Z. Yan. 2001. Study on the induction of IFN-γ and LAK cells activity in mice stimulated with sepia. Chin. J. Mar. Drugs. 20(1):37-39.

Xie, G. L. and S. He. 2001. Study about CSF activity induced by sepia in mice. Chin. J. Mar. Drugs. 20(3):25-27.

Zhang, Y. J., X. Y. Xu and B. Q. Wang. 2003. The influence of compound sepia capsules on SOD activity in mice. Chin. New. Med. 2(1):23-27.

Zheng, G. L., X. Y. Zhang, Y. G. Zhou, Q. C. Meng and W. G. Gong. 2002. Comparison between partial components and trace elements in sepia and squid ink. Chin. J. Mar. Drugs. 21(3):12-14.

Zhong, J. P., G. Wang, J. H. Shang, J. Q. Pan, K. Li, Y. Huang and H. Z. Liu. 2009. Protective effects of squid ink extract towards hemopoietic injuries induced by cyclophosphamide. Mar. Drugs 7(1):9-18.