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Estrogen in the Chick Embryo Plays a Role in Utilization of Plasma Lipids during Incubation

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An aromatase inhibitor, Fadrozole (CGS16949A), was administered into chick embryo on the 5th day of incubation to clarify the roles of endogenous estrogen in the regulation of circulating lipoprotein levels during the development. The plasma of 10-, 16- and 20-day embryos and neonatal chicks were separated by polyacrylamide gel electrophoresis. Amounts of plasma lipoproteins, high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) were then measured using a densitometer and expressed as a percentage in total lipoproteins. Changes in HDL percentages of the groups treated with the aromatase inhibitor (AI group) and propylene glycol (Vehicle group) showed similar patterns during the experimental period. This was also the case in the changes in LDL percentages. The 16-day embryos of the AI group, in which endogenous estrogen synthesis was inhibited, showed a distinctly high VLDL percentage, whereas plasma VLDL in the Vehicle group showed only a little developmental changes. The plasma concentration of triglyceride (a major component of VLDL), phospholipid and total cholesterol in the AI group showed a profile similar to that of VLDL, with the maximum level in the 16-day embryo. These results suggest that endogenous estrogen of the embryo plays an important role in the utilization and resulting clearance of plasma lipids from the circulation, in addition to its well-documented role in sex differentiation.

Key words : plasma lipoprotein, lipid utilization, chick embryo, estrogen, aromatase inhibitor

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Introduction

The chick embryo intensively utilizes lipids in the yolk as an essential nutrient from the 13th day of incubation to the hatch (Noble and Moore, 1964 ; Noble, 1987). The plasma estrogen concentration in this period is kept at higher levels than after the hatch in male as well as in female (Tanabe *et al.*, 1986). Excess amounts of extrinsic estrogen is known to affect lipid metabolism in the rat (Nishikawa *et al.*, 1984) and

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human (Walsh *et al.*, 1994 ; LaRosa, 1996 ; Tikkanen, 1996). Estrogen administration into chickens has been observed to stimulate syntheses of two components of plasma lipoprotein, apolipoprotein-B and apolipoprotein-II, in the liver and to stimulate secretion of very low density lipoprotein (VLDL) and vitellogenin from the liver. Exogenous estrogen is thus considered to increase lipid concentration in the circulation not only in the hen but also in the rooster and chick (Kudzma *et al.*, 1973, 1975, 1979 ; Luskey *et al.*, 1974 ; Chan *et al.*, 1976 ; Miller *et al.*, 1984 ; Gordon *et al.*, 1988 ; Park and Cho, 1988 ; Hermier *et al.*, 1996). In the chick embryo, plasma levels of apolipoprotein-B, a component of VLDL, have been reported to increase by the administration of exogenous estrogen (Nadin-Davis *et al.*, 1980 ; Schjeide *et al.*, 1980 ; Lazier *et al.*, 1994). However, no investigations have been conducted whether endogenous estrogen regulates lipid metabolism during the incubation. A dominant component of embryonic plasma lipoprotein changes from low density lipoprotein (LDL) to high density lipoprotein (HDL) between the 13th day of incubation and the hatch (Kelley *et al.*, 1980). This period coincides with a rapid increase period of estrogen (Woods and Brazzill, 1981). These reports provided a basis for our hypothesis that intrinsic estrogen had some contribution to the lipid metabolism in the chick embryo. In this study, we investigated the role of estrogen played in the lipid metabolism from the 10th day of incubation to the hatch using a specific inhibitor of aromatase.

Materials and Methods

Aromatase inhibitor

Fadrozole (CGS16949A, 4-(5,6,7,8- tetrahydrimidazo[1,5-a]pyridin-5-yl) benzonitrile monohydrochloride) was synthesized by the method described by Pento (1989) and used as a potent and specific aromatase inhibitor (Steele *et al.*, 1987 ; Hausler *et al.*, 1989 ; Bhatnagar *et al.*, 1990).

Treatments of eggs

Fertilized eggs of White Leghorn hens were incubated at 38±0.5℃ and 70±5% humidity. Our preliminary experiment revealed that Fadrozole should be injected before the 6th day of incubation to affect sex differentiation of gonads. According to this result, on the 5th day of incubation, 0.1 m*l* of saline containing 20% propylene glycol (Vehicle group) or 0.1 mg of Fadrozole (AI group) suspended in 0.1 m*l* saline containing 20% propylene glycol was injected into the eggs. Each of these solutions was injected through a small hole made with a 23 G needle at the pointed end of the egg. Shell holes were sealed with tapes and the treated eggs were further incubated until blood collection.

Blood collection and sex genotype determination

The blood was collected with a syringe rinsed with EDTA from the vitelline vessels of 10-, 16- and 20-day-old embryos and from the heart of neonatal chicks (7-13 eggs or chicks per group). Plasma was assayed for concentrations of lipids and lipoproteins immediately. Plasma aliquots were stored at −20℃ until analysis for estradiol concentration. After the blood collection, body weights of chicks and embryos were measured with and without yolk, respectively. Sex genotype of the embryos was

determined according to the method of Tone *et al.* (1982).

Estradiol assay

Estradiol was extracted from 50-400μl plasma with diethyl ether and measured by radioimmunoassay as described by Tanabe *et al.* (1986). The plasma obtained from embryos was pooled to extract sufficient amounts of estradiol for the assay.

Densitometric assay for plasma lipoproteins

Plasma was stained immediately after collection with Sudan Black B (saturated in ethylene glycol) for 30 minutes and electrophoresed according to the method of Dangerfield and Pratt (1970) to avoid oxidation of lipoproteins. Lipoproteins were separated on a polyacrylamide gel at 40 mA for 2 h in Tris-glycine buffer, pH 8.3. The polyacrylamide concentrations of the gel were 2% for a spacer and 2-8% gradient for a resolving gel. After the separation, HDL, LDL and VLDL were identified from their migration distances as described by Dangerfield and Pratt (1970). The optical densities at 610 nm were immediately measured and the percentage of each lipoprotein in total density of lipoproteins was calculated to standardize difference of total densities between gels.

Assays for triglyceride, phospholipid and total cholesterol in plasma.

Concentrations of triglyceride, phospholipid and total cholesterol in the plasma were measured by enzymatic methods using commercial kits (Wako Pure Chemical Co., Osaka).

Statistical analysis

The Student's *t* test was used to assess the significance of difference between two means. Percental data were arcsine-transformed before analysis.

Results

Plasma estradiol levels during development are shown in Table 1. Estradiol concentrations of the Vehicle group were more than 20-fold higher in embryos than in neonatal chicks of either sex. Estradiol concentrations in the AI group were much

Table 1. Plasma estradiol concentration (pg/ml) in embryo and neonatal chick

Age	Sex	Group			
		Vehicle	(n)	AI	(n)
(Embryo at)					
Day 16	male	138	(—)	27.3	(—)
	female	320	(—)	48.4	(—)
Day 20	male	208	(—)	3.80	(—)
	female	265	(—)	15.7	(—)
Neonatal	male	7.70±6.77	(6)	4.80±0.41*	(4)
chick	female	12.0 ±1.49	(4)	13.5 ±1.97	(6)

(n) : numbers of neonatal chicks.
(—) : pooled plasma.
* : $P<0.05$ vs. female.
AI : aromatase inhibitor.

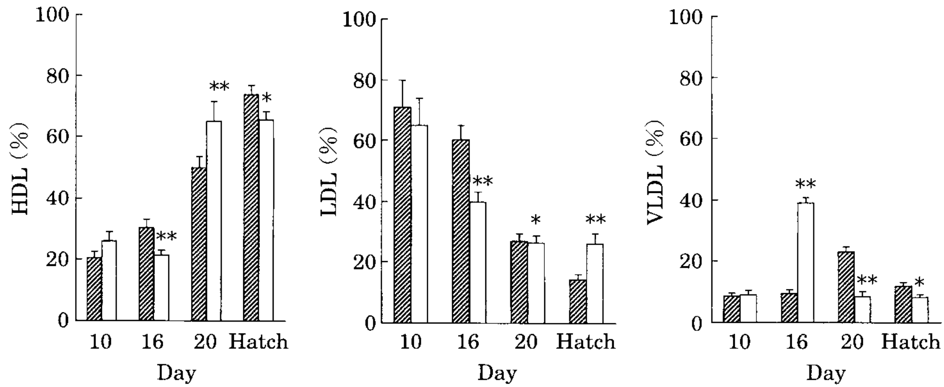

Fig. 1. Plasma HDL, LDL and VLDL ratios in total plasma lipoproteins of chick embryos and neonatal chicks. Hatched bars : Vehicle group, white bars : AI group. Means±SE (n=7–10). *P<0.05 and **P<0.01 by Student's *t* tests after arcsine transformation of percentage data.

lower than those in the Vehicle group at any age, indicating that Fadrozole inhibited aromatase sufficiently. In the AI group, the wet weights of the left and right gonads had no significant differences (data not shown), indicating that the dose of AI administered was sufficient to induce masculinization of the embryonic gonads.

Figure 1 shows developmental changes in the percentage of lipoproteins. Since data showed no significant differences between sexes, they are combined in the figure. The percentage of HDL increased and that of LDL decreased with age, regardless of the experimental group. The VLDL percentage remained almost unchanged during development, but was distinctly high in the AI group on the 16th day.

Table 2 shows developmental changes in plasma lipid concentrations, which increased to the maximum levels in 16-day embryo and tended to decrease with age. On and after the 16th day, lipid concentrations of the AI group were significantly higher or tended to be higher than those of Vehicle group. After the hatch, the lipid concentra-

Table 2. Plasma lipid concentration (mg/d*l*) in embryo and neonatal chick

Age	Group	(n)	Lipid		
			Phospholipid	Triglyceride	Total cholesterol
(Embryo at)	Vehicle	(9)	106±15	127±14	116±15
Day 10	AI	(10)	123±19	128±23	125±10
Day 16	Vehicle	(6)	228±17	229±13	238±18
	AI	(7)	281±14*	302±23*	311±17*
Day 20	Vehicle	(8)	166±18	106±11	224±20
	AI	(7)	211±25	157±17*	274±28
Neonatal chick	Vehicle	(10)	112±38	49.2±7.8	177±31
	AI	(10)	191±35	60.2±6.7	273±29*

Values are means±SE.
* : P<0.05 vs. Vehicle group.
(n) : numbers of embryos and neonatal chicks.
AI : aromatase inhibitor.

Table 3. Body weight (g) of embryo and neonatal chick

Age	Group			
	Vehicle	(n)	AI	(n)
(Embryo at)				
Day 10	1.87±0.040	(9)	1.91±0.030	(11)
Day 16	14.3 ±0.30	(9)	13.0 ±0.38*	(8)
Day 20	28.8 ±0.75	(9)	29.9 ±0.54	(10)
Neonatal chick	42.9 ±0.71	(9)	42.4 ±0.75	(13)

Values are means±SE.
* : $P<0.05$ vs. Vehicle group.
(n) : numbers of embryos and neonatal chicks.
AI : aromatase inhibitor.

tions decreased in all experimental groups : triglyceride levels decreased to 50% of the levels of 20-day embryos. These data showed no statistically significant sex differences.

Table 3 shows changes in body weights. Comparison between groups at the same age revealed that the body weight of 16-day embryos was significantly lighter in the AI group than in the Vehicle group.

Discussion

The high embryonic estradiol concentrations in male as well as in female of the Vehicle group confirmed the previous report by Tanabe *et al.* (1986), indicating that estrogen synthesis in both sexes during incubation is more active than after the hatch. A possibility therefore arises that embryonic estrogen has some role common to male and female other than the well-documented role as a sex hormone.

The increasing HDL and decreasing LDL percentages during the development of the Vehicle group were in accordance with the report by Kelley *et al.* (1980). Since VLDL contains a large amount of triglyceride, the significantly high VLDL percentage of the AI group on the 16th day is almost in accord with the result that triglyceride concentrations of the AI group were significantly higher than those of the Vehicle group in 16- and 20-day embryos. A significantly lower VLDL percentage than the Vehicle group was observed in 20-day embryos when estradiol (0.1 mg per egg) was administered instead of AI (data not shown), suggesting that the lower VLDL percentage of the AI group of 20-day embryos is not a specific effect of the AI treatment.

Plasma lipid concentrations, including those of triglyceride, of 16- and 20-day embryos, were significantly higher or tended to be higher in the AI group than in the Vehicle group, while this was not very distinct in 10-day embryos. These results suggest that AI was responsible for the increase of plasma lipid concentrations after the 10th day. In normal incubation, the lipid utilization and metabolism are active in embryos from the 13th day to the hatch (Noble and Moore 1964 ; Noble 1987) ; plasma estrogen concentrations increase at the same time (Woods and Brazzill, 1981 ; Tanabe *et al.*, 1986). It is, therefore, suggested that endogenous estrogen possibly contributes to the utilization and resulting clearance of plasma lipids after the 10th day.

This possibility could explain our result that the body weight of 16-day embryo in the AI group was significantly lighter than that in the Vehicle group. If the high plasma lipid concentration in 16-day embryos in the AI group is due to the blunting of plasma lipid uptake into the cells because of the deficiency of plasma estradiol, the body weight should be reduced, since embryos require a large amount of lipid for development. This possibility is also supported by Dewil *et al.* (1998), who reported that the wet weight of the abdominal fat pad decreased in 4-week chicks by administration of an aromatase inhibitor, Vorozole, on the 6th day of incubation. Dewil *et al.* (1998) attributed this decrease to a block of lipid accumulation in the abdomen.

From these results we concluded that endogenous estrogen in the embryonic plasma plays an important role in the utilization of lipids by the tissues and their clearance from the circulation, in addition to its well-documented role in sex differentiation. Further investigations will serve to clarify in more detail how the substance regulates lipid metabolism in chick embryos.

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