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Presence of Acute Phase Changes in Zinc, Iron, and Copper Metabolism in Turkey Embryos (42438)

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Abstract. Acute phase changes in trace mineral metabolism were examined in turkey embryos. An endotoxin injection resulted in increased concentrations of serum copper and liver zinc and decreased concentrations of serum zinc in embryos incubated either *in ovo* or *ex ovo*. Changes in zinc and copper metabolism occurred when endotoxin either was injected intramuscularly, into the amnionic fluid, or administered onto the chorioallantoic membrane. Unlike poults, embryos did not respond to an inflammatory challenge with decreased serum iron concentrations. Acute phase changes in embryo serum zinc and copper as well as liver zinc concentrations were similar to those in poults. Increased liver zinc concentrations were associated with increased zinc in metallothionein (MT). An injection of a crude interleukin 1 preparation into embryos resulted in similar increases in hepatic zinc and MT concentrations as an endotoxin injection, suggesting a role for this cytokine in mediating the acute phase changes in embryonic zinc metabolism.

Embryos and to some extent immature animals do not possess a fully active immune response. Emergence of immunocompetence is a gradual process with functions attributed to the reticuloendothelial system arising early in embryogenesis and functions attributed to lymphocytes emerging during late embryogenesis or thereafter (1–3). Aves are a good model for developmental studies because of easy access via the egg. In chick embryos, macrophage activity can be detected during the first week of incubation, with bacterial clearance being efficient by the second week (2). Specific immune functions such as antibody production and cell-mediated immune responses are not significantly active in chick embryos (3).

During the interval when the immune system is developing, a number of nonspecific defense mechanisms exist to protect the embryo (3). These mechanisms are particularly important in birds where maternal immunologic assistance in the form of newly synthesized antibodies is not available. These nonspecific factors include complement, interferon, phagocytosis, and passively acquired antibodies. In mature mammals additional nonspecific protection is provided by the metabolic events that follow an immunologic challenge. These changes are known as the acute phase response and include hepatic synthesis of acute phase proteins, alterations in trace mineral metabolism, fever, neutrophilia, and granulopoesis. Most of the various aspects of the acute phase response provide protection against bacterial and viral challenges or protect the animal against potentially toxic intermediates generated during an immune response (4–6). Little is known about the activity of the acute phase response in embryos; however, such protection would be especially valuable during this stage when specific (B- and T-cell) immune functions are suboptimal.

Changes in trace mineral metabolism are typical of acute phase responses and occur quickly after exposure of immunocompetent animals to a variety of bacteria, bacterial products, and other inflammatory agents. These changes are characterized by a redistribution of zinc from serum to liver, decreased serum iron, and increased serum Cu and ceruloplasmin (7–9). The altered trace mineral metabolism is mediated, at least in part, by interleukin 1 produced by macrophages (10). Many aspects of macrophage function are present in embryos; however, it is not known if embryonic macrophages are capable of initiating an acute phase response or if embryonic liver is capable of responding. Experiments were conducted to determine the presence of

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an acute phase response in embryos as represented by changes in trace mineral metabolism.

Materials and Methods. *Embryo culture.* Turkey (*Meleagris gallopavo*) embryos were cultured either *in ovo* or *ex ovo. In ovo* incubation was accomplished in a humidified, temperature-controlled (37.5°C) environment with frequent turning of the eggs. *Ex ovo* culture technique has been described in detail by Richards (11). Briefly, eggs were incubated for 4 days *in ovo* at which time embryos, yolk, and white were aseptically excised from the shell and placed in specially designed culture dishes. Incubation was continued in a humidified water-jacketed incubator at 37.5°C. These *ex ovo* cultured embryos develop normally for at least 18 days and provide easy access to the embryo as well as extraembryonic membranes.

Tissue sampling and analysis. Ex ovo cultured embryos were bled from the vitelline vessels. Poults and *in ovo* cultured embryos were bled from the heart. Livers were dissected from embryos, dried overnight at 110°C, and digested in nitric acid. Diluted serum and liver samples were analyzed for metal content by atomic absorption spectrophotometry. For metallothionein (MT) analysis, livers from embryos were pooled and homogenized (1:1) in 10 m*M* Tris, pH 8.6. Cytosols were prepared by centrifugation at 50,000*g* for 30 min. Cytosols were heat treated at 60°C for 10 min and recentrifuged before applying to a Sephadex G-75 column (16 × 650 mm) eluted with 10 m*M* Tris (pH 8.6) and collected in 5-ml fractions. The total concentration of zinc in the fractions containing MT (V_e/V_0 = 1.8–2.2) was used to quantitate total MT concentrations in each sample. The low molecular weight, Zn-containing peak was verified to be MT by rechromatographing on a Sephadex-DEAE anion-exchange column utilizing a 10–300 m*M* Tris continuous gradient (pH 8.6). Data were statistically analyzed by analysis of variance. Duncan's multiple range test was used to determine significant differences between means.

Interleukin 1 production. Production of turkey interleukin 1 (IL-1) was accomplished by methods similar to those described previously (8). Peritoneal monocytes were isolated from 4-week-old poults 24 h after an injection of Sephadex G-50 superfine. Monocytes were incubated in RPMI 1640 (GIBCO, Grand Island, N.Y.) with 5% fetal bovine serum, 50 μg/ml gentamicin sulfate (media), and 20 μg/ml endotoxin (*Escherichia coli* 0111:B4) for 4 h at 39°C and 5% CO_2. Cells were then washed three times and resuspended in fresh media without endotoxin for an additional 24 h. The conditioned media was concentrated on an Amicon PM10 ultrafiltration membrane and then passed through a PM30 membrane. The resulting 10,000–30,000 mol wt fraction was membrane sterilized and examined for mitogenic activity in a poult thymocyte comitogen assay with 4 μg/ml phytohemagglutinin-P (Difco, Detroit, Mich.). Ten microliters of this crude IL-1 preparation resulted in half-maximal proliferation of 5×10^6 poult thymocytes. Part of this preparation was heated at 90°C for 1 h and served as a heat-denatured control (heated IL-1). Heated IL-1 did not express any IL-1 activity as determined by induction of thymocyte mitogenesis. Another control was prepared by subjecting media to the same ultrafiltration steps as the conditioned media.

Experiment 1. The first experiment was designed to determine if trace mineral metabolism is altered in embryos after an inflammatory stress and to compare the response between *in ovo* and *ex ovo* embryo cultures. On Day 21, embryos incubated either *in ovo* or *ex ovo* were injected with either 0.1 ml of 0.9% saline (control), 0.25 μg endotoxin (*Escherichia coli:* 0111:B4)/0.1 ml 0.9% saline, or 2.5 μg endotoxin/0.1 ml 0.9% saline (15 embryos/treatment) directly under the chorioallantoic membrane (CAM).

Experiment 2. To determine the influence of route of exposure on inflammatory agents, embryos cultured *ex ovo* to 16 days of age were injected with 0.9% saline or 2.5 μg endotoxin/0.1 ml 0.9% saline into one of three locations: amnionic fluid, thigh muscles, or CAM. After 12 or 24 h, 14 embryos/treatment were bled via the vitelline vessels and livers were removed.

Experiment 3. A comparison of acute phase changes in trace mineral metabolism was made between embryos and young poults. Embryos cultured *ex ovo* to 16 days of age (15/treatment) were injected (im) with 0.1 ml and poults (10/treatment) with 1.0 ml of either 0.9% saline, 2.5 μg endotoxin/ml 0.9% saline,

or 5% Sephadex G-75 superfine in 0.9% saline (w/v). After treatments were administered, poults were fasted until the end of the experiment.

Experiment 4. A fourth experiment examined the possibility that liver zinc accumulates as MT in embryos injected with endotoxin and that IL-1 may be partially responsible for this induction. Embryos cultured to 16 days *ex ovo* were injected (im) with 0.1 ml of either 0.9% saline, 2.5 μg endotoxin/0.1 ml 0.9% saline, the crude IL-1 preparation, heated IL-1, or culture media treated in the same manner as that containing IL-1 (media). Fifteen embryos were injected per treatment and livers from 5 embryos were pooled for MT analysis.

Results. An injection of endotoxin beneath the CAM resulted in altered Zn and Cu metabolism in embryos incubated either *in ovo* or *ex ovo* (Table I). Added endotoxin resulted in significantly decreased concentrations of serum Zn and increased concentrations of serum Cu 24 h after injection beneath the CAM. Liver Zn concentrations were significantly increased by endotoxin; liver copper concentrations were not influenced. Neither the low (0.25 μg) nor high (2.5 μg) dose of endotoxin affected iron levels in serum or liver, though a trend (not significant) toward reduced serum iron was observed as a result of endotoxin treatment. The overall effect of endotoxin on trace mineral metabolism was similar in embryos incubated *in ovo* and *ex ovo;* therefore, *ex ovo* incubation was used in the following studies to provide easy access to the embryo and to extraembryonic membranes.

Experiment 2 was conducted to determine the effect of injection site on serum and liver trace mineral concentrations (Table II). When 2.5 μg endotoxin was applied on top of the CAM, zinc concentrations tended to be decreased in serum and were significantly increased in liver compared to saline-injected controls. Endotoxin application to the CAM did not influence iron or copper concentrations in serum or liver. Injection of endotoxin into amnionic fluid did not significantly affect serum zinc or copper concentrations at either 12 or 24 h postinjection, although at 24 h serum Zn concentrations tended to be depressed ($P \leq 0.1$). At 12 h postinjection, amnionic fluid administered endotoxin resulted in a significant increase in serum iron. After 24 h, liver zinc and copper concentrations were significantly increased. An intramuscular injection of endotoxin resulted in the largest changes in trace mineral metabolism of any injection site. Serum zinc was decreased by 24.2% at 12 h and serum copper was increased by 15.7% 24 h after injection im. Liver copper concentrations were increased by 43.4 and 46.7% at 12 and 24 h, respectively, and liver zinc concentrations were increased by 71% at 24 h post-im injection. Liver iron concentrations were not significantly changed at 12 or 24 h by endotoxin regardless of injection route.

Experiment 3 compared endotoxin and Sephadex-induced changes in trace mineral metabolism in four ages of turkeys: embryos at Day 16 of incubation (−12 days) and poults at 1, 10, and 42 days of age (Table III). Injections of either inflammatory agent resulted in decreased serum zinc at all ages, with the rel-

TABLE I. COMPARISON OF ENDOTOXIN-INDUCED CHANGES IN TRACE MINERAL METABOLISM IN EMBRYOS INCUBATED *IN OVO* VERSUS *EX OVO*

		Serum (μg/ml)			Liver (μg/g)		
Incubation	Treatment	Zn	Cu	Fe	Zn	Cu	Fe
In ovo	Control	1.30 ± 0.08^{a}	0.20 ± 0.01^{a}	2.10 ± 0.18	128 ± 7^{a}	35 ± 3	643 ± 25
In ovo	0.25 μg Endo	1.04 ± 0.06^{b}	$0.23 \pm 0.01^{a,b}$	1.82 ± 0.13	168 ± 14^{b}	33 ± 2	641 ± 52
In ovo	2.25 μg Endo	$1.07 \pm 0.06^{a,b}$	0.25 ± 0.01^{b}	1.94 ± 0.18	162 ± 8^{b}	39 ± 4	587 ± 55
Ex ovo	Control	1.20 ± 0.02^{a}	0.19 ± 0.01^{a}	1.97 ± 0.13	151 ± 3^{a}	27 ± 2	648 ± 26
Ex ovo	0.25 μg Endo	1.01 ± 0.06^{b}	0.23 ± 0.01^{b}	1.61 ± 0.13	193 ± 4^{b}	29 ± 2	632 ± 42
Ex ovo	2.25 μg Endo	0.91 ± 0.07^{b}	0.24 ± 0.01^{b}	1.68 ± 0.15	205 ± 6^{b}	28 ± 3	664 ± 41

Note. Embryos (21 day) were injected with treatments (Control, 0.9% saline; endotoxin (Endo)) beneath the chorioallentoic membrane 24 hr prior to bleeding and collecting livers. Data expressed as means ($n = 15$) ± SEM.

^{a,b} Means in a column, within incubation type, with different superscripts are significantly different ($P < 0.05$).

TABLE II. EFFECT OF SITE AND TIME OF INJECTION ON CHANGES IN TRACE MINERAL METABOLISM IN TURKEY EMBRYOS

			Serum (μg/ml)			Liver (μg/g)		
Treatment	Site[a]	Time[b]	Zn	Cu	Fe	Zn	Cu	Fe
Control	im	12	1.65 ± 0.09	0.17 ± 0.01	0.69 ± 0.06	143 ± 11	12.2 ± 0.6	354 ± 32
Endo	im	12	1.25 ± 0.09*	0.15 ± 0.01	0.77 ± 0.05	154 ± 11	18.1 ± 0.9*	345 ± 69
Control	AF	12	1.72 ± 0.11	0.18 ± 0.01	0.48 ± 0.03	142 ± 6	15.2 ± 0.6	299 ± 24
Endo	AF	12	1.66 ± 0.11	0.16 ± 0.01	0.78 ± 0.08*	156 ± 11	15.4 ± 0.4	268 ± 24
Control	im	24	1.52 ± 0.09	0.19 ± 0.01	0.42 ± 0.04	128 ± 7	12.2 ± 0.6	377 ± 62
Endo	im	24	1.30 ± 0.10	0.22 ± 0.01*	0.49 ± 0.03	219 ± 6*	17.9 ± 0.8*	342 ± 40
Control	AF	24	1.50 ± 0.07	0.17 ± 0.01	0.72 ± 0.09	127 ± 6	13.1 ± 0.6	395 ± 28
Endo	AF	24	1.30 ± 0.09	0.19 ± 0.01	0.58 ± 0.04	174 ± 13*	16.7 ± 0.4*	350 ± 32
Control	CAM	24	1.72 ± 0.13	0.18 ± 0.01	0.69 ± 0.08	122 ± 6	15.2 ± 0.6	395 ± 22
Endo	CAM	24	1.57 ± 0.08	0.18 ± 0.02	0.45 ± 0.07	170 ± 9*	17.4 ± 0.9	388 ± 28

[a] Embryos (16 days) were injected with either 0.9% saline (Control) or 2.5 μg endotoxin (Endo) into one of the following injection sites: im, muscle; AF, amnionic fluid; CAM, chorioallantoic membrane. Data expressed as means (n = 14) ± SEM.

[b] Hours post-injection.

* Significantly different from control value ($P < 0.05$).

ative size of the decrease being greatest at 1 day of age (a 46% decrease). Inflammation-induced decreases in serum zinc were similar in magnitude for embryos and poults at 1 and 10 days. Serum copper concentrations were markedly increased after exposure to inflammatory agents at all ages, although to a greater extent in the poults than the embryos. Endotoxin injection resulted in a significantly greater increase in serum copper than did Sephadex injection in 1- and 10-day poults. Increases in serum copper concentrations in embryos were of similar magnitude as in 42-day poults but smaller than in 1- and 10-day

TABLE III. EFFECT OF AGE ON TRACE MINERAL METABOLISM FOLLOWING AN INFLAMMATORY STRESS

		Serum (μg/ml)			Liver (μg/g)		
Treatment	Age (days)	Zn	Cu	Fe	Zn	Cu	Fe
Control	−12	1.94 ± 0.05[a]	0.16 ± 0.01[a]	0.63 ± 0.08[a]	123 ± 6[a]	15 ± 1	383 ± 17
Endotoxin	−12	1.47 ± 0.08[b]	0.23 ± 0.01[b]	1.10 ± 0.10[b]	215 ± 9[c]	17 ± 1	405 ± 25
Sephadex	−12	1.65 ± 0.08[b]	0.21 ± 0.01[b]	0.68 ± 0.10[a]	159 ± 10[b]	17 ± 1	375 ± 22
Control	1	2.00 ± 0.17[a]	0.17 ± 0.01[a]	2.41 ± 0.12[a]	95 ± 8[a]	34 ± 3	256 ± 17
Endotoxin	1	1.10 ± 0.15[b]	0.39 ± 0.02[b]	1.87 ± 0.26[b]	105 ± 9[a,b]	24 ± 3	217 ± 24
Sephadex	1	1.06 ± 0.12[b]	0.29 ± 0.03[c]	1.71 ± 0.26[b]	121 ± 11[b]	29 ± 3	244 ± 17
Control	10	2.96 ± 0.16[a]	0.17 ± 0.01[a]	1.04 ± 0.20	121 ± 6[a]	23 ± 1	197 ± 9
Endotoxin	10	2.09 ± 0.09[b]	0.41 ± 0.03[b]	0.88 ± 0.05	146 ± 4[b]	29 ± 4	233 ± 25
Sephadex	10	2.10 ± 0.06[b]	0.28 ± 0.02[c]	0.89 ± 0.15	143 ± 6[b]	23 ± 3	182 ± 9
Control	42	2.47 ± 0.13[a]	0.18 ± 0.02[a]	1.19 ± 0.11[a]	177 ± 9[a]	31 ± 5	266 ± 21
Endotoxin	42	1.74 ± 0.07[b]	0.37 ± 0.04[b]	0.80 ± 0.05[b]	201 ± 7[b]	30 ± 4	279 ± 15
Sephadex	42	1.88 ± 0.09[b]	0.24 ± 0.02[b]	1.04 ± 0.05[a]	211 ± 9[b]	31 ± 3	260 ± 27

Note. Poults (1, 10, and 42 days) and embryos (−12 days) were injected with treatments (Control, 0.9% saline; endotoxin, 2.5 μg; sephadex, 5%) 24 h prior to bleeding and removing livers. Data expressed as means ± SEM.

[a–c] Means within a column with different superscripts are significantly different ($P < 0.05$).

poults. Endotoxin caused decreased serum iron concentrations in 1- and 42-day poults but increased concentrations in embryos.

Endotoxin injection resulted in a significant increase in the concentration of zinc in embryonic liver (Table III), liver cytosol, and MT (Table IV). The crude IL-1 preparation resulted in even greater increases in zinc concentrations in liver cytosol and MT. Neither heated IL-1 nor media induced MT synthesis or increased cytosolic Zn.

Discussion. These experiments demonstrate that inflammatory agents such as endotoxin and Sephadex induce characteristic acute phase changes in zinc and copper metabolism of avian embryos. These changes can be detected in embryos incubated *in ovo* as well as in shell-less cultures and include decreased serum zinc, increased serum copper, and increased liver zinc and MT. Many reports have demonstrated similar acute phase changes in zinc and copper metabolism of adult or adolescent mammals (7, 9, 12, 13) and chicks (8, 14–17); however, there is little information on these changes in neonates and especially embryos. Several investigators have examined the hepatic synthesis of acute phase proteins and demonstrated that the neonatal liver is capable of increased synthesis of acute phase plasma proteins such as C-reactive protein, α2-macroglobulin, α1-acid glyoprotein, and haptoglobin (18–21).

Other aspects of the acute phase response are absent in neonates. Neonatal humans, lambs, rabbits, and guinea pigs do not develop fever in response to endotoxin as do juveniles and adults (22–24). Fever as well as many components of the acute phase response are mediated by the monokine IL-1 (endogenous pyrogen, leukocytic endogenous mediator). Blatteis (22) showed that the lack of fever development in neonatal guinea pigs is not due to the inability to produce IL-1 but rather from a lack of response to IL-1 at normal pyrogenic levels due to insufficient development of the central intrapreoptic fever-activating mechanism. Dinarello *et al.* (25) demonstrated that stimulated leukocytes from infants born after natural onset of labor produce adult levels of IL-1. Leukocytes from infants taken by Caesarean section and whose mothers had not experienced natural onset of labor produced substantially less IL-1, suggesting the inability of fetal leukocytes to produce IL-1. Although our experiments did not measure IL-1 production by embryonic leukocytes, our results indirectly indicate that IL-1 can be produced, as demonstrated by typical IL-1-induced changes in trace mineral metabolism in embryos injected with endotoxin, a potent stimulator of IL-1 release. Additionally, injection of the crude IL-1 preparation resulted in the induction of hepatic metallothionein and liver zinc accumulation, demonstrating the ability of embryos to respond to IL-1 in a manner similar to chicks (8).

Acute phase changes in iron metabolism differed between turkey embryos and poults (Experiment 3). Serum iron was significantly decreased in 1- and 42-day poults and tended to be decreased in poults at Day 10 but was significantly increased in embryos after endotoxin injection. We observed a high incidence of cranial bleeding and hemolysis in endotoxin-injected embryos. Neither pathological changes nor changes in serum iron

TABLE IV. EFFECT OF ENDOTOXIN AND A CRUDE PERPARATION OF INTERLEUKIN 1 ON CONCENTRATIONS OF ZINC IN LIVER CYTOSOL AND METALLOTHIONEIN OF TURKEY EMBRYOS

Treatment	Liver cytosolic zinc (μg/ml)	Metallothionein zinc (μg/ml cytosol)
Saline	2.60 ± 0.14^a	0.57 ± 0.15^a
Media	2.81 ± 0.09^a	0.66 ± 0.21^a
Heated IL-1	2.83 ± 0.18^a	0.69 ± 0.21^a
IL-1	4.97 ± 0.26^b	3.66 ± 0.69^b
Endotoxin	3.88 ± 0.29^c	2.34 ± 0.54^b

Note. Values represent the means ($n = 3$) $\pm$ SEM. Livers from five embryos were pooled for each analysis.
$^{a-c}$ Means within a column with different superscripts are significantly different ($P < 0.05$).

concentrations were seen in Sephadex-injected embryos. Thus, increased serum iron due to endotoxin may be a result of hemolysis and may not represent part of the nonspecific acute phase response.

During normal *in ovo* incubation, exposure of the embryo to microbes increases sharply during the last half of incubation when the expanding CAM contacts the shell membrane. The CAM-shell membrane area is a common site for the multiplication and dissemination of pathogens (26). Results of this study demonstrate that application of endotoxin to the CAM can result in a metabolic response in the embryo (Experiment 2). It is not known if the change in trace mineral metabolism is due to a local response to endotoxin at the CAM followed by release of a mediator, probably IL-1, into the circulation or if endotoxin enters the circulation and is responded to in the embryo. In our experiments, injection of endotoxin directly into the embryo results in the largest and quickest change in trace mineral metabolism; however, mononuclear phagocytes from mouse and human placenta release IL-1 (27).

The protection afforded to animals by acute phase changes in trace mineral metabolism is only partially characterized. A defense system termed nutritional immunity was proposed in which the host prevents the multiplication of bacteria in body fluids by preventing their acquisition of nutritionally required trace minerals (28). This system has been well characterized for iron which is tightly chelated in extracellular fluids by transferrin and lactoferrin. Iron is made less available during a bacterial challenge by decreased circulating concentrations. It is not clear if turkey embryos utilize this defense system because inflammatory agents that simulate a bacterial challenge do not result in decreased serum iron concentrations. However, serum iron levels in embryos are considerably lower than levels found in poults and a further decrease may not be necessary. Both embryos and poults limit the concentrations of zinc in circulating fluids. The evidence for nutritional immunity involving zinc is not well substantiated (29); however, zinc concentrations in the physiologic range inhibit phagocytosis by macrophages possibly by inhibiting adenosine triphosphate pyrophosphatase (30). Optimal phagocytosis in polymorphonuclear leukocytes occurs with slightly decreased levels of zinc (31). The increased acute phase serum copper concentrations in embryos observed in this report may be associated with ceruloplasmin as was shown in chicks (15) and mammals (7). Ceruloplasmin acts as an extracellular scavenger of superoxide radicals (32) and may serve to limit tissue damage that would result from the massive release of oxygen-free radicals by activated macrophages and neutrophils. Increased hepatic metallothionein may also serve a protective role to prevent tissue injury. Metallothionein is a very efficient scavenger of free hydroxyl radicals (33). Thus circulating ceruloplasmin and hepatic metallothionein may complement each other to dispose of oxygen-free radicals generated during the inflammatory response.

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