A Diabetic-Like Condition of Turkey Embryos Maintained in Shell-Less Culture (42867)

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Abstract. **Serum insulin concentration and pancreatic insulin content were determined for turkey embryos incubated *in ovo* and in long-term shell-less culture (*ex ovo*). Insulin was undetectable (<10 pg) in serum from 87% of the *ex ovo* embryos compared with their *in ovo* counterparts. This was evident at all incubation ages, although insulin was detectable in more of the *ex ovo* embryos on Day 24. Insulin increased in the embryos incubated *in ovo* from 122 (Day 15) to levels exceeding 2000 pg/ml at hatching. Total pancreatic insulin content was greater in the cultured embryos on Days 15, 17, and 22 compared with their *in ovo* counterparts. Serum glucose was significantly greater ($P < 0.05$) in the *ex ovo* embryos at all ages. In response to an infusion of L-arginine, serum insulin increased from 566 to 1256 pg/ml in the *in ovo* embryos, whereas no change was evident in the *ex ovo* embryos (233 vs 257 pg/ml). When embryos incubated *in ovo* were injected with insulin, a significant ($P < 0.05$) reduction of serum glucose was observed at 60 min after injection. Serum glucose concentrations remained elevated in the embryos incubated *ex ovo* despite the insulin injection. Liver glucose 6-phosphatase activity, assessed on Days 15 and 22 of incubation, was found to be significantly ($P < 0.05$) lower in the *ex ovo* embryos. Turkey embryos incubated in shell-less culture exhibited chronic hyperglycemia in concert with extremely low circulating levels of insulin. The pancreatic beta cells of these embryos were not responsive to arginine or elevated glucose. Taken together these findings suggest the occurrence of a diabetic-like condition in the *ex ovo* embryos. This defect in insulin secretion may, in part, be responsible for some of the developmental abnormalities characteristic of the turkey embryo cultured *ex ovo*.** [P.S.E.B.M. 1989, Vol 190]

Insulin has been shown to be present in both fertilized and unfertilized chicken eggs as well as in the tissues of the developing embryo as early as Day 2 of incubation (1, 2). This is well before pancreatic beta cells are evident (3). Since insulin receptors are present on Day 2 of incubation, a functional role for insulin during the early stages of chick embryogenesis has been suggested (4). Exogenous insulin has been shown to influence glycogen deposition, amino acid uptake, and plasma protein synthesis in the chick embryo yolk sac membrane, as well as in cultured heart and liver cells (5–8). Insulin may also be involved in tissue differentiation and organogenesis in addition to its role in metabolic regulation (9–12). At pharmacologic levels, exogenously administered insulin induced major anatomical aberrations in the developing chick embryo such as rumplessness, beak deformities, and micromelia, some of which resulted in embryonic death. A recent study (13) suggested that the teratogenic properties of exogenous insulin are mediated by the receptors for insulin-like growth factors, while the classic metabolic functions of insulin are controlled via its own receptor.

Richards (14) has described a technique for the long-term shell-less culture of turkey embryos. Survival rates of 90% through Day 24 of incubation are routinely attainable. However, several anatomical and physiologic aberrations were noted in these cultured embryos including: (i) a significant reduction in growth rate beginning on Day 18 of incubation; (ii) failure of proper elongation of the beak and abnormal cranial growth; (iii) edema; (iv) poor feathering; (v) hypocalcemia; and (vi) polycythemia. Because of the importance of insulin to normal embryonic development, this study was undertaken to compare the ontogeny of plasma and pancreatic insulin levels in turkey embryos incubated *in ovo* and *ex ovo*.

Materials and Methods

Embryo Culture. Turkey embryos were incubated *in ovo* or *ex ovo* as previously described by Richards (14). Briefly, fertile turkey eggs were incubated at 37.5°C

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and 84% relative humidity in a commercial incubator. On Day 4 of incubation, eggs to be used for shell-less embryo culture were removed from the incubator, washed with ethanol (70%), and air dried. The developing embryo and egg contents were then carefully removed from the shell and placed in sterile culture dishes. Culture dishes consisted of an 8-oz plastic container with a pocket of kitchen plastic wrap. The culture dishes were transferred to a standard tissue culture incubator and maintained at 37.5°C and 95% relative humidity. The *in ovo* group of embryos were obtained from the same set of eggs used for shell-less culture which were maintained in the commercial incubator throughout the study.

Blood and Tissue Collection. On designated days of incubation, blood samples were taken from *ex ovo* embryos by first carefully dissecting the vitelline blood vessels free of the overlaying chorioallantoic membrane. Using microscissors, a small incision was made in the exposed blood vessels and the blood collected into disposable glass pipets. Care was taken to avoid contamination of the blood samples with allantoic fluid. Blood was obtained from the *in ovo* embryos using the same technique except a "window" was made in the blunt portion of the egg, exposing the shell membrane. The shell membrane and chorioallantoic membranes were cut away, allowing access to the underlying vitelline blood vessels. Blood was placed in microcentrifuge tubes and allowed to clot. Serum obtained by centrifugation of the clotted blood was frozen (−20°C) for later analyses. Following blood sampling, embryos were killed by cervical dislocation for tissue removal. Livers from both groups were removed, weighed, and frozen (−20°C) prior to the determination of glucose 6-phosphatase enzyme activity. Pancreatic tissue from 22- and 24-day-old embryos was dissected from the surrounding duodenal loop using a dissecting microscope and weighed. On Days 15 and 17 of incubation, the entire duodenal loop containing pancreatic tissue was removed and weighed. In order to determine pancreatic insulin content, an extract of the tissue was prepared. Each embryonic pancreas or pancreas and duodenum combination was homogenized in ice-cold phosphate-buffered saline, pH 7.4 (0.25 ml/mg wet tissue). The homogenate was allowed to extract for 4 hr at 4°C, after which it was clarified by centrifugation. The supernatant was collected and frozen (−20°C) prior to determination of insulin content.

Insulin and L-Arginine Injections. Highly purified chicken insulin was obtained from Litron Laboratories (Rochester, NY) and diluted in sterile saline for injection. Injections were made into a chorioallantoic vessel on Day 24 of incubation using a 26-gauge needle. Insulin was injected at a dose of 0.25 units/kg body wt, based on previously published turkey embryo weights for Day 24 of incubation (14). This was equivalent to 150 and 250 ng of insulin in a 10-μl injection volume for *ex ovo* and *in ovo* embryos, respectively. Controls were treated with a similar volume of sterile saline. Blood samples were taken from the vitelline vessels as described above at 60 min following either saline or insulin treatment and the serum was frozen (−20°C) prior to analysis of glucose concentration.

An infusion of arginine was employed both *in ovo* and *ex ovo* on Day 24 of incubation to determine pancreatic sensitivity to a known secretagogue. L-Arginine-HCl (Sigma Chemical Co.), at a dose of 0.5 g/kg body wt in 50 μl of sterile saline, was infused into a chorioallantoic vessel using a 26-gauge needle. The control group of embryos received an equivalent volume of sterile saline. One hour after injection, blood samples were collected from the vitelline vessels and the serum was frozen (−20°C) prior to analysis of insulin concentration.

Serum and Tissue Analyses. Serum samples and pancreatic extracts were analyzed for insulin utilizing a homologous radioimmunoassay for chicken insulin (15). Serum glucose was determined by the glucose oxidase method (16). Glucose 6-phosphatase activity was determined by the method described by Harper (17).

Statistical Analysis. A nonparametric (binomial test for small sample size) test (18) was used to determine if the number of samples which had undetectable insulin concentrations were statistically different between *ex ovo* and *in ovo* on Days 15, 17, 22, and 24. The level of significance was established by utilizing Bonferroni's procedure for multiple comparisons (19). All other data were subjected to a one-way analysis of variance. The statistical significance of the difference between the means for *ex ovo* versus *in ovo* was determined using Student's *t* test (20).

Results

A majority of the turkey embryos incubated *ex ovo* exhibited extremely low serum insulin levels compared with their *in ovo* counterparts (Table I). In fact, insulin concentrations in these embryos were below the detection limit of the assay (10 pg/tube). The number of *ex ovo* embryos which were determined to have undetectable insulin levels compared with *in ovo* was significantly different on Day 15 ($P < 0.001$) and Day 17 ($P < 0.007$) (Table I). This pattern was not evident on Days 22 ($P > 0.039$) and 24 ($P > 0.219$). Serum insulin was detectable in most of the *in ovo* embryos sampled (Table I). On Day 24 of incubation, a slight increase in serum insulin was noted in the *ex ovo* embryos. Unfortunately, due to the high mortality past this stage of development, it was impossible to determine if serum insulin continued to increase during the final stages of incubation. Circulating levels of insulin remained fairly constant *in ovo* until just prior to hatching. A maximum concentration exceeding 2 ng/ml was observed on Day 24 of incubation, which is 3 to 4 days prior to hatching.

Table I. Comparison of Serum Insulin Levels in *Ex Ovo* and *In Ovo* Turkey Embryos during Incubation and Hatching

Stage of development	Insulin (pg/ml)	
Day 15		
Ex ovo	467 (1)	UD (13)*
In ovo	122 ± 25 (6)	UD (1)
Day 17		
Ex ovo		UD (12)**
In ovo	147 ± 52 (10)	UD (2)
Day 22		
Ex ovo	205 ± 170 (2)	UD (9)***
In ovo	266 ± 63 (13)	UD (2)
Day 24		
Ex ovo	261 ± 102 (3)	UD (4)****
In ovo	2998 ± 473 (12)	UD (1)
Pipping		
In ovo	759 ± 122 (7)	UD (0)
Hatched		
In ovo	1359 ± 420 (7)	UD (0)
Day 1 post-hatching	1117 ± 105 (8)	UD (0)

Note. Data expressed as mean ± SEM. Numbers in parentheses denote number of observations. UD, undetectable; serum insulin concentrations were below the sensitivity of the immunoassay. Minimum detectable amount = 10 pg insulin/tube. Nonparametric statistical test was utilized to determine level of significance. See Materials and Methods for description. Levels of significance were: * = $P < 0.001$, ** = $P < 0.007$, *** = $P < 0.039$, **** = $P < 0.219$.

A significant ($P < 0.05$) decrease in plasma insulin was noted in the *in ovo* embryos undergoing pipping or the initial stages of emerging from the egg (Table I). Serum insulin increased to a concentration exceeding 1 ng/ml immediately following hatching and remained at this level for 1 day after emerging from the egg.

Because of the difficulty in separating the pancreas from the duodenal loop and surrounding connective tissue, only pancreatic tissue weights from older embryos (viz., Day 22 of incubation to 1-day posthatching) were recorded and used in calculating pancreatic insulin concentration (Table II). Pancreatic weight was significantly ($P < 0.05$) greater for the *in ovo* than *ex ovo* embryos on Days 22 and 24 of incubation (Table II). Insulin content of the pancreas was greater for the *ex ovo* embryos on Days 15, 17, and 22 of incubation compared with their *in ovo* counterparts. Insulin concentration of the pancreas, expressed on a per milligram tissue basis, was significantly greater ($P < 0.05$) for the *ex ovo* embryos compared with those incubated *in ovo* on Days 22 and 24 of incubation. Pancreatic insulin concentration (μg/mg tissue) decreased dramatically at pipping in the *in ovo* embryos and remained constant during the sampling period. Total pancreatic insulin content declined in these embryos at the time of pipping. These findings may be related to the dramatic accretion of pancreatic tissue mass between Day 24 and hatching (Day 28) which occurred *in ovo* (Table II).

Serum glucose levels were found to be 1.4- to 2.4-fold greater ($P < 0.05$) in the *ex ovo* embryos compared with those *in ovo* at all stages of incubation (Fig. 1). In general, serum glucose concentration increased in both groups as incubation advanced. A 1.6-fold increase in serum glucose was observed *in ovo*, reaching concentrations in excess of 200 mg/dl at the time of pipping and remaining at this level through Day 1 posthatching.

In response to an L-arginine infusion *in ovo*, serum insulin was significantly increased ($P < 0.05$) by 2-fold at 60 min after treatment (Table III). The arginine infusion was without effect on serum insulin levels in the *ex ovo* group. As previously noted (Table I), a majority of the *ex ovo* serum samples had undetectable insulin concentrations. This pattern was still evident following L-arginine treatment.

The response of serum glucose to injected insulin for both *in ovo* and *ex ovo* embryos is summarized in Table IV. A significant ($P < 0.05$) decline in serum glucose was noted 60 min after injection of insulin into the *in ovo* embryos. There was a tendency for serum glucose to decrease in the *ex ovo* embryos; however, the decline did not achieve either statistical significance or normal *in ovo* levels. Elevated serum glucose levels (>300 mg/dl) were observed prior to and following insulin treatment of the *ex ovo* embryos.

Because the *ex ovo* embryos exhibited chronic hyperglycemia (Fig. 1), we decided to ascertain if differences in the gluconeogenic properties of the livers from *ex ovo* and *in ovo* embryos existed. Glucose 6-phosphatase activity would be expected to be elevated if glycogenolysis were occurring as well as gluconeogenesis. In the absence of insulin, this might be expected. To accomplish this, glucose 6-phosphatase activity was measured on Days 15 and 22 of incubation. Significant ($P < 0.05$) differences in the activity of this enzyme were noted between the groups (Table V). Glucose 6-phosphatase activity, expressed as units/g of tissue/hr, was significantly ($P < 0.05$) greater in the livers of the *in ovo* embryos compared with their *ex ovo* counterparts on Day 15 of incubation. This difference was even greater on Day 22 (210 vs 119 units/g/hr). No increase in activity of this enzyme occurred in the *ex ovo* livers between Days 15 and 22 of incubation, whereas a 60% increase in enzyme activity was noted during this time *in ovo*. Because of the significant ($P < 0.05$) differences in liver size between the two groups of embryos on both days of incubation, the *in ovo* vs *ex ovo* differences would be greater if expressed as total activity per liver (Table V).

Discussion

In recent years long-term shell-less culture of turkey and chicken embryos has been used to study physiologic and biochemical aspects of development (14, 21, 22). However, one of the problems encountered with this technique is that of growth retardation in the later stages of development. In addition, malformations become

Table II. Comparison of Pancreatic Insulin Content in *Ex Ovo* and *In Ovo* Turkey Embryos during Incubation and Hatching

Stage of development	*n*	Pancreas weight (mg)	Pancreas insulin content Total (μg)	Pancreas insulin content μg/mg tissue
Day 15				
Ex ovo	14	—	0.74 ± 0.24	—
In ovo	7	—	0.57 ± 0.09	—
Day 17				
Ex ovo	12	—	1.57 ± 0.22	—
In ovo	12	—	1.13 ± 0.03	—
Day 22				
Ex ovo	10	6.7 ± 0.6	35.66 ± 2.32	4.90 ± 0.57
In ovo	15	15.0 ± 1.2[a]	25.83 ± 4.13[a]	1.81 ± 0.29[a]
Day 24				
Ex ovo	7	12.3 ± 0.9	35.75 ± 9.70	2.81 ± 0.20
In ovo	13	21.1 ± 1.4[a]	42.38 ± 6.37	1.94 ± 0.21[a]
Pipping				
In ovo	7	87.5 ± 6.3	27.26 ± 7.69	0.32 ± 0.04
Hatched				
In ovo	7	80.3 ± 4.8	17.37 ± 4.34	0.22 ± 0.11
Day 1 posthatching	8	71.3 ± 3.9	14.50 ± 2.62	0.21 ± 0.09

Note. Data are expressed as mean ± SEM. *n*, number of observations.

[a] Denotes a statistically significant difference ($P < 0.05$) for the *ex ovo* vs *in ovo* comparison within each stage of development.

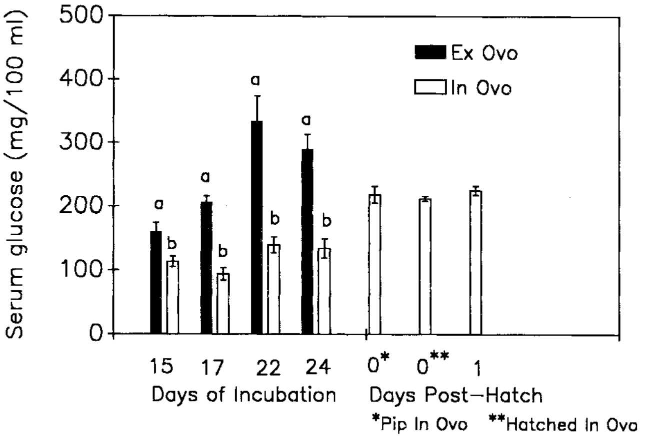

Figure 1. Comparison of serum glucose concentrations in *ex ovo* and *in ovo* turkey embryos during incubation and hatching. For each stage of development the number of observations for *ex ovo* and *in ovo* are as follows, respectively: Day 15 = 14 and 7, Day 17 = 12 and 12, Day 22 = 11 and 15, Day 24 = 7 and 13, Pip *in ovo* = 7, Hatched = 7, Day 1 posthatching = 8. a and b denote a statistically significant difference ($P < 0.05$) for the *ex ovo* vs *in ovo* comparison within each stage of development.

evident as the embryo develops (22). The etiology of these conditions is not readily evident and, indeed, is most likely multifaceted. Our study strongly suggests that a diabetic-like condition is present in the turkey embryos grown in shell-less culture. It is evident that *ex ovo* embryos exhibit hyperglycemia in concert with suppressed circulating insulin. This condition may be a contributory factor to the retarded growth and developmental abnormalities which occur in embryos cultured *ex ovo*.

Table III. Effect of Arginine on Serum Insulin Concentrations in *Ex Ovo* and *In Ovo* Day 24 Turkey Embryos Sampled 60 Min after Injection

Incubation group	Treatment group	Serum insulin (pg/ml)	
Ex ovo	Saline	232.9 ± 56.9	(3) UD (5)
	Arginine	257.2 ± 34.1	(2) UD (6)
In ovo	Saline	566.3 ± 78.2	(7) UD (1)
	Arginine	1256.5 ± 120.7[a]	(8) UD (0)

Note. Data are expressed as mean ± SEM. Numbers in parentheses indicate number of observations. UD, undetectable; serum insulin concentrations were below the sensitivity of the immunoassay. Minimum detectable amount = 10 pg insulin/tube. L-Arginine or saline were administered as described in Materials and Methods.

[a] Denotes a statistically significant difference ($P < 0.05$) for the comparison of saline vs arginine treatment groups within each incubation group.

Early studies have implicated the absence of the shell as a major cause of the growth retardation in cultured embryos, since approximately 80% of the calcium utilized by the embryo during incubation is derived from the shell (23–25). Clearly, calcium metabolism is altered in avian embryos incubated under *ex ovo* conditions (14, 21–28). Hypocalcemia develops in these embryos at about the time eggshell calcium reserves are normally utilized *in ovo* (23, 26, 27). It is known that both the synthesis and release of insulin are strongly calcium dependent (29). Observations in this study for embryos incubated *ex ovo*, including low serum insulin concentrations, the absence of a response of serum insulin to arginine, and elevated pancreatic insulin levels indicate that the release of insulin was affected. Arginine-induced insulin secretion has been shown to involve the influx of calcium into the beta cell (30). The absence of an insulinogenic response by the *ex ovo* embryos to arginine infusion suggests that the hypocalcemic state prevented the release of insulin. The synthesis of insulin was apparently unaffected since pancreatic content was greater in *ex ovo* compared with *in ovo* embryos. Together these results indicate an impairment of beta cell function. However, it is not possible to determine whether the diabetic-like state is the result of altered calcium metabolism or whether this condition contributes to abnormal calcium regulation which is characteristic of the diabetic state (31).

In addition to beta cell dysfunction, there is an indication of some degree of insulin resistance in the *ex ovo* embryo. Exogenous insulin failed to significantly lower circulating glucose in the *ex ovo* embryos (Table IV). This is a feature consistent with Type II diabetes.

Recently, it has been shown that copper deficiency can impair pancreatic function in rats (32). This may also be a factor in the etiology of the diabetic-like condition in the *ex ovo* turkey embryo as altered copper metabolism is also prevalent in shell-less embryos (33). Alternatively, it has been shown that streptozotocin-induced diabetes in rats causes abnormal copper metabolism (34). Whether the diabetic-like condition contributes to impaired copper metabolism or whether impaired copper metabolism affects pancreatic function remains unknown. Further studies are required to resolve this question.

Chick embryo hepatocytes cultured in the absence of insulin cease producing albumin (7). In the presence of insulin, the level of albumin mRNA increases, suggesting an effect of insulin on albumin gene expression (35). Cultured turkey embryos (14) and chick embryos (26) exhibit lowered levels of blood proteins. Our data suggest that in turkey embryos, the lack of adequate circulating insulin may be partly responsible for the reduced blood protein concentration.

It is unknown whether other endocrine functions are also compromised in the shell-less cultured turkey embryo. The role of hormones such as growth hormone and the somatomedins (insulin-like growth factors) in avian embryonic development and differentiation has not been extensively investigated (36, 37). Clearly, insulin is important for normal fetal growth and differentiation (38). Our study has demonstrated that normal pancreatic function is altered in the *ex ovo* turkey embryo. This defect may be in part responsible for the reduction in somatic growth in the later stages of incubation and contribute to the abnormal development (edema, poor feathering, beak deformities, etc.) commonly observed in avian embryos maintained in shell-less culture. The technique of shell-less culture may be a useful tool for investigations of the role of specific hormones in growth and differentiation of the avian embryo.

Table IV. Effect of Insulin Treatment on Serum Glucose Concentrations in *Ex Ovo* and *In Ovo* Day 24 Turkey Embryos

Incubation group	Treatment group	*n*	Serum glucose (mg/dl)
Ex ovo	Saline	8	333.7 ± 30.8
	Insulin	6	301.1 ± 15.8
In ovo	Saline	7	168.8 ± 9.0
	Insulin	7	122.9 ± 12.7[a]

Note. Data are expressed as mean ± SEM. *n*, number of observations. Chicken insulin or saline were injected as described in Materials and Methods.

[a] Denotes a statistically significant difference ($P < 0.05$) for the comparison of insulin vs saline treatment groups within each incubation group.

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Table V. Liver Glucose 6-Phosphatase Activity in *Ex Ovo* and *In Ovo* Turkey Embryos

Stage of development	*n*	Liver weight (mg)	Glucose 6-Phosphatase (units/g/hr)
Day 15			
Ex ovo	13	70.2 ± 2	115 ± 2
In ovo	10	92.5 ± 3[a]	125 ± 4[a]
Day 22			
Ex ovo	15	370.6 ± 15	119 ± 5
In ovo	10	552.8 ± 15[a]	210 ± 10[a]

Note. Data are expressed as mean ± SEM. *n*, number of observations.

[a] Denotes a statistically significant difference ($P < 0.05$) for the *ex ovo* vs *in ovo* comparison within each stage of development.

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