Steroid-Induced Short Term Diabetes in Chick Embryo: Reversible Effects of Insulin on Metabolic Changes and Cataract Formation

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Purpose. To determine the reversible effect of insulin on glucocorticoid (GC)-induced cataract formation in relation to systemic metabolic changes in the developing chick embryo.

Methods. Hydrocortisone sodium succinate (HC; 0.25 micromoles) was administered to 15-day-old embryos followed by administration of long-acting recombinant human insulin, 4 and 28 hours later. At the indicated time after HC administration, the incidence of cataractous lenses and any changes in the components of the lenses, liver, and blood were determined.

Results. At 48 hours after HC administration, the following observations were made: opacification of lenses; an elevation of glucose and lipids in the blood and lenses; an increase in lipid peroxide (LPO) in the blood, liver, and lenses; a decrease in glutathione (GSH) in the lens and liver (at 24 hours after HC administration); and a depletion of adenosine triphosphate (ATP) in the liver. These changes in response to HC administration were reversed by a double application of insulin.

Conclusions. Insulin antagonizes GC-induced gluconeogenesis, stimulates glycolysis, and ultimately leads to recovery of decreased activity in the citric acid cycle. The restoration of ATP by the recovered citric acid cycle may facilitate de novo synthesis of GSH, which in turn may diminish GC-induced elevation of LPO in the liver. Thus, the metabolic changes in response to HC-accelerated gluconeogenesis in the liver, which can be reversed by insulin, are likely to produce oxidative stress that leads to cataract formation. GC-induced metabolic changes in the liver, which are antagonized by insulin, may relate to production of one of the risk factors for cataract formation. (*Invest Ophthalmol Vis Sci.* 2000;41:1846–1852)

Glucocorticoids (GC) play an important role physiologically and have been widely used as valuable therapeutic agents for various diseases. However, high doses and long-term therapy with GC is well known to have adverse effects. For example, in addition to the appearance of GC on tissues that have GC receptors, some of these changes are observed in nontarget tissues for GC and are probably produced by changes in blood components, such as hyperglycemia and hyperlipidemia that result from the effects of GC on the main target tissue, liver.

Steroid-induced cataracts were first documented by Black et al.[1] and were reported in subsequent studies to occur in patients with rheumatoid arthritis, nephrosis, and systemic lupus erythematosus and in organ transplant recipients treated with GC.[2–4] Lorand et al.[5] reported that transglutaminase activity in cataractous lenses from GC-treated patients was higher than that observed in clear lenses. However, no other studies investigating the mechanism of cataract formation in humans have been reported, because it is usually difficult and often impossible to obtain human lenses for analysis. Very few animal model studies have investigated the underlying mechanism of steroid-induced cataracts. Despite this, Manabe et al.[6] and Bucala et al.[7] demonstrated that the formation of a Schiff base through the amine of lens protein and the C-20 carbonyl of corticoids, followed by a Heyns rearrangement with the C-21 hydroxyl, was involved in cataract formation.

We have demonstrated that steroid-induced cataracts are produced by the biologic activity of GC and not by the chemical formation of the Schiff base.[8] We have also shown that no differences exist in transglutaminase activity between clear and cataractous lenses (unpublished data, 1983). In contrast, we observed a loss of lens due to oxidative stress that was indirectly affected by an elevation of blood lipid peroxide (LPO), which was in turn due to an imbalance of redox functions in the liver after hydrocortisone sodium succinate (HC) treatment.[9–11] Specifically, glutathione (GSH) in the liver decreased until it was approximately 50% of control levels at 24 hours, whereas LPO levels in the liver and blood increased sharply from 20 hours until they were 8 to 10 times that of control levels at 30 to 48 hours after HC administration. In terms of timing, the decrease in GSH and increase in LPO in the lenses were observed 48 hours after HC was administered to a level that elicited maximum opacity. Furthermore, the administration of radical scavengers such as ascorbic acid,[12] pyrroloquinoline quinone (PQQ),[13] and sulfhydryl compounds[14,15] effectively prevents the HC-induced phenomena that we have

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described. Moreover, among intermediates of the citric acid cycle, isocitrate has shown the most potent and similar protection against HC-induced events, including cataract formation.[10] As expected, hyperglycemia was observed in HC-treated chick embryos, but the formation of sorbitol and glycation in the opaque lens was not detectable, suggesting that the osmotic theory of accumulation of polyol[16–18] and the glycation of protein theory[18] are not active in our cataract model.[8]

Based on the these observations, our cataract model was found to be similar to most other animal cataract models,[19,20] in that cataract is probably caused by oxidative stress that is induced by accelerated-gluconeogenesis and a change in metabolic activities in the citric acid cycle of the liver, which is the main target tissue of GC.[10]

In the present study, we attempted to clarify whether insulin prevents cataract formation through an improvement of hepatic gluconeogenesis, in light of the knowledge that GC and insulin possess antagonistic activities in gluconeogenesis in mammals. This improvement involves a restoration of the imbalance of redox function, LPO production, and a reversal of decrease in adenosine triphosphate (ATP) and GSH levels after GC administration.

Materials and Methods

Chemicals

HC was obtained from Sigma (St. Louis, MO). Long-acting human recombinant insulin (Novolin R U40; biological half-life 24–48 hours) was obtained from Novo Nordisk (Bagsværd, Denmark). All other reagents were of analytical grade.

Treatment of Animals

Chick embryos were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The procedure used to produce cataracts in developing chick embryos by HC has been described elsewhere.[10,21]

Preparation and Administration of Insulin

Insulin was dissolved in vehicle (17 mM sodium acetate buffer [pH 7.0] containing 0.12 M NaCl and 15 mM $ZnCl_2$), and 0.4 ml of the solution containing 2.5 to 10 units of insulin was administered at 4 and 28 hours after HC administration in the same manner as HC.

Classification of Lenses

Lenses were removed from chick embryos at 48 hours after HC administration, and visually classified as described previously, except that stages IV and V were not separated.[12]

Determination of Glucose in Blood and Lens

Blood was centrifuged at 2000*g* for 15 minutes at 4°C, and serum was collected. A 10-μl aliquot of the serum was used for the determination. Ten lenses were homogenized in 0.25 ml of redistilled water by a sonicator (Handy Sonic model UR-20P; Tomy Seiko, Tokyo, Japan) and centrifuged at 15,000*g* for 10 minutes at 4°C. After centrifugation, 10 μl of the supernatant was used for determination. Glucose concentrations were determined by a test kit (Glucose-C; Wako, Osaka, Japan) based on the method of Miwa et al.[22]

Analysis of Ketone Bodies in Blood and Allantoic Fluid

Blood and allantoic fluid were both centrifuged at 2000*g* for 15 minutes at 4°C. A 10-μl aliquot of the supernatant was blotted onto test paper for determination of ketone bodies and classified into six grades (from − to 4+) according to the manufacturer's protocol: −, not detected; ±, 5 mg/dl; +, 15 mg/dl; 2+, 40 mg/dl; 3+, 80 mg/dl; 4+, 160 mg/dl. Acetoacetic acid was used as the positive control.[23]

Determination of GSH in Lens and Liver

The method of Ellmann's reagent was used for determining GSH in the lens and liver.[24] Nonprotein sulfhydryl residue levels in chick embryo lenses, as well as in other animal lenses, can be derived mostly from the amount of glutathione present, whereas 80% of nonprotein sulfhydryl residues in the liver are glutathione, determined by the glyoxal I-methylglyoxalase method.[14] In the present study, GSH was used instead of nonprotein sulfhydryl residues.

Determination of ATP in the Liver

Livers were homogenized in two volumes of PBS by a sonicator (Handy Sonic model UR-20P). Three volumes of 12% trichloroacetic acid (TCA) were added to the liver homogenate. After a centrifugation at 15,000*g* for 10 minutes at 4°C, 0.25 ml of the supernatant was used for determination of ATP by an enzymatic method using an ATP kit (Sigma), based on the reactions described by Bucher[25] as modified by Adams.[26]

Determination of Lipids in Blood and Lens

Eight lenses from four embryos were isolated and homogenized in a test tube containing 0.08 ml phosphate-buffered saline (PBS; pH 7.4) by a microhomogenizer (Micro Multi Mixer C; Ieda, Tokyo, Japan), and centrifuged at 15,000*g* for 10 minutes at 4°C. After centrifugation, the supernatant was collected and used for assay. Serum and lenticular lipids were determined by enzymatic tests (Triglyceride [TG] E-Test, Nonesterified Fatty Acid [NEFA] C-Test, and Cholesterol [T-Chol]) E-Test, all kits from Wako).

Determination of LPO, a Thiobarbituric Acid–Reacting Substance, in Lens, Blood, and Liver

After four lenses from four embryos had been homogenized in redistilled water by a microhomogenizer, the amount of LPO in the homogenate was determined fluorometrically with thiobarbituric acid by a modification of Yagi's method, as described previously.[9] A solution of 1,1,3,3-tetraethoxypropane, which quantitatively produces malondialdehyde, was used as a standard.

Determination of Serum Insulin

Serum insulin was determined as an immunoreactive insulin (IRI) using a radioimmunoassay kit (Phadeseph; Pharmacia & Upjohn, Uppsala, Sweden).

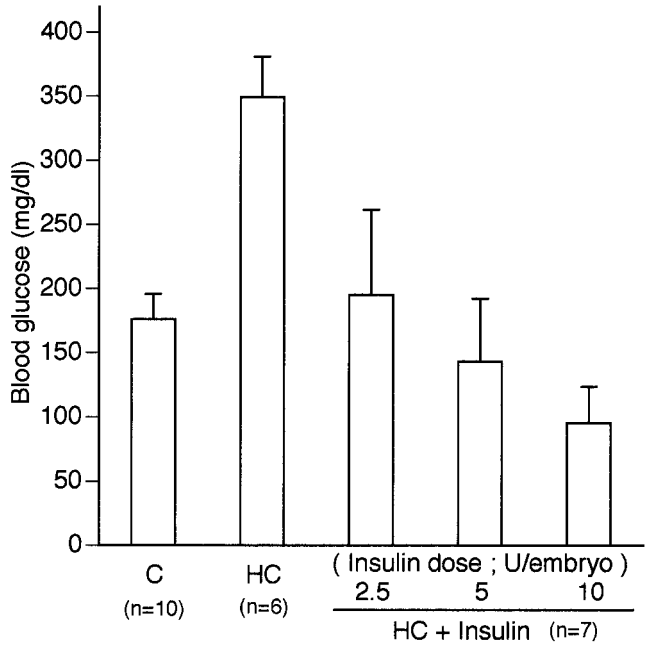

FIGURE 1. Blood glucose levels in HC-treated chick embryos after insulin administration. HC (0.25 micromoles/embryo) was administered to 15-day-old chick embryos. Insulin was administered at 4 and 28 hours after HC treatment, and was collected at 48 hours. Data are shown as means ± SD (n = 6–10). C, Control; HC, HC alone; HC + Insulin, insulin was administered twice after HC treatment.

Statistical Analysis

For determination of the lens components, a lens from one animal was used unless otherwise stated. For all experiments, an unpaired Student's t-test was used. Levels of statistical significance were set at $P < 0.05$.

RESULTS

Glucose and IRI Levels in Serum

To determine the effective doses of insulin, we first measured serum glucose (Fig. 1). The serum glucose level at 48 hours after HC administration increased to approximately two times that of controls. However, a double application of insulin after HC administration suppressed the serum glucose levels in a dose-dependent manner.

Serum insulin (IRI) levels were also significantly increased at 48 hours after HC administration (control 5.9 ± 1.6 μU/ml versus HC 22.1 ± 9.2 μU/ml; $P < 0.005$). The normal range of IRI levels in human serum were observed to be 9.4 ± 4.0 μU/ml by this method.

TABLE 2. Incidence of Cataractous Lenses in HC-Treated Developing Chick Embryos after Insulin Administration

	Stage of Lenses at 48 hours after HC Treatment			
	I	II	III	IV–V
Control	78/78 (100)	0/78 (0)	0/78 (0)	0/78 (0)
Insulin	49/49 (100)	0/49 (0)	0/49 (0)	0/49 (0)
HC	0/50 (0)	0/50 (0)	4/50 (8)	46/50 (92)
+Ins 2.5 U	4/8 (50)	0/8 (0)	1/8 (12.5)	3/8 (37.5)
+Ins 5.0 U	5/8 (62.5)	2/8 (25)	0/8 (0)	1/8 (12.5)
+Ins 10 U	51/59 (86.4)	1/59 (1.7)	4/59 (6.8)	3/59 (5.1)

Data are the number of embryos with percentages in parentheses.

Ketone Bodies in Blood and Allantoic Fluid

As shown in Table 1, when 15-day-old chick embryos were treated with HC for 48 hours, ketone bodies were detected in their serum and allantoic fluid but were not detected in either fluid from embryos treated with a double application of insulin after HC administration.

Incidence of Cataractous Lenses after Insulin Administration

When 15-day-old chick embryos were treated with HC for 48 hours, almost all the lenses (92%) were classified as stage IV–V (Table 2) A double application of insulin after HC administration effectively prevented HC-induced cataract formation in a dose-dependent manner. In particular, at 10 units insulin more than 85% of lenses cleared.

Effect of Insulin on Glucose and GSH Levels in the Lens

As shown in Figure 2A, HC-induced cataractous lenses (stage IV–V) contained glucose at a much higher concentration than control lenses, as described previously.[8,27] However, the amount of lenticular glucose in the transparent lenses (stage I) obtained from embryos treated with HC and insulin, remained at approximately the level in controls. As shown in Figure 2B, the stage IV–V lenses with HC-treatment alone had approximately 60% of the control level of GSH, whereas the stage I lenses with insulin administration showed almost the same level as the controls.

TABLE 1. The Stick Ketone Body Test in Serum and Allantoic Fluid Obtained from Control, HC-Treated, and HC-Insulin–Treated Chick Embryos

Ketone Body (mg/dl)	Serum			Allantoic Fluid		
	C	HC	HC+Ins	C	HC	HC+Ins
− (0)	7/7	1/8	7/7	7/7	3/8	7/7
± (5)	0/7	3/8	0/7	0/7	5/8	0/7
+ (15)	0/7	3/8	0/7	0/7	0/8	0/7
2+ (40)	0/7	1/8	0/7	0/7	0/8	0/7

Data are the number of embryos.

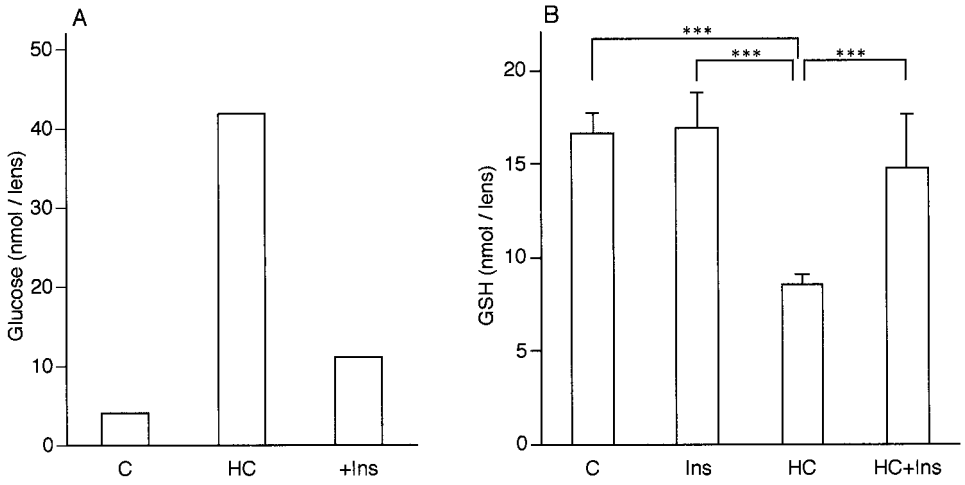

FIGURE 2. Effect of insulin on glucose and GSH levels in the lens. (**A**) Blood glucose and (**B**) GSH level in the lens after insulin treatment. Ten lenses were pooled in each group and were used for determination of glucose. C, control; HC, HC alone; +Ins, HC with insulin; Ins, insulin. Glucose amount shown as nanomoles per lens.

Effect of Insulin on GSH and ATP Levels in the Liver

The effect of insulin administration on GSH levels in the liver at 24 hours after HC treatment is shown in Figure 3A. The level of hepatic GSH was approximately 60% of the matched control level after HC treatment. However, insulin prevented the decline of hepatic GSH caused by HC.

ATP levels in the liver obtained from an HC-treated embryo with stage IV-V lenses were also approximately 60% of the control levels, whereas that in the liver obtained from insulin-treated embryo with stage I lenses had recovered to almost control levels by 48 hours after HC treatment (Fig. 3B).

Effect of Insulin on Lipids in the Serum and the Lens

During HC-induced cataract formation, we also observed not only hyperglycemia but also hyperlipidemia as an adverse effect of HC in this model. Thus, we investigated the effect of insulin administration on hyperlipidemia caused by HC. As shown in Table 3, when HC was administered to 15-day-old chick embryo, the levels of serum TG, NEFA, and T-Chol were found to have increased at 48 hours after HC treatment. However, a double application of insulin after HC administration effectively suppressed HC-induced hyperlipidemia.

The change in the content of TG and NEFA in the lenses after HC treatment with or without insulin administration was examined. As shown in Table 3, when chick embryos were given HC, the content of TG in the lenses increased to 1.8 times that of controls, whereas those of NEFA increased to 3.4 times that of controls. However, a double application of insulin after HC treatment suppressed the elevation of TG and NEFA caused by the HC treatment.

Preventive Effect of Insulin on HC-Induced Elevation of LPO

The preventive effect of insulin on the elevation of LPO caused by HC is shown in Table 4. As previously reported,[9,28,29] the levels of LPO in the lens, blood, and liver of chick embryos at 48 hours after HC treatment were approximately 1.3, 5.0, and 6.2 times that of the matched controls, respectively. However, a double application of insulin after HC administration effectively suppressed HC-induced LPO production in those tissues.

DISCUSSION

HC administration was found to produce short-term diabetes in developing chick embryos with cataracts. In particular, HC

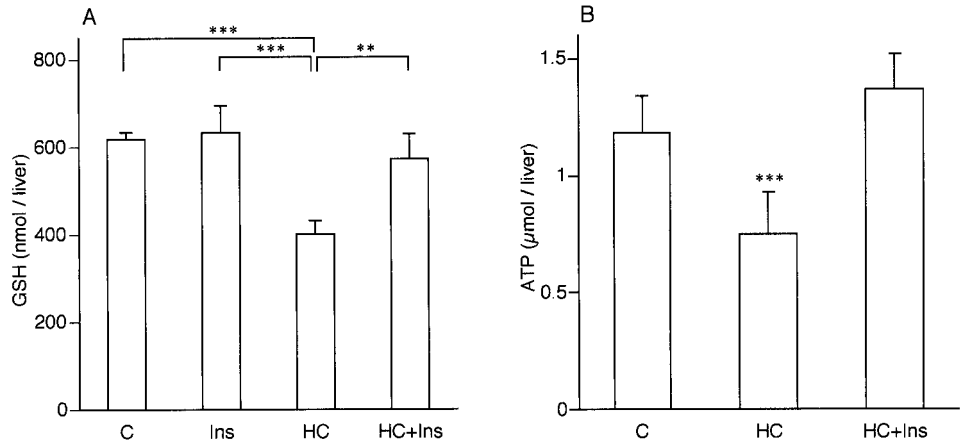

FIGURE 3. Effect of insulin on GSH and ATP levels in the liver. (**A**) GSH levels at 24 hours and (**B**) ATP levels at 48 hours after HC administration. The lens and liver were obtained from control, insulin-treated, HC-treated, and HC-insulin treated chick embryos. C, Control; Ins, insulin alone; HC, HC alone; HC + Ins, HC with insulin. $^{***}P < 0.001$ vs. control (Student's *t*-test).

Table 3. Effect of Insulin on Serum and Lenticular Lipids of HC-Treated Developing Chick Embryos

	Control	HC	HC+Insulin
Serum lipids			
TG (mg/ml)	2.6 ± 0.4 (100)	13.9 ± 4.9 (534)*	3.0 ± 1.3 (115)
NEFA (μEq/ml)	0.34 ± 0.08 (100)	0.73 ± 0.08 (215)†	0.35 ± 0.10 (103)
T-Chol (mg/ml)	3.4 ± 0.5 (100)	5.6 ± 0.5 (165)†	2.7 ± 0.6 (79)
Lenticular lipids			
TG (μg/lens)	9.4 ± 1.4 (100)	17.0 ± 2.5 (181)‡	11.4 ± 0.6 (121)
NEFA (nEq/lens)	1.1 ± 0.1 (100)	3.7 ± 1.5 (336)§	2.1 ± 0.4 (191)

Data are means ± SD with percentages in parentheses. Serum lipids, $n = 5$; lenticular lipids, $n = 3$.
* $P < 0.001$; † $P < 0.0001$; ‡ $P < 0.005$; § $P < 0.05$ vs. control; Student's *t*-test.

increased glucose and ketone bodies in blood and allantoic fluid and lipids in blood of the embryos. However, these changes were reversed by insulin administration, and application of insulin effectively prevented cataract formation through a suppression of the decline in both hepatic and lenticular GSH levels, the decline in hepatic ATP pool, and the elevation of LPO in the liver, blood, and lens after HC administration. Thus, these results demonstrate that the biological actions of GC related to cataract formation were antagonized by insulin. In the present model, GC-induced cataractous lenses represent an alteration of lens components: an elevation of glucose, a decline in GSH, and an elevation of LPO.

Glucose in the Lens

Several hypotheses have been made concerning the mechanism of sugar cataract formation, including an accumulation of polyol[16–18] and a formation of glycation between glucose and protein.[18] None of these hypotheses was supported by our findings, given that no sorbitol, fructose, or glycation of protein was detected in either control or HC-induced cataractous lenses obtained 48 hours after HC administration.[8] Because sugar cataract was caused by HC-induced hyperglycemia, it appears likely that the decrease in glucose in the lens by insulin acted as a direct trigger to prevent HC-induced cataract formation. However, the transparent stage I lenses obtained from embryos treated with HC and agents possessing anticataract activity such as isocitrate,[10] propylene glycol,[30] and ascorbic acid[12] generally but not always further decreased the amount of glucose compared with stage IV–V lenses treated with HC alone. Thus, our results and those of previous reports[10,12,30] failed to demonstrate direct correlation between high glucose levels and loss of lens transparency.

GSH and LPO in the Lens

A decline in GSH levels has been observed during formation of most cataracts. However, in developing chick embryos treated with buthionine sulfoximine (an inhibitor of GSH synthesis), although the levels of GSH in lenses were much lower than those in HC-treated embryos with severe cataracts, the lenses were clear, and the amount of LPO did not increase.[31] One of the mechanisms proposed for cataract formation is that oxidative changes in the lens leading to GSH consumption may be involved in the loss of lens transparency.[19] As described in the Results section, insulin prevented the elevation of LPO and formation of HC-induced cataracts. The same results were obtained when agents possessing anticataract activity were used.[9,10,12] Because the marked elevation of LPO in the blood and liver after HC administration is suppressed by VC[9] and isocitrate,[10] we speculated that LPO produced in the liver flows through the blood stream and aqueous humor to reach the lens, and ultimately leads to a loss of lens transparency and consumption of GSH. Thus, we concluded that the regulation of LPO production in the liver is very important for clarifying the mechanism of GC-induced adverse effects, including cataract formation.

Gluconeogenesis, GSH, and LPO in the Liver

Insulin treatment after HC administration effectively prevented the induced decline of GSH and elevation of LPO in the liver and the elevation of LPO and glucose in the blood. We have suggested that LPO synthesis occurred at an accelerated rate in the liver of HC-treated chick embryos, given the decrease in the superoxide dismutase, catalase, and glutathione peroxidase activities and the increase in hydroxidase activity in the liver.[11] The present results assumed that the stimulation of gluconeogenesis and its associated metabolic changes in the liver of chick embryos by HC involve a mechanism similar to that seen in mammals, and may have produced an imbalance of redox functions that induced a decrease in GSH and an overproduction of LPO as a result of oxidative stress in the liver.

Gluconeogenesis by GC is demonstrated by inducing glucose-6-phosphatase, fructose-1,6-bisphosphatase, pyruvate calboxylase, and phosphoenolpyruvate carboxykinase.[32] These metabolic changes suggest that the amount of oxaloacetate in

Table 4. Preventive Effect of Insulin on the Elevation of LPO Caused by HC Treatment

	Control	Insulin	HC	HC+Insulin
Liver (nmol/liver)	142.9 ± 28.6	171.4 ± 108.2	884.6 ± 131.6*	294.0 ± 75.0
Blood (nmol/ml)	4.8 ± 1.3	6.0 ± 0.9	24.2 ± 5.9*	5.3 ± 1.4
Lens (pmol/lens)	41.6 ± 3.1	NT	54.8 ± 4.7†	43.8 ± 6.5

Data are presented as mean ± SD ($n = 4–6$). NT, not tested.
* $P < 0.0001$; † $P = 0.0002$ vs. control; Student's *t*-test.

the liver decreased as phosphoenolpyruvate carboxykinase converted oxaloacetate to phosphoenol pyruvate. In fact, Agius et al.[33] demonstrated that dexamethasone (one of the potent derivatives of glucocorticoids) decreases the reduced nicotine adenine dinucleotide (NADH)/ nicotine adenine dinucleotide (NAD) ratio that acts as an indicator of a mitochondria redox state in hepatoma cells. Accordingly, the lower levels of oxaloacetate may decrease the metabolic activities of the citric acid cycle, leading to a decline in the NADH/NAD ratio, which in turn reduces ATP production through a respiratory chain reaction in the liver. These metabolic changes seem to take place in chick embryos, evidenced by our observation of the decline of the hepatic ATP pool after HC administration in this study.

In contrast, HC administration elevated ketone bodies in addition to hyperlipidemia and elevated NEFA, TG, and T-Chol, a finding that has been observed in chick embryos as well as mammals. Accordingly, the production of ATP was probably due to β-oxidation of fatty acids. However, in a recent study Letteron et al.[34] reported that GC inhibited β-oxidation of fatty acids in the livers of mice. In the present study, we also observed a decline in the hepatic ATP pool after HC administration (Fig. 3B). This depletion appears to have been the result of HC-induced systemic changes in glucose and lipid metabolism, as mentioned above.

De novo synthesis of 1 mole glutathione requires 2 mole ATP and 1 mole each glutamic acid, cysteine, and glycine. Although glutathione biosynthesis is influenced by various other factors, ATP is one of the important factors in the liver. In our experiment, a double application of insulin after HC administration prevented cataract formation and promoted the recovery of ATP depletion in the liver. We postulate that the recovery of ATP depletion may facilitate de novo synthesis of glutathione consumed by the scavenging of reactive oxide substances such as LPO in the liver.

However, the biological activity of GC is complex and not well understood. Therefore, it is impossible to draw conclusions based on our limited results, given the absence of any direct evidence. Based on the present findings, we speculate that the acceleration of gluconeogenesis and related metabolic changes by HC suppressed de novo synthesis of GSH, resulting in an imbalance in redox activity and a tendency to produce oxidative stress.

In conclusion, we demonstrated that GC produced a short-term diabetic condition in the developing chick embryo with cataracts. The processes underlying cataract formation by GC were closely related to acceleration of GC-induced gluconeogenesis, which can be recovered by insulin. Further research is currently under way.

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