

## Evaluation of Steviol Injection on Chicken Embryos: Effects on Post-hatch Development, Proportional Organ Weights, Plasma Thyroid Hormones and Metabolites

Mohsen Daneshyar<sup>1</sup>, Jan M.C. Geuns<sup>2</sup>, Johan G. Buyse<sup>3</sup>, Hassan Kermanshahi<sup>4</sup>, Hilke Willemsen<sup>3</sup>, Zarbakt Ansari<sup>5</sup>, Eddy Decuypere<sup>3</sup> and Nadia Everaert<sup>3</sup>

<sup>1</sup> Department of Animal Science, Faculty of Agriculture, Urmia University, Urmia, Iran

<sup>2</sup> Laboratory of Functional Biology, K.U.Leuven, Kasteelpark Arenberg 31, B-3001 Leuven, Belgium

<sup>3</sup> Laboratory of Livestock Physiology, Immunology and Genetics, Department of biosystems, K.U.Leuven, Kasteelpark Arenberg 30, B-3001 Leuven, Belgium

<sup>4</sup> Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

<sup>5</sup> Department of Animal Science, University of Agricultural Science and Natural Resources of Sari, Sari, Iran

At day 7 of incubation, eggs were injected with high levels of steviol (1.5 and 3 mg per egg). At hatch, no effect of steviol injection was observed on plasma thyroid hormone levels, hatchability, body weight and chick quality. Proportional liver weight of chicks receiving the highest steviol dose was significantly higher, while the proportional bursa weight of the steviol injected groups was significantly lower compared to that of the control. Moreover, plasma lactate dehydrogenase activity of chicks from the highest steviol injected group was significantly higher compared to control values. At 7 days of age, proportional liver weight of chicks of the 3 mg steviol injected group was significantly lower compared to that of the control treatment. It is concluded that injection of a high dose of steviol has no effect on plasma thyroid hormones and has only slight and temporary metabolic effects on the chicken embryo.

**Key words:** chicken embryo, metabolic parameters, steviol, thyroid hormones

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### Introduction

Stevia (*Stevia rebaudiana Bertoni*) is a perennial shrub indigenous to Brazil and Paraguay where it has been used as a natural sweetener for hundreds of years. In recent years, stevia has been grown commercially and used as natural sweetener in many countries including Brazil, Paraguay, Japan, China, Korea, the United States, Canada and parts of Europe. The sweetening property of stevia and its extracts (stevioside and rebaudioside A) is well documented in humans (Mizutani and Tanaka, 2002; Geuns *et al.*, 2004). Stevioside is the main sweet compound and dulcoside A, steviolbioside, rebaudioside A, B, C, D and E are present in lower concentrations. In Brazil, Korea and Japan, stevia leaves, stevioside and highly refined extracts are officially used as a low calorie sweetener (Mizutani *et al.*, 2002; Kim, 2002).

Stevioside is a high-intensity sweetener that tastes about 300 times sweeter than sucrose (0.4% solution). In many

countries, it is used as a low calorie sweetener in a wide range of food products and beverages (Kinghorn, 2002). The advantages of stevioside as a dietary supplement for human subjects are manifold: it is stable, it is noncalorific, it maintains good dental health by reducing the intake of sugar, and it opens the possibility for use by diabetic and phenylketonuria patients and obese persons (Geuns *et al.*, 2003a).

Steviol, the aglycone of stevioside, is one of the major metabolites of stevioside during its enzymatic hydrolysis (Hutapea *et al.*, 1997). It has been demonstrated that stevioside is completely converted to its aglycone steviol after incubation with human colon microflora *in vitro* (Hutapea *et al.*, 1997; Gardana *et al.*, 2003), and by colon bacteria of pig *in vivo* (Geuns *et al.*, 2003a) and human (Simonetti *et al.*, 2004). In humans, part of this steviol is absorbed by the colon and transported to the liver by portal blood where it is transformed into steviol glucuronide that is filtered from the peripheral blood by the kidneys and excreted into urine (Geuns *et al.*, 2007). In contrast, in meat type chickens and laying hens, about 21.5 and 7.3% were converted to steviol respectively (Geuns *et al.*, 2003c).

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Correspondence: Dr. N. Everaert, K.U. Leuven, Kasteelpark Arenberg 30, B-3001 Leuven, Belgium. (E-mail: nadia.everaert@biw.kuleuven.be)

Many papers have been published regarding the safe use of stevioside in humans (Geuns *et al.*, 2007) and animals (Akashi and Yokoyama, 1975; Geuns *et al.*, 2004). The safety of steviol glycosides was confirmed by JECFA (2008) that proposed an acceptable daily intake (ADI) of 0–4 mg/kg BW steviol equivalents. However, it is known that there are some negative effects about steviol. Steviol was mutagenic in *Salmonella typhimurium* TM677 (Pezzuto *et al.*, 1985), and showed a clastogenic effect in cultured Chinese hamster lung in combination with Arochlor 1254-induced liver supernatant 9000 g fraction (S9) (Matsui *et al.*, 1996). Chromosome breakage was observed in mice, rats and hamsters, and a reduced ratio of polychromatic over normochromatic erythrocytes in female hamsters, mice and rats after steviol administration (Temcharoen *et al.*, 2000). A significant decrease of maternal body weight gain and high maternal mortality was observed at 0.75 and 1.0 g steviol/kg BW/day in hamsters (Wasuntarawat *et al.*, 1998). In zebrafish embryos, 200  $\mu$ M steviol induced developmental delays, pericardial edema, circulatory defects and lethality (Crawford *et al.*, 2008). Although there is some information about the negative effects of steviol on developmental stage of hamster and zebra fish, it is not clear if this is due to changes in thyroid hormone metabolism.

The embryonic stage is the most critical and sensitive period in the development of organisms. Embryos are much more susceptible to lower doses of a toxic compound as compared to the postnatal period (Geuns *et al.*, 2003b). Chicken embryos have been used successfully as a screening technique to assess the safety of food additives, drugs, pesticides, herbicides, fungicides, and the potency of viruses. This technique differs from feeding trials in that the chemical is in direct contact with the embryo during its development and, therefore, may indicate more readily teratogenic or toxic effects.

In a previous study (Geuns *et al.*, 2003b), injection of three doses of steviol (0.025, 0.25 and 1.25 mg per egg) at embryonic day (ED) 7 had no effect on day-old chick quality and proportional organ weights. However further knowledge of plasma metabolites and thyroid hormones is lacking. The purpose of this study was to investigate the effect of higher doses of injected steviol (1.5 mg and 3 mg on ED7) on chick quality, chick and organ weights (hatch and day 7) and plasma parameters such as lactate dehydrogenase (LDH) activity (as a liver toxicity indicator), total protein, glucose, total cholesterol and thyroid hormone concentration.

## Materials and Methods

### Chemicals

Steviol was prepared according to Minne *et al.* (2004) and repeatedly crystallized from methanol to a purity of over 98%. Solvents of high performance liquid chromatography grade were from Acros (Geel, Belgium) (H<sub>2</sub>O, acetonitrile, CHCl<sub>3</sub>), BDH Chemicals (Haasrode, Belgium) (methanol, ethanol, N,N-dimethylformamide), and Bio-

solve (Valkenswaard, The Netherlands) (acetone). N,N-diisopropylamine was from Acros (Geel, Belgium), and 4-(bromomethyl)-7-methoxycoumarin was from Fluka (Bornem, Belgium).

### Experimental Set-Up

The experiment was approved by the Ethical Commission for Experimental Use of Animals of the K.U.Leuven.

Two hundred fifty two fertile eggs (weighing on average 65 g) from the same broiler breeder flock (Cobb) (Belgabriod, Merksplas, Belgium) were put in an incubator (PasReform, Zeddam, The Netherlands), and incubated under standard conditions (T=37.8°C, RH=55%, rotation over 90° once an hour).

At ED7, eggs were randomly divided into three groups, each containing 84 eggs: corn oil injected group, 1.5 mg steviol injected and 3 mg steviol injected group, where corn oil served as a solvent. The eggs were injected with 100  $\mu$ l corn oil (containing no, 1.5 mg or 3 mg steviol) with a pipette with a tip just going into the air cell. To avoid precipitation, solutions were kept at 40°C. Before injection, the top of the eggs was sterilized with 70% ethanol, and a small hole was made with a needle of 18G. After injection, the holes were sealed with tape and the eggs returned to the incubator. ED7 was chosen as a suitable moment for injection in this and previous (Geuns *et al.*, 2003b) experiment as injection occurs then during the critical developmental stages of the embryo (organogenesis) (Willier, 1954). Steviol was injected in the air cell, since the embryo lies between the yolk and air cell at that moment (Freeman and Vince, 1974).

On the ED18, eggs were candled and those with evidence of living embryos were transferred to hatchery baskets.

Chicks were reared under standard conditions until day 7. Water and a commercial starter diet (21.5% crude protein; 2958 kcal ME) (Research Diet Services BV, Wijk Bij Duurstede, The Netherlands) were provided for *ad libitum* consumption. Lighting schedule provided 23 hours of light per day. The temperature was set at day-old at 34°C and was decreased by 1°C every day until a final temperature of 29°C was reached.

### Sampling

At the end of the ED21, eggs that did not hatch were opened to identify infertile or dead embryos. Dead embryos were classified as early (those that died between ED1 to ED7), mid (those that died between ED8 to ED14) and late dead embryos (those that died between ED15 to ED21). Age of mortality was determined according to Hamburger-Hamilton criteria (Hamburger and Hamilton, 1951). Hatching percentage was calculated as number of hatched chicks to fertile eggs.

At hatch, all chicks were classified according to chick quality parameters as previously described (Tona *et al.*, 2003).

At hatch and day 7, all chicks were weighed and 15 chicks of each group were randomly selected and sacrificed by decapitation to take blood samples and to dissect

and weigh organs (liver, heart, bursa and spleen). Blood samples were collected into heparinized tubes, stored on ice and centrifuged. Plasma was collected after centrifugation and stored at  $-20^{\circ}\text{C}$  until further analysis. In addition, three blood samples from chicks of the 3 mg steviol injected group were kept at  $-20^{\circ}\text{C}$  for steviol determination.

#### Measurement of Steviol

Samples of about 50 mg dried and pulverized blood were measured as described by Geuns *et al.* (2003b). The detection limit was 50 pg of steviol per injection.

#### Plasma Analysis

Glucose, cholesterol, total protein concentration and lactate dehydrogenase activity of plasma were determined using the Vet Test 8008 analyzer (Idexx laboratories Inc. U.S.A). The triiodothyronine ( $T_3$ ) and thyroxine ( $T_4$ ) concentration in the plasma samples were determined by radioimmunoassay using the procedure described by Darras *et al.* (1992).  $T_3$  and  $T_4$  standards were purchased from Byk-Belga (Brussels, Belgium). All samples were analyzed in the same assay in order to avoid inter-assay variability.

#### Statistical Analysis

The data were subjected to one-way analysis of variance using SAS statistical package version 9 (SAS Institute Inc., Cary, NC, USA). When the overall model was statistically significant, the means were further separated using the Tukey-Kramer multiple comparison test. Significance was set at 0.05. Data are presented as means  $\pm$  SEM. The Kolmogorov-Smirnov test, at significance level of 0.01 was used to assess the normality of the distribution of chick quality score and proportional organ weights. Normalized data in the case of abnormality were used for statistical analysis. Hatchability, cumulative dead embryos,

division of chick quality scores and post-hatch mortality of treatments were compared using chi-square test.

## Results

### Hatchability and Mortality

There was no effect of steviol (1.5 or 3 mg) on the hatchability and number of dead embryos (Table 1). Post-hatch mortality did not differ between treatments ( $P=0.059$ ) (Table 1).

### Chick Quality

There was no difference in occurrence of severe anomalies between chicks of the different treatments, as shown by the amount of chicks of quality score 100 (Table 2). The average quality score of chicks from the 1.5 mg steviol injected group was significantly higher than that of the 3 mg steviol injected group. The steviol injected groups did however not differ from the corn oil (control) injected chicks. Moreover, chicks with a quality score lower than 100 did not differ on average between treatments (Table 2) ( $P=0.057$ ).

### Body and Proportional Organ Weights

As shown in Table 3, at hatch there was no significant difference in mean body weight between the treatments. Proportional liver weight of chicks receiving the highest steviol dose was significantly higher compared to 1.5 mg steviol and control treatments. Proportional bursa weight at hatch was significantly lower in both steviol treated groups compared to control group. There were no significant differences in proportional heart and spleen weight between treatments. At day 7, chicks from the 3 mg steviol injected group were significantly heavier than chicks from the 1.5 mg steviol injected group. However, chick weights of corn oil injected eggs did not differ from that of steviol injected groups. At day 7, proportional liver weight of

Table 1. Hatchability (number of chicks/number of fertile eggs), embryo mortality and post-hatch mortality (number of dead embryos or chicks) of corn oil injected, 1.5 mg steviol and 3 mg steviol injected groups

	Corn oil (n=84)	1.5 mg Steviol (n=84)	3 mg Steviol (n=84)
Hatchability	56/78 (72%)	58/82 (71%)	60/81 (74%)
Early dead embryos (1-7 days)	2	7	4
Mid dead embryos (8-14 days)	1	4	2
End dead embryos (15-21 days)	19	13	15
Post-hatch mortality	7	1	2

Table 2. Effect of steviol injection on chick quality

Quality parameters	Corn oil	1.5 mg Steviol	3 mg Steviol
Chicks with score 100	4/20	7/20	4/20
Average score (n=20)	89 $\pm$ 2 <sup>ab</sup>	94 $\pm$ 1 <sup>a</sup>	86 $\pm$ 3 <sup>b</sup>
Average score of chicks with score < 100	86 $\pm$ 2	90.3 $\pm$ 1	83 $\pm$ 3

Values of average quality score are mean  $\pm$  SEM, and those with different superscripts (a and b) are significantly different ( $P < 0.05$ )

Table 3. Body weight (g) and proportional organ weights (%) for steviol (1.5 or 3 mg per egg) or corn oil injected groups at hatch and day 7

Parameters	Hatch			Day 7		
	Corn oil	1.5 mg Steviol	3 mg Steviol	Corn oil	1.5 mg Steviol	3 mg Steviol
Body weight (g)	45.4±0.6 (n=56)	45.8±0.4 (n=58)	45.1±0.4 (n=60)	134±4 <sup>ab</sup> (n=33)	132±3 <sup>b</sup> (n=43)	146±4 <sup>a</sup> (n=36)
Proportional liver weight (%)	2.0±0.1 <sup>b</sup>	2.1±0.1 <sup>b</sup>	2.3±0.1 <sup>a</sup>	4.2±0.2 <sup>a</sup>	4.0±0.1 <sup>ab</sup>	3.6±0.1 <sup>b</sup>
Proportional heart weight (%)	0.75±0.02	0.70±0.02	0.72±0.03	0.85±0.04	0.78±0.02	0.81±0.03
Proportional bursa weight (%)	0.14±0.01 <sup>a</sup>	0.11±0.01 <sup>b</sup>	0.10±0.01 <sup>b</sup>	0.15±0.01	0.16±0.01	0.16±0.01
Proportional spleen weight (%)	0.043±0.001	0.045±0.001	0.041±0.001	0.103±0.009	0.107±0.006	0.097±0.008

Means±SEM are given (n=15 for proportional weight), and per age, those with different superscripts (a and b) are significantly different ( $P<0.05$ )

Table 4. Effect of Steviol (1.5 or 3 mg per egg) or corn oil injection on plasma triiodothyronine (T<sub>3</sub>), thyroxine (T<sub>4</sub>) concentrations (ng/mL) and T<sub>3</sub>/T<sub>4</sub> ratio, glucose (mmol/L), cholesterol (mmol/L) and total protein (g/L) concentrations at hatch and lactate dehydrogenase (LDH) activity at hatch and day 7

Parameters <sup>2</sup>	Corn oil (n=15)	1.5 mg Steviol (n=15)	3 mg Steviol (n=15)
T <sub>3</sub> (ng/mL)	1.2±0.1	1.2±0.1	1.1±0.1
T <sub>4</sub> (ng/mL)	7.3±0.6	8.1±0.7	8.5±0.5
T <sub>3</sub> /T <sub>4</sub> ratio	0.16±0.01	0.15±0.01	0.14±0.01
Glucose (mmol/L)	12.9±0.3	12.8±0.4	12.8±0.1
Cholesterol (mmol/L)	10.0±0.4	9.9±0.4	10.4±0.5
Total Protein (g/L)	14.2±0.7	15.1±1.2	16.4±0.7
LDH activity (U/L) at hatch	1624±62 <sup>b</sup>	1753±76 <sup>ab</sup>	1869±53 <sup>a</sup>
LDH activity (U/L) at 7 <sup>th</sup> day of age	1722±119	1721±108	1962±102

Means±SEM are given (n=15), and those with different superscripts (a and b) are significantly different ( $P<0.05$ )

chicks that were injected with 3 mg steviol was significantly lower than that of the control group, whereas the 1.5 mg steviol injected chicks did not differ from the other groups. There were no significant differences in proportional heart, bursa and spleen weight between treatments at this age.

#### Plasma Hormones and Metabolites

Results of plasma hormones and biochemical indices are shown in Table 4. There were no significant differences in T<sub>3</sub>, T<sub>4</sub> and T<sub>3</sub>/T<sub>4</sub> ratio between treatments at hatch. Moreover, no significant differences in plasma glucose, total protein and cholesterol concentration were observed. Only plasma LDH activity of chicks from the 3 mg steviol injected group was significantly higher than that of the control group at hatch while no significant differences between treatments for LDH activity were observed at 7 days of age (Table 4).

No free steviol could be detected in the plasma of day-old chicks that hatched from the steviol treated eggs.

#### Discussion

*In ovo* injection of 1.5 mg or 3 mg steviol at ED7 did not influence day-old chick weight or hatchability, which is in agreement with previous results (Geuns *et al.*, 2003b),

where lower doses of steviol (0.025, 0.25 and 1.25 mg per egg) were used. Corn oil as a solvent decreased the hatchability compared to standard incubation (normally around 90%), as already observed by Dewitt *et al.* (2005). There was no negative effect of the embryonic steviol injection on the chick performance at hatch, as shown by an equal or even somewhat higher chick quality score at hatch in the steviol injected groups, confirming previous results (Geuns *et al.*, 2003b). These results are also in agreement with another study (Oliveira-Filho *et al.*, 1989) where no effect of concentrated leaf extracts on male fertility nor on the growth of prepubertal male rats were observed.

Since steviol was shown to delay the development of zebrafish embryos (Crawford *et al.*, 2008) and hamster fetus (Wasuntarawat *et al.*, 1998), it was hypothesized that steviol may affect the development by changing the thyroid hormone metabolism. However, in our study, steviol injection did not change plasma T<sub>3</sub>, T<sub>4</sub> concentrations or T<sub>3</sub>/T<sub>4</sub> ratio significantly between treatments at hatch. In contrast, supplementation of 130 ppm of steviol or 2% dried stevia leaves for 6 weeks in a broiler diet, lowered the T<sub>3</sub> level with 27% or 55% respectively (Atteh *et al.*, 2008). However, no differences in plasma thyroid hormone concentrations of pre-pubertal male rats were

found by gastric tubing (2 ml/rat) of crude water extracts from stevia leaves (66.7 g dried leaves/100 ml final solution) given twice a day during 60 days (Oliveira-Filho *et al.*, 1989). Of course, attention should be paid between the different additives (stevia leaves, stevioside or steviol), supplementation ways (oral, injection) and treatments (single embryonic injection or chronic ingestion) between experiments.

In our study, a significantly higher proportional liver weight at hatch was observed for the 3 mg steviol injected group. This may indicate a liver challenge by steviol and is further supported by higher level of LDH activity. Indeed, an increased LDH activity of ducks by toxic effects of fumonisin B1 in maize has been observed (Tardieu *et al.*, 2004). Additionally, decreased bursa and spleen weights along with reduced antibody production have been observed in poult fed moniliformin in the diet (Li *et al.*, 2000). Consequently, the decreased proportional bursa weight at hatch in both steviol injected groups of our study might indicate a decreased immune function due to the embryonic exposure to steviol. However, information about immunological effects of steviol in chickens is lacking, while it is known that steviol suppressed TNF- $\alpha$ -induced IL-8 release in human colon carcinoma cell lines (Boonkaewwan *et al.*, 2008).

Since the glucose, cholesterol and total protein concentration in the plasma of chicks before they had access to feed, did not differ between groups, it is assumed that the single prenatal steviol injection did not have an important effect on the energy metabolism of the embryos or chicks. In the same line are the results where no differences were found in glycaemia of prepubertal male rats, given extracts of stevia leaves twice a day (Oliveira-Filho *et al.*, 1989). Chatsudthipong and Muanprasat (2009) on the other hand noted that stevioside seems to have the ability to lower plasma glucose only when these parameters are abnormally elevated. In addition, stevioside was able to lower the plasma cholesterol level after 5 weeks of treatment in the diabetic Goto-Kakizaki Rat (Jeppesen *et al.*, 2006), while no adverse effects on lipid or glucose were reported in non-diabetic subjects, treated with stevioside for two years (Hsieh *et al.*, 2003).

At day 7, chicks from the high steviol dose groups were significantly heavier and had significantly lower proportional liver weights, with an equal absolute liver weight. At day 7, proportional bursa and spleen weight and LDH activity did not differ between treatments, suggesting that there were only slight and temporary metabolic effects of the steviol injection during embryonic development. Noteworthy are the histopathological changes of the kidneys of pregnant hamsters receiving steviol at high doses (Wasuntarawat *et al.*, 1998), which were the same as seen in stevioside-treated animals, that also had increased blood urea nitrogen and creatinine concentration (Toskulkao *et al.*, 1997).

No free steviol could be detected (detection limit of 50 pg) in blood of chickens of the highest steviol dose, which

is in agreement with previous results (Geuns *et al.*, 2003 b). Since the increased LDH activity at hatch indicates that the steviol injection into the air cell was an efficient administration method for toxicological research, it might indicate that the steviol was metabolized during embryonic development. It is important to note that ED7 was chosen as a suitable moment for injection in this and previous (Geuns *et al.*, 2003b) experiments as injection occurs then during the critical developmental stages of the embryo (organogenesis) (Willier, 1954). Steviol was injected in the air cell, since the embryo lies between the yolk and air cell at that moment (Freeman and Vince, 1974), so we can assume the injected product could directly reach the embryo.

In conclusion, no significant differences in hatchability, body weight or total quality score were found in day-old hatchlings treated with a single steviol injection during embryonic development. At hatch, chicks of the 3 mg steviol injected group showed a higher proportional liver weight and LDH activity and lower proportional bursa weight as compared to control chicks. However, it was only a slight and temporary effect that disappeared after 1 week of raising the chicks. Therefore, it is concluded that the highest dose of steviol had a temporary metabolic effect on chicks without effects on thyroid hormone metabolism.

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