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Effect of Stevioside and Steviol on the Developing Broiler Embryos

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At day 7 of incubation, fertile broiler eggs were injected with different amounts of stevioside and steviol of 0.08, 0.8, or 4 mg stevioside/egg and 0.025, 0.25, or 1.25 mg steviol/egg. At hatch (day 21) and 1 week later, not any influence of the different treatments could be found on embryonic mortality, body weight of the hatchlings, deformations (e.g., bone, beak, and head malformations, abnormal feathering, open vent), or abnormal development of the gonads. No stevioside or steviol could be detected in the blood of the hatchlings. The hatchlings developed normally. It is concluded that prenatal exposure to stevioside and steviol is not toxic for the chicken embryo.

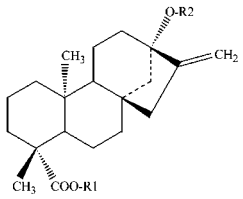
KEYWORDS: ***Stevia rebaudiana*** **(Bertoni) Bertoni (Compositae); stevioside; steviol; chicken embryo toxicology**

INTRODUCTION

Stevioside, the main sweet component in the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (Compositae), is a high-intensity sweetener that tastes about 300 times sweeter than sucrose (0.4% solution). Structures of the sweet components of *S. rebaudiana* (Bertoni) Bertoni occurring mainly in the leaves are given in **Figure 1**. Their content varies between 4 and 20% of the dry weight of the leaves depending on the cultivar and growing conditions. Stevioside **3** is the main sweet component. Other compounds present but in lower concentration are dulcoside A (**9**), steviolbioside (**2**), rebaudioside A (**4**), B (**5**), C (**6**), D (**7**), and E (**8**). The presence of steviolbioside and rebaudioside B in extracts might be due to artifacts of the extraction procedure (*1*).

Both the *Stevia* plant, its extracts, and stevioside have been used for several years as a sweetener in South America, Asia, Japan, China, and in different countries of the EU. In Brazil, Korea, and Japan, *Stevia* leaves, stevioside, and highly refined extracts are officially used as a low calorie sweetener. In the U.S.A., powdered *Stevia* leaves and refined extractives from the leaves have been used as a dietary supplement since 1995. Since 2000 in the European Union, *Stevia* leaves and stevioside are not approved as foods (*2*; Geuns, unpublished). 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.

Many papers describe the safety of stevioside used as a sweetener (see *3* for a review). In humans, an acceptable daily intake (ADI) of 7.9 mg stevioside/kg body weight (BW) was calculated (*4*). However, this ADI should be considered as a minimum value as the authors did not test concentrations of stevioside higher than 793 mg/kg BW (safety factor 100). Considering many reports from the literature, an ADI of more than 20 mg stevioside/kg BW is likely (*3*). However, mutagenic effects of steviol, the aglycone of stevioside, and/or its metabolites were reported in tests using *Salmonella typhimurium*



	Compound name	R1	R2
1	steviol	H	H
2	steviolbioside	H	β-Glc-β-Glc(2→1)
3	stevioside	β-Glc	β-Glc-β-Glc(2→1)
4	rebaudioside A	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)
5	rebaudioside B	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)
6	rebaudioside C (dulcoside B)	β-Glc	β-Glc-α-Rha(2→1) | β-Glc(3→1)
7	rebaudioside D	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1) | β-Glc(3→1)
8	rebaudioside E	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)
9	dulcoside A	β-Glc	β-Glc-α-Rha(2→1)

Figure 1. Structures of stevioside and related compounds. In rebaudioside A–D, an additional sugar moiety is added on carbon 3 of the first β-Glc.

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TM677 (*5*−*9*). After metabolic activation, it was shown that so far unknown steviol metabolites caused mutations in *S. typhimurium* TM677, i.e., transitions, transversions, duplications, and deletions at the guanine phosphoribosyltransferase (*gpt*) gene (*9*). However, stevioside and steviol were inactive in *S. typhimurium* TA strains, *Escherichia coli* WP2 *uvrA*/pKM101, and the *rec*-assay using *Bacillus subtilis* even when activation S9 mix was present (*9, 10*). The direct mutagenic activity of 15-oxo-steviol was refuted by ref *11*. The activity of steviol in *S. typhimurium* TM677 was only about 1/3000 that of 3,4-benzopyrene and that of steviol methyl ester 8,13-lactone was 1/24 500 that of furylfuramide (*7*). Although a weak activity of steviol and some of its derivatives was found in the very sensitive *S. typhymurium* TM677 strain, the authors concluded that the daily use of stevioside as a sweetener is safe. Moreover, the presence in the blood of the chemically synthesized steviol derivatives after feeding stevioside is not proven at all. Very high doses of steviol (90% purity) intubated to hamsters (4 g/kg bw), rats, and mice (8 g/kg BW) did not induce micronucleus in bone marrow erythrocytes of both male and female animals. However, these doses showed some cytotoxic effect to the female but not to the male of all treated animal species (*12*).

Studies on the effects of oral stevioside or *Stevia* extracts on reproduction and reproductive organs could not reveal any harmful effects (*13*−*15*). In contrast to these results, a possible decrease of the fertility of male rats was suggested (*16*). However, these results are in contradiction with the above and below cited studies that could not reveal any effect on fertility of male or female animals. The results of a decrease of live birth rate between 20 and 30% in rats (*17*) by *Stevia* decoctions were refuted by Shiotsu (*18*) who did more reliable experiments with many more animals using methods as similar as possible to the methods used in ref *17*. No effect on general condition, BW, water consumption, live birth rate, or litter size was found.

The embryonic stage is the most critical and sensitive period in the development of organisms. A number of studies aiming to test possible toxicological effects of environmental or food contaminants are performed during the embryonic stage. Embryos are much more susceptible to lower doses as compared to the postnatal period. The avian egg is already used for testing the toxicity of several environmental compounds such as polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls (*19*−*22*) by injecting the components into the egg or as a result of natural exposure of wildlife birds to these environmental contaminants. LD50 values of 0.6, 8.8, and 5592 μg/kg egg were published for PCB IUPAC numbers 126, 77, and 105, respectively (*21*) and 75 mg/kg and 6×10^{-7} mg/kg for 2,4,5-T and TCDD, respectively (*19*). This research aims to investigate whether stevioside or steviol are toxic to chicken embryos that react very sensitively to administered toxicants.

MATERIALS AND METHODS

Chemicals. Experiments were performed with stevioside (from Stevita Co. Inc., Arlington, TX) that was purified by repeated crystallization from MeOH to a purity of more than 96%. Steviolbioside (around 3%) and rebaudioside A (around 0.5%) were the main impurities. Steviol was prepared according to ref *23* and repeatedly crystallized from MeOH to a purity of over 98%. Solvents of high-performance liquid chromatography (HPLC) grade were from Acros (H_2O, acetonitrile, $CHCl_3$), BDH (MeOH, EtOH, *N*,*N*-dimethylformamide) (DMF), and Biosolve (acetone). *N*,*N*-Diisopropylethylamine was from Acros, and 4-(bromomethyl)-7-methoxycoumarin was from Fluka.

Experiments with Fertilized Eggs. Six hundred fertile broiler eggs (Ross) were purchased from a local hatchery (Avibel, Zoersel, Belgium) and incubated at 37.6 °C and 50% relative humidity with rotation once each hour. At day 7 of incubation, the eggs were injected into the yolk through a small hole in the shell at the blunt end (air chamber) by inserting the needle 30 mm into the egg. After injection, the eggs were sealed with tape and incubation was continued under standard incubation conditions. Stevioside (96% purity) was dissolved in 20% glycerol. Steviol was dissolved in corn germ oil. Two series of controls were run as follows: 20% glycerol and pure corn germ oil. At the moment of the injection (day 7), the embryos weighed less than 1 g.

Each egg was injected with 100 μL of solution. Treatments were carried out as follows: (i) injection with 20% glycerol (controls); (ii) injection with pure corn germ oil (controls); (iii) injection with stevioside: 0.08, 0.8, and 4 mg per egg; and (iv) injection with steviol: 0.025, 0.25, and 1.25 mg per egg.

On day 18 of incubation, the eggs were candled, and those with evidence of living embryos were recorded and transferred to hatcher baskets. The remaining candled eggs were broken for macroscopic analysis to distinguish the fertile from the infertile eggs. At the end of incubation, the hatched chicks and dead embryos were recorded, and the numbers were used to calculate the hatchability in relation to fertile eggs. Dead embryos were recorded and classified as early dead embryos, those estimated to have died during less than 18 days of incubation, and late dead embryos, those estimated to have died after 18 days of incubation (**Tables 2** and **3**).

At hatch, all chicks were weighed and macroscopically examined to classify chick quality. Chick quality was defined to encompass several qualitative characteristics such as activity, feathering, yolk absorption, remaining membrane, and the aspects of leg, beak, navel, etc. These characteristics were scored according to their importance within a total scale of 100 (**Table 1**; *24*).

A number of 1 day old chicks were sacrificed in order to take blood samples and to dissect several organs for weighing. The gonads were macroscopically inspected to look for possible deformities. The remaining chicks were reared under standard conditions and weighed after 7 days.

Table 1. Allocation of Scores to Different Parameter Observations of 1 Day Old Chicks According to the Method Described by Ref *24*

parameters	characteristics	scores	max score
activity	good	6	6
	weak	0	
downs appearance	clean and dry	10	10
	wet	8	
	dirty and wet	0	
swallowed yolk	body with normal swallowed yolk	12	12
	body with swallowed large yolk and rather hard on touch	0	
eyes	opened and bright	16	16
	opened and not bright	8	
	closed eyes	0	
legs	normal legs and toes	16	16
	one infected leg	8	
	two infected legs	0	
navel	completely closed and clean	12	12
	not completely closed and not discolored	6	
	not closed and discolored	0	
remaining membrane	no membrane	12	12
	small	8	
	large	4	
	very large	0	
remaining yolk	no yolk	16	16
	small	12	
	big	8	
	very big	0	
max score = good quality			100

Table 2. Effects of the Injection of Different Amounts of Stevioside on the Parameters Studied[a]

	control (20% glycerol)	0.08 mg/egg	0.8 mg/egg	4 mg/egg
egg weight (in g)	65.8 ± 0.63	64.8 ± 0.58	66.4 ± 0.63	66.3 ± 0.59
	$n = 51$	$n = 55$	$n = 55$	$n = 55$
not fertilized	15.7%	5.4%	14.5%	12.7%
early embryonic mortality	11.6%	11.5%	21.3%	7.2%
late embryonic mortality	20.9%	13.5%	7.3%	18.2%
hatched chicks	67.4%	75.0%	70.2%	71.8%
day old chick weight (in g)	48.3 ± 0.74	47.3 ± 0.67	49.3 ± 0.67	48.1 ± 0.61
	$n = 29$	$n = 37$	$n = 32$	$n = 33$
chick weight at day 7	125.1 ± 4.74	125.1 ± 4.17	132.8 ± 4.65	131.5 ± 6.33
	$n = 15$	$n = 18$	$n = 20$	$n = 17$
chick quality score	81.67 ± 3.80	85.14 ± 3.49	91.33 ± 1.72	86.83 ± 2.80
	$n = 24$	$n = 28$	$n = 30$	$n = 29$
weight of heart (in g)	0.209 ± 0.009	0.209 ± 0.008	0.221 ± 0.011	0.205 ± 0.008
	$n = 14$	$n = 18$	$n = 12$	$n = 16$
weight of spleen (in g)	0.015 ± 0.001	0.014 ± 0.0008	0.015 ± 0.001	0.015 ± 0.001
	$n = 14$	$n = 18$	$n = 12$	$n = 16$
weight of bursa (in g)	0.042 ± 0.003	0.044 ± 0.004	0.044 ± 0.004	0.039 ± 0.003
	$n = 14$	$n = 18$	$n = 12$	$n = 16$
weight of liver (in g)	0.964 ± 0.029	0.909 ± 0.038	1.007 ± 0.031	0.973 ± 0.034
	$n = 14$	$n = 18$	$n = 12$	$n = 16$
heart as % of BW	0.434 ± 0.022	0.439 ± 0.015	0.433 ± 0.020	0.433 ± 0.019
	$n = 14$	$n = 18$	$n = 12$	$n = 16$
spleen as % of BW	0.032 ± 0.002	0.029 ± 0.002	0.031 ± 0.001	0.031 ± 0.002
	$n = 14$	$n = 18$	$n = 12$	$n = 16$
bursa as % of BW	0.086 ± 0.005	0.093 ± 0.008	0.085 ± 0.007	0.083 ± 0.006
	$n = 14$	$n = 18$	$n = 12$	$n = 16$
liver as % of BW	2.005 ± 0.072	1.916 ± 0.086	1.984 ± 0.079	2.05 ± 0.077
	$n = 14$	$n = 18$	$n = 12$	$n = 16$

[a] Means ± SEM are given.

Table 3. Effects of the Injection of Different Amounts of Steviol on the Parameters Studied[a]

	control (corn oil)	0.025 mg/egg	0.25 mg/egg	1.25 mg/egg
egg weight (in g)	66.2 ± 0.69	66.7 ± 0.56	65.6 ± 0.66	65.7 ± 0.60
	$n = 51$	$n = 55$	$n = 55$	$n = 55$
not fertilized	10.9%	14.5%	9.0%	16.4%
early embryonic mortality	11.1%	17.0%	7.3%	10.9%
late embryonic mortality	24.4%	17.0%	12.0%	15.2%
hatched chicks	64.4%	66.0%	80.0%	73.9%
day old chick weight (in g)	48.3 ± 0.68	47.9 ± 0.74	48.3 ± 0.63	48.7 ± 0.68
	$n = 29$	$n = 31$	$n = 37$	$n = 32$
chick weight at day 7	126.9 ± 5.96	130.3 ± 6.30	121.9 ± 5.02	140.2 ± 3.45
	$n = 15$	$n = 16$	$n = 18$	$n = 15$
chick quality score	81.04 ± 3.93	85.62 ± 3.28	84.81 ± 1.99	89.73 ± 1.96
	$n = 27$	$n = 26$	$n = 32$	$n = 30$
weight of heart (in g)	0.215 ± 0.007	0.212 ± 0.011	0.210 ± 0.007	0.219 ± 0.007
	$n = 14$	$n = 15$	$n = 19$	$n = 17$
weight of spleen (in g)	0.015 ± 0.001	0.014 ± 0.002	0.013 ± 0.001	0.015 ± 0.001
	$n = 14$	$n = 15$	$n = 19$	$n = 17$
weight of bursa (in g)	0.045 ± 0.004	0.042 ± 0.006	0.040 ± 0.003	0.046 ± 0.003
	$n = 14$	$n = 15$	$n = 19$	$n = 17$
weight of liver (in g)	0.975 ± 0.028	0.932 ± 0.045	0.943 ± 0.033	1.005 ± 0.026
	$n = 14$	$n = 15$	$n = 19$	$n = 17$
heart as % of BW	0.439 ± 0.019	0.444 ± 0.022	0.427 ± 0.016	0.453 ± 0.014
	$n = 14$	$n = 15$	$n = 19$	$n = 17$
spleen as % of BW	0.030 ± 0.003	0.029 ± 0.003	0.027 ± 0.002	0.032 ± 0.003
	$n = 14$	$n = 15$	$n = 19$	$n = 17$
bursa as % of BW	0.0925 ± 0.007	0.089 ± 0.013	0.082 ± 0.006	0.096 ± 0.007
	$n = 14$	$n = 15$	$n = 19$	$n = 17$
liver as % of BW	1.985 ± 0.059	1.965 ± 0.107	1.927 ± 0.077	2.086 ± 0.064
	$n = 14$	$n = 15$	$n = 19$	$n = 17$

[a] Means ± SEM are given.

Measurement of Stevioside. Freeze-dried blood samples were pulverized in a dismembrator. Weighed aliquots of about 100 mg were extracted with methanol (4 × 1 mL). Then, the pooled extract was diluted with 6 mL of water (end concentration 40% MeOH). This solution was quantitatively applied to a conditioned 500 mg C-18 cartridge (Alltech, Deerfield, IL) and washed with 40% MeOH. Stevioside was eluted with 3 mL of a 70% methanol solution. Under these conditions, steviol can be eluted with 3 mL of a 90% solution. However, as the recovery of steviol was better when the samples were extracted with acetone, separate extractions were done for steviol

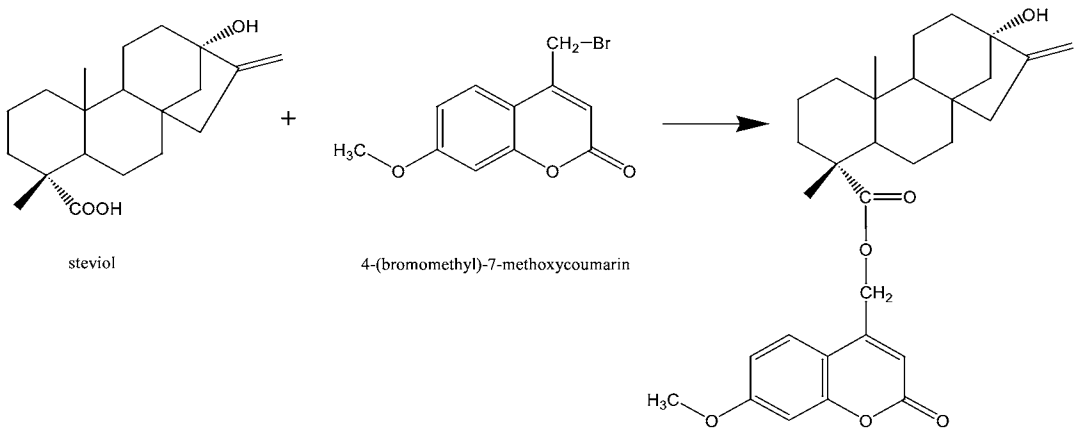

Figure 2. Derivatization of steviol with 4-(bromomethyl)-7-methoxycoumarin and *N*,*N*-diisopropylethylamine in an aprotic solvent (*N*,*N*-DMF).

analysis. The extracts were evaporated at 50 °C under a flow of nitrogen and purified by thin-layer chromatography (TLC) (solvent: ethyl acetate:EtOH:water; 130:27:20; R_f stevioside = 0.20). It is very important to rinse the sample vials containing the stevioside extracts three times to allow for a quantitative transfer of stevioside to the TLC plates. The stevioside bands (R_f = 0.20) were eluted by MeOH.

Measurement of Steviol. Samples of about 100 mg dried and pulverized blood were extracted four times with 1 mL of acetone. The extracts were concentrated and purified by TLC ($CHCl_3$:MeOH, 90:10). The bands corresponding to steviol (R_f = 0.35) were eluted with $CHCl_3$:MeOH (50:50) and completely dried (presence of traces of water influence the subsequent derivatization reaction). To enable measuring very minute amounts of steviol in biological samples, steviol was derivatized with 4-(bromomethyl)-7-methoxycoumarin in an aprotic solvent (**Figure 2**) (*25*). The residue was solubilized in 200 μL of *N*,*N*-DMF containing 25 μg of reagent and 1 μL of diisopropylethylamine. After warming for 20 min at 75 °C, the reaction mixture can directly be injected onto the HPLC column (ODS silicagel, 25 cm × 4.6 mm i.d., AcCN:water, 80:20; detection: fluorescence detector, exc. at 321 nm, em. 391 nm). The detection limit was about 50 pg of steviol per injection.

HPLC Analysis. Stevioside extracts were separated by HPLC on ODS-silica columns (25 cm length, 4.6 mm i.d.) with 35% AcCN as solvent. Detection of stevioside was by UV at 210 nm. The detection limit was 50 ng per injection.

The steviol coumarate was separated by HPLC on ODS-silica columns (25 cm length, 4.6 mm i.d.) with 80% AcCN as solvent. Detection of the steviol−coumarate derivative was by fluorescence (exc. at 321 nm, em. 391 nm). The detection limit was about 50 pg per injection. This way, steviol can be measured at a sensitivity that is about 1000× higher than that of stevioside.

Statistics. In the tables, the means ± SEM are given. Results were analyzed by Student's *t*-test ($p < 0.05$).

RESULTS AND DISCUSSION

The results of the effects of stevioside and steviol after in ovo injection at day 7 of incubation on chick parameters are given in **Tables 2** and **3**. As the test substances were injected into the yolk, the highest doses in the yolk were about 0.3 and 0.25 mM for stevioside and steviol, respectively. The general chick quality score is calculated as indicated in **Table 1**. No significant effect of steviol or stevioside administration in ovo could be observed on 1 day old chick quality, and no abnormalities were observed. Hatchabilities were in all groups somewhat lower as compared to noninjected eggs (which is normally around 90%). This is a known phenomenon, which is due to the injection itself. The 1 day old chick weight was around 48 g, after 1 week around 125 g, i.e., a weight increase of about 160%. No differences in BWs were found between the different treatments. The absolute weights of the heart, the spleen, the bursa, or the liver were not significantly different, nor was their weight as percentage of the BW. From these results, it is clear that injection of steviol or stevioside at day 7 of incubation, i.e., during the critical developmental stages of the embryo (organogenesis), has no detrimental effects on hatchability and does not affect BW or organ weights of the 1 day old chicks. These results are in good agreement with those previously obtained with rats and suggest the lack of harmful effects on the chicken embryo.

Table 4. Estimation of the Maximum Amount of Steviol that Might Be Present Undetected in the Animals

weight of hatchlings	total blood volume (mL)	volume analyzed (mL)	max amount of SV (in ng/animal)
	stevioside (4 mg/egg)		
48.1 ± 0.61	3.37	0.12	280
	steviol (1.25 mg/egg)		
48.7 ± 0.68	3.4	0.12	283

The occurrence of stevioside and steviol was investigated in blood samples of hatchlings from the eggs treated with the highest administered concentration of stevioside (4 mg/egg) and steviol (1.25 mg/egg). Thourough purification and analysis of the extracts did not reveal the occurrence of stevioside or steviol. As the detection limit is 50 pg of steviol, the maximum amount of undetected steviol in the blood can be estimated to be around 280 ng/hatchling or about 0.26 μM concentration in the blood (**Table 4**).

The lack of toxic effects of stevioside and steviol on chicken embryos agrees with literature data obtained with other species. High doses of 525 mg/kg of stevioside equivalent aqueous *Stevia* extract were daily administered to male and female mice during copulation and pregnancy periods. The authors could not observe any difference in the copulation and conception averages and any change in the fetus and broods when compared with control groups (*26*). Male and female Wistar rats received feed containing 0.15, 0.75, and 3% stevioside before and at the start of pregnancy (*27*). Consumption of stevioside in the three treated groups was 100, 480, and 2100 mg/kg per day in males and 120, 530, and 2400 mg/kg per day in females. Males received stevioside from 60 days before mating and females from 14 days before mating to day 7 of pregnancy. No differences could be found between test and control groups regarding sexual cycle, mating, or pregnancy, nor was there any abnormality in the test groups regarding implantation and survival of the fetus. There were no abnormalities found in the growth, general appearance, viscera, or skeleton of the fetus. The final BW showed no differences between the groups, although a small reduction in

BW was observed at first in the 3% group in both sexes. This might have been due simply to an adaptation of the rats to the extreme sweetness of the feed, avoiding it in the beginning. The authors concluded that the addition of stevioside to feed up to a concentration of 3%, from which an intake can be calculated of about 300 times the ADI of 7.9 mg/kg BW (*4*), had no effect on mating, fertility, or the development and the state of fetuses in rats given the diet prior to and during the early stage of pregnancy. No effects of concentrated leaf extracts (0.667 g dried leaves/mL, 2 mL/rat twice a day) were found on male fertility nor on the growth of prepubertal male rats (*15*).

One month old male and female hamsters were daily force-fed with stevioside (0, 0.5, 1, and 2.5 g/kg BW, respectively (*28*)). No abnormalities were found in growth and fertility in both sexes. All males mated with females efficiently and successfully. Females showed normal 4 day estrus cycles and became pregnant after mating. Each female was mated and allowed to bear three litters during the period of the experiment. The duration of pregnancy, number of fetuses, as well as number of young delivered each time from females in the experimental groups were not significantly different from those in the control group. The young F1 and F2 hamsters continuously receiving stevioside via drinking water until they were 1 month old and daily force-fed afterward at the same doses as their parents showed normal growth and fertility. Histological examination of reproductive tissues from all three generations revealed no evidence of abnormality, which could be linked to the effects of consuming stevioside. It was concluded that stevioside at a daily dose as high as 2.5 g/kg BW does not affect the growth or reproduction in hamsters.

Applied stevioside has no effect on fertility, mating performance, pregnancy, number of fetuses, or the growth and fertility of the offspring (*13*, *15*, *18*, *27*−*29*). In our experiments, no effects of administered steviol were found. However, when steviol (the aglucone of stevioside) was daily given to hamsters on day 6−10 of pregnancy at doses of 500−1000 mg/kg BW, it induced toxicity (*30*). The number of live fetuses per litter and mean fetal weight decreased. The maternal kidneys showed a dose-dependent increase in severity of convoluted tubules in the kidneys. The no observable effect level (NOEL) for maternal and fetal toxicity was 250 mg/kg BW. This study with steviol is not relevant for the use of stevioside as a sweetener. When stevioside was fed to hamsters up to 2.5 g /kg BW, no toxic effects were found, not even in three successive generations (*28*). When steviol is given in the feed, it can be resorbed directly by the intestines, whereas stevioside is not. Stevioside is transformed only by the bacteria of the caecum or the colon from which steviol eventually may be resorbed or taken up by coprophagy. Moreover, hamsters are known to be very sensitive to steviol and stevioside (*31*); this is the reason that hamsters were chosen in this study. The NOEL of steviol was 250 mg/kg BW (*30*), which corresponds to 625 mg stevioside/kg BW. Even under these very unfavorable conditions, an ADI of 2.5 mg steviol/kg BW, which corresponds to 6.25 mg stevioside/kg BW, can be calculated, which is close to 7.9 mg/kg BW obtained for stevioside (*4*). In conclusion, from our results, it is obvious that prenatal exposure to stevioside and steviol is not toxic for the chicken embryo.

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