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The effect of fumonisin B_1 on developing chick embryos: correlation between de novo sphingolipid biosynthesis and gross morphological changes*

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Fumonisins, mycotoxins produced by *Fusarium moniliforme* and a number of other fungi, are potent inhibitors of the sphinganine-*N*-acyltransferase, a key enzyme of sphingolipid biosynthesis, and cause neuronal degeneration, liver and renal toxicity, cancer and other injury to animals.

In this study we investigated the effect of fumonisin B_1 on the sphingolipids of developing chick embryos. After yolk sac injection of fumonisin B_1 a concentration and time dependent increase of the sphinganine-over-sphingosine ratio of the embryos could be demonstrated. Studies were done to evaluate the effect of fumonisin B_1 on the glycosphingolipid pattern of the chick embryos. In the presence of 72 μg fumonisin B_1 per egg the incorporation of [^{14}C]galactose and of [^{14}C]serine into embryonic glycosphingolipids was reduced by about 70%, although the mass of glycosphingolipids was not affected by the toxin. However, a reduction of the wet weight of the treated embryos was observed. Additionally, histological examinations of whole embryo sections of control and fumonisin B_1 treated embryos are presented. Fumonisin B_1 caused haemorrhages under the skin as well as in the liver of treated embyros. A close correlation between disruption of sphingoid metabolism and light microscopic detectable tissue lesions could be observed.

Keywords: chick embryo, fumonisin B_1, glycosphingolipid biosynthesis, sphingoid bases

Abbreviations: Cer, ceramide (*N*-acylsphingosine); FB_1, fumonisin B_1; GM3, NeuAcα2→3Galβ1→4Glcβ1→1Cer; GD3, NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1→1Cer; GD1a, NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1Cer; GT1b, NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→8NeuAcα2→3)Galβ1→4Glcβ1→1Cer; HPLC, high pressure liquid chromatography; PBS, phosphate buffered saline; PDMP, 1-phenyl-2-dodecanoylamino-3-morpholino-1-propanol; Sa, sphinganine; So, sphingosine; Sa/So, sphinganine-over-sphingosine; TLC, thin layer chromatography; Tris, Tris(hydroxymethyl)aminomethan.

Introduction

Fumonisins are toxic metabolites of the fungus *Fusarium moniliforme* and other fungi that grow on corn. Consumption of contaminated corn has been associated with oesophageal cancer in humans in southern Africa, China and other countries [1–3], and with numerous diseases of veterinary animals, including equine leucoencephalomalacia and hepatotoxicity [4], porcine pulmonary oedema [5], renal toxicity, hepatotoxicity and liver cancer in rats [6].

Much effort is being committed to determine the minimum dietary concentration of fumonisins necessary to cause deleterious effects in different animal species, for example, in young broiler chicks [7–9]. Fumonisin B_1 causes morphological and functional changes in chicken

*Dedicated to Dr Sen-itiroh Hakomori in celebration of his 65th birthday.
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macrophages *in vitro*, which implies an immunosuppressive effect [10].

The predominant member of the fumonisins is fumonisin B_1 (FB_1). Fumonisin B_1 bears a remarkable structural resemblance to sphinganine and sphingosine, metabolites in the biosynthesis and degradation of complex sphingolipids (Fig. 1). It could be demonstrated *in vitro* and *in situ* that fumonisin B_1 blocks sphingolipid biosynthesis by specifically inhibiting sphinganine-*N*-acyltransferase [11–13]. This enzyme catalyses the transfer of an acyl chain to sphinganine, thus yielding dihydroceramide. This reaction is followed by the introduction of the double bond leading to ceramide, the precursor of sphingomyelin and complex glycosphingolipids [14]. A consequence of the fumonisin B_1 block is an accumulation of the sphingoid bases. Elevated levels of free sphinganine and an elevated sphinganine-over-sphingosine ratio could also be detected in the sera of ponies, pigs and rats fed contaminated corn or culture material from *Fusarium moniliforme* containing known levels of fumonisin B_1 [15–17].

Since sphingolipids are involved in cell growth, differentiation, morphogenesis and apoptosis [18, 19], disruption of their metabolism by fumonisin B_1 might be the mechanism for the toxicity and carcinogenicity of these mycotoxins [20]. Sphingolipids function in cell type specific adhesion processes as ligands for selectins and serve as binding sites for viruses, bacteria, and toxins [21–23]. Recently, the role of sphingosine as well as that of its metabolite sphingosine-1-phosphate as second messengers have been discussed [24, 25].

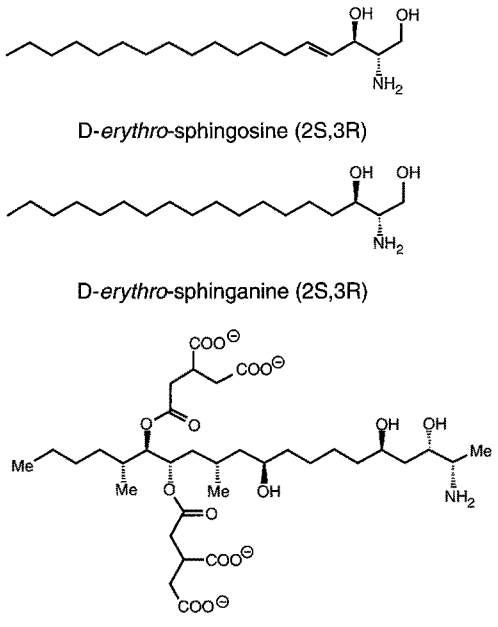


Figure 1. Structures of fumonisin B_1, sphingosine, and sphinganine.

The chick embryo is a well-documented system to test the toxic effects of mycotoxins on embryonic development. Recently embryopathical and embryocidal effects of fumonisin B_1 on the chick embyro have been reported [26]. However, no studies concerning the mechanism of action of the toxin have been conducted.

In this study, fumonisin B_1 was used to inhibit biosynthesis of embryonic glycosphingolipids and to investigate the consequences of this inhibition on embryonic development.

Materials and methods

MATERIALS

Fumonisin B_1 (F-2643), D-sphingosine and DL-*erythro*-sphinganine were supplied by Sigma, Germany. The synthetic C20-sphinganine standard for HPLC was prepared as described previously [30]. D-*erythro*-[4,5-^{3}H]sphinganine was made according to Schwarzmann and Sandhoff [33] with a specific activity of 750 Ci mol^{-1}. [^{14}C]FB_1 (1.13 Ci mol^{-1}) obtained by spiking corn cultures of *F. moniliforme* with L-[methyl-^{14}C]methionine during the active production phase of the mycotoxins [34] was available in Dr Merrill's laboratory as a gift from Dr David Miller (Canada). D-[1-^{14}C]-Galactose (2.04 GBq mmol^{-1}) and L-[3-^{14}C]serine (2.13 GBq mmol^{-1}) were purchased from Amersham Corp., Germany. Thin layer Silica Gel 60 plates and LiChroprep RP18 were supplied by Merck, Germany. All other reagents and solvents used were of analytical grade quality.

Chick embryos

White Leghorn eggs were purchased locally in Atlanta, Georgia (USA). Eggs were kept refrigerated at 2–8 °C (approximately 24 h) until candled for fertility, and were incubated at 37 °C and at 40–60% relative humidity. On the day of inoculation, living embryos were selected by candling.

METHODS

Yolk sac injection

For inoculation into the yolk sac of the embryo, the egg was placed upright (air sac upwards), and the shell above the air sac was nicked in the centre. A 23-gauge 0.6 × 25 mm needle was inserted down the long axis of the egg through the air sac and the chorioallantoic membrane to the full length of the needle approximately to the centre of the egg. Each egg in a treatment group was inoculated with up to 100 μl of liquid. Control embryos were treated with phosphate-buffered saline (PBS); the fumonisin B_1 treated embryos received the indicated concentration of the mycotoxin dissolved in PBS. Sphinganine was dissolved in PBS containing 4 mg

of BSA per ml. Eggs were sealed with paraffin wax and embryos were kept at 37 °C. Death was established under candle by breakdown of vascular structure and (or) appearance of disseminated coagulation, blood leakage into allantoic or yolk fluid. Embryos that died because of the injection procedure or differed in their developmental stage from controls (the prevailing occurrence) were not taken into consideration.

Labelling and isolation of sphingolipids

Sphingolipids were labelled by injecting [^{14}C]galactose (10 μCi per 50 μl) or [^{14}C]serine (10 μCi per 50 μl) into the yolk sac. After the indicated times the embryos were killed and lipids extracted from homogenized embryos with 10 ml of chloroform:methanol:water:pyridine (60:30:6:1, by vol) for 48 h at 48 °C. Phospholipids were degraded by mild alkaline hydrolysis with methanolic NaOH (50 mM) for 2 h at 37 °C. Then the lipid extracts were desalted by reverse-phase chromatography on silica gel LiChroprep RP18, applied to TLC, and chromatographed with chloroform:methanol:0.22% aqueous $CaCl_2$ (60:35:8, by vol); sphingolipids were visualized by fluorography.

Identification and quantitative evaluation of labelled glycosphingolipids

All glycosphingolipids were identified from their R_f values [27, 28]. Radioactive bands were scraped from the TLC plate and measured by liquid scintillation counting.

Mass measurements of free long-chain (sphingoid) bases

The free sphingosine and sphinganine content in base-treated chloroform-methanol extracts of chick embryo homogenate was determined by HPLC as described previously [29] with C20-sphinganine [30] as an internal standard for the recovery of long-chain bases. C18-sphinganine and C18-sphingosine were identified by comparison of their retention times with standards. To prepare the homogenate the chick embryo was taken from the egg, washed with PBS and weighed. Then, four volumes (w/v) of 0.05 M potassium phosphate buffer were added and the embryo was minced using a motor driven tissue grinder.

Mass measurements of total sphingolipids

Total sphingolipids were determined by acid-hydrolysing chloroform-methanol extracts of chick embryo homogenate and HPLC analysis of the released sphingosine and sphinganine as described previously [31]. Acid hydrolysis releases all sphingosine and sphinganine from complex sphingolipids (ceramides, sphingomyelin, glycosphingolipids, etc.). The values obtained for sphingosine and sphinganine released after acid hydrolysis were then corrected for free sphingosine and free sphinganine, as determined from base hydrolysed samples, to give the complex sphingolipid content (total minus free). All samples for sphingolipid analysis were kept frozen at −20 °C.

Assay of acyl-CoA:sphinganine-N-acyltransferase

To assay for sphinganine-*N*-acyltransferase, D-*erythro*-[4,5-^{3}H]sphinganine and stearoyl-CoA were used as substrates. The reaction mixture in a total volume of 50 μl contained 0.1 M Tris (pH 7.4, 37 °C), 0.5 mM dithiothreitol, 0.2 mM stearoyl-CoA, 0.2 mM sphinganine (0.8 μCi), 2 mM phosphatidylcholine, and 200 μg of chick embryo homogenate. The mixture of sphinganine and phosphatidylcholine was dried, Tris and dithiothreitol were added, and the sample was sonicated for several minutes at 4 °C. Then, stearoyl-CoA and the chick embryo homogenate were added. After incubation for 15 min at 37 °C, the lipids were extracted, separated on TLC (chloroform:-methanol:water, 160:20:2), and the radiolabelled ceramide was determined by scanning, cutting out the regions of interest, and scintillation counting.

Histological examination

Whole embryos collected for histological examination were fixed in 10% formalin, sectioned, and stained with Haematoxylin and Eosin [32].

Presentation of data and statistics

All experiments were conducted in duplicate and standard deviations never exceeded 15%. HPLC analysis of sphingoid bases of each homogenate were conducted in triplicate. All values are expressed as means ± SD and '*n*' refers to the number of chick embryos in a certain treatment group. Differences between treatment groups were analysed by Student's *t*-test. Differences were considered significant with $p < 0.05$.

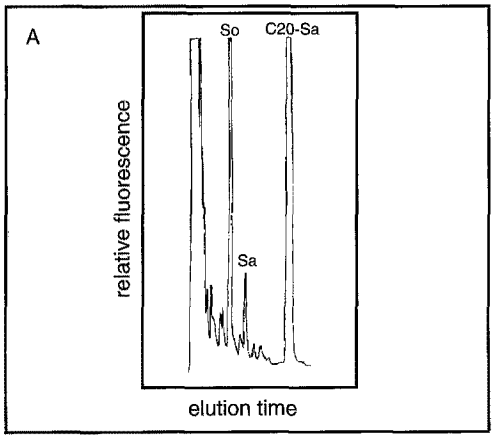
Results

The effect of fumonisin B_1 on the profile of long-chain bases in chick embryos

Chick embryos were treated with fumonisin B_1 (inoculation into the yolk sac), harvested on the indicated days and HPLC analysis of the o-phthalaldehyde derivatives of the free long-chain bases were conducted. As shown in the elution profile of long-chain sphingoid bases from the homogenate of a control embryo (Fig. 2, panel A), C18-sphingosine and C18-sphinganine are the predominant free long-chain bases. The free sphingosine content is six times higher than the free sphinganine content in controls (So: 1.25 nmol g^{-1} wet weight; Sa: 0.22 nmol per g wet weight). The ratio sphinganine-over-sphingosine, which has been used as a biomarker for FB_1-exposure [16, 17], amounts to 0.18. The HPLC profile of sphingoid bases from the homogenate of a fumonisin B_1 treated embryo

(360 μg per egg) shows pronounced alterations, especially the sphinganine peak, as compared to untreated controls (Fig. 2, panel B).

To quantify this effect, chick embryos were treated either from day 3 to day 7 of development (Fig. 3) or from day 4 to day 7 (data not shown) with different concentrations of fumonisin B_1. In both experiments, a concentration dependent increase in the free sphinganine content could be observed. From developmental day 3 to 7, a three-fold increase in the sphinganine content at 72 μg fumonisin B_1 per egg, and at 360 μg fumonisin B_1 per egg a six-fold elevation of the sphinganine content was observed compared to controls. An increase in the sphingosine level could also be observed at concentrations from 72 μg fumonisin B_1 per egg upwards.



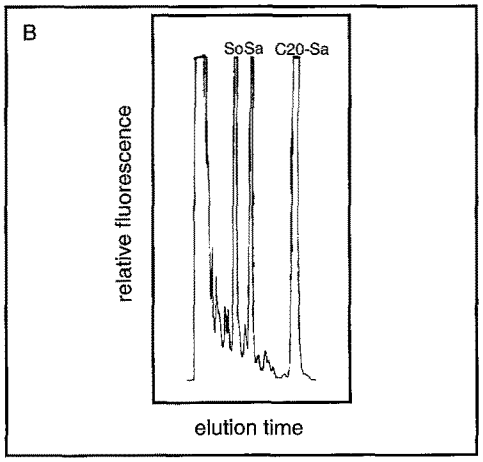


Figure 2. Analysis of the free long-chain bases of whole chick embryos. Chick embryos were treated from embryonic day 3 until day 7 in the absence (upper panel) or presence of 360 μg per egg fumonisin B_1 (lower panel). The elution profiles of the o-phthalaldehyde derivatives of the free long-chain bases with C20-sphinganine (C20-Sa) as an internal standard (eluting at 14.2 min) are shown. The elution times for sphingosine (So) and sphinganine (Sa) were 6.4 and 8.5 min, respectively.

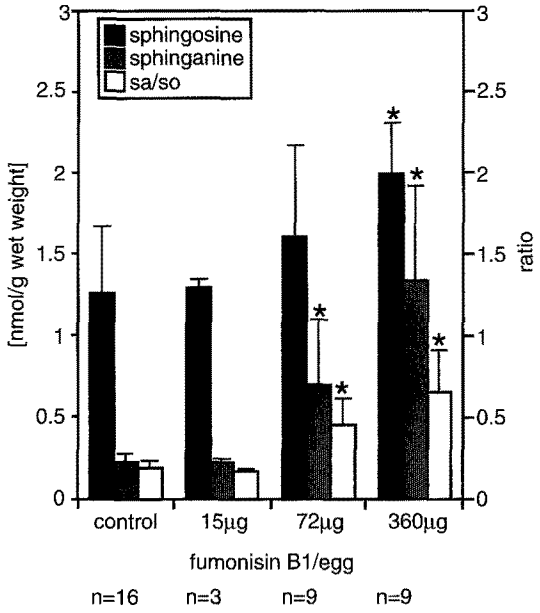


Figure 3. Effect of different concentrations of fumonisin B_1 on the free long-chain bases of chick embryos. Chick embryos were treated from embryonic day 3 until day 7 with PBS (control) or the indicated amounts of fumonisin B_1. Then HPLC analysis of the free long-chain bases were conducted as described in Materials and methods. Each bar represents the mean ± SE, n = number of chick embryos in each treatment group. *Significantly different from controls ($p < 0.05$).

However, only the elevation of the sphingosine level in embryos treated with 360 μg fumonisin B_1 per egg was statistically significant, raising from 1.25 to 1.99 nmol g^{-1} wet weight. Relatively high standard deviations were seen in all groups and are probably due to individual differences.

Even though both sphinganine and sphingosine increase, the alteration in the sphingosine level was not as pronounced as the change in free sphinganine. As a consequence, the sphinganine-over-sphingosine relationship increased in a fumonisin B_1 concentration dependent manner. In the experimental group injected at day 4 with different concentrations of fumonisin B_1, there was only a two-fold and four-fold increase of free sphinganine at 72 μg fumonisin B_1 per egg and 360 μg fumonisin B_1 per egg, respectively (data not shown). The concentration of free sphingosine remained unchanged compared to controls, thus leading to a clearcut increase of the sphinganine-over-sphingosine ratio.

To investigate if the effect of fumonisin B_1 (72 μg per egg) is time dependent, embryos were injected on embryonic day 3 and harvested at different time points (Fig. 4). A lag in the change of the profile of the free long chain bases was observed. There was no difference in the sphingosine and sphinganine level in the homogenate of treated embryos taken out of the egg on day 5 and on day 6 compared to control embryos. The first

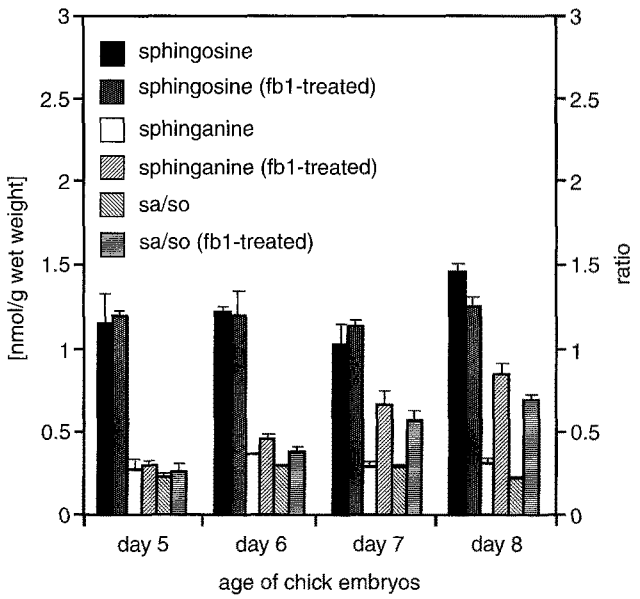


Figure 4. Time course of the effect of fumonisin B_1 on the free long-chain bases of chick embryos. Chick embryos were treated from embryonic day 3 until the indicated day of development with 72 μg fumonisin B_1 (fb 1-treated) or with PBS (control). Then HPLC analysis of the free long-chain bases were conducted as described in Materials and methods. Each bar represents the mean ± SE of two different chick embryos.

change in the sphinganine value could be observed in the homogenate of embryos harvested on developmental day 7. The sphinganine level rose from 0.28 nmol g^{-1} wet weight in controls to 0.65 nmol g^{-1} wet weight in treated embryos. In the homogenate of embryos which were harvested on developmental day 8 an even more pronounced elevation of the sphinganine level (0.84 nmol g^{-1} wet weight) was measured compared to controls (0.3 nmol g^{-1} wet weight). As the sphingosine content is not influenced under these conditions, the sphinganine-over-sphingosine ratio changed correspondingly. Further treatment of the embryos led to death.

Analogous results were obtained when the injection of 72 μg fumonisin B_1 was carried out on day 4 (data not shown). Increased sphinganine levels in the homogenates of treated versus untreated embryos could be seen only after 3 days of treatment with fumonisin B_1: whereas the homogenate of control embryos contained 0.22 nmol g^{-1} wet weight sphinganine, in the fumonisin B_1 treated embryos 0.53 nmol g^{-1} wet weight sphinganine were determined. There was no difference in the sphingosine content of control and treated embryos on this day.

The effect of fumonisin B_1 on sphingolipid biosynthesis of chick embryos

Taken together, our experiments demonstrate that after 3 days of treatment higher concentrations of fumonisin B_1 (72 μg fumonisin B_1 per egg) cause a significant increase (two-fold) of the free sphinganine level in chick embryos. These increased levels of sphinganine are most probably due to the inhibition of sphinganine-*N*-acyltransferase by fumonisin B_1. *In vitro*, this enzyme from chick embryo homogenate is indeed inhibited by about 65% in the presence of 25 μM fumonisin B_1 (corresponding to 72 μg (100 nmol) fumonisin B_1 per egg) (data not shown). Now the question arises: does the blocking of sphinganine-*N*-acyltransferase lead to changes in the mass of chick embryo sphingolipids? Therefore, the lipid extracts were subjected to acid hydrolysis, thus releasing all long-chain bases from ceramides, sphingomyelin and glycosphingolipids. As illustrated in Fig. 5, no significant differences in the nmol g^{-1} wet weight of the released long-chain bases could be observed in the different treatment groups compared to control embryos. However, the wet weight of the treated embryos was reduced by about 25% compared to controls.

To get more information on the effect of fumonisin B_1, we studied the glycosphingolipid pattern of treated and untreated embryos by injecting radioactive precursors of their biosynthesis. Table 1 clearly shows that the biosynthetic labelling of sphingolipids is reduced by about 76% and 67% in the presence of FB_1 using serine or galactose as a radioactive precursor, respectively.

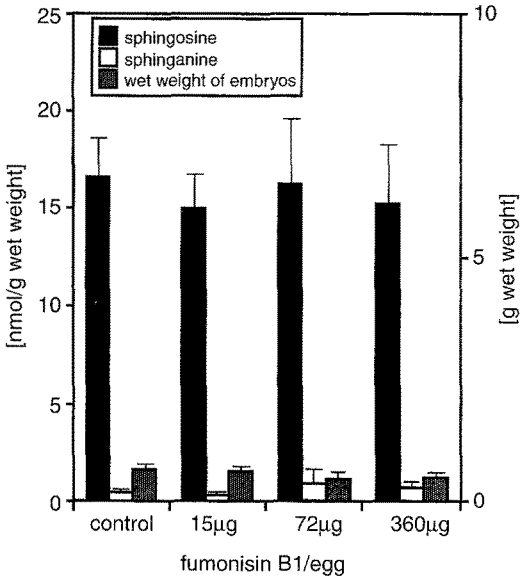


Figure 5. The effect of fumonisin B_1 on the mass of chick embryo sphingolipids. Chick embryos were treated from embryonic day 3 until day 7 with PBS (control) or the indicated amounts of fumonisin B_1. After acid hydrolysis HPLC analysis of the long-chain bases were conducted as described in Materials and methods. These values were corrected for the amount of free sphingosine or free sphinganine (total minus bound). Each bar represents the mean ± SE of three different chick embryos.

Table 1. Inhibition of sphingolipid labelling in chick embryos by fumonisin B_1. Chick embryos were inoculated with 50 μl PBS (control) or with 72 μg fumonisin B_1 per egg (dissolved in 50 μl PBS) on embryonic day 4. On embryonic day 7 either [^{14}C]serine (10 μCi per 50 μl) or [^{14}C]galactose (10 μCi per 50 μl) were also injected. After 24 h embryos were collected, homogenized and lipids were extracted and separated by thin-layer chromatography. Sphingolipids were identified by radioscanning and comparison with known standards. Radiolabelled sphingolipids were recovered from the plates by scraping and radioactivities were determined by liquid scintillation counting. The experiment was conducted in duplicate and standard deviations never exceeded 15%.

Sphingolipids	*[^{14}C]Serine-labelling (cpm × 10^3 per g wet weight)*		*[^{14}C]Galactose-labelling (cpm × 10^3 per g wet weight)*	
	Control	*Fumonisin B_1 (72 μg)*	*Control*	*Fumonisin B_1 (72 μg)*
Gangliosides	3.7	1.0	19.6	5.9
Sphingomyelin	6.7	1.0		
LacCer	1.0	0.6	3.3	1.6
GlcCer	0.9	0.3	2.7	1.0
	12.3 (100%)	2.9 (24%)	25.6 (100%)	8.5 (33%)

Uptake of [^{14}C]fumonisin B_1

To determine the amount of fumonisin B_1 that was taken up by the embryo, experiments with [^{14}C]-labelled fumonisin B_1 were carried out (data not shown). After injection of 72 μg [^{14}C]fumonisin B_1 on day 3 and harvesting on day 7, only 0.2% of dose radioactivity could be detected in the homogenate of whole embryos, while most of the radioactive FB_1 was found unchanged in the yolk and extraembryonic membranes as analysed by TLC.

Uptake of [^{14}C]sphinganine

To find out whether the toxic effects of fumonisin B_1 might be due to the accumulation of sphinganine, we injected sphinganine instead of fumonisin B_1 into the yolk sac of embryos. In analogy to the fumonisin B_1 experiments, embryos were subjected to 100 nmol sphinganine from day 4 to day 7, 8 and 9 of development. At the end of incubation only 0.2–0.5% of the radioactivity applied could be detected in the homogenate of whole embryos. Weight and outer appearance of treated embryos were unchanged when compared to control embryos. No haemorrhages were visible. Thus, exogenously applied sphinganine did not mimic the effect of fumonisin B_1.

Gross morphological changes of chick embryos induced by fumonisin B_1

The measured biochemical differences were paralleled by morphological changes of the embryos. Thus, a close correlation between disruption of sphingolipid metabolism and light microscopic detectable tissue lesions could be observed. Sectioning of embryos treated either with 72 μg or 360 μg fumonisin B_1 for 4 days (embryonic day 3 to day 7) showed subcutaneous haemorrhages all over the body of the embryos and in visceral organs, especially in the liver. The photographs in Fig. 6 give a qualitative impression. Correspondingly, an increased amount of erythrocytes could be seen in the liver and under the skin of treated embryos. Moreover, a different shape of the erythrocytes was observed. Red blood cells appeared to be round, thus resembling the morphology of immature rather than of adult cells.

Discussion

In the present work, a concentration and time dependent increase of the sphinganine level in the homogenate of whole chick embryos in response to fumonisin B_1 has unambiguously been demonstrated. These changes in sphinganine accumulation after fumonisin B_1 treatment resemble the effects that have been observed earlier. For examples, in cultured neuronal cells fumonisin B_1 [25 μM] caused a 20-fold increase of sphinganine [13], and *in vivo* studies of animals eating fumonisin-containing feed have revealed 96-, 17-, and 26-fold increases in free sphinganine concentrations in pig liver, lung, and kidney, respectively [16]. Compared to these systems, only a small effect was observed in chick embryos (maximum 6-fold increase), suggesting an active sphinganine catabolism. On the other hand, the inhibition of sphinganine-*N*-acyltransferase *in vitro* in chick embryos was not as high as reported for other tissues: 25 μM FB_1 caused 65% inhibition of this enzyme from chick embryos. In primary cultured neurons, sphinganine-*N*-acyltransferase was completely inhibited by 5 μM FB_1 [13]; the apparent IC_{50} for inhibition of this activity in rat hepatocytes was approximately 0.1 μM for FB_1 *in vitro* [11].

Another reason for the relatively weak effect could be the slight uptake of the fumonisin B_1 by the embryo. Only 0.2% of the administered radioactivity of fumonisin B_1 could be detected in the homogenate of chick embryos after 4 days of treatment. However, it cannot be excluded that more fumonisin B_1 was taken up at an earlier timepoint and then shedded back into the yolk. The [^{14}C]FB_1 was found metabolically unchanged in the yolk under these conditions. Correspondingly, other studies conducted with radioactive fumonisin B_1 showed

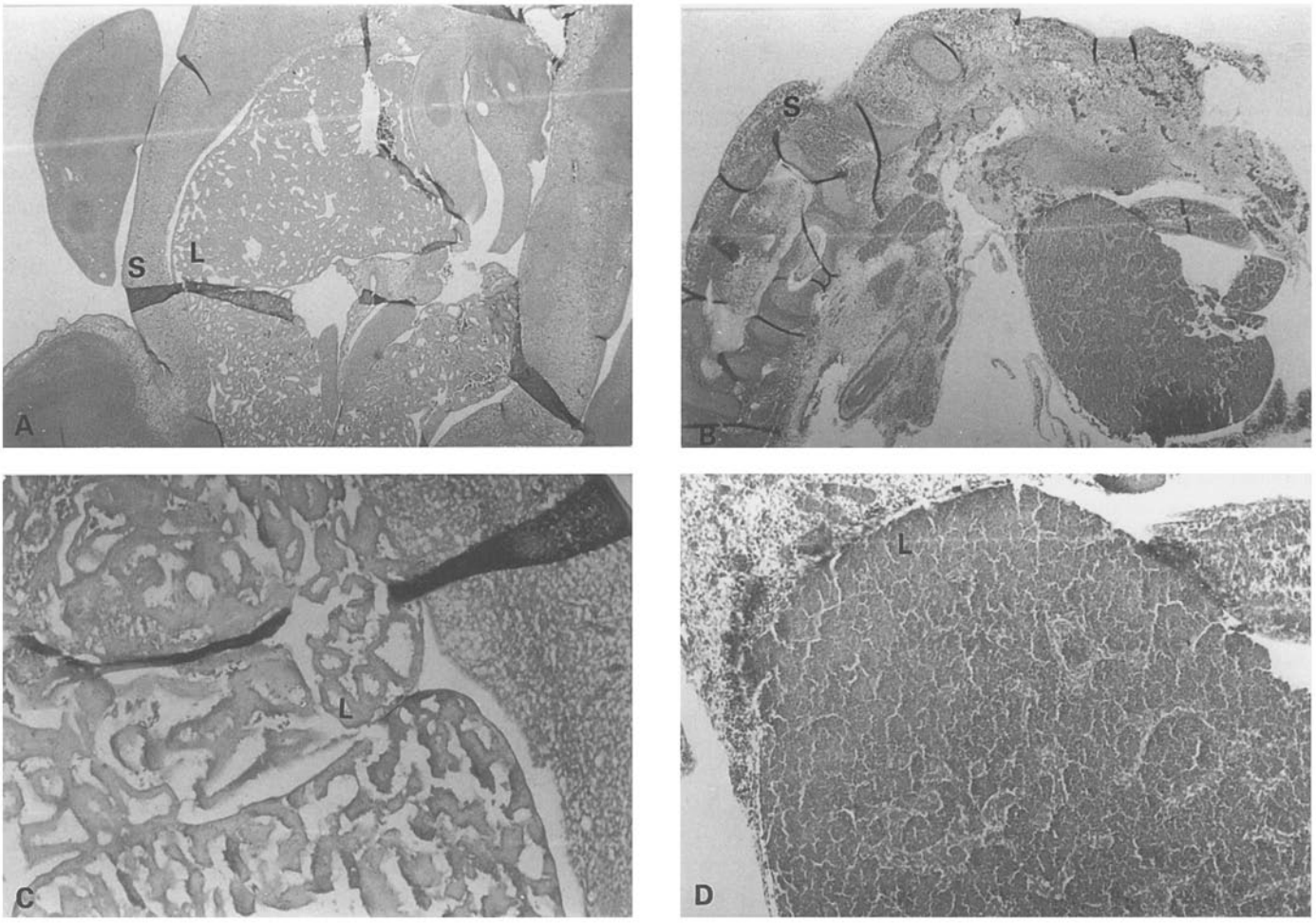


Figure 6. The effect of fumonisin B_1 on the morphology of skin and liver of chick embryos. Chick embryos were inoculated on day 4 with PBS (control A, C) or fumonisin B_1 (D, 72 μg) (B, 360 μg). On day 7 embryos were harvested, sectioned and stained as described in Materials and methods. Magnification 17× A, B; 68× C, D. L, liver; S, skin.

that most of the fumonisin B_1, which was administered intragastrically to rats, was found in faeces and urine, while in blood, liver and kidney only 0.1–0.5% of the administered dose could be detected [34]. Additionally, a study using rat hepatocytes demonstrated that only 0.01% of the [^{14}C]FB$_1$ was associated with these cells; most of the applied toxin was found unchanged in the culture medium [35].

Studying the time course of the sphinganine accumulation in the chick embryo after fumonisin B_1 administration, a lag period of 2 days was observed. Similar lag periods of fumonisin B_1 action were also found in feeding studies with ponies and pigs [15, 17]. The sphinganine level starts to rise from the third day after the fumonisin B_1 injection and rises with the time of treatment. After 5 days the treatment of chick embryos with 72 μg fumonisin B_1 per egg caused embryonic death.

Although sphingosine is only an intermediate of sphingolipid catabolism [14], it is a substrate for sphinganine-*N*-acyltransferase (ceramide synthase). Thus, reacylation of sphingosine generated from the breakdown of complex glycosphingolipids, sphingomyelin or ceramide is also blocked by fumonisin B_1. As a consequence, its level should also increase after fumonisin B_1 treatment. In our experiments, however, the increase in the free sphingosine content was statistically significant only in one treated group (day 3–day 7 at 360 μg fumonisin B_1 per egg). This may indicate that little sphingolipid turnover to free sphingosine occurs during development of chick embryos.

There was no significant difference in the effect of fumonisin B_1 on the level of sphingoid bases when the injection was done either on day 3 or day 4 of embryonic development. At this timepoint the blood vessels, which are the best indicators for the fertility of the egg, and the skeleton start to form. Injections done on developmental day 2 or day 1 with 72 μg fumonisin B_1 per egg led to an early necrotizing of the embryos (data not shown). However, at this timepoint it was impossible to

discriminate between embryos that died from the injection itself or due to the action of fumonisin B_1.

Sugar structures of glycoproteins on the cell surface are known to be essential for embryogenesis. Treatment of preimplanted mouse embryos with tunicamycin to inhibit protein glycosylation causes them to remain irreversibly uncompacted, and to not develop to the blastocyst stage [36]. It is well known that glycolipids also undergo qualitative and quantitative modifications during ontogenesis. In general there is a shift from the synthesis of the simplest gangliosides (GM3 and GD3) to synthesis of more complex gangliosides (GD1a, GT1b) [37–39]. However, to our knowledge, the involvement of glycolipids in embryogenesis has not, so far, been clearly demonstrated. Fish embryos cultured in the presence of 1-phenyl-2-dodecanoylamino-3-morpholino-1- propanol (PDMP), an inhibitor of glucosylceramide synthase, exhibited a dramatic decline in glycolipid synthesis and cell surface expression, but despite these changes they were fully viable with no evidence of developmental abnormality [40]. In this study, we have tried to determine if there is any correlation between glycosphingolipid biosynthesis and development of the chick embryo. The incorporation of [^{14}C]galactose and of [^{14}C]serine into glycosphingolipids of the developing whole chick embryo was reduced by about 70% in the presence of 72 μg fumonisin B_1. In spite of this reduced labelling, the mass of sphingolipids, calculated as nmol g^{-1} wet weight, was not influenced under the described conditions. There are several possible explanations for this finding. The remaining sphinganine-*N*-acyltransferase activity may be sufficient to maintain the specific glycosphingolipid content; or, the embryo may be able to utilize gangliosides, neutral and sulfated glycosphingolipids (which have been detected in the yolk) to compensate for a reduction in biosynthetically derived glycosphingolipids [28, 41]. One must take into account, however, that the wet weight of the treated embryos was reduced by about 25%, suggesting that the absolute lipid mass was also reduced by the same order. However, we cannot exclude that the mass of glycosphingolipids was limiting for the growth of the embryos as indicated by the reduced wet weight. This would imply that the inhibition of sphingolipid synthesis leads, indeed, to retarded growth. Moreover, the mass of glycosphingolipids may have been reduced in special cell types at an appointed timepoint leading to the arrestment of development and ultimately to embryonic death.

To investigate if the toxic effects of fumonisin B_1 were a result of the elevated levels of sphingoid bases rather than of the inhibition of complex sphingolipid biosynthesis, embryos were directly treated with sphinganine. The cytotoxic potential of long chain bases has already been demonstrated in Chinese hamster ovary cells [42]. However, embryogenesis was not affected by sphinganine. No differences in size and outer appearance could be noticed. Moreover, no bleeding was visible. The low amount of radioactivity found in the embryo after injection of labelled sphinganine could be either free sphinganine (and then it would correspond exactly to the sphinganine level accumulated after treatment with 72 μg fumonisin B_1) or metabolic products of this long-chain base. It appears, nonetheless, that the toxicity of long-chain bases depends on the cell type because even high concentrations [50 μM] of exogenously applied long-chain bases were not toxic for primary cultured neurons which rapidly use these compounds as precursors for their glycosphingolipid biosynthesis [43].

It has been shown that the catabolism of sphinganine accumulated in the presence of fumonisin B_1 is increased [Smith ER, Merrill AH, unpublished results]. Thus, we cannot exclude that in chick embryos catabolic products such as sphingosine-1-phosphate are responsible for the toxic effect of fumonisin B_1.

The possibility that the distribution of the exogenous sphinganine in the embryo is different from that of the endogenous accumulated sphinganine, could also explain its uneffectiveness. Very recently Javed *et al.* published a study on the embryopathical and embryocidal effects of fumonisin B_1 in chicken embryos [26]. The gross pathological changes noted in liver, kidney, musculoskeletal system and brain could be fully confirmed in our study using 72 μg fumonisin B_1 per egg, a concentration which significantly impeded sphingolipid biosynthesis. The most striking morphological change observed in our study was, however, the immigration of red blood cells in tissue of embryos exposed to the toxin. The erythrocytes looked immature and occurred predominantly in liver and under the skin. We do not know at the present time if the erythrocytes under the skin had not yet reached the blood vessels during erythropoeisis or if the vessels were leaky, thus liberating red blood cells. It has been shown recently in studies of porcine endothelial cells in culture that fumonisin B_1 disrupts the permeability barrier; therefore, the subcutaneous hemorrhaging may be due to injury to the vasculature [Ramasamy S, Wang E, Hennig B, Merrill AH, Jr, unpublished results].

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