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Evaluation of the Reproductive Toxicity of Dietary Fumonisin B_1 in Rats

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Abstract

The toxicity of dietary fumonisin B_1 (FB_1), a mycotoxin from the common maize fungus *Fusarium verticillioides*, on serum gonadotropins, ovarian histopathology, and fertility were examined in female Wistar rats. Thirty-nine female rats were randomly assigned to two test diets containing 10.0 and 20.0 mg FB_1/kg and a control diet. After 14 days of feeding, blood samples were obtained by intracardiac puncture from 4 rats in each treatment for gonadotropins evaluation, and were then killed by cervical dislocation to collect samples of ovaries for histopathology. Also, each of the remaining nine females in each treatment was mated to one healthy adult male rat. The serum LH concentrations of rats fed diets containing 10 and 20 mg FB_1/kg were significantly lower (P<0.05) than those fed the control diet, while the serum FSH level of rats fed diet containing 20 mg FB_1/kg only was significantly lower (P<0.05) compared with the controls. Dietary FB_1 however failed to induce histopathological changes in the ovaries of the rats. Fertility, gestation lengths and foetal weights of the rats decreased significantly (P<0.05) with increased dietary FB_1. The concentration of daily dietary FB_1/kgBW of 1.74 in this study is less than five times the estimated probable daily fumonisin intake of 355 μg/kg BW for person eating 'mouldy' maize in the high oesophageal cancer area of the Transkei region, South Africa. The apparent significant "safety factor" of about five times relative to human FB_1 exposure may be a cause for concern in areas where maize is a dietary staple.

Keywords: Fertility, Fumonisin B_1, Gonadotropins, Mycotoxin, Rats, Toxicity.

1. Introduction

Mycotoxins are natural contaminants of cereals and other food commodities throughout the world and they significantly impact human and animal health. Animals, as well as humans, are exposed to mycotoxins through consumption of contaminated diets, which can be considered the gateway to cases of natural intoxication by these fungal secondary metabolites (Gutema, 2000; Hennigen, 2000).

The economic consequences of mycotoxin contamination are profound, and exposure of people and livestock to mycotoxin-contaminated foods is particularly a serious problem in the tropics (Reddy and Raghavender, 2008). According to Lawlor and Lynch (2001), 25% of the global crop is contaminated with mycotoxins. Crops with large amounts of mycotoxins often have to be destroyed. Alternatively, contaminated crops are sometimes diverted into animal feeds. Giving contaminated feeds to susceptible animals poses a serious threat to the health and productivity of the animals and cause great economic losses (Griessler and Encarnação, 2009), by acting directly or indirectly on fertility. Reproductive inefficiency is recognized as the most costly limiting constraint to efficient animal production as reproduction is the bedrock of animal production (Gbore, 2009; Ewuola and Egbunike, 2010). This makes the assessment of the effects of mycotoxins on livestock reproduction a unique challenge in an effort to improve livestock production.

Fusarium verticillioides (Sacc) Nirenberg (= *F. moniliforme* Sheld.), one of the most prevalent mycotoxigenic fungi reported to be associated with dietary staples such as maize intended for human and animal consumption throughout the world (Nelson *et al*., 1991; Kedera *et al*., 1992), produces the mycotoxin, fumonisin. *F. verticillioides* is present in virtually all maize samples (Marasas *et al*., 2001). Maize is the major cereal utilized in the formulation of livestock feeds in several parts of the world, hence, the potential for fumonisins to be found in feeds and feedstuffs is high. Several naturally occurring fumonisins are known; FB_1 has been reported to be the most abundant and most toxic which represents approximately 70% of the total concentration in naturally contaminated foods and feeds, followed by fumonisins B_2 (FB_2) and B_3 (Murphy *et al*.,

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1993; Norred, 1993). Consequently, toxicological studies on the fumonisins have been concentrated on FB_1.

The carcinogenicity, hepatotoxicity and mutagenicity as well as the effects on feed intake, live weight gain and blood abnormalities of fumonisin in animals have been well documented (Marasas *et al*., 1988; Harrison *et al*., 1990; Kellerman *et al*., 1990; Gelderblom *et al*., 1991, 1994; Colvin and Harrison, 1992; Voss *et al*., 1998; Ewuola and Egbunike, 2008; Ewuola *et al*., 2008; Gbore and Egbunike, 2008, 2009; Gbore, 2009). However, studies on depression of fertility and reproductive processes in rats by the toxin are rare.

Flynn *et al*. (1996) reported growth inhibition of rat embryos exposed *in vitro* to FB_1 on gestation day (GD) 9.5. Although useful as screens, *in vitro* methods allow direct foetal exposure and avoid maternal gastrointestinal absorption, pharmacokinetics, placental transfer, and other potential barriers of *in utero* exposure. *In vivo* assessment of the effects of fumonisin is therefore necessary.

Consumption of lesser amounts of fungal toxins at levels below those that cause overt toxicity may result in impaired fertility and decreased reproduction in animals. In a preliminary study, Gbore and Olorunfemi (2009) reported significant concentration-dependent decline conception rates in rabbits exposed to dietary fumonisin prior to mating. Fumonisins inhibit sphingolipids metabolism, and a variety of biological activities for sphingolipids have been reported (Wang *et al*., 1992). It was therefore hypothesized that fumonisin could alter the release of gonadotropins from the pituitary because the brain contains high levels of sphingolipids. Studies on the alterations of gonadotropins and subsequent fertility in rats exposed to dietary FB_1, to our understanding, are scarce. It is essential, therefore, to assess the gonadotrophic and ovarian histopathological effects of *F. verticillioides*-contaminated maize-based diets and subsequent fertility and reproductive processes in rats.

2. Materials and Methods

2.1. Experimental Site and Animals

Eight weeks old male and female Wistar rats (*Rattus norvegicus*) obtained from a commercial breeder of Wistar rats in Benin City, Nigeria were used. Females weighed 168.92 ± 1.41 g. Male rats (202 ± 2.21 g) were used as sires only and were not exposed to dietary fumonisin. Thirty-nine mature females were individually housed under standard housing conditions (22°C, light/dark cycle 12/12 hours) in wire mesh rat cages at the Animal House of the Department of Biochemistry, Adekunle Ajasin University, Akungba Akoko, Nigeria. Further laboratory analyses were carried out at the Department of Chemical Pathology of the University College Hospital, and Department of Veterinary Pathology, all of the University of Ibadan, Ibadan, Nigeria. This study was approved by the local Institutional Animal Ethics Committee and was performed in accordance with "Guide for the care and use of Laboratory Animals" (National Research Council, 1996).

2.2. Fumonisin B_1 Production and Experimental Diets

Maize grits in 500 g quantities were placed into autoclavable polypropylene bags and soaked with 200 ml of distilled water for 2 h, then autoclaved for 1 h at 121°C and 120 kPa. The autoclaved maize grits were then cultured with a toxigenic strain of *F. verticillioides* (MRC 286) obtained from the Plant Pathology Laboratory of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria to produce FB_1 as described previously (Nelson *et al*., 1994). Uncultured maize grits and the cultured maize grits were used to formulate three diets. Samples of homogenously mixed diets were quantified in replicates for FB_1 and other common *Fusarium* mycotoxins including deoxynivalenol (DON, vomitoxin), T-2 toxin, and zearalenone using mycotoxin quantitative CD-ELISA test kits (Neogen, Lansing, MI, USA) and reconfirmed by using HPLC analyses as described by Shephard *et al*. (1990). The concentrations of FB_1 in the diets were adjusted to 0.2, 10.0 and 20.0 mg/kg constituting diet 1 (= control diet, which had no *Fusarium*-contaminated maize grits), diets 2 (medium FB_1-contaminated diet) and 3 (high FB_1-contaminated diet), respectively. The concentrations of all other common *Fusarium* mycotoxins screened were below the detection limit of 0.2mg/kg for the toxins. The dietary FB_1 doses in this study were based on a preliminary dose-range-finding study by Collins *et al*. (1998a). Lower doses were selected for this study, the highest dose being 20 mg/kg. The pelleted diets provided ~20% crude protein, 5% crude fibre and 2.9 kcal of digestible energy/g.

2.3. Experimental Model

After 3 weeks of physiological adjustment period, the rats were randomly allocated to each of the three diets (n = 13 rats per treatment). The rats were provided with fresh clean water and appropriately weighed feed daily, and the weights of feed portions given and left uneaten after 24 h were determined. The body weight was determined weekly on a weighing scale (Ohaus Corp., Pine Brook, NJ, USA) with a precision of 0.05 g. The body weight gain of each rat was determined weekly as the weight difference in comparison to the weight in the previous week.

After 14 days of feeding, blood samples were obtained by intracardiac puncture from 4 rats from each treatment and were killed by cervical dislocation to collect samples of ovaries. Blood samples were collected into vacutainer tubes, covered and centrifuged at 4000 rpm for 10 minutes. The separated sera were decanted and deep-frozen for serum gonadotropins analyses at the Chemical Pathology Unit of the University College Hospital, Ibadan, Nigeria.

2.4. Evaluation of Reproductive Performance

To evaluate reproductive performance, after 2 weeks of feeding the respective experimental diets, each of the remaining nine females in each treatment was placed in a cage with one healthy adult male. The animals were kept together overnight, and then separated the following morning. Immediately after each separation, a vaginal smear examination was carried out to determine if sexual intercourse had occurred. When intercourse was positive (presence of spermatozoa in the vaginal smear), the night/day routine was discontinued and the female was housed individually during the estimated period of

gestation. At parturition, percentage of fertility with respect to the number of positive smears, gestation length, number of litters, mean litter size, number of live foetus per litter, and foetal weights were determined.

2.5. Examination of Ovaries

For examination by light microscopy, ovary samples collected were fixed in 10% neutral buffered formalin (pH 7.2) before dehydration in ten changes in ethanol of different concentrations ranging from 70 to 100% at 1-hr interval. After dehydration, the tissues were cleared in two changes of chloroform before infiltration and embedding in molten wax (60°C) for 12 h. Thereafter, the tissues were blocked in paraffin wax and later sectioned using a microtome. Paraffin sections (4µm) of the ovary samples were stained with haematoxylin and eosin. Photomicrographs were taken with a Zeiss Axiophot instrument using Kodak Plus-X pan (PX 135 to 24) film.

2.6. Determination of Serum Gonadotropins

Serum levels of gonadotropins; follicle-stimulating hormone (FSH) and luteinizing hormone (LH), were measured using a commercial ELISA kit (Habersham, Buckinghamshire, UK). All samples were run in duplicate in a single assay.

2.7. Statistical Evaluation

Data from this study were analyzed by one-way analysis of variance procedure of SAS (2001). The treatment means were compared using the Duncan procedure of the same software and results giving *P* values of <0.05 were considered significantly different.

3. Results

3.1. Feed Consumption and Body Weight Gain

Daily observation through the 35 days did not indicate detectable alterations in the general state of any of the animals. The average food consumptions were 17.64, 16.03, and 18.77 g for rats on diets 1, 2, and 3 respectively. Significant ($P<0.05$) differences in feed consumption and final weights were not observed (Figures 1 and 2), although the mean weights of the dams given 10 and 20 mg FB_1/kg (216 ± 17.6 to 218 ± 15.0 g) were 2.5 - 3.4% lower than that of the controls (223.56 ± 11.0 g) on day 35. Based upon feed consumption and body weight data, the diets provided 0.08, 0.74 and 1.74 mg FB_1/kg BW per day to the rats on diets 1, 2, and 3, respectively.

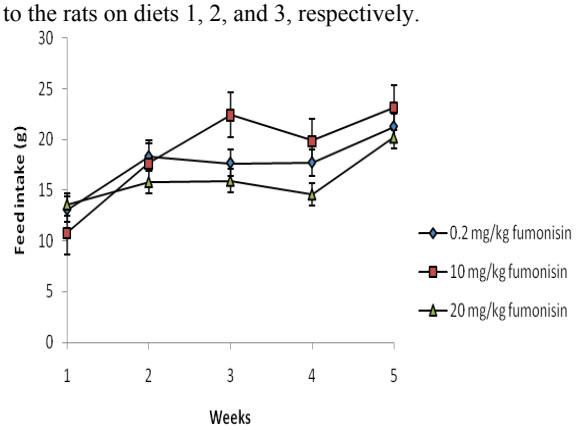


Figure 1. fed intake of rats fed different concentrations of dietary fumonision B1.

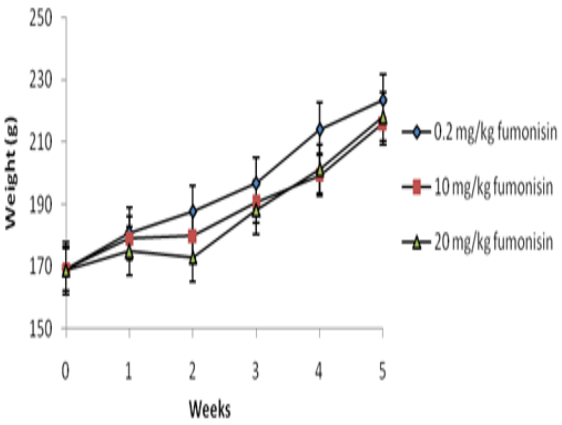


Figure 2. weights of rats fed different concentrations of dietary fumonision B1.

3.2. Serum Gonadotropins Levels

Dietary FB_1 significantly depressed serum gonadotropins in rats (Table 1). The serum LH concentrations of rats fed diets containing 10 and 20 mg FB_1/kg were significantly lower ($P<0.05$) than those fed the control diet. Similarly, the serum FSH levels of rats fed diet containing 20 mg FB_1/kg were significantly lower ($P<0.05$) than those fed the control diet and diet containing 10 mg FB_1/kg.

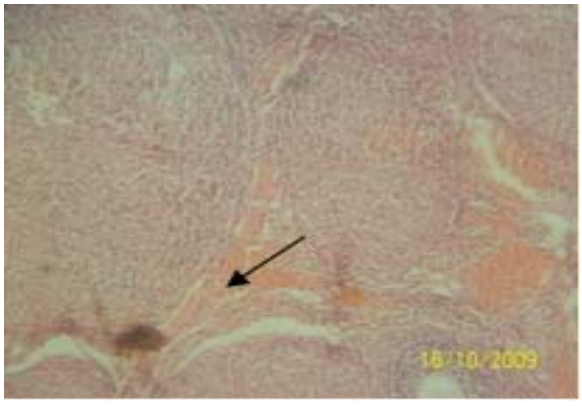
Table 1. Serum Gonadotropins (ng/ml) of Rats Exposed to Different Concentrations of Dietary Fumonisin B_1 (Mean ± SEM)

	Dietary fumonisin B_1 concentrations (mg/kg)		
Parameters	0.2 (Control Diet)	10.0 (Diet 1)	20.0 (Diet 2)
Luteinizing Hormone	23.00 ± 0.01[a]	10.90 ± 2.50[b]	10.03 ± 2.80[b]
Follicle Stimulating Hormone	215.00 ± 21.01[a]	214.90 ± 20.07[a]	199.80 ± 22.16[b]

[ab]: Means on same row with different superscripts differ significantly ($P < 0.05$)

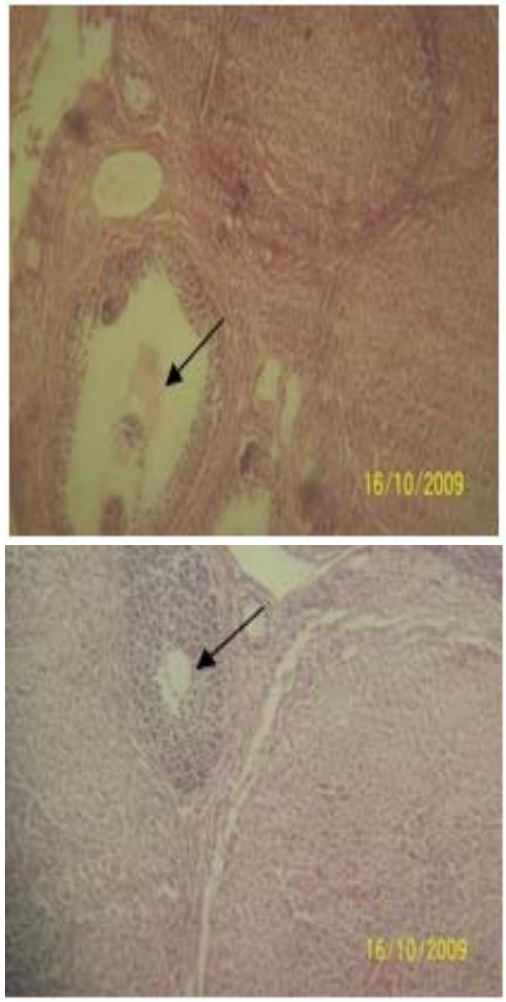
3.3. Histopathology of the Ovaries

The histopathological examination of the ovaries showed no modification by the dietary FB_1 concentrations. No visible lesion was observed in the ovaries (Figures 3a, b and c) for the control rats, those fed 10 and 20 mg FB_1/kg diets, respectively.



a

b



c

Figure 3. Haematoxylin-and-eosine stained sections of rat ovaries exposed to diets containing different concentrations of dietary FB_1. No visible lesion was observed in the ovaries of rats fed diets containing different concentrations of FB_1 as shown in Figures 3a, b and c for the control animals, those fed 10 and 20 mg/kg dietary FB_1 respectively. Note the oocytes (arrowed). Mag. X 200.

3.4. Fertility Assessment

The summary of the reproductive indices of female rats exposed to different concentrations of dietary FB_1 are shown in Table 2. The results revealed that fertility, gestation length and foetal weight/litter were dose-dependent. These parameters decreased with increased dietary FB_1, which were significant (P<0.05) at both feed contamination levels. Out of the 9 rats mated in each group (as determined using the vaginal smears), only 5 had pups in the group fed diet containing 20 mg FB_1/kg as against 8 and 7 for rats fed the control diet and diet containing 10 mg FB_1/kg, respectively. No statistically significant difference was found across the treatment groups for number of live foetus/litter. The total number of pups obtained from the dams resulted in relative number of pups/dam of 6.78 for those fed the control diet as against 6.11 and 3.78 for those fed 10 and 20 mg FB_1/kg diet, respectively.

Table 2. Reproductive Performance of Rats Exposed to Different Concentrations of Dietary Fumonisin B_1 (Mean ± SEM)

	Dietary fumonisin B_1 concentrations (mg/kg)		
Parameters	0.2 (Control Diet)	10.0 (Diet 1)	20.0 (Diet 2)
Fertility (%)	88.89 ± 33.33[a]	77.78 ± 44.10[b]	55.56 ± 52.70[c]
Gestation length (days)	21.60 ± 0.53[a]	23.33 ± 0.51[b]	23.00 ± 0.74[b]
No of live foetus/litter	7.67 ± 0.33	8.00 ± 0.33	7.25 ± 0.38
Foetal weight/litter (g)	5.33 ± 0.01[a]	5.23 ± 0.04[b]	5.22 ± 0.04[b]
No of litters	8	7	5
Total no of live foetus	61	55	34
No of dead foetus	0	1	2
Rel.* no of live foetus/dam	6.78	6.11	3.78

*Relative to total no of rats with positive scrapes in each treatment.

[abc]: Means on same row with different superscripts differ significantly (P < 0.05).

4. Discussion

Although, initial studies using laboratory animal (Voss *et al*., 1996; LaBorde *et al*., 1997; Collins *et al*., 1998a, b) provided no evidence that FB_1 is teratogenic. However, more recent observations and experimental findings have again drawn attention to FB_1 as a possible risk factor for birth defects (Merrill *et al*., 2001, Marasas *et al*., 2004, Voss *et al*., 2006). In this present study, the decrease in fertility of 77.78 and 55.56% for the rats fed diets 2 and 3 respectively compared with 88.89% for the control rats revealed adverse effect of the dietary fumonisin on fertility processes in the rats. The total number of pups obtained from the 9 mated female rats in each treatment showed that the 34 pups obtained from rats fed 20 mg/kg dietary FB_1 was only 55.74% of the pups obtained from the controls. This is quite significant in breeding programmes.

Effects of fumonisin on developing foetuses were expressed most often by foetal death and resorption (Floss *et al*., 1994). Reduced weight gain is often the earliest indicator of maternal illness in developmental toxicity studies, and would be expected as a sign of maternal effect. While the cause of foetal deaths in rats exposed to diets containing 10 and 20 mg FB_1/kg was not evident in this study, it was clear that the dose-dependent significant decline in foetal weight observed in this study was as a result of developmental toxicity produced by FB_1 and not secondary to maternal toxicity. In a study (LaBorde *et al*., 1997), the observed reduced foetal weight from rabbits gavaged daily on GD 3 – 19 with purified FB_1 at 0.5 – 1 mg/kg/day was ascribed to maternal toxicity, rather than any developmental toxicity produced by FB_1. Dose-responsive significant decrease in foetal weight observed in this study correlates with reports of other studies. Pregnant rats dosed by gavage on GD 8 – 12 with a

semipurified extract of culture material containing FB_1 with a purity of 80% resulted in lower foetal weight at dose of 60 mg/kg (Lebepe-Mazur *et al*., 1995). Similarly, decreased body weight of live foetuses obtained from pregnant Syrian hamsters gavaged with FB_1 in a dose-dependent manner was reported in a study (Penner *et al*., 1998). Voss *et al*. (1996) reported lower litter weights from rats fed *F. moniliforme* culture material providing 1 – 55 ppm FB_1 from two weeks before mating compared to the control group.

The dose-dependent significant decrease in fertility observed in this study may be due to an increase in gonadal steroid inhibition or suppression of the hypothalamus and/or pituitary gland resulting in a decline in serum FSH and LH levels. These two gonadotropins are the most important regulatory hormones of ovarian and uterine function (Everett, 2006). Suppression in secretion of hypothalamic gonadotropin releasing hormone (GnRH) causes reduced secretion of LH and FSH from pituitary (Rai *et al*., 2004). Fumonisins are structurally similar to sphinganine and sphingosine and inhibit sphingosine metabolism in tissues, leading to an accumulation of sphingoid bases, which are intermediates in sphingolipid biosynthesis (Wang *et al*., 1991). Because the brain contains high levels of sphingolipids, the disruption of sphingosine metabolism was speculated to be the mechanism behind the degeneration of neuronal cells seen in equine leukoencephalomalacia (Wang *et al*., 1991). A variety of biological activities for sphingolipids have been reported (Wang *et al*., 1992). Alterations in the amounts of any of these by fumonisins could potentially result in a variety of biological and pathological effects (Penner *et al*., 1998). These may be responsible for the significant decline in serum gonadotropins resulting in reduced fertility with increased dietary FB_1 observed in this study. The mechanisms involve is not clear further investigations are therefore necessary to elucidate the mechanism.

The elongated gestation lengths observed in this study further leads credibility to the fact that FB_1 potentially affect reproductive development. As in this study, seemingly longer gestation length from 21.9 ± 0.35 days was observed in rats fed control diet to 22.7 ± 1.00 days in rats fed *F. moniliforme* culture material providing 10 ppm FB_1 from two weeks before mating (Voss *et al*., 1996). Adverse effects of mycotoxins on sexual and reproductive developments have been reported. Green *et al*. (1990) and Rainey *et al*. (1990) observed that 1.5 mg zearalenone/kg diet disturbed the hypothalamo-hypophyseal function of prepubertal gilts, but after withdrawal of the contaminated diets, the animals attained puberty without delay, and their fertility was unimpaired. Also, dietary zearalenone levels as low as 0.05–0.06 mg/kg DM have been shown to increase the number of ovarian follicles and to decrease the serum concentration of the gonadotropic hormone FSH in female piglets (Döll *et al*., 2003), thus potentially affecting their sexual development. Recently, dietary FB_1 have been reported to delay attainment of sexual maturity in growing pigs (Gbore, 2009) and rabbits (Ewuola and Egbunike, 2010).

Impaired action of FSH and LH on the ovary has as a primary consequence, a concomitant alteration in the capacity of this organ to synthesize ovarian reproductive hormones; mainly estrogens from follicular cells and progesterone from luteal cells (Everett, 2006). Since the histopathological examination of the ovary did not reveal any leision, it is suggested that the decline in fertility rates could not have resulted from impaired ovaries but impaired action of the gonadotropins on the organs.

The data from this study suggest that dietary FB_1 of ≥10 mg /kg significantly reduced serum gonadotropin levels and lowered fertility without adverse effect on the ovarian histology of rats. The concentration of daily dietary FB_1/kgBW (1.74) which resulted in significant decline in fertility of female rats in this study is less than five times the estimated probable daily fumonisin intake of 355 µg/kg BW (Gelderblom *et al*., 1996) for person eating 'mouldy' maize in the high oesophageal cancer area of the Transkei region, South Africa. The apparent significant "safety factor" of about five times relative to human FB_1 exposure may be a cause for concern in areas where maize is a dietary staple. However, further studies on whether the toxin disrupts the hypothalamic production or release of GnRH or the absorbed FB_1 acts directly on the pituitary, at doses that are not maternally toxic, to lower serum gonadotropins are warranted.

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