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ABSTRACT

The fumonisins, produced by fungi that infest foodstuffs, in particular corn, are responsible for a series of illnesses and toxicosis in various species of animals, including humans. There is still not detailed information about the genotoxic and mutagenic activity of Fumonisin B1 (FB1), but it is clear that it interferes with growth control, differentiation and cellular apoptosis. The purpose of this study was to assess the genotoxic potential of Fumonisin B1 using *in vitro* cultures of bovine lymphocytes, through the calculation of the 'mitotic index' (MI), the frequency of 'sister chromatid exchange' (SCE) and the 'micronucleus test' (MN). The bovine lymphocytes were exposed to different concentrations of FB1 (25, 50 and 100 μM) in order to find out which amount is sufficient to cause a reduction in the mitotic potential of the cells, the onset of MN and a higher frequency of SCE. The results obtained show a considerable reduction in the 'mitotic index' with a FB1 concentration of 50 μM, an increase in the frequency of MN with a concentration of 50 μM and a significant increase in the SCE with a concentration of 100 μM.
In the light of the information we have obtained, compared with that of other Authors, we feel that the genotoxic potential of FB1 has been underestimated until now and should, therefore, be reconsidered.

Key Words: Bovine lymphocytes, Genotoxicity, Mitotic index, Micronucleus test, Sister chromatid exchange.

RIASSUNTO
ATTIVITÀ GENOTOSSICA DELLA MICOTOSSINA FUMONISINA B1 IN COLTURE DI LINFOCITI BOVINI

Le fumonisine sono micotossine che contaminano preferibilmente i cereali, e, soprattutto, il mais e i suoi derivati, responsabili dell'insorgenza di patologie, anche gravi, nell'uomo e negli animali allevati.
La Fumonisina B1 (FB1) è la forma più tossica e diffusa: l'Agenzia Internazionale per la Ricerca sul Cancro, l'ha inserita nel gruppo 2b (cancerogena per gli animali e possibili cancerogeni per l'uomo), per l'accertato legame con l'insorgenza del cancro esofageo dell'uomo riscontrato nelle popolazioni maggiormente esposte alla contaminazione.
Non sono ancora del tutto spiegati gli eventuali effetti genotossici e mutageni della FB1, mentre è accertata l'interferenza con la crescita cellulare, la differenziazione e l'apoptosi. Lo scopo di questo lavoro è di ottenere maggiori informazioni sul potenziale genotossico della FB1 in colture di linfociti bovini, proprio perché i ruminanti sembrano meno suscettibili all'azione della micotossina, anche se l'ipotesi sulla capacità detossificante del rumine non è condivisa da tutti gli Studiosi. A tal fine si sono esposti i linfociti bovini a diverse concentrazioni di FB1 (25, 50 e 100 μM) e, si è calcolato l'indice mitotico (MI), gli scambi intercromatidici (SCE) e la frequenza dei micronuclei (MN), considerate tra le migliori metodiche di indagine per la valutazione dell'insulto genotossico.

I nostri dati indicano una riduzione considerevole della potenzialità di divisione cellulare e un aumento nella frequenza dei MN alla concentrazione 50 μM, mentre si è osservato un aumento significativo della frequenza di SCE alla concentrazione 100 μM. Confrontando i nostri risultati con quelli di altri Autori riteniamo che il potenziale genotossico della FB1 è stato, fino a questo momento, sottostimato e che andrebbe riconsiderato.

Parole chiave: *Linfociti bovini, Genotossicità, Indice mitotico, Micronuclei, Scambi tra cromatidi fratelli.*

Introduction

Mycotoxins are produced by numerous fungi, found all over the world, in different types of fodder and cereals. They are substances that are toxic for animals and man. The contamination of foodstuffs is a matter of great interest at the moment because of the risks of exposure to toxic agents by cattle and man and regarding environmental health, especially as a result of the wider distribution of food products all over the world. Contamination concerns various types of foodstuffs, but among the most susceptible are cereals due to the high level of carbohydrates they contain. These ferment easily thereby forming an ideal substrate for fungi.

The assessment of the risk of these substances in the environment is complicated as currently no exposure limits for certain mycotoxins (e.g. fumonisins in Italy) have been fixed for Italy (or other countries).

Among the mycotoxins of primary interest are the fumonisins, secondary metabolites of the *Fusarium* species, which are highly toxic for animals and are also harmful for humans. The effects of ingestion of the toxin differ according to which species has eaten the contaminated food, for how long, the amount of toxins.

Currently there are seven known different fumonisins (FA1, FA2, FB1, FB2, FB3, FB4, and FC1), but FB1 is the most widespread and toxic in nature (Thiel *et al.*, 1992; Musser and Plattner, 1997; Food and Drug Administration, 2001).

According to a study developed by Meister *et al.* (1996), samples of cereals grown in Germany, such as wheat, rye, maize, barley and oats, and maize imported from Argentina, were analysed for fumonisins. The only cereal infected by fumonisins was maize, which is a fundamental part of animal feed and widely used in the nourishment of some human populations.

Fumonisins are responsible for the onset of leucoencephalomalacia, a neuro-degenerative disease, in horses (Marasas *et al.*, 1988), pulmonary edema and cardiovascular toxicity in pigs (Smith *et al.*, 2000), cancer of the esophagus in laboratory mice (Norred and Voss 1994; Marasas 1996; Riley *et al.* 1996; Ueno *et al.*, 1997), nephropathy in rabbits and sheep (Gumprecht *et al.*, 2001), damage to liver, kidney, heart and lungs in chickens (Norred and Voss, 1994) and are associated with the spread of cancer of the esophagus in humans, which was noted in a particular part of South Africa - Transkei - (Sydenham *et al.*, 1990; Rheeder *et al.*, 1992) and in China (Chu and Li, 1994; Rumbeiha and Oehme, 1997). However, Gumprecht *et al.* (2001) suggest that certain other species are predisposed to its toxic effects.

In a recent paper by Ehrlich *et al.* (2002) on FB1 effects in human-derived hepatoma cells, Fumonisin B1 was tested in micronucleus (MN) and single cell gel electrophoresis assay. The mycotoxin caused a dose-dependent genotoxic effect at exposure concentrations of 25 μg/ml. The results indicate that FB1 is clastogenic in human-derived cells and may act as a genotoxic carcinogen in humans.

There is still controversy about the mutagenic activity of Fumonisin B1 (FB1) but Sakakura *et al.*, (1996) are certain that the mycotoxin interferes with growth control, differentiation and cellular apoptosis, so the purpose of this study has been to acquire more knowledge about the possible genotoxic and mutagenic effects of Fumonisin B1 in cultures of cells that belong to the bovine species, which, compared with other species, in fact seem to be less susceptible to the effects of the mycotoxin. According to a study by Dutton (1996) bovine given feed which is contaminated with large amounts of Fumonisin B1 have shown only slight lesions to the liver, while the administration via rumen of Fumonisin B1 in sheep has resulted in damage to the liver and kidneys, and in some cases, death. Thanks to the ruminal fermentation that de-activates the various mycotoxins at least

partially, the ruminants appear to be more resistant compared with monogastric species.

Notwithstanding this reduced susceptibility to mycotoxins, Johnson and Sharma (2001) document the immune-depressive activity of FB1 in bovines with significant repercussions on the health and productive performance of this species.

The molecular mechanism which renders the FB1 toxic must still be studied in depth. The purpose of this paper has been to estimate possible mutagenic effects of Fumonisin B1 on bovine lymphocytes by using 'mitotic index' (M.I.), 'micronuclei frequency' (MN) and 'sister chromatid exchange' (SCE) as cytogenic indicators of genotoxicity. The micronucleus test is a valid indicator of chromosomic breakdown and dysfunction of the spindle. The micronuclei are fragments of chromosomes or whole chromosomes that are not incorporated in the main nuclei during the mitosis and, therefore, appear in those cells which have gone through a division process.

These methods permit us to assess how the mycotoxins interfere with the division of the cells, to evaluate, by early diagnosis, the onset of changes in chromosomes and to check any variation in the number of sister chromatid exchanges in cultures which have suffered genotoxic damage.

Material and methods

Animal management

In this study blood samples were taken from ten Italian Friesian heifers at first delivery. The animals were fed a diet based on maize silage, gramineous hay and a feedstuff for lactating cows, and reared in free stall housing condition. Blood was taken from the jugular vein using vacutainers containing sodium heparin as the anticoagulant and immediately carried to laboratory to prepare cellular cultures.

Test chemicals

Fumonisin B1 (Sigma, St. Louis, MO, USA) and Cytochalasin-B (Cyto-B, Sigma, St. Louis, MO, USA) were diluted in methanol and dimethylsulfoxide, respectively, (DMSO, Sigma, St. Louis, MO, USA) in order to obtain concentrations of 25, 50 and 100 μM for the Fumonisin B1 and 6 μg/ml for the Cyto-B.

Lymphocyte cultures

All cultures were prepared, according to the method of De Grouchy *et al.* (1964) which was slightly modified, from peripheral blood taken from the jugular vein of ten young animals of the Italian Friesian breed using vacutainers containing sodium heparin as the anticoagulant. Whole blood (0.5 ml) was cultured in 4 ml of RPMI 1640 medium (Gibco, New York, NY, USA), with the addition of 1% of L-glutamine 200 mM (Gibco, New York, NY, USA), 1% of kanamicine solution 10,000 μg/ml (Gibco, New York, NY, USA), 1% of nystatin suspension 10000 U/ml (Gibco, New York, NY, USA), 1 ml of Foetal Calf Serum (FCS) and concanavalin A (*Canavalia ensiformis* type IV, Sigma, St. Louis, MO, USA) (25 μg/ml of culture). The cells were grown at 37°C for 72 hours.

Microscopic examination of the preparations showed that 50 μl of absolute methanol (necessary to dilute the FB1) per 5 ml culture flask is the maximum amount that can be added without causing toxicity to the cells and without interfering with the cell cycle. Toxicity was determined by counting at least 1000 cells for each subject and each treatment. The FB1 was therefore diluted so as not to exceed this amount of diluent.

For each subject examined, three test cycles were performed (with the above-indicated three concentrations of Fumonisin B1), preparing two cultures at the same time: one used as the test and the other as the control, in order to eliminate variables due to the conditions of the cultures. FB1 was added immediately after stimulation with concanavalin in the test cultures and left for the entire culture period.

'Mitotic Index': metaphase identification and counting

The 'mitotic index' was assessed by counting at least 1000 cells for each subject and each treatment, and comparing the number of metaphases identified in the microscopic preparation with the total number of cells scored, using the Leica IM 50 software (Image Manager) and the optical microscope (Leica DM R), connected to a digital camera (Leica DC 250). To count the number of cells in metaphase, 10 not overlapping fields of view (magnification 10X), were acquired through the microscope. These 10 fields of view were sufficient for scoring at least 1000 cells for treatment.

Treatment with Cytochalasin B

Micronuclei are fragments of chromosomes or whole chromosomes produced by a range of cellular damage other than spindle dysfunction such as centromere/kinetochore damage.

In order to calculate the exact number of micronuclei in a cell population it is necessary to identify which cells have separated only once. The most widely used method to block them at the first mitotic division calls for the administration of cytochalasin B (Cyto-B), at a concentration of 6 μg/ml, after 44 hours of culture, according to the technique introduced by Fenech and Morley in human lymphocytes in 1985 and called 'cytokinesis-block (CB)'. Cytochalasin B inhibits the polymerisation of the actin by working on the microtubulus of the mitotic spindle, and prevents the cytodieresis without jeopardising the nuclear division, so that the cells treated are bi-nucleated and the micronuclei in them can easily be identified.

The test of the micronuclei also makes it possible to estimate the dose of toxic agent that has been absorbed by the 'target' (dose-response curve).

Harvesting, slide preparation and staining. Micronucleus identification and counting

Once the incubation time was over, the cultures were spun at 1040 rpm for 10 minutes, resuspended successively in PBS in a hypotonic solution and fixed many a time with a solution of ethanol-acetic acid 5:1, and finally coloured with a solution of Giemsa 5% (pH=6.8) for 10 minutes.

The micronuclei were counted under the microscope on a total of 500 bi-nucleated cells. The criteria of Countryman and Heddle (1976) and Heddle *et al.*, (1978) were used in order to identify the MN.

SCE treatment, identification and counting

In the cultures prepared to calculate the frequency of the 'sister chromatid exchange' (SCE), 30 hours before harvesting, 10-15 μg/ml of 5-Bromo-2'-deoxyuridine (BrdU, Sigma, St. Louis, MO, USA) were added taking care to protect the samples from light. Colcemid was added (Gibco, New York, NY, USA, 10 μg/ml of culture) 2 hours prior to the termination of the cultures in order to block the mitosis at the metaphase stage. The setting-up of the preparations for the microscope followed the method used for the calculation of the 'mitotic

index'. The slides obtained were coloured for 20 minutes in bisBenzimide (Hoechst 33258, Sigma, St. Louis, MO, USA) (50 μg/ml H_2O), dried, mounted in 2XSSC and exposed to U.V. rays for 45 minutes, incubated in 2XSSC at 65°C for one hour, dehydrated in successive baths of alcohol at 70/80/96% and coloured with Giemsa at 10% for 10 minutes.

For the two concentrations of Fumonisin B1 which were tested (100 and 50 μM) and for each of the 10 subjects, 50 complete and well spread metaphases were analysed as previously described including the evaluation of the SCE, followed by the calculation of the average SCE per metaphase of each subject.

Statistical analysis

All data were analysed by ANOVA according to General Linear Model of the S.A.S. package (SAS 2000) in order to evaluate the effect of Fumonisin B1 (FB1). The statistical model employed for 'mitotic index' (M.I.) 'micronuclei test' (MN) and 'sister chromatid exchange' (SCE), was the following:

$$Yij = \mu + \alpha i + \varepsilon ij$$

where μ was the general average, α was the effect of the treatment and ε was the error.

Results and discussion

Our results indicate that the genotoxic effect of Fumonisin B1 (FB1), the most toxic and universally diffused form, administered to in vitro cultures of bovine lymphocytes obtained from blood of 10 Friesian cows, is associated with a statistically significant reduction in the 'mitotic index' in all the subjects treated with concentrations of 50 and 100 μM (P< 0.05), whilst it is not significant with a concentration of 25 μM, even though average values were found to be 2.67 in the cultures treated compared with 3.38 in the control cultures (Table 1).

Figure 1 shows how FB1 induces a dose-dependent reduction in the proliferation of cells in which the higher the dose of mycotoxin is administered. Although the trend is also decreasing in the control cultures, it is most incisive in those treated. This trend is confirmed by high R^2 value ($R^2 = 0.997$).

The reduction we have noted in the values of the 'mitotic index' of the lymphocytes is in line with data of Ciacci-Zanella *et al.* (1998) which indicate

Table 1.   Mitotic index (%) at different FB1 levels.

		Control	Treated	SE	P
FB1 25 μM	%	3.38	2.67	0.35	ns
FB1 50 μM	"	2.72	1.74	0.26	P<0.05
FB1 100 μM	"	2.57	0.95	0.23	P<0.001

ns: not significant

Table 2.   Frequency of Micronuclei (%) at different FB1 levels.

		Control	Treated	SE	P
FB1 25 μM	%	3.80	3.67	0.27	ns
FB1 50 μM	"	5.98	7.24	0.51	ns
FB1 100 μM	"	8.97	19.59	1.98	P<0.01

Table 3.   Frequency of Sister Chromatid Exchange (%) at different FB1 levels.

		Control	Treated	SE	P
FB1 50 μM	%	3.75	3.64	0.13	ns
FB1 100 μM	"	3.46	5.31	0.18	P<0.001

that FB1 is capable of preventing the cell cycle in CV-1 cells (renal cells of the African green monkey).

The spontaneous frequency of the micronuclei (MN, Figure 2), calculated as the number of MN/500 bi-nucleated cells in the 10 animals under examination in all control samples, varies from 2.8 to 14.8 (average 6.54 ± 3.02) while Scarfi *et al.* (1993) indicated a slightly higher average of 12.3 ± 4.1. This could be due to the difference in age of the animals in question and the different environmental breeding conditions, but in his experiment Scarfi *et al.* (1993) did not specify. The results obtained by us in the three trials show an increase in the frequency of the micronuclei starting with a concentration of FB1 50 μM (Table 2) although only at 100 μM are the differences statistically significant.

Figure 3 shows how FB1 induces a dose-dependent increase in the micronuclei frequency the higher the dose of mycotoxin administered.

It is clear that although the trend is also increasing in the control cultures, it is most incisive in those treated. This trend is confirmed by
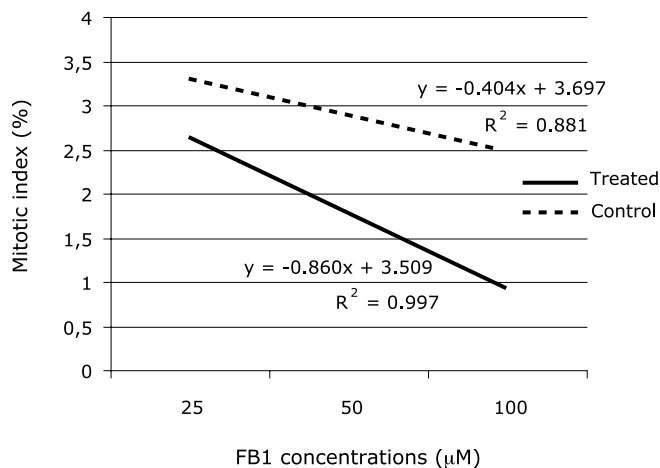
high R^2 value ($R^2 = 0.994$).

The variations in the number of 'sister chromatid exchanges' (SCE, Figure 4) with FB1 50 μM are not statistically significant, whilst those with a concentration of 100 μM are. With this concentration the FB1 prevents the complete success of the cell cultures; with such high doses of mycotoxin, the capacity to repair the DNA of the cell is probably compromised (Table 3).

For this reason no trial was made with a concentration of mycotoxin of 25 μM.

As the fumonisins have a chemical structure which is very similar to sphinganine, Speigel and Merrill (1996), Guzman *et al.* (1997), Gumprecht *et al.* (1998) and Haschek *et al.* (2001) arrived at the conclusion that their principal cell target is the ceramide-synthase enzyme; therefore, the inhibition of this enzyme results in an accumulation of sphinganine and sphingosine with a subsequent alteration of the metabolism of the sphingolipids. It seems that this is the mechanism attributable to the carcenogenicity of the mycotoxin (Mobio *et al.*, 2000).

Figure 1.   Trend of proliferation of cells related to FB1 level.

$y = -0.404x + 3.697$

$R^2 = 0.881$

—— Treated

- - - - Control

$y = -0.860x + 3.509$

$R^2 = 0.997$

Mitotic index (%)

FB1 concentrations (μM)

To date, few studies have been conducted by Tolleson *et al.* (1996), Knasmuller *et al.* (1997), Sahu *et al.* (1998), Atroshi *et al.* (1999), Mobio *et al.* (2000) concerning potential damage to DNA from Fumonisin B1.

It is important to emphasize that when a comparison is made of the results of the various Authors about the genotoxic-mutagenic activity of Fumonisin, consideration should be given to the differences between experiments *in vitro* and *in vivo*, species examined, dosages used and exposure times. In the light of the information we have obtained, compared with that of other Authors, we feel that the genotoxic potential of FB1 has been underestimated until now and should therefore be reconsidered.

Conclusions

The purpose of this study was to assess the genotoxic potential of Fumonisin B1 using *in vitro* cultures of bovines.

Nowadays it is very important to acquire information about fumonisins, mycotoxins which have been discovered recently and have assumed a significant importance, especially since the Ministry of Health has classified them as a possible human carcinogenic substance. In addition, they have been considered to be responsible for the majority of contamination in 2004.

Through this *in vitro* experiment using bovine

lymphocytes as a biological dosimeter of the genotoxic injury to animals exposed to the Fumonisin B1, it turns out that this mycotoxin has a genotoxic effect on the cells of the organism at concentrations greater than or equal to 50 μM. It is also evident that the Fumonisin B1 has a mutagenic effect at concentrations greater than 50 μM, which is consistent with Knasmuller *et al.* (1997) and Abado-Becognee *et al.* (1998). The rumen could carry out a detoxification action in the comparisons of the mycotoxin, although this effect would require further study.

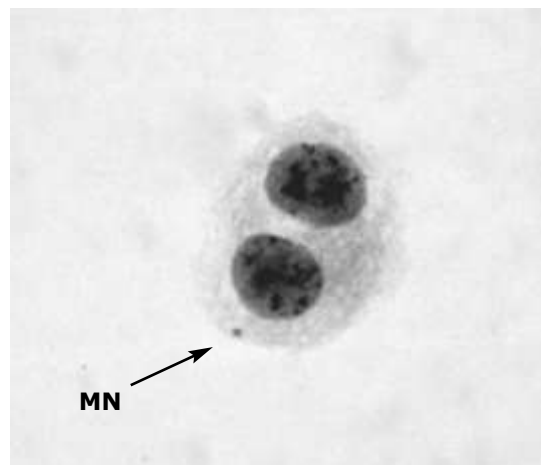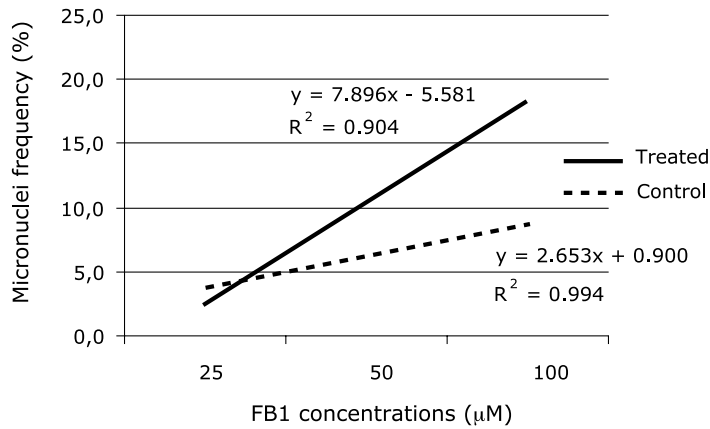Figure 2.   Binucleated cell with micronucleus (MN).

MN

Figure 3.   Trend of micronuclei frequency related to FB1 level.

$$y = 7.896x - 5.581$$
$$R^2 = 0.904$$

$$y = 2.653x + 0.900$$
$$R^2 = 0.994$$

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Figure 4.   Bovine metaphase after SCE treatment.

SCE

SCE
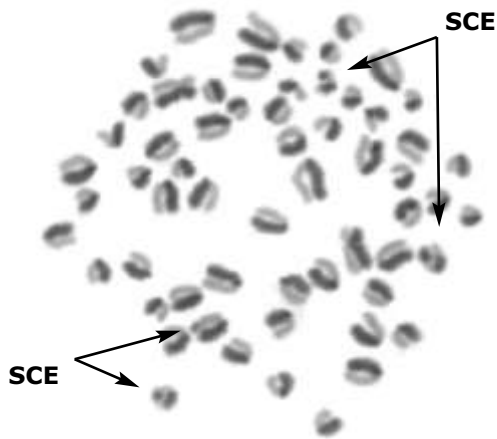
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