

Chronic Fumonisin Intake Induces Erythrocyte Osmotic Fragility

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Abstract

Fumonisin B₁ (FB₁) is a mycotoxin that appears as a frequent maize and maize-based foods contaminant in Argentina. It has an inhibitory effect of ceramide synthetase, a key enzyme in the biosynthesis of sphingolipids, it can affect cells in different ways since sphingomyelin is required for the stability of cells membranes. In Argentina, has been reported a high percentage of contamination in freshly harvested and stored maize. The aim of this work was to determine the effects of FB₁ on human erythrocytes “*in vitro*” and to evaluate the effect of a chronic intake of fumonisin on erythrocyte function and on hematological and hepatic parameters in rats.

Male 1-month Wistar rats were separated into two groups of 5 rats. The control group was given saline solution while the other group received orally for 60 days 1mg/kg/day of FB₁ from a culture of *Fusarium proliferatum*. On day 60, blood was collected in EDTA tubes for haematological or heparin for hepatic parameters and osmotic fragility. The osmotic fragility study was performed by two tests: immediate erythrocyte osmotic resistance (ROE i), where the erythrocytes from control or treated rats were placed in solutions of different concentrations of ClNa between 0 and 0.9 g/% and other ROE 24 hs, where the erythrocytes were preincubated 24 hrs at 37° C and then placed in hypotonic ClNa solution (0.4 g%). Hematological parameters showed a significant decrease of treated vs. control rats in the number of platelets (393.3 ± 70.6 x 10³/mm³ vs 926.0 ± 23.5 x 10³/mm³, in hemoglobin and hematocrit: 12.9 ± 0, 4 g% vs 14.1 ± 1.1 g% and 38.2 ± 0.0% vs 40.7 ± 3.1% and liver damage parameters showed significant differences between treated vs controls for phosphatase alkaline (1144±89 vs 608.3.3±96.4). In the studies of osmotic resistance, sodium chloride concentrations of 0.5 g% and 0.4 g% showed differences in the values of percentage of hemolysis in treated vs controls (97% vs 9.8%, 0.5g% ClNa and 100 vs. 74%, 0.4g% ClNa) with ROEi values of 0.53 g% for the treated and 0.41 g% for the controls; while for 24 hs ROE, the differences were for sodium chloride concentration 0.4g% (77 vs 47%). “*In vitro*” experiments, human erythrocytes incubated with FB₁ during 24 hs showed an altered morphology; while no significant differences were observed in % of hemolysis between controls and treated erythrocytes.

The results show that the chronic intake of fumonisin in rats leads to alterations in erythrocytes in either their shape or size, which manifests itself in the increase of their osmotic fragility. Differences were observed in hemoglobin concentrations although it was not reflected in the mean corpuscular volume (MCV) between both groups. Further investigations should be done to clarify the effect of fumonisins on erythrocyte cellular membrane and their function.

Keywords: Osmotic Fragility; Fumonisin; Chronic Intake; Liver Injury; Mycotoxin

Abbreviations: FB₁: Fumonisin B₁; ROE: Erythrocyte Osmotic Resistance; LC: Liquid Chromatography; OPD: O-Phthalaldehyde; SGPT: Serum Glutamate-Pyruvate Transaminase; SGOT: Serum Glutamate-Oxaloacetate Transaminase; PBS: Phosphate Buffer Saline; ANOVA: Analysis of Variance; MCV: Mean Corpuscular Volume; PE: Phosphatidylethanolamines and FA: Fatty Acid.

Introduction

Fumonisin B₁ (FB₁) is a mycotoxin that appears as a frequent contaminant of maize and maize-based foods in Argentina. The percentage of maize samples contaminated by fumonisins was between 90 and 100% for all years studied and with levels of contamination between 1773 to 9093 µg kg⁻¹ for freshly harvested maize and from 2525 to 11,528 µg kg⁻¹ for storage samples. In 35 samples of corn flour and corn grits, fumonisin B₁ (FB₁) and fumonisin B₂ (FB₂) were determined with levels of contamination between nd and 4987 ng/g for FB₁ and to 1818 ng/g for FB₂ [1,2,3]. A survey in durum wheat has shown the presence of total fumonisins with different levels of contamination depending on the year of harvest [4]. It has an inhibitory effect of ceramide synthetase, a key enzyme in sphingolipid biosynthesis, which affects cells in different ways since sphingomyelin is required for the stability of plasma membranes.

In previous studies, it has been reported that rabbits fed a diet contaminated with FB₁ (10 mg / kg diet) present an alteration in the fatty acid profile of the erythrocyte membrane together with an increase in the activity of the sodium pump probably associated with altered metabolism of ceramide [5]. Taking into account that the erythrocyte membrane is semipermeable and that an alteration in its composition leads to changes in the ionic solutes (Cl⁻, K⁺ and Na⁺), it is suggested that these erythrocytes from animals treated with fumonisins could present an alteration in their surface/volume ratio.

The measurement of the ability of a population of erythrocytes to resist the effect of a hypotonic medium is called erythrocyte osmotic resistance (ROE) and depends on the surface/volume ratio. Due to this, when the red blood cell is incubated in an ionic concentration medium superior to the one existing in its cytoplasm (hypertonic medium), a passage of water from the inside to the outside of the cell that causes its dehydration is produced by osmotic effect. In contrast, if the medium has an ionic concentration lower than that of the red blood cell (hypotonic medium), the water flows through the membrane in the opposite direction and the red blood cells are hydrated. This passage of water in either direction, depending on the ionic concentration of the medium, will occur until equilibrium is reached between both sides of the membrane. Thus, when the erythrocyte is incubated in a medium of absolute hypotonia (distilled water), hemolysis is observed in all red cells, but when it is relative, there is always a certain number of red cells capable of resisting the osmotic effect.

The aim of this work was to determine the effects of FB₁ on human erythrocytes *in vitro* and to evaluate the effect of a chronic intake of a fumonisin (from *Fusarium proliferatum*) culture on erythrocyte function and on hematological and hepatic parameters in rats.

Materials and Methods

Experimental Design

Male 1-month Wistar rats were separated into two groups of 5 animals. The control group was given saline solution while the other group received orally for 60 days 1mg/ kg/day of FB₁ [6] from a culture of *Fusarium proliferatum*. All rats were sacrificed in the CO₂ chamber and cardiac puncture was performed to obtain blood collected in EDTA or heparin tubes for osmotic fragility and haematological and hepatic parameters measurement.

Production of Fumonisin and Determination of the Fumonisin Content in the Culture

Fumonisin were produced from a highly toxicogenic strain of *Fusarium verticilloides* coming from the Mycotoxin Research group of the Faculty of Exact and Natural Sciences of Buenos Aires University. The strains were cultivated into petri dishes containing sabouraud media and incubated at 25°C for 7 days. Twenty-five grams of yellow corn were inoculated with a subculture of 1 cm² and grown at 25°C for 28 days. Fumonisin extraction was performed according to the protocol of Sheppard et al. [7].

Briefly, the fumonisins were extracted with 100 ml of 1:1(V/V) acetonitrile: water by stirring overnight; the crude extracts were filtered and concentrated by centrifugation 10 minutes at 500g and then filtered with vacuum pump and in a bath at 60°C. Analyses of samples were performed by liquid chromatography (LC) using a Shimadzu Prominence (LC Solution software) equipped with a fluorescence detector by setting the excitation wavelength to 335nm and the emission wavelength at 440 nm with a thermostated column compartment. The mobile phase was methanol: 0.05 M NaH₂PO₄ (70:30, v/v) acidified to an apparent pH of 3.3 with orthophosphoric acid and run isocratically at a flow rate of 0.8 ml min⁻¹. The samples were derivatized with a solution of O-phthalaldehyde (P 0657, Sigma-Aldrich, St. Louis, MO, USA) (OPD) by incubating at room temperature for 15 minutes 75 ul of the OPD solution with 25ul of the samples and injected 20 ul of the mixture. The OPD solution was prepared with 13.3 mg of OPD, 300ul of methanol, 1700ul of 0.1 M sodium tetraborate and 17ul of beta mercaptoethanol. The samples were previously filtered with PVDF filters, Millex GV. Measurements were made by peak area

Haematology

The blood was collected in tubes with EDTA. Hematologic parameters were determined in an Abbott Cell Dyn1200 hematological counter.

Hepatic and Renal Parameters

Serum glutamate-pyruvate transaminase (SGPT), serum glutamate-oxaloacetate transaminase (SGOT), alkaline phosphatase, urea and creatinine were determined in BT 2000 automated analyzer.

Erythrocytic Osmotic Resistance Study

The osmotic fragility study was performed by two tests: immediate erythrocyte osmotic resistance (ROE i), and erythrocyte osmotic resistance single tube post incubation at 37 ° C for 24 h (ROE S24 hs). ROE i: Five milliliters of decreasing concentrations of sodium chloride (0.9 to 0 g%) in phosphate buffered saline buffer pH 7.4 were incubated at room temperature for 30 minutes with 50 µl of heparinized venous blood and centrifuged at 1200 g for 5 min. The percentage of lysis was determined by reading on a spectrophotometer with a wavelength set at 540 nm. Tube supernatant (NaCl 0.9 g%) was used as a blank while a 100% lysis value was assigned to the supernatant reading of tube 16 (water) and expressing the readings of the other tubes as a percentage of value of the tube 16. ROETS24: The heparinized blood sample was incubated at 37° C for 24 h. Then the same ROETUi technique was used. The results were plotted with Microsoft Excel. The concentration of NaCl corresponding to 50% of hemolysis was obtained by extrapolating from the curve of % hemolysis vs concentration of sodium chloride [8,9].

"In Vitro" Studies

Samples were obtained from two individuals healthy volunteers, from each individual by 4ml venipuncture of heparinized peripheral blood, which is centrifuged at 2500 rpm for 15 minutes to separate the plasma and leukocytes of erythrocytes. The pellet of erythrocytes, washed twice with 2.5ml of phosphate buffer saline (PBS) at 5% pH 7.4 and centrifuged at 2500 rpm for 12 minutes at room temperature. The erythrocytes were resuspended in (PBS) at 5% with pH 7.4 until reaching a final volume of 6 ml. For "*in vitro*" studies, human red blood cells 5g% were incubated 24hrs temperature 37°C with increasing amounts of FB₁ (0-200 ng); 30µl was taken from each of the previous treatments of the erythrocyte pellet and resuspended in 30µl of PBS, to be observed under an optical microscope. Quantification of the hemoglobin of the supernatant by spectrophotometric reading at 540 nm

Statistical Analysis: All data are expressed as mean ± SD and analyzed by one-way analysis of variance (ANOVA). Exact Test of Fisher was performed. Differences between means were considered significantly different when values of $P \leq 0.05$.

Results

The hematological parameters showed significant differences in the number of platelets ($393 \pm 70.6 \times 10^3/\text{mm}^3$ vs $926.0 \pm 23.5 \times 10^3/\text{mm}^3$), hematocrit ($38.2 \pm 0.0\%$ vs $40.7 \pm 3.1\%$) and hemoglobin ($12.9 \pm 0.4 \text{ g\%}$

vs $14.1 \pm 1.1 \text{ g\%}$) in the treated rats compared to controls, whereas for leukocytes ($6.2 \pm 0.9 \times 10^9/\text{l}$ vs $4.4 \pm 1.2 \times 10^9/\text{l}$) and mean corpuscular volume (MCV) differences between treated and controls were not significant (Table 1).

Haematological parameters	Controls		FB ₁ Treated		Value P
	Average	Average deviation %	Average	Average deviation %	
Platelets	926.0±23.5	2.5%	393.3±70.6	18.0%	0.005
Hemoglobin	14.1±1.1	7.5%	12.9±0.4	3.1%	0.05
Hematocrit	40.7±3.1	7.7%	38.2±0.0	0.1%	0.05
MCV	55.8±0.3	0.5%	56.4±1.4	2.4%	ns
Leukocytes	4.4±1.2	28.1%	6.2±0.9	13.9%	ns

Table 1: Haematological parameters of the control and FB₁-treated rats.

Serum chemical indications of hepatocellular injury, showed significant differences for the alkaline phosphatase values in treated and controls (1144.0 ± 89.0 vs 608.3 ± 96.4), for GOT (417.3 ± 43.9 vs $226.5 \pm 7.3 \text{ U/l}$) and GPT (202.0 ± 53.0 vs $133.9 \pm 17.5 \text{ U/l}$) (Table 2).

the differences were for sodium chloride concentration 0.4 g\% (77 vs 47%) (Figure 1).

Hepatic parameters	Controls	FB ₁ treatment	p value
Alkaline phosphatase (U/L)	608.3±96.4	1144±89	0.0005
GOT (U/L)	226.5±7.3	417.3±43.9	0.001
GPT(U/L)	133.9.0±17,5	202±53	0.001
GOT/GPT	1.69±2,6	2.1±0.9	ns
Urea (mg%)	38±1.3	58.7±6.0	0.05
Creatinine (mg%)	0.4±0.0	0.6±0.0	ns

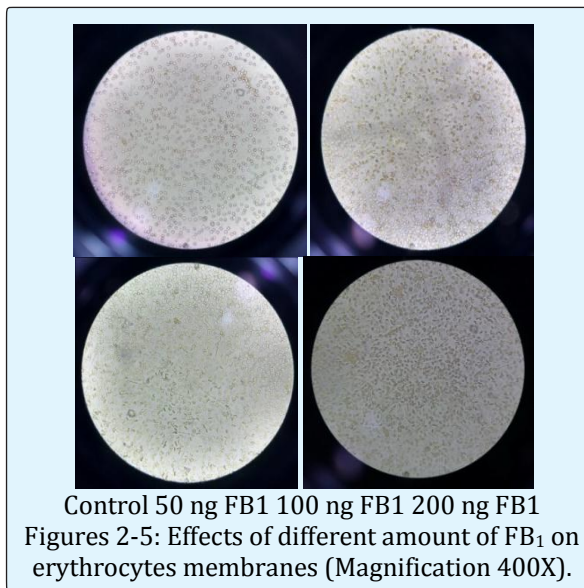
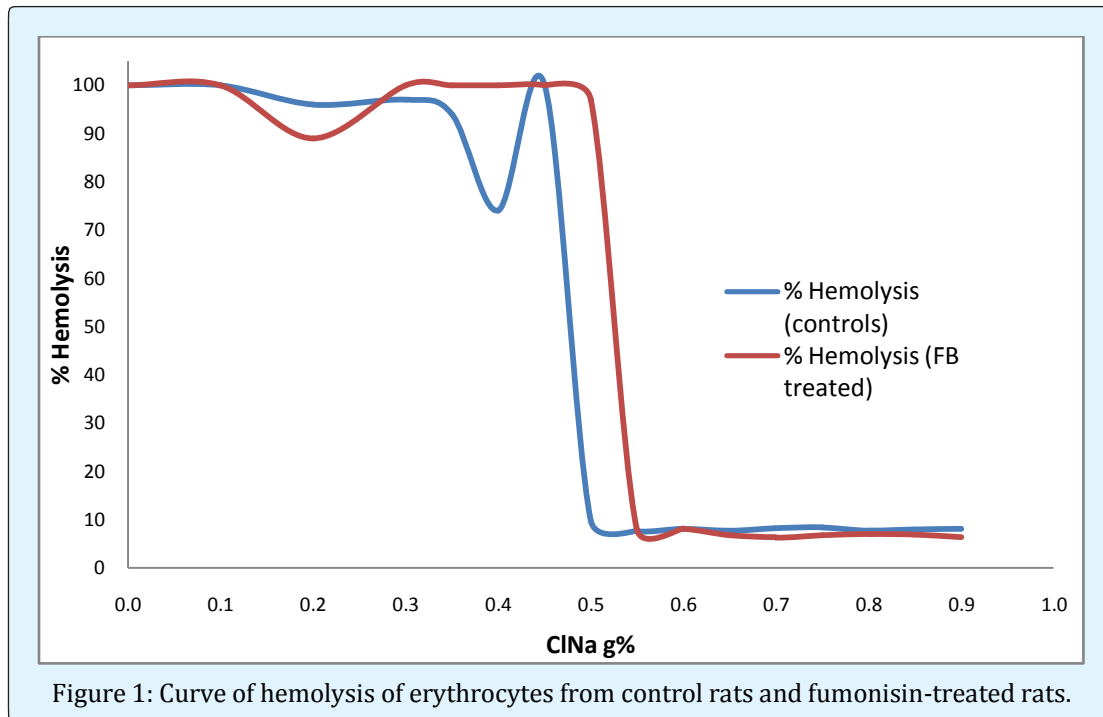
Table 2: Hepatic parameters of the control and FB₁-treated rats.

The percentages of hemolysis in treated vs controls were for 0.5 g\% ClNa, 97% vs 9.8% , and for 0.4 g\% ClNa, 100% vs 74% . The results obtained from the ROEi at the level of 50% of hemolysis were 0.53 g\% ClNa for the treated and 0.41 g\% for the controls; while for ROE24 hs

“*In vitro*” studies, human erythrocytes incubated with FB₁ during 24 h, showed significant morphological alterations of varying degree and were evident among erythrocytes treated with FB₁ irrespective of the concentration. Some of the major alterations were mild to moderate distortion in shape (anisocytosis) significant ruptured membranes (Figures 2, 3, 4 and 5). No significant differences were observed between controls and FB₁ treated erythrocytes in % haemolysis (Table 3).

Experiment	0 ng FB ₁	50 ng FB ₁	100 ng FB ₁	200 ng FB ₁
1	11	Nd	6	Nd
2	44	24	36.3	40.5
3	10.1	17.8	20.9	24.8
4	11.4	10.4	24.7	17.7
Mean ± SD*	19.1 ± 14.4	17.4 ± 5.6	22±10.8	27.7 ± 9.5

Table 3: Percentage hemolytic activity induced by FB₁ (50, 100 and 200 ng) on a suspension of human erythrocytes in PBS pH 7.4. Nd: not determined. *No significantly different ($p < 0.05$, test ANOVA).



Discussion

Among the clinical chemical parameters examined, the significant differences ($p < 0.001$) in the serum concentration of hepatic enzymes between both groups of rats, revealed hepatic injury though the GOT/GPT ratio did not show the same. The toxicological and pathological effects of fumonisins have been extensively studied in

laboratory animals. The liver and kidney are the major target organs but species-, strain-, and sex-dependent differences in dose-response occur. Histopathologic effects in rats, which have been referred to by various terms such as hepatopathy, hepatosis, or toxic hepatitis, have been reported by several research groups [10,11]. Other studies showed accumulation of FB₁ in the liver after chronic administration of contaminated feed with FB₁ in rats [12]. Serum chemical indications of hepatocellular injury, including increased alanine and aspartate transaminase, alkaline phosphatase, and lactate dehydrogenase activities, as well as increased cholesterol and triglyceride concentrations has been reported previously and histopathological examinations confirmed that 150 ppm FB₁ was hepatotoxic to both sexes [13].

The reduction observed in the number of platelets, hemoglobin concentration and hematocrit in the treated rats could indicate decrease stability of erythrocytes.

Even more the results obtained in the experiments of osmotic resistance curves revealed that 50% of haemolysis was achieved with higher sodium chloride concentrations in treated rats compared to the controls, which would indicate a higher osmotic fragility of the erythrocytes in rats treated with FB₁ versus controls. Above results suggested that an alteration of the erythrocytes membrane could occurred post treatment with FB₁.

Previous studies revealed lipid-compositional changes underlying microscopic alterations caused by FB₁; in particular phosphatidylethanolamines (PE) were significantly increased in the hepatic mitochondrial and even in the plasma membrane fractions [14]. In a recent study, Szabó et al. [15], confirmed these changes in the fatty acid (FA) composition of the hepatic mitochondrial phospholipids (PL) of rats treated during 28 days with 10 mg/kg FB₁ supplementation in the feed. In the same way, Fodor et al [11] reported some changes compatible with lipid peroxidation, as a reduction in glutathione content in blood plasma and in red blood cell haemolysate of weaned piglets after oral administration of *Fusarium verticillioides* culture mixed into the experimental feed for 10 days. Ferrante et. al [16] evaluated the modifications of fluidity, endocytosis and peroxidative damage of plasma membrane induced by FB₁ in macrophage cell line and concluded that FB₁ induced a membrane peroxidative damage that might enhance membrane disruption and oxidative stress and Garbetta et al. denoted lipid peroxidation induced by FBs on ex vivo rat intestine [17].

Conclusions

Preliminary results showed that the chronic intake of fumonisins in rats would lead to possible alterations in the shape or size of erythrocytes that manifested in the increase of their osmotic fragility and were an indication of changes in biological properties of plasma membrane as a consequence of ceramide synthase inhibition and alteration in its phospholipids composition. The toxic effect on erythrocytes "*in vitro*" showed an alteration in their morphology that could affect the erythrocytes physiological functions. Although there were no significant differences in mean corpuscular volume (MCV) between the two groups, differences in hemoglobin and hematocrit concentrations in both groups could indicate the above. Further investigations should be done to clarify the effect of fumonisins on erythrocyte cellular membrane and their function.

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