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RESEARCH ARTICLE

Fumonisin FB1 treatment acts synergistically with methyl donor deficiency during rat pregnancy to produce alterations of H3- and H4-histone methylation patterns in fetuses

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Scope: Prenatal folate and methyl donor malnutrition lead to epigenetic alterations that could enhance susceptibility to disease. Methyl-deficient diet (MDD) and fumonisin FB1 are risk factors for neural tube defects and cancers. Evidence indicates that FB1 impairs folate metabolism.
Methods and results: Folate receptors and four heterochromatin markers were investigated in rat fetuses liver derived from dams exposed to MDD and/or FB1 administered at a dose twice higher than the provisional maximum tolerable daily intake (PMTDI = 2 μg/kg/day). Even though folate receptors transcription seemed up-regulated by methyl depletion regardless of FB1 treatment, combined MDD/FB1 exposure might reverse this up-regulation since folate receptors transcripts were lower in the MDD/FB1 versus MDD group. Methyl depletion decreased H4K20me3. Combined MDD/FB1 decreased H4K20me3 even more and increased H3K9me3. The elevated H3K9me3 can be viewed as a defense mechanism inciting the cell to resist heterochromatin disorganization. H3R2me2 and H4K16Ac varied according to this mechanism even though statistical significance was not consistent.
Conclusion: Considering that humans are exposed to FB1 levels above the PMTDI, this study is relevant because it suggests that low doses of FB1 interact with MDD thus contributing to disrupt the epigenetic landscape.

Keywords:
Epigenetic modifications / Folates / Fumonisin / Methyl donor deficiency / Prenatal nutrition

1 Introduction

The fumonisin mycotoxins are produced by several Fusarium species and are contaminant of corn and corn-based diet worldwide. About 30 types of fumonisins have been identified [1] with the most prevalent being fumonisin B1 (FB1) which was found to be a complete carcinogen [2–4]. In humans, FB1 is associated with esophageal cancer, particularly in areas with high levels of FB1 in corn such as the Transkei region in South Africa [5] or in China [6]. Recently, in Tanzania, a correlation between exposure to fumonisins and growth retardation was made for the first time in infants consuming corn-based food [7]. Moreover, maternal intake of fumonisin contaminated tortillas increases the risk of neural tube defects (NTDs) in offspring along the Texas–Mexico border [8].

FB1 disrupts sphingolipids biosynthesis by inhibiting the enzyme ceramide synthase thus leading to accumulation of free sphingoid bases and reduced formation of glycosphingolipids among which is sphingomyelin [9]. Sphingomyelin is a major component of the plasma membrane and is required

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Abbreviations: **FB1**, fumonisin B1; **FR**, folate receptor; **MDD**, methyl-deficient diet; **SAH**, *S*-adenosylhomocysteine; **SAM**, *S*-adenosylmethionine

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for the proper function of glycosylphosphatidylinositol-anchored (GPI) proteins, such as the folate receptor (FR) [10]. We have reported that FB1 reduces the expression of the two ubiquitous folate cell transporters, the FR and the reduced folate carrier (Slc19a1) in the human hepatocarcinoma HepG2 cells [11]. Dosing pregnant dams with FB1 results in complex sphingolipids temporary decrease in placenta [12] and folate depletion in liver fetuses [13]. FR binds to folates with high affinity and internalizes folates by an endocytic mechanism, while Slc19a1 functions as an anion exchanger [14]. Several types of experiments in vitro [11,15], ex utero [16], and in vivo [13] hint that FB1 alters folate uptake. These results suggest that the toxicity of FB1 may be mediated, at least in part, through its effects on folate status and methyl donor metabolism.

FB1 contamination depends on several factors including climate conditions (temperature, humidity), insect infestation, and pre- and postharvest handling. In some European countries, corn-based food was found to be contaminated by fumonisin at an overall incidence of 62% and fumonisin was detected at levels exceeding the legal limits in 7% of the analyzed samples [17]. In this context, the influence of FB1 on cellular uptake of folate may be partly neutralized in Northern America, where food is fortified with folate. This assumption is based on several lines of evidence. In LM/Bc mice, maternal FB1 exposure results in a dose-dependent increase in NTDs that could be prevented by maintaining adequate folate levels [13]. In HepG2 cells, 5-methyltetrahydrofolate attenuates FB1-induced apoptosis [11]. However, in other countries, the combination of folate deficiency and FB1 exposure strengthens the threat for public health.

Inside the cell, the role of folate is to transfer one-carbon units between compounds that act as donors or receivers of one-carbon moieties. Folate is a methyl group donor in the reaction that methylates homocysteine to methionine. The methionine produced is subsequently converted to *S*-adenosylmethionine (SAM), which in turn is converted to *S*-adenosylhomocysteine (SAH) by transferring its methyl group onto a variety of substrates. SAM is required for cytosine methylation and methylation of histones both of which are components of epigenetic mechanisms essential in many cell functions such as transcription, DNA repair, and replication [18]. The folate nutritional status and/or genetics have been linked to a vast array of pathologies including cancer. Overwhelming evidence has established that periconceptional fortification with folate has a protective effect on NTDs [19].

Histone modifications together with DNA methylation are involved in shaping the chromatin architecture, which can forbid or authorize gene accessibility to regulatory proteins. These epigenetic modifications play a critical role in the control of cell processes including embryonic development, transcription, X chromosome inactivation, and genomic imprinting [18]. Several cases have been reported indicating that the maternal fetal environment can change the epigenetic state in adult offspring and at least in some cases this change seems to be reversible [20]. Methyl-deficient diet (MDD) can induce liver carcinogenesis in rodents and the development of the tumor is characterized by reduced repetitive DNA LINE-1 methylation and linear decline in H4K20me3 whereas global H3K9me3 decreases at the early stages but increases at the advanced stages of carcinogenesis [21]. To our knowledge, the role of FB1 in epigenetic events has not been addressed yet.

We used a rat model to investigate the synergic impact of prenatal methyl donor deficiency and low dosage of FB1 administration on the pattern of global histone modifications. We found that MDD decreased significantly H4K20me3, which is a marker of assembled heterochromatin. Amplifying the metabolic stress by adding FB1 to the methyl depletion triggered a defense mechanism in the fetuses that attempted to resist heterochromatin disorganization by increasing H3K9me3.

2 Material and methods

2.1 Experimental design

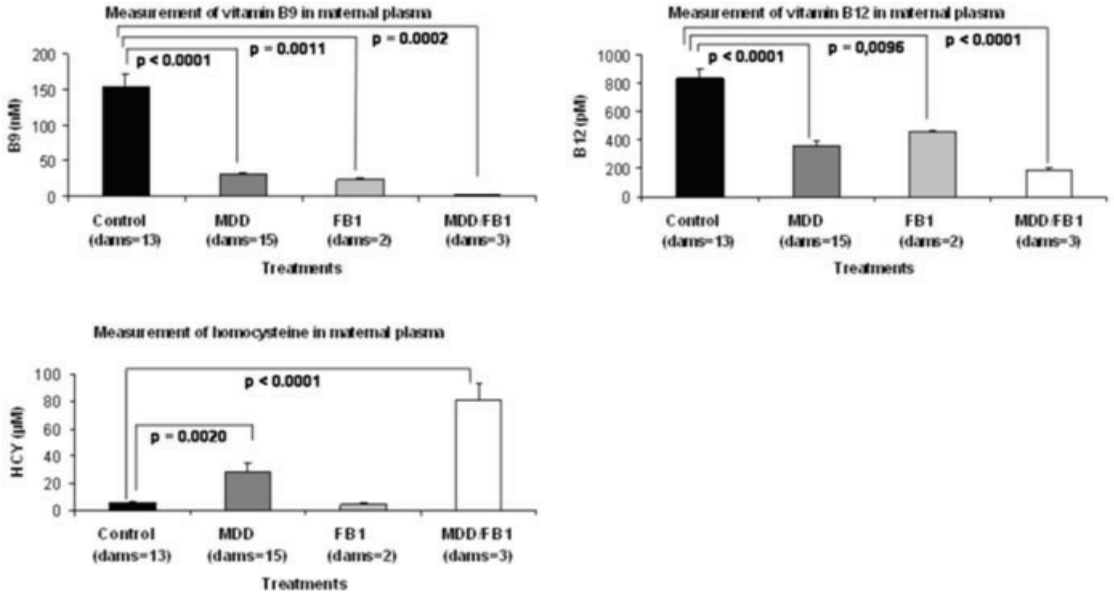
Animal treatments were conducted under institutional approval and in accordance with international guidelines for animal care and housing (Authorization n°54–547-22 delivered by the Departmental Direction for Populations Protection, Meurthe et Moselle, France).

Three-month-old sexually mature virgin Wistar female and male rats (Charles River, l'Arbresle, France) were left to acclimatize during 2 weeks under standard laboratory conditions. One month before mating, dams were exposed daily to a control (folates: 0.9 mg/kg, vitamin B12: 0.04 mg/kg, choline: 2100 mg/kg; SAFE, Augy, France) or methyl deficient (folates: 0.01 mg/kg, vitamin B12: 0 mg/kg, choline 0.06 mg/kg; Special Diet Service, Saint Gratien, France) diet with or without fumonisin FB1 (Sigma-Aldrich F1147–1 mg, St Louis, MO, USA) administered daily by gavage at 4 μg/kg body weight, which amount to twice the provisional maximum tolerable daily intake (PMTDI), defined at 2 μg/kg/day, by the Joint Expert Committee on Food Additives (JECFA). The four female groups were established as follows: (i) animals exposed to control diet (Control, $n = 13$); (ii) animals exposed to MDD ($n = 15$); (iii) animals exposed to FB1 alone (FB1, $n = 2$); (iv) animals exposed to MDD and FB1 (MDD/FB1, $n = 3$). Number of females in the control and MDD groups is large because those females were dispatched on other teams in the laboratory working on topics related to methyl depletion but unrelated to FB1. The whole number of control and MDD dams was used to assess methyl depletion but only a subset was included in the analysis of FB1 and MDD interactions in fetuses. Females were paired with males overnight and successful mating was confirmed by the presence of sperm in the vaginal smears and used to determine the beginning

of pregnancy. The respective diets were maintained during mating and gestation and body weights and food consumption were recorded daily.

Dams were killed with excess halothane on day 20 of gestation; gravid uteri were removed and weighed. Blood samples and livers were collected from all dams for the measurement of vitamin B12, folate and homocysteine concentrations, and morphological study (Fig. 1). In order to analyze FB1 and MDD interactions, 2/13 control dams, 3/15 MDD dams, 2 FB1 dams, and 3 MDD/FB1 dams were utilized (Table 1). Fetuses were removed from the uterus, blotted dry, measured, weighed, and examined for gross malformations. Total numbers of fetuses derived from dams in each group were as follows: control group, $n = 23$; MDD group, $n = 39$; FB1 group, $n = 25$; MDD/FB1 group, $n = 25$ (Table 2). From each group, eight embryos were randomly selected and their livers were retrieved and used in all subsequent experiments (Figs. 2–4)

A



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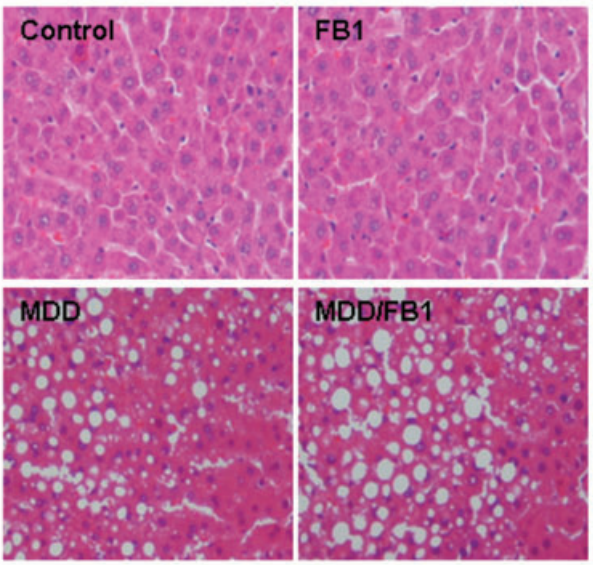

Figure 1. **(A)** The vitamins concentrations are reduced and homocysteine concentration is significantly increased in dams exposed to isolated MDD or to combined MDD/FB1 administration, compared to the control group. Each sample was analyzed in duplicate in three independent runs and values were compared by ANOVA. Histograms represent means and error bars standard errors of the means. **(B)** The morphological study reveals that the pericentrolobular steatosis caused by the MDD is aggravated by FB1 administration. MDD, methyl donor deficieny; FB1, fumonisin.

Table 1. Effects of methyl donor deficiency (MDD) and fumonisin (FB1) on various developmental and nutritional parameters. FB1 administration and/or exposition to MDD did not affect the number of fetuses per rat, maternal body weight, food intake, nor gravid uterus weight

Treatments	No. of fetuses/rat	Gain in maternal body wt. (g)	Feed intake (acclimatization) (g)	Feed intake (gestation) (g)	Gravid uterus wt. (g)
Control (dams = 2)	11.5 ± 0.5	121.5 ± 17.5	501.7 ± 16.4	284.9 ± 7.5	66.8 ± 3.6
MDD (dams = 3)	14.7 ± 1.8	137.7 ± 9.8	424.6 ± 36.5	264.0 ± 8.8	81.5 ± 12.8
FB1 (dams = 2)	13.0 ± 0	141.5 ± 0.5	448.5 ± 10.2	289.1 ± 0.3	71.6 ± 0.8
MDD/FB1 (dams = 3)	13.3 ± 1.5	139.7 ± 1.2	445.0 ± 20.9	254.8 ± 2.4	67.8 ± 6.7

± represents standard error to the mean.

2.2 Determination of vitamin, homocysteine, and hepatic metabolites concentrations

Plasma concentrations of vitamin B12 and folate were determined by radio-dilution isotope assay (simulTRAC-SNB, ICN Pharmaceuticals, New York, NY, USA). Homocysteine concentrations were measured by ultra performance liquid chromatography coupled to mass spectrometry (Api 4000 Qtrap Applied Biosystems, Courtaboeuf, Essonne, France). SAM and SAH concentrations were measured by solid-phase extraction and high-performance liquid chromatography using a Nova-pack column C18, 150 × 3.9 mm, 4 μm [22, 23].

2.3 Morphologic study of pregnant dams liver

Tissue samples were fixed in 4% (m/v) formaldehyde pH 7.4 for 24 h. Tissue were routinely processed and embedded in paraffin. Paraffin blocks were used to generate 5-μm thick hematoxylin and eosin-stained sections. By using the nonalcoholic steatohepatitis scoring system [24], histological features were graded 0–3 (0 as < 5% parenchymal involvement by steatosis, 1 between 5–33%, 2 between 33–66%, and 3 as > 66%). The slides were examined by two different investigators blinded to samples origins.

Table 2. Effects of methyl donor deficiency (MDD) and fumonisin (FB1) on fetal body weight and height. Fetuses exposed to isolated MDD or to combined MDD/FB1 administration show significantly decreased weight and height, compared to the control group, whereas isolated FB1-treated fetuses show no significant change

Treatments	Average fetal body weight (g)	Average fetal height (cm)
Control ($n = 23$)	3.64 ± 0.05^{a}	3.60 ± 0.03^{a}
MDD ($n = 39$)	3.34 ± 0.07^{b}	3.50 ± 0.03^{b}
FB1 ($n = 25$)	3.60 ± 0.08^{a}	3.58 ± 0.03^{a}
MDD/FB1 ($n = 38$)	3.22 ± 0.05^{b}	3.47 ± 0.02^{b}

n represents the total number of fetuses derived from all litters born in a given group. ± represents standard error to the mean and means in the same column not sharing a common superscript letter are significantly different.

2.4 FRs quantitation by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using RNeasy Mini extraction kit (Qiagen, Courtaboeuf, Essonne, France). First strand cDNA was synthesized using Quantitect Reverse Transcription kit (Qiagen, Courtaboeuf, Essonne, France). The qPCR was performed using the Quantitect SYBR Green PCR kit from Qiagen and iCycler (BioRad, Marnes La Coquette, Auvergne, France). *FR* (NM_133527.1) and the Reduced Folate Carrier Protein (*Slc19a1*, NM_017299.2) were amplified with *Tceb1* (Transcription Elongation Factor B polypeptide 1 NM_022593.3) used as the reference gene, with the oligonucleotides described in Supporting Information Table 3. The qPCR was performed according to the following program: 15 min at 95°C to activate the enzyme, followed by 45 cycles at 94°C for 15 s, 56°C for 20 s, and 72°C for 15 s. Then melting curve analyses were performed by increasing temperature from 64 to 89°C. Relative gene expression was calculated by the ΔΔCT method [25].

2.5 Histone extraction

Histones were extracted as previously described [26]. Acidic cell extracts were prepared from frozen liver tissues using a lysis buffer containing Tris 10 mM, 1.5 mM $MgCl_2$, 1 mM KCl, dithiothreitol 1 mM (DTT) (v/v), 1 mM phenylmethanesulfonyl fluoride (PMSF) (v/v), protease inhibitor cocktail 2% (v/v), sodium orthovanadate 1 mM ($NaVO_3$) (v/v), sodium fluoride 2 mM (NaF) (v/v), sodium butyrate 5 mM ($C_5H_8O_2Na$) (v/v). The lysate was passed ten times through a 20 Gauge $1^1/_2$ inch syringe needle and incubated during 30 min at 4°C in a rotator. Cell lysates were centrifuged at 10 000 × *g* at 4°C for 10 min. The supernatant was eliminated and the pellet resuspended in 400 μL of sulphuric acid (H_2SO_4) 0.2 M. The mixture was incubated 2 h/4°C in a rotator and centrifuged at 14 000 × *g*/4°C/10 min. The acid insoluble pellets were discarded. The supernatant fractions, which contain the acid soluble proteins, were purified by sequential dialysis (Slide-A-Lyser Dialisys Cassette, 3500 MWCO, 0.1–0.5 mL capacity, Pierce, Brebiéres, Nord-Pas-de-Calais, France) against 200 mL acetic acid 2.5%. Then proteins were removed, transferred to an Eppendorf tube, and frozen at –80°C during

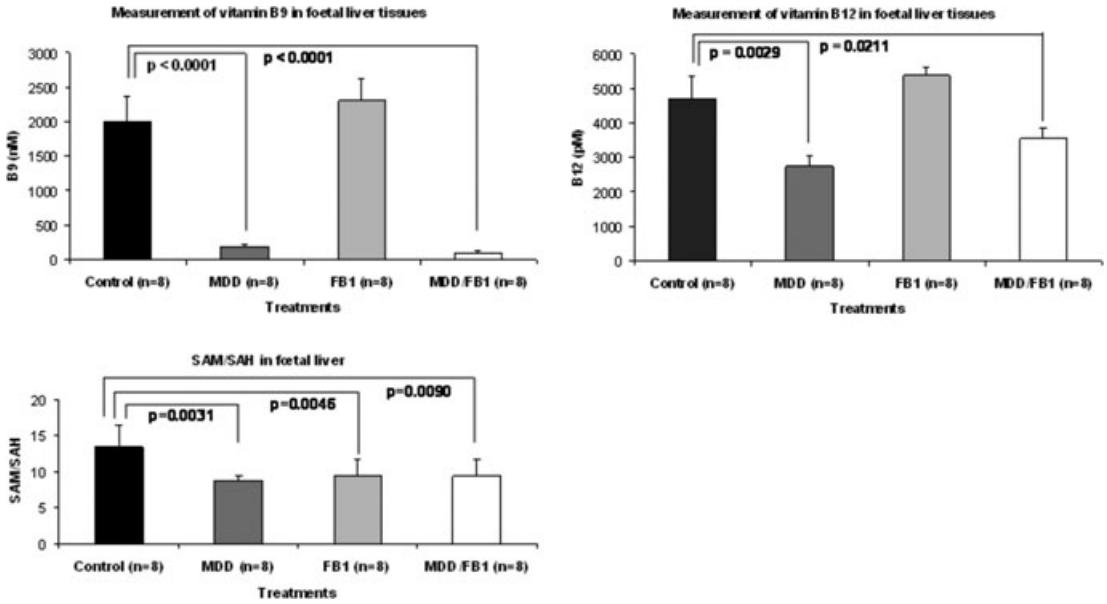

Figure 2. Folate and vitamin B12 are reduced in the isolated MDD and combined MDD/FB1 groups. The SAM/SAH ratio is significantly reduced in fetuses from dams exposed to isolated MDD, isolated FB1 administration, or to both factors in combination, compared to control fetuses. *n* represents the number of fetuses randomly selected and analyzed in each group. Histograms represent means and error bars standard errors of the means. Each fetus sample was tested in duplicate in three independent runs and averaged values of parameters per fetus were compared by ANOVA. MDD, methyl donor deficieny; FB1, fumonisin.

1 h. Extracts were lyophilized using speed vac during 4 h minimum. Finally, lyophilisate was resuspended in 100 μL H_2O. Protein concentrations were determined using the BCA protein assay kit (Interchim Montluçon, Auvergne, France) and aliquots of total histones were stored at –80°C.

2.6 Analysis of histones modifications

For Western blot analyses proteins were resolved on sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with nonfat dried milk, probed with antibodies, developed with the enhanced chemiluminescence protocol (ECL kit, VWR Strasbourg, France), and analyzed by Image J software. All membranes were stained with Coomassie Blue and probed with antihistone H3 and antihistone H4 antibodies to confirm equal protein loading. The selected histone modifications are markers of heterochromatin assembly (see Discussion for details): H4K20me3 and H3K9me3 [27], H3R2me2 [28], H4K16Ac [29]. Antibodies

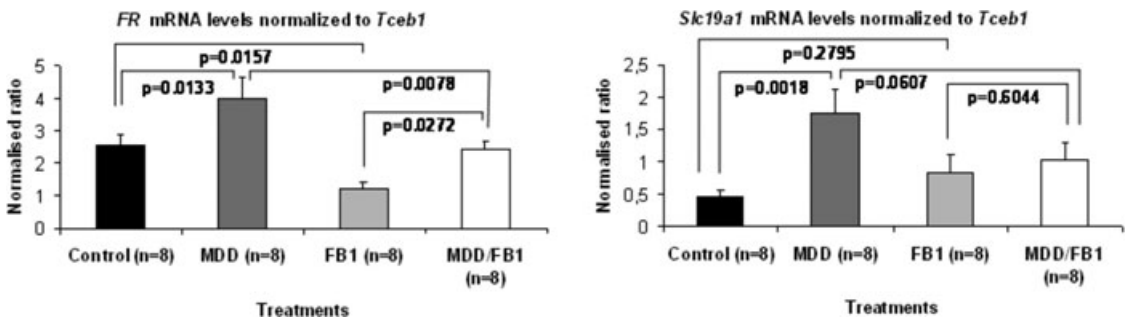

Figure 3. By comparing MDD and control groups and MDD/FB1 and FB1 groups, mRNA levels indicate that folate receptor transcription is up-regulated regardless of fumonisin treatment. However, combined methyl group deficiency and fumonisin exposure might neutralize folate receptors up-regulation since FR and Slc19a1 transcripts are diminished in MDD/FB1 group compared to MDD group. Isolated FB1 exposure reduces FR transcripts but not Slc19a1 transcripts. Representation and analyses of data are similar to Figure 2. MDD, methyl donor deficieny; FB1, fumonisin.

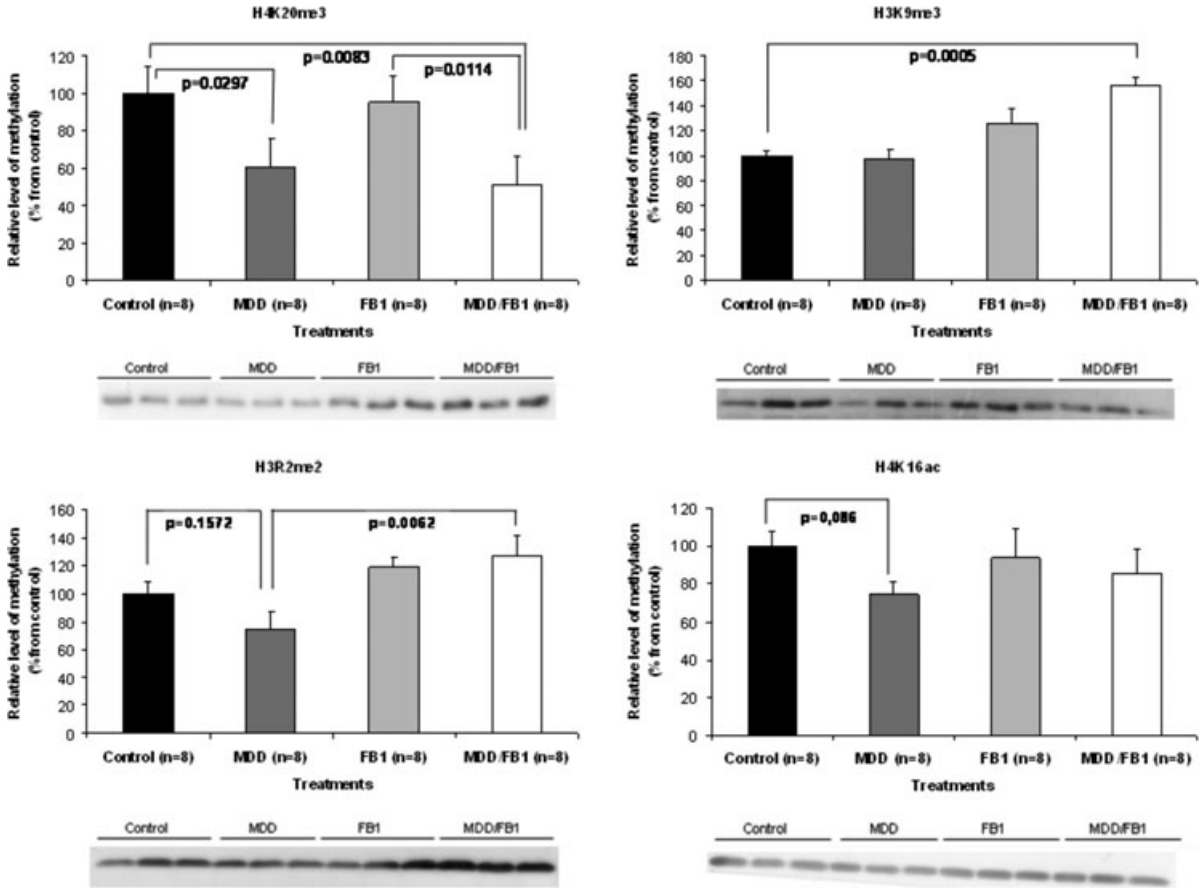

Figure 4. MDD results in decreased H4K20me3 and H4K16Ac in comparison to the control group. Combined exposure to MDD/FB1 decreases H4K20me3 and increases H3K9me3. This opposite variation is a defense mechanism to resist heterochromatin disorganization. Combined exposure to MDD/FB1 also increases H3R2me2 and H4K16Ac in agreement with the defense mechanism. Representation and analyses of data are similar to Figure 2. MDD, methyl donor deficieny; FB1, fumonisin.

characteristics are described in Supporting Information Table 4.

2.7 Statistical analysis

Maternal parameters were compared by one-way ANOVA. All fetal analyses were performed including the litter of origin as a covariate in order to account for the possible variance between and within dams [30]. For the following parameters in fetus—folate, vitamin B12, SAM/SAH, FR, Slc19a1, and histone modifications—the expression data sets in each group were generated from eight fetuses selected randomly from the corresponding joint litters. For a given parameter, each fetus was tested in duplicate in three independent runs and the averaged values per fetus and per parameter were compared in statistical analyses. Statistical analyses were done with Statistica (http://www.statsoft.com) by using one-way ANOVA tests; p-values < 0.05 were judged significant. Error bars in figures represent standard error of the mean.

3 Results

3.1 MDD but not FB1 decreased fetuses weight and length

None of the groups showed differences concerning the number of fetuses/litter, maternal body gain, food intake, gravid uterus weight (Table 1). No maternal nor fetal death or implant occurred. Isolated MDD yielded significant decreased fetal body weight ($p = 0.0020$) and length ($p = 0.0089$). An identical result was observed for the combined exposure MDD and FB1 but not for FB1 administration alone (Table 2).

3.2 MDD-induced plasma homocysteine increase and induced hepatic steatosis in dams

MDD efficiently decreased plasma folate ($p < 0.0001$) and vitamin B12 ($p < 0.0001$) and increased homocysteine ($p = 0.002$) in comparison to the control diet. FB1 by itself also

decreased folate ($p = 0.0011$) and vitamin B12 ($p = .0.0096$) without significantly elevating homocysteine. However, combined MDD/FB1 resulted in significantly increased homocysteine ($p < 0.0001$) to a much greater extent than MDD. MDD yielded a pericentrolobular steatosis grade 1 in the liver of pregnant dams, aggravated to grade 2 by combined FB1 administration (Fig. 1B).

3.3 Unlike isolated MDD, isolated FB1 administration did not correlate with low folate concentrations in liver fetuses but both conditions resulted in reduced SAM/SAH ratio

In fetal liver tissues, MDD induced decreased folate ($p < 0.0001$) and vitamin B12 ($p = 0.0029$) concentrations in comparison to the control diet (Fig. 2). Combined MDD and FB1 administration also decreased folate ($p < 0.0001$) and vitamin B12 ($p = 0.0021$) significantly. FB1 by itself did not affect folate nor vitamin B12 levels but decreased SAM/SAH ratio ($p = 0.0046$) to the same extent as MDD ($p = 0.0031$) or combined MDD and FB1 ($p = 0.009$).

3.4 FB1 decreases FR transcripts

Methyl donor deficiency (MDD) up-regulated both *FR and Slc19a1* transcripts in animals fed control diet or exposed to FB1. FR was elevated in the MDD compared to the control group ($p = 0.0133$) and in the MDD/FB1 group compared to the FB1 group ($p = 0.0272$). Slc19a1 was elevated in the MDD compared to the control group ($p = 0.0018$) and it was also elevated, but not significantly, in the MDD/FB1 group compared to the FB1 group ($p = 0.6044$) (Fig. 3).

However, it cannot be ruled out that combined exposure to methyl deficiency and FB1 reverses FR and *Slc19a1* transcripts up-regulation. This mechanism displays a more pronounced impact on FR than *Slc19a1* transcripts. The data show that FR transcripts have similar concentrations in the control group and the MDD/FB1 group. Moreover, FR transcripts are lower in the MDD/FB1 group compared to the isolated MDD group ($p = 0.0078$). Slc19a1 transcripts variations exhibit a similar profile but with no statistical significance ($p = 0.0607$). Interestingly, isolated exposure to FB1 resulted in decreased FR mRNA ($p = 0.0157$) whereas Slc19a1 mRNA was increased but not significantly ($p = 0.2795$). This result reinforces the hypothesis that one of the mechanisms by which FB1 alters folate transport might be the interference with sphingolipids metabolism.

3.5 Added stress generated by combining MDD and FB1 leads to loss of H4K20me3 accompanied by increased H3K9me3

The histone modifications were compared between the three treated groups and the control group (Fig. 4). MDD induced significant decreased H4K20me3 ($p = 0.0297$) with no significant H3K9me3 alteration. Combined exposure to MDD/FB1 generated a bivalent mark associating significant decreased H4K20me3 ($p = 0.0083$) and increased H3K9me3 ($p = 0.0005$). Thus, amplifying the metabolic stress by adding FB1 to methyl donor depletion might trigger in the cell a defense mechanism that tries to resist heterochromatin disorganization.

H3R2me2 was low in the MDD group compared to the control group, but the difference between the two groups was not significant ($p = 0.1572$). However, combined MDD/FB1 exposure yielded significantly elevated H3R2me2 ($p = 0.0062$) in comparison to MDD only. H4K16Ac pattern was similar to H3R2me2 but the variations were not statistically significant. Isolated MDD resulted in decreased H4K16Ac in comparison to the control group. MDD/FB1 exposure slightly increased H4K16Ac in comparison to MDD only.

4 Discussion

As expected, the MDD used in this study induced reduced plasma folate and vitamin B12 and increased homocysteine in pregnant dams. This model of gestational methyl donor deficiency was established in our laboratory: as we have previously described in several publications, it is efficient in depleting methyl donors from the cells and slowing down enzymes involved in the methionine cycle [31, 32]. FB1 by itself did not increase homocysteine in pregnant dams; however, combined MDD/FB1 increased homocysteine to a much greater extent than isolated MDD. Moreover, FB1 aggravated the MDD-induced pericentrolobular steatosis in pregnant dams liver indicating that the association of both factors is more deleterious than each factor alone.

Isolated maternal MDD was associated with diminished fetal body weight and length and resulted in decreased vitamin B12 and folate levels and SAM/SAH ratio in fetuses liver. It is noteworthy that FB1 by itself produced a decreased SAM/SAH ratio in fetuses liver. That FB1 by itself did not alter folate and vitamin B12 levels can be attributed to the low dosage of FB1 administered (4 μg/kg/day). Low dosage was intentionally used because FB1 is present at low levels in maize throughout the world. Humans are chronically exposed to FB1 concentrations not largely exceeding the current recommended PMTDI set at 2 μg/kg/day. Using high FB1 dose does not mimic the exposure likely to be achieved in humans. Using low FB1 dose offers the opportunity to characterize a threshold level beyond which cellular effects are observable.

A molecular mechanism of adaptation to folate deficiency [33] was triggered by activating *FR* and *Slc19a1* transcription in fetal liver. Overall, FR and Slc19a1 transcription were increased by MDD regardless of FB1 treatment. However, FB1 exposure may introduce subtle changes in FRs transcription since combined MDD/FB1 might antagonize the adaptation to folate deficiency. Methyl depletion by itself significantly

increased FR and Slc19a1 in comparison to the control diet but these two receptors did not vary significantly between the MDD/FB1 and control groups. Furthermore, both FR and Slc19a1 transcripts were significantly diminished in the MDD/FB1 group compared to the isolated MDD group. This neutralizing effect was found to be stronger for FR transcripts consistent with a preferential interaction between FB1 and FR. Adding FB1 exposure to methyl deficiency might aggravate the maternal adverse environment and makes it harder for the fetus to adapt to the folate depletion.

Most importantly, FB1 reduced FR transcription in fetuses fed control diet but did not reduce Slc19a1 transcription. FR is a GPI-anchored protein associated with membrane domains enriched in sphingolipids [15]. Thus, this result lends support to previous reports showing that FB1 is involved in disruption of sphingolipid metabolism [12, 13]. Two restraints should be pointed out. First, only FRs transcripts were measured but it is not known if the proteins were preserved or not. Second, FR is associated with membrane domains enriched with sphingolipids but also cholesterol and plasminogens. Therefore, possible interactions between FB1 and these complex structures should also be considered.

Of all histone modifications investigated, isolated MDD induced a significant decrease in H4K20me3 only. H4K20me3, together with H3K9me3, heterochromatin protein 1 (HP1) and DNA methylation, is an essential feature of constitutive heterochromatin. H4K20me3 is a conserved mark of constitutive heterochromatin that contributes to the maintenance of genome stability [34, 35]. Loss of H4K20me3 induced by MDD may contribute to weaken heterochromatin organization and interfere with the appropriate expression of genes thus leading to impaired cell function. One of the molecular mechanisms explaining the DNA instability observed in rodents exposed to MDD [36] may be the low levels of H4K20me3.

The HP1 family of proteins is a major constituent of heterochromatin. HP1α and HP1β bind to H3K9me3 to establish condensed transcriptionnally inactive chromatin [37, 38]. It was reported that H4K20me3 occurs only after HP1 binds to H3K9me3 [27, 39]. We show in this work that when pregnant dams are exposed to MDD and FB1, fetuses display significant decrease in H4K20me3 and significant increase in H3K9me3. It looks like the elevated H3K9me3 is a part of a rescue attempt to restore H4K20me3 to normal levels. FB1, even at moderately elevated concentrations, adds up metabolic stress on the cell that tries then to escape these dire straits and attempts to preserve its functions by developing a defense mechanism that resists heterochromatin disorganization. This reciprocal interaction between H4K20me3 and H3K9me3 has been described before for adult rat males exposed to MDD but for a sustained period of 54 weeks at the end of which liver tumors develop [21]. In the maternal diet deprivation described here, dams are exposed to methyl donor depletion for a relatively short period, 1 month before mating then during gestation until fetuses are 20 days old. The diet-induced metabolic stress during this period may not be sufficient enough to drive the fetuses to a defense reaction; however, addition of FB1 strengthens the threat and moves the cell faster toward the defense threshold.

Heterochromatin assembly depends on a large number of proteins that implement several distinctive marks other than H4K20me3 and H3K9me3. H3R2me2 was found to be enriched through heterochromatic loci and inactive euchromatic genes [28]. Isolated MDD yielded diminished H3R2me2 where as combined MDD/FB1 exposure yielded elevated H3R2me2. The difference between these two exposures was statistically significant. This result is in agreement with the defense threshold mechanism. The added metabolic stress represented by exposure to both MDD and FB1 constitutes a "second hit" that drives the cell to increase H3R2me2 in order to resist heterochromatin disorganization.

An additional histone residue, H4K16, may be viewed as a docking site that recruits the complex Sir2-Sir3-Sir4 necessary to establish heterochromatin (Sir: Silent information regulator) [29]. Indeed, acetylated chromatin allows Sir2/Sir4 binding. Sir2 is a NAD-dependent histone deacetylase that specifically deacetylates H4K16. Sir3 binds to deacetylated H4K16 and turns chromatin off. The pattern of H4K16Ac was similar to H3R2me2 but the variations did not reach statistical significance. Levels of H4K16Ac were lower in the methyl deficient versus control group but were higher in the methyl-deficient group/fumonisin-exposed group versus methyl deficient only group.

The fact that environmental factors do not affect in the same way histone modifications marking heterochromatin is not surprising. Microarray analysis of global gene expression in human nasopharyngeal carcinoma KB cells grown in folate deplete and folate replete medium revealed that expression of only eight genes responded to variation of folate levels [40]. Among those genes, some were up-regulated and some were down-regulated, some were hypermethylated and some were hypomehtylated. Thus, methyl deficiency is gene specific and does not alter epigenetic modifications uniformly. It is likely that this is the case for other environmental factors such as FB1.

Animal models and epidemiological studies show that maternal FB1 exposure increases the risk of NTDs that could be prevented by maintaining adequate folate levels [8, 13]. The interactions between FB1 and folate are complex and not fully elucidated and the role of FB1 and folate in NTD etiology still needs clarifications. NTDs are multifactorial in origin and involve complex gene–environment interactions. The beneficial effect of folate in NTDs may be ascribed to improved DNA methylation [41]. Another epigenetic mechanism may be at stake since emerging evidence show that histone modifications participate in the etiology of NTDs [41]. In *Splotch* mutant embryos (*Pax3*) which develop NTDs, H3K27me is increased [42]. Cranial NTDs are observed among knock-out embryos for p300 [43] and CITED [44] and knock-in embryos for Gcn5 [45, 46]. All three enzymes have acetyltransferase activities. Conversely, several histone deacetylases (*Hdac*) control the progression from neural precursors to neurons [47].

Particularly, NTDs were found to occur in a subset of *Hdac4* and *Sirt1* mutants [48, 49]. *Sirt1* is the mammalian homolog of *Sir2* that deacetylates H4K16. Moreover, Hdac inhibitors, such as valproic acid and trichostatin A, display teratogenic effect and can induce NTDs as well as axial skeletal defects [50, 51]. Accumulating evidence also underpin the global changes in histone modification patterns in many cancers [18]. Notably aberrant epigenetic alterations imposed by nutritional factors appear to be an essential mechanism leading to hepatocarcinogenesis in rodents [21].

The main interest of this study is to report that FB1, at low doses likely to contaminate food, can alter histone modifications. A common pathway through which folate and FB1 are involved in NTDs could be epigenetic regulation. One limitation of this study is the small number of animals analyzed. Additional investigations are needed to confirm on a larger scale that methyl deficiency and FB1 interact to cause a disruption of the epigenetic landscape potentially contributing to the onset of developmental disease or tumor proliferation.

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