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Original Article

Lipid Metabolism Disorders, Lymphocytes Cells Death, and Renal Toxicity Induced by Very Low Levels of Deoxynivalenol and Fumonisin B_1 Alone or in Combination Following 7 Days Oral Administration to Mice

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ABSTRACT

Scope: In our previous study focused on in vitro interactive effect of Fusarium mycotoxins deoxynivalenol (DON) and fumonisin B1 (FB1), we reported that these toxins tested at low level and in association could lead to additive or synergistic cytotoxic effect. The aim of the present study is to confirm those findings by in vivo study. **Materials and Methods:** Swiss mice were orally administered with low doses of DON (45 μg/kg bw/day), FB1 (110 μg/kg bw/day), and their mixture (DON + FB1) for 7 days. **Results:** As results, no death or abnormal symptoms were observed in all groups. The significant of loss of weight was observed in females group treated with FB1 and its association with DON. Serum chemistry examinations revealed that disorders in lipid metabolism, renal filtration perturb and a rhabdomyolysis. DON has been found as particular inducer of kidney cell deoxyribonucleic acid (DNA) methylation and blood lymphocytes cell death as measured by lymphocytes DNA fragmentation. Female mice were more sensitive and the mixture of DON and FB1 led to additive or more than additive effect particularly for their target kidney which showed different pattern of toxicity. **Conclusion:** Based on the results of this study, the no-observed-adverse effect level (NOAEL) of both DON and FB1 should be low than 45 μg/kg bw/day and 110 μg/kg bw/day, respectively in mice.

Key words: Combined effect, Fusarium toxins, mice

INTRODUCTION

Mycotoxins are secondary metabolites produced by different fungal species that can contaminate agricultural commodities in fields, during harvest and/or in storage, and can reach human beings through contaminated food. Aflatoxin, ochratoxin A, patulin, and the Fusarium toxins are well-investigated mycotoxins because of their prevalence in agricultural commodities. Plant fusariose is recognized as a great agricultural problem, due to the variety of mycotoxins Fusarium species can produce, including trichothecens, zearalenone, and fumonisins; which have been identified as important contaminants in foodstuffs.[1,2] Since, Fusarium mycotoxins are often found in combination in infested cereal grains, several studies have been focused on their interactive effect in human or animal cells lines.[3-5] Thus, it has been demonstrated that the toxic effect of mixtures of mycotoxins could be greater than the single mycotoxins. For example, in our previous study focused on mixture of deoxynivalenol (DON) and fumonisin B_1 (FB_1), using several endpoints such as lipid peroxidation, protein and deoxyribonucleic acid (DNA) synthesis, cell viability, DNA methylation, and DNA fragmentation; we have reveled FB_1 and DON display synergistic or additive

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effects in lipid peroxidation, inhibition of protein synthesis, and DNA methylation and fragmentation.[5] DON and FB_1 are known to cause numerous toxic effects in animals and human exposed via diet.[5]

DON produced abundantly by *Fusarium graminearum*[6] when ingested, can induce a decrease in food intake or refusal to eat food, vomiting, and digestive disorders; with subsequent losses of weight gain in animals who ingest this mycotoxin.[7] The gastrointestinal system is the target organ of the toxin.[8] DON induced hematological effects, lesions in the nonglandular stomach, and caused thymic lymphoid depletion, increased incidences, and mean severity of spleenic hematopoiesis, and increased mean severity of sternal bone marrow adipocyte deposition in rats at the highest dose.[9]

FB_1 causes equine leukoencephalomalacia, porcine pulmonary edema, nephrotoxicity, hepatotoxicity, cardiotoxicity, and hepatocarcinogenicity in laboratory animals and of esophageal carcinoma in humans.[6] It had been reported hepatotoxicity and nephrotoxicity in lambs, rabbits, and mink, as well as an increased rate of apoptosis in liver and kidney.[10,11]

According to our previous *in vitro* study in human intestinal cells line Caco-2, combination of FB_1 and DON lead always to toxic effect greater than FB_1 or DON alone.[5] In addition, although each component has been shown to cause tissue toxicity in experimental animal, there is no evidence regarding the effect of mixture of DON and FB_1 *in vivo*. For example, DON and FB_1 could exert their *in vivo* toxic effect on the same target. So the aim of the present study is to determine whether association of DON and FB_1 cause an additive or synergistic toxic effect on their systemic targets in mice.

MATERIALS AND METHODS

Materials

Chemicals

DON and FB_1 were obtained from Sigma Chemical Company (St Louis, MO, USA) and were dissolved in ethanol/water (50:50). All other chemicals used were of analytical grade. For the study, the mix solvents (ethanol/water) was dried under an air stream at 50°C and reconstituted in water which became the vehicle in the experimentation.

Animals

Male and female Swiss mice (7-8 week old), weighing between 20 and 25 g were obtained from Society of DEPRE (France). Mice were housed in environmentally protected transparent polypropylene cages with stainless steel wire tops for a period of 1 week before induction of different treatments. The mice had free access to water. Experimental diets were placed in special containers to minimize spillage. Environmental conditions included 23-25°C, relative humidity of 45-55%, and a 12-h light: dark cycle. The protocol for this study was approved by the Committee of Bioethics of Nangui Abrogoua University, Abidjan, Ivory Coast.

Methods

Forty mice (20 male and 20 female) were divided into four groups (n = 10 per group with five female + five male): (a) Normal control group, mice received water; (b) DON dosed group, mice received 45 μg/kg bw/day of DON; (c) FB_1 dosed group, mice received 110 μg/kg bw/day of FB_1; and (d) DON + FB_1 dosed group, mice receive 45 (DON) + 110 (FB1) μg/kg bw/day. Each group received the appropriate vehicle (water), DON, FB_1, or DON + FB_1 daily by oral administration for 7 days. Every day the weight of the mice was observed using a Sartorius balance (ED 224S Germany) with 0.0001 mg accuracy. Urine samples were collected refrigerating metabolic cages (Tecniplast, Buguggiate, Italy) and at the end of the experiment, the mice were sacrificed to collect serum and tissues.

Assay for serum and urine chemistry

The serum biochemistry parameters including aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein (TP), triglyceride (TG), total cholesterol (TC), creatinine, gamma glutamyl transpeptidase (γ-GT) and creatine kinase (CK) were evaluated spectrophotometrically, using commercially available diagnostic kits supplied by BioMerieux (France). Similarly, unary TP and creatinine were evaluated spectrophotometrically using commercially available diagnostic kits supplied by BioMerieux (France).

Renal DNA methylation

The DNA methylation examination was performed as previously described with okadaic acid.[12] DNA was extracted using the Wizard genomic extraction kit (Promega, France) which is designed for isolation of DNA from white blood cells, tissue culture cells and animal tissue, plant tissue, yeast, and gram positive and gram negative bacteria. Then, DNA was quantified by ultraviolet (UV) spectrophotometry at 254 nm. Ten micrograms of purified DNA dissolved in 10 ml of water was incubated at 100°C for 2 min and then treated with 1 ml of 250 mM potassium acetate buffer (pH 5.4), 1 ml of 10 mM zinc sulfate, and 2 ml of nuclease P1 (6.25 U/ml; Sigma, France) overnight at 37°C, and then treated with 2 ml 0.5 M Tris-HCl (pH 8.3), plus 2 ml of the buffer containing alkaline phosphatase (0.31 U/ml). This mixture was incubated at 37°Celsius (37°C) for 2 h. Analysis of DNA base composition was performed on an

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Instrumentation Consommable Service (ICS; Toulouse, France) chromatograph equipped with a UV Spectra Focus 3-D at room temperature using a C18-phenyl-nucleosyl column (250 × 3.4 mm). Elution was carried out with 6.5 mM H_2PO_4 (NH_4), pH 3.95, and 4% (v/v) methanol at a flow rate of 1 ml/min. Eluates were monitored at 254 nm. Standard bases (dC, dT, dG, dA) (100 mg/ml) and m5dC (10 mg/ml) were obtained from Sigma Chemical and used for quantification, after sequential injection into the high-performance liquid chromatography (HPLC) system. The surface under the curve adjusted to the known concentration of each base was used with computer aided software (Pic3, ICS, France) to quantify the bases. The results in micrograms were used to calculate the rate in percentage of m5dC as compared to m5dC + dC × 100. These rates were presented as mean of the four independents experiments ± standard error of mean (SEM), prior to statistical analysis.

Evaluation of blood lymphocytes DNA fragmentation induced by DON

For agarose gel electrophoresis, genomic DNA was isolated from 300 µl of blood using the Wizard® Genomic DNA Purification Kit as previously described. According to manufacturer's instructions, the isolation of DNA from white blood cells, involves firstly lysis of the red blood cells in the cell lysis solution, followed by lysis of the white blood cells and their nuclei in the nuclei lysis solution. A ribonuclease (RNase) digestion was carried out by addition of RNase solutions. The cellular proteins are then removed by a salt precipitation step, which precipitates the proteins but leaves the high molecular weight genomic DNA in solution. Finally, the genomic DNA is concentrated and desalted by isopropanol precipitation. The DNA was collected by centrifugation (13,000 g) dissolved in DNA rehydrating solution. Total DNA was quantified using the ratio of absorbance at 260/280. Qualitative evaluation of DNA fragmentation was carried out by electrophoresis of the DNA in 1% agarose gel containing ethidium bromide (60 V, 1 h).

Statistical analyses of data

Results are presented as mean ± SEM and analyzed using a nonparametric statistical test, Mann-Whitney test for significance of differences. Acceptable limit is set from $P = 0.05$*.[13]

RESULTS

Evolution of the weight of mice

During the study, no remarkable changes in general appearance were observed and all mice survived until scheduled necropsy. Likewise, we observed significant differences in body weight of male mice between control and treatment group only at day 3 (D_3) but there were no significant differences at day 7 (D_7) [Figure 1a]. In females, body weight significantly decreased in mice administrated with DON, FB1, or their combination during the 7 days of mice treatment [Figure 1b].

Serum and urine chemistry

The results of biochemical examinations were summarized in Tables 1 and 2. TGs and TC were significantly increased in mice treated with mycotoxins DON or FB_1 or their mixture only for male mice in contrast to female mice in which there were no remarkable changes [Tables 1 and 2]. FB_1 provoked an increasing of level of blood TPs in male mice, but a decreasing of those of both unary creatinine and TPs in males and females mice [Table 3]. DON increased the level of blood creatinine in both females and males mice, but CK only in male mice. The mixture of DON and FB_1 leaded to an increasing of blood TPs and creatinine, but a decreasing of unary creatinine in both males and females. In addition, DON + FB_1 increased blood AST level (very notably) and ALT (slightly) only in female mice.

Effects of mycotoxins on DNA methylation in kidney cells

Methylation of deoxycytosine (percentage of m5dC) in the DNA of kidney cells was evaluated by HPLC-UV method in the presence of individual toxin (DON, FB_1) and their mixture (FB_1 + DON). DON alone slightly increased the level of m5dC from 3.6 to 4.07%, but not FB1. Interestingly, association of FB_1 and DON increased level of m5dC from 3.6 to 7.2% [Figure 2].

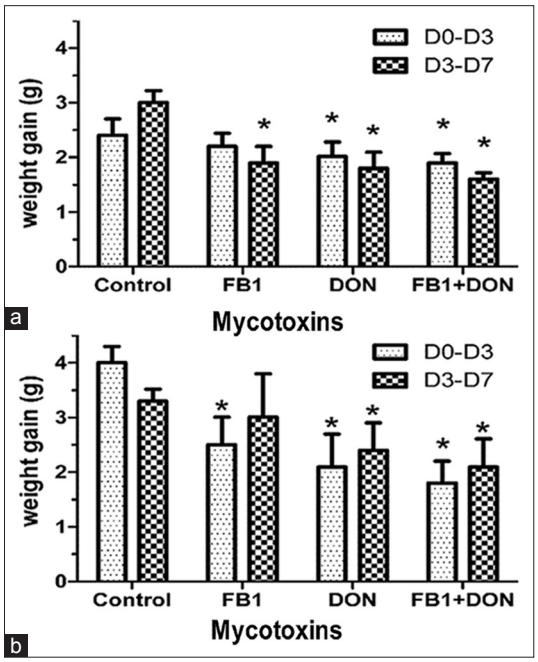


Figure 1: Evolution of body weight (g) after oral treatment mice by deoxynivalenol (DON; 45 µg/kg bw/day), fumonisin B_1 (FB_1; 110 µg/kg bw/day) and their mixture; $P < 0.05$ (*) with (a) for males mice and (b) females mice

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Table 1: Blood parameters for male mice after oral treatment by DON (45 µg/kg bw/day), FB_1 (110 µg/kg bw/day), and their mixture; *P*<0.05(*)

	Cholesterol (mg/dl)	Triglycerides (g/l)	ALT (IU/l)	AST (IU/l)	γ-GT (IU/l)	Creatine kinase (IU/l)	Creatinine (mg/dl)	Proteins (g/dl)
Control	188±5	1.07±0.05	15±4	50±4	3.05±0.3	27±4.2	0.38±0.01	5.07±0.023
FB_1	211*±6 (a)	1.36*±0.06 (a)	16±4.4	48±6	3.5±0.42	33±2	0.58* ±0.04 (a)	5.23*±0.03 (a)
DON	173±5	1.52*±0.07 (b)	17±3	51±2	3.67±0.5	45*±5(a)	0.53*±0.03 (a)	5.06±0.02
FB_1+DON	214*±4.4 (a)	1.62*±0.04 (c)	22±4.3	48±3	3.25±035	46*±3(a)	0.7*±0.08 (b)	5.30*±0.02 (a)

*Significantly different from the control group at the level of *P*<0.05. (a), (b), and (c) used to indicate the significant differences between values of animals treated. FB_1 = Fumonisin B_1, DON = Deoxynivalenol, ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, γ-GT = Gamma glutamyl transpeptidase

Table 2: Blood parameters for females mice after oral treatment by DON (45 µg/kg bw/day), FB_1 (110 µg/kg bw/day), and their mixture; *P*<0.05(*)

	Cholesterol (mg/dl)	Triglycerides (g/l)	ALT (IU/l)	AST (IU/l)	γ-GT (IU/l)	Creatine kinase (IU/l)	Creatinine (mg/dl)	Proteins (g/dl)
Control	172±4	0.89±0.05	23±3	42±5	2.48±0.2	32±5	0.32±0.02	5.08±0.02
FB_1	171±4	1.04* ±0.07 (a)	25±5	48±4	2.65±0.4	32±3	0.53* ±0.04 (a)	5.15±0.05
DON	190* ±5 (a)	0.98* ±0.09 (a)	23±2	47±3	2.5±0.43	41* ±4 (a)	0.41±0.025	5.17±0.03
FB_1+DON	180* ±3 (b)	1.33* ±0.05 (b)	30±5.5	62* ±4	2.48±0.4	51* ±2 (b)	0.8* ±0.06 (b)	5.27* ±0.02

*Significantly different from the control group at the level of *P*<0.05. (a), (b), and (c) used to indicate the significant differences between values of animals treated. FB_1 = Fumonisin B_1, DON = Deoxynivalenol, ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, γ-GT = Gamma glutamyl transpeptidase

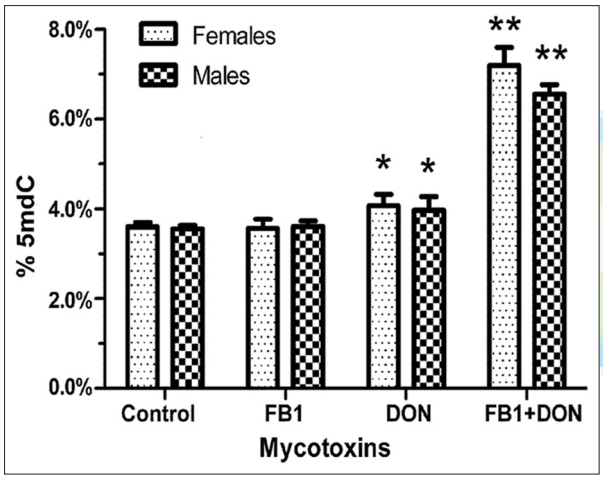


Figure 2: Deoxyribonucleic acid (DNA) methylation as measured by determination of the ratio m5dC on (dC + m5dC) × 100 in the DNA of kidney cells after oral administration of DON (45 µg/kg bw/day), FB_1 (110 µg/kg bw/day), and their mixture; $P < 0.05$ (*)

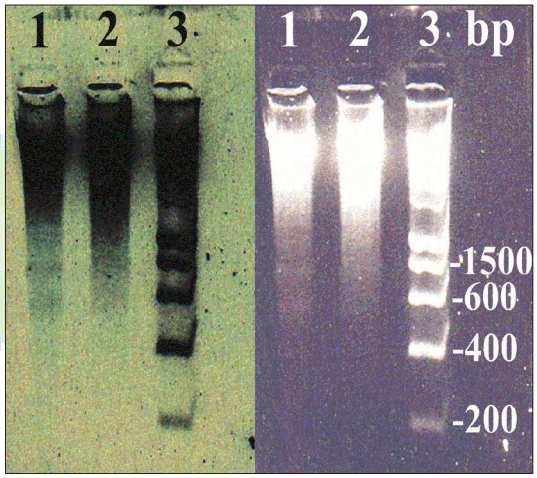


Figure 3: Agarose gel electrophoresis of DNA extracted from kidney cells after treatment of mice (only females) by DON at dose of 45 µg/kg bw/day. Lane 1 = Ladder, Lane 2 = control mice, Lane 3 = DON

Effect of DON on DNA fragmentation in blood lymphocytes of mice

We next evaluated fragmentation of the DNA of blood lymphocytes cells by agarose gel electrophoresis. There was an increase in DNA fragmentation as evidenced by the appearance of DNA ladders [Figure 3]. The sizes of DNA fragments appear to be above 250 bp in majority.

DISCUSSION

In our previous study performed on Caco-2 cells line, we have reported that the mixtures of Fusarium mycotoxins zearalenone, FB_1, and DON tested at low levels (10 µM for each mycotoxin); led to additive, antagonism, or synergistic effect.[5] These results should be confirmed by *in vivo* test. Thus, in the present study, we use low levels of FB_1 (110 µg/kg bw/day) and DON (45 µg/kg bw/day) defined following the concentrations of 10 µM previously used in our *in vitro* study.[5,14] Moreover, after an examination of toxicity data relevant for hazard characterization for DON, it had been proposed a tolerable daily Intake (TDI) of 1 µg/kg bw for DON[15] which might correspond to 100 µg/kg bw for experimental animals. More recently, in a study on induction of food refusal by DON, it has been reported that the no-observed adverse effect level (NOAEL) for DON is 0.5 and 1.0 mg/kg bw following intraperitoneal (ip) and oral exposure, respectively.[7] The doses of DON and FB_1 used in the previous both studies were higher than those used in the present study, so the toxic effects induced by DON and FB_1 or their mixture could be considered as precocious effects.

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Table 3: Unary creatinine and total proteins for females and males mice after oral treatment by DON (45 µg/kg bw/day), FB_1 (110 µg/kg bw/day), and their mixture; *P*<0.05 (*)

	Creatinine (mg/dl)		Proteins (g/dl)	
	Female	Males	Females	Males
Control	86±5	66±6	0.78±0.02	1.22±0.01
FB_1	39*±6 (a)	46*±5 (a)	0.82±0.01	0.40*±0.03
DON	86±3	62±5	0.81±0.015	1.23±0.02
FB_1+DON	30*±4 (b)	20*±5 (b)	0.81±0.01	1.12±0.01

(a) and (b) used to indicate the significant differences between values of animals treated. FB_1 = Fumonisin B_1, DON = Deoxynivalenol

Table 4: Renal clearance by serum and unary creatinine, (Ccr (ml/min) ×10^{-4}) for females and males mice after oral treatment by DON (45 µg/kg bw/day), FB_1 (110 µg/kg bw/day), and their mixture; *P*<0.05 (*)

	Control	FB_1	DON	FB_1+DON
Females	22.5±4.4	8.43±2*(a)	19±4.1	4.3±1.4*(b)
Males	21±2	10±3*(a)	20±2	5.3±2*(b)

(a) and (b) used to indicate the significant differences between values of animals treated. FB_1 = Fumonisin B_1, DON = Deoxynivalenol

An analysis of results showed that general toxic effects of DON and FB_1 appeared at very low doses of 45 µg/kg bw/day and 110 µg/kg bw/day for DON and FB_1, respectively. Regarding the body weight, female mice have been found more sensitive comparatively to males mice, but this decreasing weight seems to be explaining by the reduction in food intake. In blood biochemistry examinations, the significant elevation of TC and TGs induced by both DON and FB_1 and their mixture revealed a plausible lipid and lipoprotein (elevation of serum proteins) metabolism disorders for all animals treated. In addition, the elevation of AST and ALT for female mice could mean a slight liver cell lyses which were more marked for female mice. But, for all mice treated by both mycotoxins and their mixture, we also observed elevation of rhabdomyolysis biomarker CK[16] which combined to elevation of AST but not for ALT (more specific to liver damage) could signify massive destruction of muscular tissue.[16] These observations should be confirmed by histological examinations.

On the other hand, we observed an evident elevation of serum creatinine which was in correlation with decrease of unary creatinine. Similarly, serum TPs were increased; but we observed a decrease of unary TPs. The renal clearances of creatinine for all mice treated (females and males) by FB_1 and FB_1 + DON (but not DON alone) were twice or four times inferior in comparison to those of control mice [Table 4]. These observations revealed evident disorders in renal filtration caused by mycotoxin FB_1 and its association with DON. Our results were supported by previous studies focused on FB_1, FB_2, and FB_3 performed in rats and mice.[17,18] But macroscopic examinations did not reveal damage of the kidney of mice treated by FB_1 and FB_1 + DON. However, we think that a prolongation of duration of our experiments in order to show an evident damage of kidney induced by FB_1 and DON tested at very low doses should lead to marked histopathological effects. In the meantime and in order to preliminarily explore mechanism of such kidney toxicity and regarding *in vitro* high DNA methylation induced by mycotoxins DON and FB_1,[14] we projected to determine whether DON, FB_1, and their mixtures provoke DNA methylation in kidney cells. Surprisingly, our results revealed high DNA methylation by DON (not FB_1) and more intensively by DON + FB_1. Evidently, FB_1 enhanced toxic action of DON which confirmed its *in vitro* effect on DNA methylation in intestinal cells line Caco-2.[5,14] However, this DNA methylation in kidney cells was not linked to renal filtration because FB_1 did not impact on DNA methylation, but has been found an effective toxic for renal filtration in contrast to DON. Previously, it had been reported that DNA methylation elevation could be linked to radical oxygen species (ROS) produced by oxidative stress.[19] In fact, the lipid, proteins, or specially DNA oxidation induced by ROS could lead to elevation of DNA methylation.[5,19] In addition, the oxidation of cell membrane lipids led to loss of maintenance of cell homeostasis or cell death by necrosis. Thus, by a high oxidative stress induced in the kidney cells, DON and its association with FB_1 induced an elevation of serum CK which is a consequence of the destruction of the kidney tissue by necrosis.[8,20,21] Moreover, the methylation of DNA revealed the capability of DON to provoke early *in vivo* DNA modifications or damage. Thus, an examination of blood lymphocytes DNA has been performed specially for DON. The DNA fragmentation induced by DON confirmed not only its precocious toxic action in immune system,[19] but its tendency to impact negatively cell DNA *in vivo*.

CONCLUSION

The oral repetitive administration of low dose of DON and FB_1 during only 7 days has revealed disorders in lipid metabolism, renal filtration disturb and renal cell DNA methylation, rhabdomyolysis, and` blood lymphocytes cell deaths. These effects could be considering as precocious effect of both mycotoxins DON and FB_1 in mice. Female mice were more sensitive and the mixture of DON and FB_1 led to additive or more than additive effect as previous reported.[18] Finally, our results demonstrated that the NOAEL of both DON and FB_1 were low than 45 µg/kg bw/day and 110 µg/kg bw/day, respectively and these doses were lower than those previously reported.[7,22]

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