Effects of aflatoxin B₁ on the cell cycle distribution of splenocytes in chickens

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Abstract: The purpose of the present study was to evaluate effects of aflatoxin B₁ (AFB₁) on the cell cycle and proliferation of splenic cells in chickens. A total of 144 one-day-old Cobb male chickens were randomly divided into 2 equal groups of 72 each and were fed on diets as follows: a control diet and a 0.6 mg/kg AFB₁ diet for 21 days. The AFB₁ diet reduced body weight, absolute weight and relative weight of the spleen in broilers. Histopathological lesions in AFB₁ groups were characterized as slight congestion in red pulp and lymphocytic depletion in white pulp. Compared with the control group, the expression levels of ataxia–telangiectasia mutated (ATM), cyclin E₁, cyclin-dependent kinases 6 (CDK6), CDK2, p53, p21 and cyclin B₃ mRNA were significantly increased, while the mRNA expression levels of cyclin D₁, cdc2 (CDK1), p16, p15 were significantly decreased in the AFB₁ groups. Significantly decreased proliferating cell nuclear antigen (PCNA) expression and arrested G₀G₁ phases of the cell cycle were also seen in the AFB₁ groups.

In conclusion, dietary AFB₁ could induce cell cycle blockage at G₀G₁ phase and impair the immune function of the spleen. Cyclin D₁/CDK6 complex, which inhibits the activin/nodal signaling pathway, might play a significant role in the cell cycle arrest induced by AFB₁. (DOI: 10.1293/tox.2018-0015; J Toxicol Pathol 2019; 32: 27–36)

Key words: AFB₁, G₀G₁ phase, cell cycle arrest, mechanism, spleen, chicken

Introduction

Aflatoxin B₁ (AFB₁) is a type of mycotoxin that is classified as a Group 1 carcinogen to humans by the International Agency for Research on Cancer and is a mycotoxins produced typically during storage of crops¹. It is commonly encountered and has potent carcinogenic, genotoxic, immunotoxic and other adverse effects in many animal species including poultry²–³. An active intermediate products of AFB₁, AFB₁-exo-8, 9-epoxide, can bind with DNA to form the predominant trans-8, 9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB₁ (AFB₁-N7-Gua) adduct, which causes DNA lesions⁴. Aflatoxin exposure is one of the multiple of human hepatocellular carcinoma (HCC) development⁵.

Many researchers have reported that aflatoxin could affect many biological characteristics of intestine⁶–⁸, cecal tonsil⁹, thymus¹⁰,¹¹, spleen¹²,¹³, bursa of Fabricius¹¹,¹⁴ and kidney¹⁵ morphology in poultry, such as by causing histological lesions, oxidative stress, apoptosis, changes in T-cell subsets, and changes in cellular and humoral immune function¹⁶,¹⁷.

AFB₁–8, 9-exo-epoxide can affect every period of the cell cycle with its potent biological activity¹⁸,¹⁹. The researches has demonstrated that AFB₁ caused chick jejunum cells to be arrested at G₂/M phase²⁰, renal cells to be arrested at G₀/G₁ phase²¹, increased percentages of chick thymocytes in the G₂/M phase, and dose dependent accumulation in S phase in vitro in human cell lines²². AFB₁ can impact a variety of cell cycle regulation gene, protein and related enzyme activities including p53 gene mutations and p16 gene methylation in tissue²³, affecting activities of CDK4, CDK6 and Rb¹⁹,²⁴,²⁵, and reducing the amount of
PCNA-positive cells\textsuperscript{8,26}. Bressac \textit{et al.}\textsuperscript{27} and Hollestein \textit{et al.}\textsuperscript{28} have demonstrated that dietary intake of aflatoxins was closely related to p53 gene mutations.

The spleen is the most important organ for antibacterial and antifungal immune reactivity\textsuperscript{29}. AFB\textsubscript{1} could decrease the number of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in the spleens of mice\textsuperscript{30} and induce biomolecular oxidative damage and decrease cell proliferation of spleen mononuclear cells in rats\textsuperscript{31–33}. Cell cycle arrest of splenocytes in chickens fed aflatoxin-contaminated corn has been reported\textsuperscript{34}, but the exact molecular mechanism of cell cycle arrest in the spleen of chickens induced by AFB\textsubscript{1} diet has not been elucidated. The results could help us to understand the molecule mechanism for the spleen immunosuppression attributable to AFB\textsubscript{1}.

**Materials and Methods**

**Animals and diets**

The experiment was performed with male Cobb chickens weighing 45 ± 5 g that were purchased from a commercial rearing farm (Wenjiang poultry farm, Chengdu, Sichuan Province, China).

A total of 144 one-day-old chickens were randomly divided into 2 groups, namely control group (0 mg/kg AFB\textsubscript{1}) and AFB\textsubscript{1} group (0.6 mg/kg AFB\textsubscript{1}). All of the chickens were put into cages with three replicates per group and 24 birds per replicate. AFB\textsubscript{1} was purchased from A6636, Sigma-Aldrich, St.Louis, MO, USA. Nutritional requirements were adequate according to National Research Council (National Research Council, 1994)\textsuperscript{35}. The AFB\textsubscript{1}-contamin diet was produced by method similar to described by Kaoud\textsuperscript{36}. In short, 27 mg AFB\textsubscript{1} fumonisin solid was dissolved into 30 mL methanol completely, and then the 30 mL mixture was mixed into 45 kg corn-soybean basal diet to formulate AFB\textsubscript{1} diet containing 0.6 mg/kg AFB\textsubscript{1}. Equivalent methanol was mixed into corn-soybean basal diet to produce a control diet. Then, methanol in diets was evaporated at 98°F (37°C). The concentrations of AFB\textsubscript{1} were analyzed by HPLC (Waters, Milford, MA, USA) with fluorescence detection 2475 Fluorescence Detector (Waters, Milford, MA, USA) and were determined to be <0.001 mg/kg in the control diet and 0.601 mg/kg in the AFB\textsubscript{1} diet. Chickens were housed in cages with electrically heated units and provided with water as well as the diets ad litem for 21 days.

**Body weight and absolute and relative weight of spleen**

At 7, 14, and 21 days of age, six chickens in each group were weighed and euthanized. The spleen was dissected from each chick immediately and weighed with electronic balance after removing the surrounding fat and connective tissue. The following formula was used to calculate the relative weight:

Relative weight (g/kg) = organ weight (g) / fasting weight of chick (kg)

**Pathological observation**

After weighing, spleens were fixed in 4% paraformaldehyde for 24 h and routinely processed in dehydration, transparent disposal, paraffin embedded and sectioned at 5 μm. Slides were stained with hematoxylin and eosin Y (H.E). Paraffin sections were also collected to perform immunohistochemistry. The histological structures of the tissues were observed and photographed with a digital camera (DS-Ri1, Nikon Instech Co., Ltd., Tokyo, Japan).

**Cell cycle of the spleen by flow cytometry method**

At 7, 14, and 21 days of age, six chickens in each group were euthanized, and the spleen was dissected from each animal an immediately minced with surgical scissors. The cell suspension was filtered through a 300-mesh nylon mesh. Then, the cells were washed and suspended in phosphate buffer at a concentration of 1 × 10\textsuperscript{8} cells/mL (PBS, pH 7.2–7.4). 1 mL suspension was transferred to a 500 μL culture tube and centrifuged at 200× g for 5 min at 4°C. The supernatant was separated and discarded. Then, propidium iodide (5 μL, 51-66211E, BD Pharmingen, San Diego, CA, USA) was added into 100 μL cell suspension and incubated for 30 min at 4°C in a dark room. Finally, 500 μL PBS was added to each tube, and cells were analyzed by flow cytometry (FACSCalibur, BD, Franklin Lake, NJ, USA). The results were analyzed using the ModFit LT for Mac V3.0 computer program.

Proliferating index (PI) = (S + G\textsubscript{2}M) / (G\textsubscript{1}G\textsubscript{0} + S + G\textsubscript{2}M)

**PCNA detection by the immunohistochemical method**

The method of immunohistochemistry was applied according to the report by Fang \textit{et al.}\textsuperscript{37} Spleen paraffin sections were dewaxed in xylene, rehydrated through a graded series of ethanol solutions and washed three times in distilled water and PBS (0.1 M, pH 7.2–7.4). Endogenous peroxidase activity was blocked by incubation with 3% H\textsubscript{2}O\textsubscript{2} in methanol for 15 min. Following a wash with PBS, the sections were exposed to normal 10% goat sera for 30 min at 37°C to block nonspecific antibody binding. In a humidified chamber, 10 μg/mL primary antibodies rabbit anti-PCNA (bs-0754R, Wuhan Boster Bio-Engineering Limited Company, Wuhan, China) was applied sections for 20 h at 4°C. After three washings in PBS, the slices were exposed to 1% biotinylated goat anti-rabbit/mouse IgG secondary antibody (SA1030, Wuhan Boster Bio-engineering Limited Company, Wuhan, China) for 1 h at 37°C, and then incubated with streptavidin-biotin complex (SABC; Wuhan Boster Bio-engineering Limited Company, Wuhan, China) for 30 min at 37°C. Slides were visualized with 3,3′-diaminobenzidin. The slices were monitored microscopically and stopped by immersion in distilled water, as soon as brown staining was visible. Slices were lightly counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and cover slipped. For negative control purposes, representative sections were processed in the same way by replacing primary antibodies in PBS. The stained sections were photographed with a digi-
tal camera (DS-Ri1, Nikon Instech Co., Ltd., Tokyo, Japan). Within a range of 1,000 times lens, 6 fields of view were randomly selected for each slice, and photos were taken for determination of integral optical density (IOD) value. The results were analyzed using Image-Pro Plus 6.0 software.

Quantitative real-time PCR

In order to comprehend the molecular mechanism of cell cycle arrest induced by AFB$_1$, we continued our research to detect the mRNA expression of cell cyclins by quantitative real-time PCR (qRT-PCR). The spleens from six chickens in each group were removed at 7, 14, and 21 days of age and stored in liquid nitrogen. The spleens were crushed with pestle to homogenize them until they powdery, respectively. As previously described, total RNA was extracted from the powder of the spleens using RNAiso Plus (9108/9109, Takara, Kusatsu, Japan). RNA concentrations and purity were checked by NanoDrop™ One (Thermo Fisher Scientific, Waltham, MA, USA). Next, cDNA was synthesized using a PrimScript RT reagent Kit (RR047A, Takara, Kusatsu, Japan) according to the manufacturer’s protocol. The cDNA product was used as a template for qRT-PCR analysis. Sequences for target genes were obtained from the NCBI database. Oligonucleotide primers were designed using Primer 5 software and synthesized at Takara (Dalian, China; Table 1). All qRT-PCR reactions were performed using the SYBR® Premix Ex Taq™ II system (DRR820A, Takara, Kusatsu, Japan) and a C1000 Touch (Bio-Rad Laboratories, Hercules, CA, USA). Chicken β-actin was used as an internal reference housekeeping gene. All data output from the qRT-PCR experiments were analyzed using the 2$^{-\Delta\Delta CT}$ method.

Statistical analysis

The significance of differences between two groups was analyzed by variance analysis, and results were expressed as mean ± standard deviation (X ± SD). The analysis was performed using the independent sample test in the IBM SPSS Statistics for Windows, Version 20.0 software (IBM Corp, Armonk, NY, USA). Difference were considered statistically significant at p<0.05 and markedly significant was considered at p<0.01.

Results

Body weight and absolute weight and relative weight of spleen

The effects of dietary AFB$_1$ on body weight and absolute and relative weight of the spleen of chickens are shown in Fig. 1. No significant differences were observed in body weight or absolute weight and relative weight in the AFB$_1$ group at 7 days of age. Compared with the control group, the AFB$_1$ diet reduced body weight at 14 and 21 days of age (p<0.05). Meanwhile, the absolute and relative weight of the spleen in the AFB$_1$ group were significantly lower than those in the control group at 14 and 21 days of age (p<0.05 or p<0.01).

### Table 1. Sequence of Primers Used in qRT-PCR

| Gene symbol | Accession number | Primer | Primer sequence (5′-3′) | Product size (bp) | Tm (°C) |
|-------------|------------------|--------|-------------------------|------------------|---------|
| ATM         | NM001162400.1    | Forward| TGCCACACTTTCCCATGT      | 110              | 60      |
|             |                  | Reverse| CCACTGCAATTTCCCTCCCAT  |                  |         |
| P53         | NM205264.1       | Forward| ACCGTACTTACTCCCCCGGT    | 127              | 59      |
|             |                  | Reverse| TCTTAGACGCCACGCCGCG     |                  |         |
| P21         | AF513031.1       | Forward| TCCCTGCCCTGTACTCTCAA    | 123              | 60      |
|             |                  | Reverse| GCGTGGGCTTCTCTTATAAT    |                  |         |
| PCNA        | AB053163.1       | Forward| GATGGCTCTTCGTTGTTAGGAG  | 104              | 60      |
|             |                  | Reverse| CAGTGCAATGAAACCTTCC    |                  |         |
| P16         | NM_204434.1      | Forward| GCGATGGAAAGTACAGCAG     | 120              | 59      |
|             |                  | Reverse| CAAATACATGCCGGTGTAAAG  |                  |         |
| Cyclin D$_1$| U40844.1         | Forward| CGGTGCCGTTGGAATATAGCTG  | 108              | 60      |
|             |                  | Reverse| GATCTGTTTGTTGCTCTGCTC  |                  |         |
| CDK6        | NM_001007892.2   | Forward| ACCGCCAGAGAGGTACATG     | 132              | 60      |
|             |                  | Reverse| GAAGAAATACGGCACAAAACCT  |                  |         |
| Cyclin E    | NM_001031358.1   | Forward| GACACCAAAAGCAGTAAGA    | 100               | 60      |
|             |                  | Reverse| AACCCAGAAGAGGCACTT      |                  |         |
| CDK2        | EF182713.1       | Forward| CTGGCTCTTGTGGCTCTGCAC   | 179              | 60      |
|             |                  | Reverse| CGGTGGATAGGAGGTTCCTC   |                  |         |
| P15         | NM204443.1       | Forward| GTTGGCCGTTGACATACAGC    | 113               | 60      |
|             |                  | Reverse| GTGCGCCGCTGACATCCTT    |                  |         |
| Cyclin B$_1$| NM205239.2       | Forward| ATCCAAACAGCTCAACAGAAC   | 171               | 59      |
|             |                  | Reverse| AGCCCTCCAGAACAATCTG     |                  |         |
| cdc2        | NM.205314.1      | Forward| TCTGGCTCTTATCTCCTCTG    | 144               | 60      |
| (CDK1)      |                  | Reverse| ATTGTTGGGTGCCCTCCTACA  |                  |         |
| β-actin     | L08165           | Forward| TGCTGTTCCTGCCACATCATG   | 178               | 62      |
|             |                  | Reverse| TTGTTGACAAATACCGTCT    |                  |         |
Pathological observation on spleen

In the AFB$_1$ group, lymphocyte density was mainly reduced in the white pulp, and loose arrangement of histocytes was observed when compared with those in the control group (Fig. 2). The main lesions of spleen were statistically analysed (Table 2). The results showed that AFB$_1$ treatment led to spleen congestion in the red pulp in 1/6, 2/6 and 3/6 chickens at 7, 14, and 21 days of age, respectively, and loosely lined histocytes within the white pulp caused by a reduction in lymphocyte density in 6/6 chickens at 7–21 days of age.

Cell cycle phase of splenocytes

The percentages of cells in the G$_2$M phase in the AFB$_1$ group were significantly lower than those in the control group at 14 and 21 days of age (p<0.05). The percentages of cells in S phase in the AFB$_1$ group were significantly lower than those in the control group at 7 and 21 days of age (p<0.05 or p<0.01). Furthermore, changes of the G$_0$G$_1$ phase were obvious. The percentage of cells in the G$_0$G$_1$ phase in the AFB$_1$ group was markedly increased at 21 days of age, when compared with the control group (p<0.01). The proliferating index (PI) value was significantly decreased in the AFB$_1$ group at 14 and 21 days of age (p<0.05) when compared with the control group (Fig. 3). Histograms obtained by cytometer analysis show that the cell peaks of S and G$_2$M phases are obviously lower and that the cell peaks of G$_0$G$_1$ phase are higher in the AFB$_1$ group than those in the control group, especially at 14 and 21 days of age.

Table 2. Incidence of Major Lesions in Spleen (n=6)

| Pathological lesions | Time  | Control group | AFB$_1$ group |
|----------------------|-------|---------------|---------------|
| Congestion in red pulp | 7 days | 0/6 | 1/6 |
| 14 days | 0/6 | 2/6 |
| 21 days | 1/6 | 3/6 |
| Blank within the white pulp | 7 days | 1/6 | 2/6 |
| 14 days | 1/6 | 6/6 |
| 21 days | 0/6 | 6/6 |
Fig. 2. Photomicrographs of hematoxylin and eosin stained chicken spleen. Notes: (A) the control group at 7 days of age; (B) the control group at 14 days of age; (C) the control group at 21 days of age; (D) the AFB1 group at 7 days of age; (E) the AFB1 group at 14 days of age; (F) the AFB1 group at 21 days of age; (Triangles) congestion in the red pulp; (Stars) lymphocytic depletion in white pulp. Bars = 50 μm.

Fig. 3. Cell cycle phase distribution of splenic cell. a: Effect of AFB1 on cell cycle phase distribution of spleen in chicken (%). b: Histogram of splenocyte cell cycle by flow cytometry. Notes: Data are presented with the means ± standard deviation (n = 6). *p<0.05 compared with control group, **p<0.01 compared with control group.
Dietary AFB₁; Chickens Spleen; Cell Cycle Blockage

PCNA expression by immunohistochemical method

Brownish-yellow staining indicated the expression of PCNA expression. Control sections showed a negative reaction, as shown in Fig. 4. The PCNA protein expression in the spleen of the AFB₁ group was obviously lower than that in the control group. PCNA expression was slightly increased at 21 days compared to 14 days treatment. When compared with the control group, the Integrated optical density (IOD sum) of PCNA protein expression in the AFB₁ group was significantly decreased at 7, 14, and 21 days of age (p<0.05 or p<0.01).

The mRNA expression of cell cyclins by qRT-PCR

At 7 days of AFB₁ treatment, the mRNA expression levels of ATM, CDK2, cdc2, p53, p21, cyclin E₁, and cyclin B₁ were obviously increased (p<0.05 or p<0.01) compared with the control group, and the mRNA expression levels of cdk6 were increased. Furthermore, the mRNA expression levels of cyclin D₁ in the AFB₁ group were obviously decreased (p<0.05), whereas the mRNA expression levels of P15, P16, and PCNA showed no obvious changes (P>0.05).

At 14 and 21 days of AFB₁ treatment, the mRNA expression levels of ATM, CDK2, cdc2, CDK6, p53, p21, cyclin E₁, and cyclin B₁ were obviously increased (p<0.05 or p<0.01). Furthermore, the mRNA expression levels of cyclin D₁, P15, P16, and PCNA in the AFB₁ group were obviously decreased (p<0.05 or p<0.01; Fig. 5).

Discussion

Compared with the control group, the AFB₁ diet reduced chicken weight at 14 and 21 days, indicating that AFB₁ diet inhibited chicken growth performance. In the present study, the relative weight of the spleen was used to judge the development status and degree of pathologic change in spleen. At 14 and 21 days of age, the relative weight of the spleen in AFB₁ group was significantly lower than that in the control group, which was consistent with the results of Chen et al.¹² and Quist et al.⁴⁰ However, it

Fig. 4. Immunohistochemistry of the spleen. a: Expression of PCNA protein by immunohistochemistry in the spleen. b: Integrated optical density (IOD sum) of PCNA protein expression in the spleen. Notes: A, B, and C showing the control group at 7, 14, and 21 days respectively; E, F, and H showing the AFB₁ group at 7, 14, and 21 days respectively; D and H showing negative control in the control group and the AFB₁ group respectively. Bars = 10 μm. Data are presented with the mean ± standard deviation (n = 10). * means p<0.05 compared with the control group, ** means p<0.01 compared with the control group.
was not consistent the results of Peng et al.34 Who showed obvious congestion red pulp of the spleen in chicks exposed to corn with containing of AFB1 and AFB2. In this study, the results of histopathological observation suggested that the decreased weight and relative weight of the spleen might be due to lymphocytes depletion, as a decreased number of lymphocytes and loosely lined histocytes in the lymphatic nodules and periartral lymphatic sheath were observed. The results of histopathological observation were also similar to the results of other researchers12, 34. Numerous studies and reports have documented that aflatoxin could inhibit the development of thymus10, 11, 14, 41. The loosely lined histocytes within the white pulp were suspected of resulting from the decreased number of lymphocytes.

In the present study the S phase and G2M phase were obviously decreased, and the G0G1 phase was markedly increased at 21 days of age. These results indicated that 0.6 mg/kg AFB1 could induce G0G1 phase arrest in chickens splenocytes, which was in line with previous research34. However, feeding with AFB-contaminated could lead to G2M and G0G1 phase blockage in the spleens of chickens44. Zhang et al.8 demonstrated that AFB1 caused jejunal cells to be arrested at G2/M phase. Scott et al.3 reported that AFB1 treatment increased the percentages of chick thymocytes in the G2/M. Ricordy et al.22 showed that human cell lines exposed AFB1 toxin for 24 h caused dose-dependent accumulation in S phase in vitro. Differences in animal species, viscera, cell type and mycotoxin type may cause different characteristics of cell cycle arrest.

Several reports have already suggested cellular and humoral immune function changes induced by AFB1 exposure6, 7, 9–14, 41 and AFB1-induced cell arrest, which can be impacted by a variety of cell cycle regulation genes. However, the exact molecular mechanism of AFB1-induced cell cycle arrest in the spleen of chickens has not been elucidated so far. The experimental results from this study could enrich the knowledge concerning the molecular mechanism of AFB1-induce immunosuppression. Further studies are needed on protein levels.

The active intermediate products of AFB1 causes DNA lesions and DNA damage (including double-strand breaks), which can activate ATM42. ATM initiates cell cycle arrest, DNA repair, or apoptosis by phosphorylating downstream targets. When ATM phosphorylates p5342, which is a tumour suppressor gene integrating numerous signals to control cell life and death, the p53 protein can directly stimulate
the expression of p21WAF1/CIP1, which is an inhibitor of cyclin-dependent kinases (CDKs) and p21WAF1/CIP1, and inhibit the G1 to S and G2 to M phases. Besides, p53 can indirectly sequester cyclin B1/CDK1 complexes and help to maintain a G2 block. The ATM(ATR)/CHK2(CHK1)–p53/MDM2–p21 pathway is the dominant checkpoint response to DNA damage in mammalian cells traversing through the G1 phase. In this study, our results showed that AFB1 caused an increase in ATM, p53, and p21 mRNA expression. Therefore, we speculated that dietary AFB1 could induce G0/G1 arrest by activating ATM(ATR)/CHK2(CHK1)–p53/MDM2–p21 pathway. Meanwhile, p21WAF1 complexes with CDKs can inhibit their kinase activity. p15 inhibits cyclin D/CDK6 without causing the dissociation of this complex. The P16 family inhibits cell growth by inhibiting the activities of CDK6 and CDK4 kinases. P21WAF1 bound to PCNA can directly inhibit DNA synthesis. Progression through the G1 is mediated by cyclin D/CDK6, and CDK2/cyclin E are thought to exclusively promote the G1/S transition, and the cyclin B/CDK1 kinase is a G2 checkpoint. Our results showed that AFB1 caused an increase in CDK2, CDK6, cyclin E, and cyclin B3 mRNA expression and a decrease in cyclin D1, p15, p16, PCNA, and CDK1 mRNA expression as well as PCNA proteins, indicating that AFB1 induced G0/G1 phase arrest via ATM-p53-p21-cyclin D/CDK6 route.

Conclusions
In summary, 0.6 mg/kg AFB1 in the diet inhibited development of the chicken’s spleen by causing G0/G1 cell cycle arrest. The mRNA expression levels of ATM, p53, p21, and CDK6 were all increased, while the mRNA expression of cyclin D1 was decreased, suggesting that cyclin D1 mRNA expression may play an important role during G0/G1 cell cycle arrest of splenocytes induced by AFB1. The results and above mention discussion suggest that G0/G1 phase arrest of splenocytes could be induced via activation ATM-p53-p21-cyclin D/CDK6 route, and the proposed mechanisms are shown in Fig. 6.

Disclosure of Potential Conflicts of Interest: The authors declare that they have no conflict of interest.

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