ORIGINAL ARTICLE

# Effects of long- and short- term of fumonisin B1 orally administration on esophagus tissue in animal model

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## ABSTRACT

***Background:*** Numerous epidemiologic studies have shown that esophagus cancer is more common in areas with grains containing mycotoxin such as fumonisin B1 (FB1). The aim of this research is to study the effects of long- and short-term of esophagus tissue to FB1 orally administered in animal model.

***Materials and Methods:*** Forty-four female mice have been divided in two short- and long-term groups that further subdivided into control and therapeutic subgroups. FB1 (25 mg/50 g Body Weight) has been gavaged for 4 weeks in the short-term therapeutic subgroups and FB1 (10 mg/L) has been used in drinking water for 12 months in the long-term group. At the end of study, liver and esophagus tissues have been studied for histopathological changes. Also genes expression of c-myc، TGF-α، HGF AFP and P53 were detected by using RT-PCR method.

***Results:*** In short- and long-term groups (6 and 9 months) no macroscopic and microscopic sign were observed in the various organs. But in the microscopic examination of the liver (9 and 12 months) mild dysplasia of hepatocytes with aniso nucleus, increased Kupffer cells and nucleolus deposit of hyalonoid amorphous in liver media layer were observed. In immunohistochemical study, nuclear dysplastic of hepatocytes were detected with increased staining and hyalonoid amorphous deposit.

***Conclusion:*** Our results indicate that even though no pathologic changes in the esophagus tissue have been found in the short- and long-term exposure to FB1, however, metabolic effects of FB1 in animal's parenchyma organs especially liver, kidney and lung warrants further study.

***Keywords: Esophagus, liver, Fumonisin B1, rat***

**زمینه و هدف:** مطالعات متعدد اپیدمیولوژیک نشان داده اند که سرطان مری در مناطقی که حاوی میکوتوکسینهایی همانند فومونیسین B1 هستند شایعتر است. هدف این مطالعه بررسی اثر کوتاه و بلند مدت تجویز خوراکی فومونیسین B1 بر روی بافت مری در مدل حیوانی است.

**مواد و روشها:** ٤٤ موش ماده در دو گروه کوتاه و بلند مدت قرار گرفتند که هر کدام از آنها نیز به دو زیرگروه، درمانی و شاهد، تقسیم شدند. فومونیسین B1 به میزان ۲۵ میلیگرم به ازای هر ۵۰ گرم وزن به مدت ٤ هفته در زیرگروه درمانی گروه کوتاه مدت و به میزان ۱۰ میلیگرم در هر لیتر آب نوشیدنی به مدت ۱۲ ماه در گروه بلندمدت تجویز شدند. در پایان مطالعه بافتهای مری و کبد تحت بررسی مطالعات آسیب شناسی قرار گرفتند. همچنین بیان ژنهای c-myc، TGF-α، P۵۳ و HGF AFP با استفاده از روش RT-PCR ارزیابی گردیدند.

**یافته ها:** در هیچکدام از گروههای کوتاه و بلند مدت نشانه های تغییرات ماکروسکوپی و میکروسکوپی در بافتهای مورد بررسی مشاهده نگردید. اما در بررسی میکروسکوپی کبد دیسپلازی خفیف سلولهای کبدی همراه با هسته های ناهمسان ، افزایش تعداد سلولهای کوپفر و رسوب هسته ای بی شکل هیالونوئید در لایه میانی کبد مشاهده گردید. در مطالعه ایمنوهیستوشیمیایی سلولهای کبدی تغییرات دیسپلازی هسته با افزایش رنگ پذیری و رسوب هسته ای بی شکل هیالونوئید یافت گردید.

**نتیجه گیری:** یافته های این مطالعه نشان داد اگرچه با وجود عدم مشاهده تغییرات آسیب شناختی در بافت مری بدنبال تماس کوتاه و بلند مدت فومونیسین B1، ایجاد اثرات متابولیک آن در بافتهای پارانشیمی مانند کبد، کلیه و ریه مطالعات بیشتری را فرامیخواند.

**واژه های کلیدی:** فومونیسین B1، مری، کبد، rat



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## Introduction

Fumonisin, a member of mycotoxin family is a toxic and carcinogenic metabolite that is mainly produced by F. verticillioides fungi. (1) Some fumonisin species were extracted from this family but only B1, B2 and B3 subtypes were naturally found in contaminated foods. (2) Fumonisin B1 (FB1), the most toxic form is one of the secondary metabolites commonly contaminates corn and other agricultural productions such as rice and wheat. (3) The most important mechanism of FB1 is meddling with synthesizing of sphagnum lipids that leads to the collection of sphagnum and sphagnosin and as a result, insufficiency in function of cells membrane. Biochemical objectives of sphagnonin include inhibition of protein kinase C and phosphatidic phosphates acid, stimulation of Phospholipase D, and also its cellular effects include regulating growth, differentiation and apoptosis, change in cell penetration and morphology.(4, 5) In the other words, frequent collection of free sphagnoidi acts as cancer promoters and causes cell mutation. (6) FB1 biologic effects have been studied during recent years indicate both toxic and carcinogenic effects. Many studies have shown that FB1 has hepatotoxic, nephrotoxic and carcinogenic effects in renal, hepatic and esophagus. (7, 8, 9) Short term exposure to FB1 causes hepatotoxicity while prescription of FB1 for a long term leads to fibrous and chronic hepatitis that can ultimately result in liver cirrus and even sometimes in hepatic carcinoma. (10, 11)

Epidemiological studies in China and South Africa have also shown a correlation between mycotoxin contaminated corn foodstuffs and esophageal cancer. In addition, higher incidence of esophagus cancer has been found in people who consume a lot of corn compared to those who use less. (12,13) Such a pattern has been observed in some regions with high prevalence of esophagus cancer such as Iran, Italy, Kenya, USA and Brazil. (14,15,16) In this study, we aimed at investigating the effects of short- and long-term exposure to FB1 on esophagus tissue in animal model.

## Materials and Methods

Forty-four female mice (25±5 g) were divided into short-term (n=12) and long-term (n=32) groups and each group further subdivided into control and therapeutic subgroups. All animals were kept in a controlled temperature environment on a 12:12 h light/dark cycle with free access to food and water. The procedures were in accordance with the guidelines for the care and use of laboratory animal of Tehran University Medical Science.

**Animals treated with FB1**

FB1 (25 mg/50 g Body Weight) has been gavaged for 4 weeks in the short-term therapeutic subgroups and FB1 (10 mg/L) has been used in drinking water for 12 months in the long-term group.

**Study evaluation**

All animals were killed after 4 weeks in short-term period, and in long-term group each 3 months 4 of animals were killed in the end of study. Then, all organs of animal's body were obtained for the study of macroscopic and microscopic changes. Esophagus and liver tissues were assessed by hematoxylin and eosin (H & E) staining. Immunohistochemistry study with cyclin D1 marker carried out on samples that showed histo-pathological changes. Also, gene's expression of c-myc, transforming growth factor alpha (TGF-α), hepatocyte growth factor (HGF), alpha-fetoprotein (AFP) and p53 were studied in liver and esophagus samples by using Real Time PCR and ΔΔCT-2 (2 to the power of minus Delta Delta CT) methods. Tissue's RNA was at first purred with derivation kit of Qiagen. Then, cDNA was formed by using RT-PCR reaction and each gene with PCR reaction was tested by the special primers trying to make the equal efficiency in entire experiment by the Real Time PCR and cycle threshold (CT). Then, the expressions of genes were calculated on control and therapeutic samples by CT (cycle number) and ΔΔCT-2 formula (difference in expression of gene in therapeutic to control group). Briefly, about half of the tissue was fixed in 4% buffered formalin for histological examination. Paraffin sections were evaluated using hematoxylin and eosin. The rest of the tissue was either snap-frozen or stored at -80°C for RNA ex-

traction and Real Time PCR analysis. RNA was extracted using an RNA extraction kit (Qiagen, Hilden, Germany) from at least 10 mg of homogenized the tissue. After homogenization DEPC-75% ethanol was added to the lysate to provide ideal binding conditions. The lysate was then loaded onto the RNeasy silica membrane ("RNeasy Mini spin column"). After binding of RNA all contaminants, including genomic DNA, were efficiently washed out. Pure, concentrated RNA was eluted in water and stored at -70°C until further analyses. The amount of total RNA was determined by measuring absorbance at 260 nm. The purity of the total RNA was established by confirming that the 260 nm: 280 nm ratio was within a 1.8-2.0 range, indicating that the RNA preparations were free of protein contaminants.

Also, mRNA expression (c-myc, TGF-α, HGF, AFP and p53) were analyzed in specimens by Real Time PCR. Experiments were done 4-6 times per animal. RNA was extracted as described above. cDNA was prepared using 2 µg of heat-denatured RNA. Primer sets from Quiagen (Hilden, Germany) were used for analysis. Optimum primer concentration was determined by titration. Real Time quantitative PCR was performed in a two-step RT-PCR using SYBR-Green PCR Master Mix (PE Biosystems, Foster City, CA) with 100 ng cDNA and 300 nM of primers in a total reaction volume of 50 µl. PCR thermal cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Gene specific products were continuously measured by an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) and relative quantification was performed following the manufacturer's instructions.

**Statistical analysis**

Man-Whitney test was used for comparison of two group considering P-value<0.05 as statistical significance.

## Results

No pathological changes were observed in short- and long-term groups (3 and 6 months). However, little changes, including darkness and wrinkling, were observed in 9 and 12 month periods in liver and a little light paleness observed in kidney. Microscopic changes have been found in examination of kidney, lung, and liver tissues.

In kidney tissue, glomerular changes in the form of mesangial matrix stretch and global hyalinization with progression toward fibrous has been observed in some areas (**Fig. 1**).

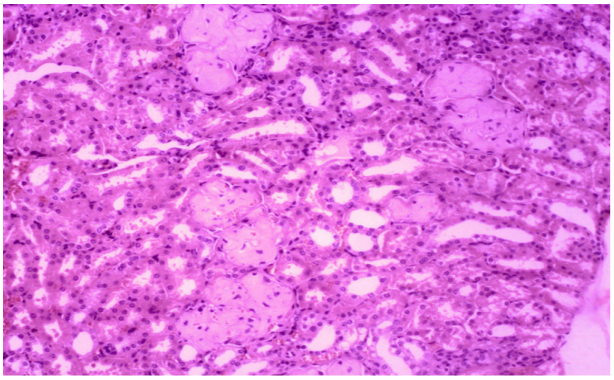

**Figure 1- Kidney tissue in treated sample with FB1**
**It shows the glummer issuance changes in the form of Mesangial matrix stretch and global hyalinization along with intact tubules (H & E staining, X100).**

In lung tissue, lobular abrade was observed through high scatter of edema cells, especially around bronchioles, and pus aggregation in lung bronchioles.

In liver tissue, we observed low hepatocytes dysplasia was observed with Anzio nucleus, increase in nucleus and cytoplasm anklozions, increased Kupffer cells and stretched sinusoidal spaces, generalized cells edema, and hyalinoid amorphous deposit in media layer (**Fig. 2**).

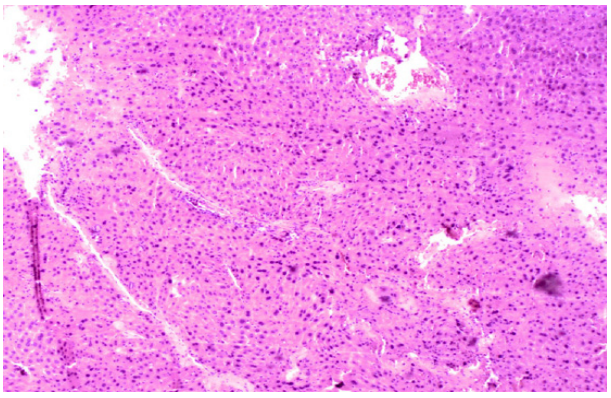

**Figure 2- Liver tissue in treated sample with FB1.**
**Lobular morphological change, minor hepatocytes dysplasia with aniso nucleosis, increased nuclear and intra nuclear cytoplasm inclusions, increased Kupffer cells and dilation of sinusoidal spaces are shown. Inflammatory cells were infiltrated mostly in lymphocyte form focally (H & E staining, X100).**

In immunohistochemistry study of liver tissue, by using cyclin D1 marker, cell's nucleus displays the color enhancement with variable intensity. In addition, hyaloids amorphous deposits were seen in liver cells which lead to sinusoidal dilatation (**Fig. 3-5**).

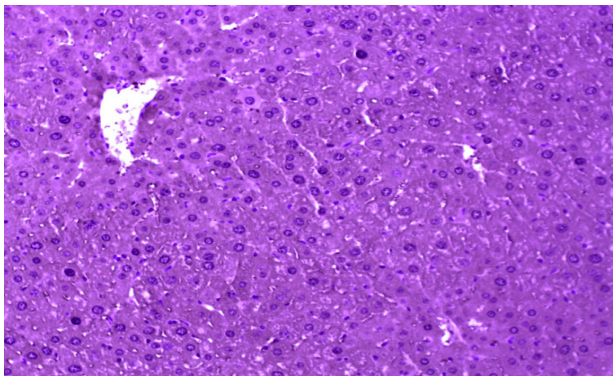

**Figure 3- Liver tissue in treatment sample, hepatocytes were stained with immunohistochemistry for cyclin D1 marker and enhancements of coloration with variable intensity in some nucleuses are shown (X100).**

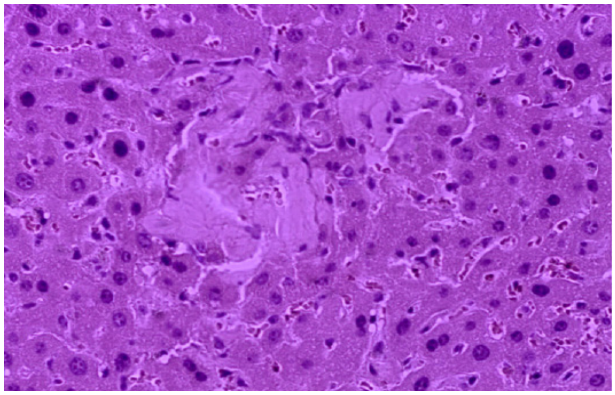

**Figure 4- Liver tissue in treated sample with cyclin D1 marker Hyaloids amorphous deposit is seen in hepatocytes resulted in sinusoidal dilation. These changes are also observed in veins (X 400).**

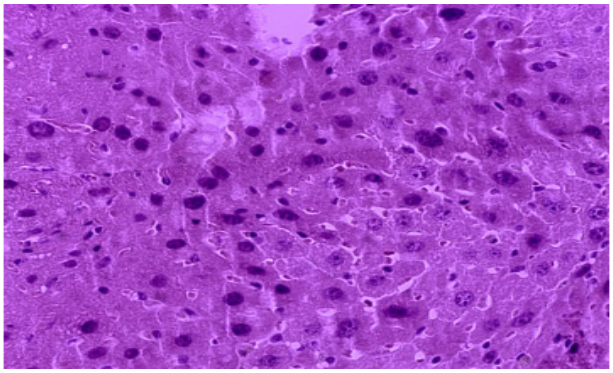

**Figure 5- Liver tissue in treated sample was immuno-histochemically stained with cyclin D1 marker (X 630) and dysplastic nucleuses with different coloration are shown.**

In genes expression of c-myc, TGF-α, HGF, AFP and P53 in samples of esophagus and liver tissues by Real Time PCR and ΔΔCT-2 methods, no difference in remarkable henes was found among therapeutic and control groups.

## Discussion

In present study, short- and long-term exposure to FB1 had no effect on histopathology of esophagus tissue but its long-term exposure lead to tissue changes in liver, kidney and lung.

In previous study, we reported that fumonisin contamination in corn and rice samples collected from Golestan farmlands in northern Iran during 2007 (Under review). F. verticillioides and Aspergilus niger were the most frequent fungal contamination in corn grains, while the most abundant ones in rice were Aspergilus niger, Aspergilus Flavus and F. verticillioides. Presence of Fusarium spp can be a potential risk for human and animal health due to their ability to produce fumonisins, a group of toxic and carcinogenic metabolites.(17, 6) Fumonisin is readily absorbed from gastrointestinal tract. It causes sphynganine and sphyngosine accumulation and cell membrane dysfunction. Sphyngoid frees bases in return, acts as cancer promoter and mutations.(18) Food-borne contamination by mycotoxins can produce cancer, liver and renal cell necrosis, neural disturbances, infertility, fetal teratogenicity and irreversible side effects in human and animal societies.(19) According to our previous study, the highest fumonisin level of corn samples in HPLC method (6.37 µg/g, $p<0.05$) was seen in the east and south Gorgan regions. According to FAO/WHO common committee, the highest tolerable daily intake of fumonisin B1, B2 and B3 individually or in combination is 2 µg/kg body weights.(20) Bolger et al. showed that average fumonisin intake is variable from 0.2 up to 2.4 µg/g body weights in European and African countries respectively. (21) Daily intake of fumonisin from various foodstuffs in different countries has been reported between 0.00024 up to 440 µg/g body weight. FB1 contaminated animal wheat foodstuffs in Mazandaran province during 1998 and 1999 has been shown to be 2.27 and 3.18 mg/kg body weight, respectively. While, in Isfahan province human

wheat contamination during same period has been 0.22 and 0.17 mg/kg, respectively.(22) Marasas et al. showed a relation between corn contamination by F. verticillioides and esophageal cancer around the world.(19) Esophageal cancer has been shown to have a dispersed incidence in Iran with the lowest in Isfahan and the highest in Northern provinces. Therefore, we can reach to this conclusion that exposure to fumonisins is a probable risk factor for esophageal cancer in this region.

Results of present study have shown that no macroscopic changes in organs were observed in any of the animals. No pathologic changes were observed in mice's esophagus tissue in two short- and long-term period. However, microscopic changes in found in lung, kidney and liver tissues. The previous studies showed that the nutrition of rat with FB1 (484 ppm) and female rat (more than 99 ppm) for about 4 weeks induced liver cell hyperplasia and apoptosis which is in agreement with the results of our study in long-term group.[23]

It should be noted that in present study, there might be different reasons to prevent fumonisin affecting on esophagus tissue. Some of these reasons are as follows: First, dose of FB1, as it hasn't effect in low doses; second, because of non existence of glands in esophagus tissue; the effects of chemical materials such as FB1 will be lower in comparison to other tissues such as liver and kidney; and third, the occurrence of pathologic changes in some tissues is needed more time. It should be noted that, up to now, no experimental study had showed the direct effects of fumonisin on esophagus tissue, and it seems that experimental role of FB1carcinogenicity, unlike epidemiologic studies, in different species needs more investigation.

## Conclusion

The present study shows that no pathologic changes due to exposure to FB1 were observed in animals in short- and long-term periods in the animals' esophagus tissue. However, metabolic effects of FB1 in animals' parenchymal organs especially in liver, kidney and lung have been found.

## Acknowledgements

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