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Evaluation of *STAT5A* Gene Expression in Aflatoxin B1 Treated Bovine Mammary Epithelial Cells

Ali Forouharmehr[1]*, Taher Harkinezhad[2]*, Babak Qasemi-Panahi[3]

[1] *Department of Animal Science, Faculty of Agriculture, University of Zanjan, Zanjan, Iran.*

[2] *Reaserch Institute of physiology and Biotechnology (RIPB), University of Zanjan, Zanjan, Iran.*

[3] *Department of Animal Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran.*

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ABSTRACT

Purpose: Aflatoxin B1 (AFB1) is a potent mycotoxin which has been produced by fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* as secondary metabolites due to their growth on food stuffs and induces hepatocellular carcinoma in many animal species, including humans. In the present study, the effect of AFB1 on *STAT5A* gene expression was investigated in bovine mammary epithelial cells using real time RT-PCR. ***Methods:*** Bovine mammary epithelial cells were seeded in a 24-well culture plate for three-dimensional (3D) culture in Matrigel matrix. After 21 days of 3D culture and reaching the required number of cells, cells were treated with AFB1 and incubated for 8 h. For real time PCR reaction, total RNA from the cultured and treated cells was extracted and used for complementary DNA synthesis. ***Results:*** The expression of *STAT5A* gene was significantly down regulated by AFB1 in dose- dependent manner and led to the reduction of proliferation and differentiation of epithelial cells, which has direct effect in milk protein quantity and quality. ***Conclusion:*** According to the results, it seems that down regulation of *STAT5A* gene in AFB1-treated cells maybe due to DNA damage induced by AFB1 in bovine mammary epithelial cells.

Introduction

Aflatoxins are toxic compounds which have been produced by fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* as secondary metabolites owing to their growth on food stuffs. These compounds can be carcinogens, mutagens, immune systems attenuator in animals and humans.[1-5] Among various types of aflatoxins, the most toxic and carcinogenic effect has been reported for aflatoxin B1 (AFB1, Figure 1).[3,6] The presence of AFB1 and other toxic compounds have side effects on bovine mammary epithelial cells and can affect milk quantity and quality.[1] After maturity, the mammary gland tissue reaches to its maximum growth and its proliferation and differentiation cycle is repeated in each pregnancy. The main functional glandular unit in mammary tissue is alveolar and the most influential gene in the development of mammary lobular-alveolar system is *STAT5A*.[7]

In mammals, *STAT* family genes consists of *STAT*1, 2, 3, 4, 5A, 5B and they can be activated in response to a wide range of stimuli such as growth hormone, prolactin, cytokines and oncoprotein that result in growth, anti-apoptotic effects, cell division and differentiation.[8] AFB1 can influence the growth of mammary epithelial cells by altering *STAT5A* gene expression and ultimately milk production can be affected as well.[8]

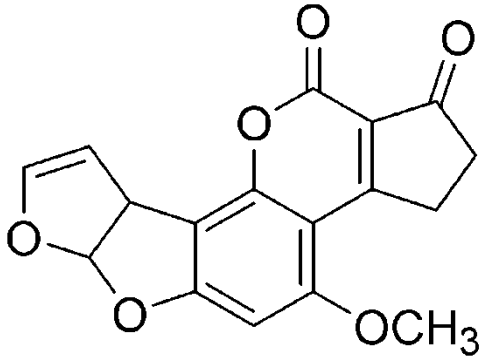

Figure 1. Chemical structure of Aflatoxin B1.

Since, toxicology tests on animals have some obstacles such as ethical issues, accuracy and cost; experiments on cell cultures are regarded as alternatives for *in vivo* experiments. Three-dimensional (3D) culture offers conditions the same as tissue culture conditions biochemically and biomechanically but monolayer cell culture could not provide tissue culture conditions *in*

***Corresponding author:** Ali Forouharmehr and Taher Harkinezhad. Tel: +98 (241) 5154246, Emailes: Forouharmehr@yahoo.com and Taher.harkinezhad@znu.ac.ir

vitro. Therefore, toxicology effects based on 3D culture eliminates restrictions on work both with the animals and a monolayer cell culture and provides more information about the effects of various toxins on the tissues in the long term.[1,9,10] The present study was designed to determine the effect of AFB1 on *STAT5A* gene expression in the mammary epithelial cell at 3D cell culture conditions.

Materials and Methods

Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffer solution (PBS), 0.25% trypsin-EDTA solution, AFB1, Penicillin, streptomycin and amphotericin B were obtained from Sigma–Aldrich Co, USA. RPMI-1640 Medium (RPMI) and fetal bovine serum were purchased from Invitrogen, USA. DNA ladders, RNeasy Mini Kit, random hexamer primer, dNTP Mix, RevertAid™ Reverse Transcriptase and real-time mix master were purchased from Fermentas Co.

Cell culture

A sample was prepared from normal bovine mammary epithelial tissue in sterile condition. After washing with PBS, samples were put in falcon containing 9 mL DMEM and 1mL FBS medium and transferred to the lab in ice. Monolayer cell culture was performed at 25cm propylene flasks at laboratory. To avoid potential contaminations, cell culture was carried out in medium containing 1% of penicillin, streptomycin and amphotericin B. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.[9] The medium was changed every 4 days and the cells confluency observed within 2 weeks.[6] After full cell confluence, 3D cell culture was performed. To carry out 3D cell culture, 2 mL of Matrigel with 1 mL of DMEM were mixed and after thirty minutes of keeping the mixture in the fridge, 200 µL of Matrigel and DMEM mixture was poured to 24 well plates. Then bovine mammary epithelial cells cultured at a seeding density of 30×10^3 cells per cm^2 into 24 well plates as described by previous studies.[1,11-13].

Quantitative real-time PCR

For real time PCR reaction, total RNA from the cultured and treated cells was extracted with the RNeasy Micro Kit (Qiagen, Hilden, Germany) exactly according to the manufacturer's instructions. After that, the integrity of the extracted RNA was evaluated by agarose gel electrophoresis and purity of RNA was examined by optical density measurement (A260/A280 ratio) with NanoDrop®1000 Spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). For complementary DNA synthesis the same amount of each extracted RNA sample was used and reverse polymerase chain reaction was followed by random hexamer primers and revert Aid TM MMulv Reverse transcriptase (fermentase co. Lituania).[4,14]

Results

Morphology of bovine mammary epithelial cells

To establish a culture system approximately the same as a mammary cell microenvironment *in vivo*, we utilized 3D cell culture instead of the regular 2D monolayer culture. Then we let bovine mammary epithelial cells grow in culture wells precoated with Matrigel. Primary cell masses had grown after four days of the 3D cell culture, but it took longer to form cellular mass in proper shape. However, in 21 days full deployment of cell growth has been observed in 3D culture at Matrigel and the multi-cell spherical colonies formed in the 3D culture appeared to be embedded at different layers in the medium containing Matrigel, whereas standard 2D-cultured cells formed the typical monolayer (Figure 2). These embedded bovine mammary epithelial cell spheroids may provide a more relevant microenvironment for analyzing the effect of AFB1 on cell morphology and various genes function *in vivo* spatially and physiologically. Therefore 3D culture system for the further studies on bovine mammary epithelial cells had been selected.

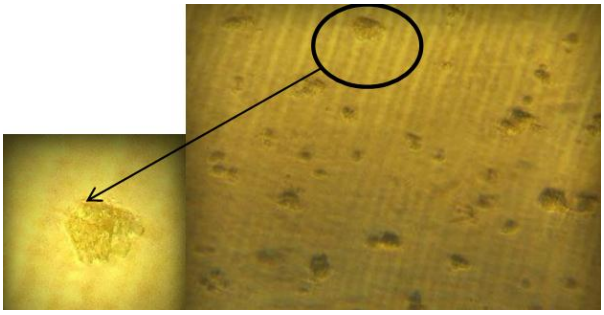

Figure 2. Bovine mammary epithelial cell mass at the first stage of three-dimensional culture condition.

Real time PCR assessment

Bovine mammary epithelial cells were incubated with AFB1 (25 and 35µl) for 8 h. Then expression levels of *STAT5A* gene were detected using real time PCR. Interpretation of the results was performed using the Pfaffle method and finally the CT values of gene were normalized to the expression behavior of GAPDH as a housekeeping gene. All reactions were performed in triplicate and negative control included in each experiment and quantified as a view of SYBR Green emitted fluorescent intensity.

We carried out a quantitative PCR approach to evaluate the effect of AFB1 on the expression of *STAT5A*, which is involved in development of mammary lobular-alveolar system.[8] The mRNA expression of these genes versus GAPDH expression is demonstrated in Figure 3. The respective ratio of expressed gene over GAPDH expression was used for representation of gene expression changes and illustrated quantitatively in Figure 3. Normalization of our data to the expression rate of GAPDH as a housekeeping gene, revealed significant downregulation of *STAT5A* gene in cells in

dose - dependent manner. This downregulation can affect mammary health and milk production.

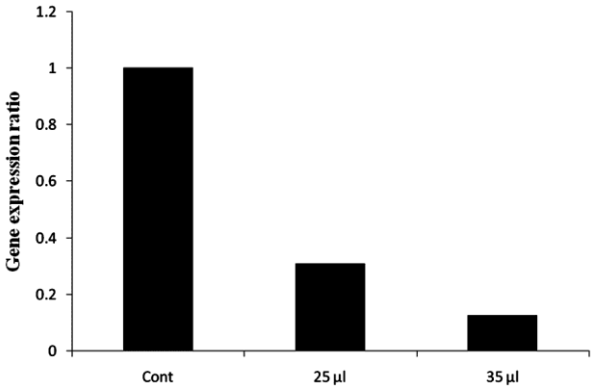

Figure 3. Down-regulation of *STAT5A* gene expression with increasing concentration of AFB1. Considerable gene expression reduction has been observed in AFB1 treated-bovine mammary epithelial cells in dose-dependent manner.

Discussion

Owing to the need of early recognition of chemicals, which could be carcinogenic in humans, the early molecular events of chemical carcinogenesis need to be clarified. AFB1 can cause cell necrosis and apoptosis and led to DNA fragmentation, mutations and chromosomal abnormalities.[1,5,15] Therefore, to reveal the cytotoxic mechanism of the AFB1, we looked at the gene expression profile of *STAT5A* gene involved in development of mammary lobular-alveolar system. *STAT5A* gene is located downstream of prolactin gene and has very important role in the growth and proliferation of mammary epithelial cells and influence the quantity of milk proteins and gap junction between epithelial cells. After the release of prolactin from the pituitary, it results in JAK2 dimerization by binding to its receptors on the cell surface and the dimerization of JAK2 led to *STAT5A* phosphorylation. Then *STAT5A* transfers to the nucleus after phosphorylation and bind to the *Bcl-x* gene promoter and caused binding of other related transcription factors. Finally, this process results in gene expression level increasing that led in the proliferation and differentiation of epithelial cells. Therefore, In short time *STAT5A* gene expression deficiency caused the reduction of proliferation and differentiation of epithelial cells.[16-19] Miyoshi et al. showed that *STAT5A* gene silencing in mouse decreased lobular-alveolar system up to 30 percent due to defects in proliferation and differentiation of epithelial cells.[16,20] Since the quantity of milk production in cows directly correlates with the number of mammary epithelial cells; decreasing of the mammary epithelial cells number due to AFB1 can reduce the amount of produced milk.

Also *STAT5A* gene expression may be declined due to DNA damage caused by AFB1 in mammary epithelial cells. This DNA damage may have a direct relationship with concentration of the AFB1. Previous study results showed that AFB1can bind to DNA and cause cell mutation in G and T bases and DNA fragmentation. This damage can result in cell death and if did not lead to cell necrosis or apoptosis, can damage programmed cell death. Ultimately, it can lead to the cancer in mammary tissue.[1,20] But in bovine due to the short period of economical life and periodical characteristic of mammary growth in each pregnancy, cancer of mammary tissue is rare. It should be noted that AFB1 can be easily transferred to human food chain through contaminated milk and in long time, can cause similar effects on human health.[3] Therefore, contaminated milk should be taken into the consideration for accurate analysis.

Conclusion

Overall, the results of this investigation indicate that *STAT5A* gene expression downregulated in AFB1-treated mammary epithelial cells in dose- dependent manner and led to the reduction of proliferation and differentiation of epithelial cells and milk protein quantity. Since the number of epithelial cells has direct and essential effect in milk quantity and quality, AFB1 can lead in financial loss for poulterer. In addition, transfer of this poison to human food stuff, can put human health at risk. Therefore, it may have the same effects on human health and it is worthy to make a comprehensive analysis on AFB1-contaminated diets.

Table 1. Real time PCR genes and their forward/reverse primers.

Gene name	Primer sequence	Tm (°C)
GAPDH	F:5′-AAGGGTGGTGCTAAGCGTGT-3′ R:5′-GTGATGGCATGGACAGTGGT-3′	60
STAT5A	F:5′-GAAACATCACAAGCCCCATT-3′ R:5′-TGAAGCGCAACAAGAAGGTA-3′	56

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Conflict of Interest

The authors report no conflicts of interest.

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