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Effect of Aflatoxin B1 on Growth of Bovine Mammary Epithelial Cells in 3D and Monolayer Culture System

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ABSTRACT

Purpose: Many studies have been showed transfer of aflatoxins, toxins produced by *Aspergillus flvaus* and *Aspergillus parasiticus* fungi, into milk. These toxins are transferred into the milk through digestive system by eating contaminated food. Due to the toxicity of these materials, it seems that it has side effects on the growth of mammary cells. Therefore, the present work aimed to investigate possible toxic effects of aflatoxin B1 (AFB1) on bovine mammary epithelial cells in monolayer and three-dimensional cultures. ***Methods:*** Specimens of the mammary tissue of bovine were sized out in size 2×2 cm in slaughterhouse. After disinfection and washing in sterile PBS, primary cell culture was performed by enzymatic digestion of tissue with collagenase. When proper numbers of cells were achieved in monolayer culture, 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, the concentrations of 15, 25 and 35 µL of AFB1 were added to the culture in quadruplicate and incubated for 8 hours. Cellular cytotoxicity was examined using standard colorimetric assay and finally, any change in the morphology of the cells was studied by microscopic technique. ***Results:*** Microscopic investigations showed necrosis of the AFB1-exposed cells compared to the control cells. Also, bovine mammary epithelial cells were significantly affected by AFB1 in dose and time dependent manner in cell viability assays. **Conclusion:** According to the results, it seems that AFB1 can induce cytotoxicity and necrosis 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 due to their growth on food stuffs. These compounds are known as carcinogens, mutagens, immune systems attenuator in animals and humans.[1-5] Of the 18 known types of aflatoxins, aflatoxins B1, B2, G1 and G2 have been regarded as a group A carcinogens by the International Agency for Research on Cancer (IARC). Among them, the most toxic and carcinogenic effect has been reported for aflatoxin B1 (AFB1, Figure 1).[5] Aflatoxin M1 (AFM1) is a 4-hydroxylated metabolite of AFB1, which has been excreted into milk in the range of 0.3-6.3% in mammals that ingest AFB1-contaminated diets and it has not been omitted even with pasteurization.[6,7] Even though, toxicity and carcinogenic effects of AFM1 is less than AFB1 but since milk and dairy products are one of the healthiest and most consumed food stuffs, it should be free of any toxins such as AFM1. Therefore, the presence of AfM1 in these products should not exceed the standard limit for consumer. Also due to the high toxicity of AFB1, it can have side effects on the growth of epithelial cells in cow mammary and reduces milk production.[8]

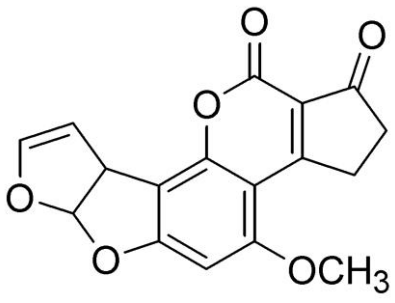

Figure 1. Chemical structure of Aflatoxin B1.

In terms of cost, accuracy and ethical issues, toxicology tests on animals have some problems. Therefore, experiments on cell cultures are considered as

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alternatives for *in vivo* experiments. Monolayer cell culture could not provide tissue culture conditions *in vitro* but three-dimensional (3D) culture conditions are approximately the same as tissue culture conditions biochemically and biomechanically. Therefore, toxicology tests based on 3D culture omit restrictions on work both with the animals and a monolayer cell culture and this type of cell culture gives us more information about the effects of toxins on the tissues in the long term.[8,9] The present study was designed to determine cytotoxicity of AFB1 on the mammary epithelial monolayer and 3D cell cultures.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffer solution (PBS), 0.25% trypsin-EDTA solution, AFB1, Penicillin, streptomycin, amphotericin B were purchased from Sigma–Aldrich Co, USA. RPMI-1640 Medium (RPMI) and fetal bovine serum were obtained from Invitrogen, USA.

Cell culture

A sample was prepared from normal bovine mammary epithelial tissue with size of 2×2 cm in sterile condition. After washing with PBS, samples were placed in falcon containing 9 mL DMEM and 1mL FBS medium and transferred to the lab on ice. Immediately after taking of samples to laboratory, monolayer cell culture was carried out at 25cm propylene flasks. To avoid potential contaminations cells were grown in medium containing 1% of penicillin, streptomycin and amphotericin B. The cultures were maintained at 37 ºC in a humidified atmosphere of 5% CO_2.[8] The medium was changed every 4 days and the cells reached confluence within 2 weeks.[10] After full cell confluence 3D cell culture was performed. To perform 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.[11-13]

Cell viability assessment

For evaluating the effect of AFB1 toxicity on growth of mammary epithelial cells on the 3D culture, cells were exposed to different doses of the freshly prepared toxin. Three treatments with AFB1 in the concentrations of 15, 25 and 35 µL were used in the quadruplicate manner compared to the control cells.

Also the cultured mammary epithelial cells in monolayer at 40–50% confluency were subjected to MTT assay in the 24-well plates. The cells were treated with the concentrations of 15, 25 and 35 µL AFB1 and were incubated for 24 and 48 hours at 37 ºC. Then cells were washed once with phosphate buffered saline (PBS) and the normal culture medium was replaced with 150 µl fresh media and then 50 µl MTT reagent (2 mg/ml in PBS) was added to each well. Following a 4 hours incubation period with MTT at 37 ºC, medium was removed and the cells were exposed to 200 ml DMSO and 25 ml of Sorenson buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) [8]. Cultures were incubated for 30 min at 37ºC for formazan crystals dissolving and then UV absorbance was measured at 570 nm using a spectrophotometric plate reader, ELx 800 (Biotek, CA, USA).

Morphological study

Morphological study of the bovine mammary epithelial cells has been carried out using microscopic technique. In the present study, microscopic pictures of different magnification and cell growth stages were used for assessment of morphological change of the cells.

Results

Cell culture

After four days of the 3D Cell culture, primary cell masses had grown but it took longer to form cellular mass in good shape (Figure 2). However, in 21 days full deployment of cell growth has been seen in 3D culture at Matrigel.

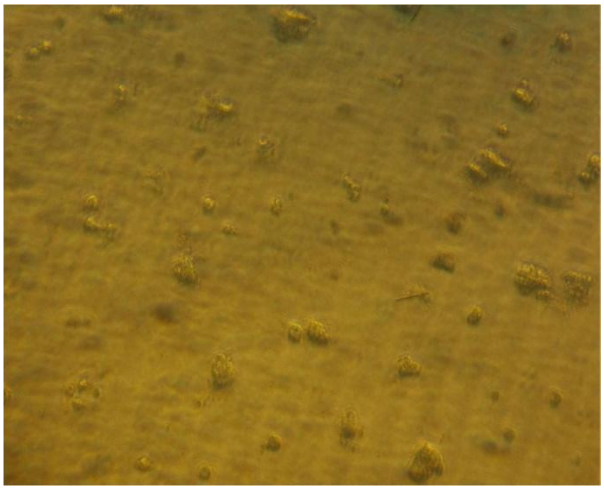

Figure 2. Bovine mammary epithelial cell mass at the first stage of three-dimensional culture.

Cytotoxicity assays

8 hours after treatment of cells with AFB1 at various concentrations, test cells showed necrosis with increasing of the concentrations of AFB1 compared to untreated cells. In fact, in the control group, no necrosis was observed and in the cell line treated with 15 µL of AFB1 necrosis was observed only in cells located on the cell mass center. But in the treatment with 25 µL, cell necrosis were more noticeable, being seen in the half of the cells; and the highest necrosis was observed for cells treated with 35 µL of AFB1 (Figure 3).

MTT survival assay as a standard colorimetric cell viability assay was undertaken to assess the cytotoxicity potential of the AFB1 within bovine

mammary epithelial cells in monolayer cell culuture system.[8] Upon treatments with AFB1, mammary epithelial cells showed cytotoxicity that appeared to be largely dependent on concentration (Figure 4). In the view of statistics, reduction in number of cells was observed in dose and time dependent manner. The highest cytotoxicity has been observed for cells treated with 35 µL AFB1 compared to control group.

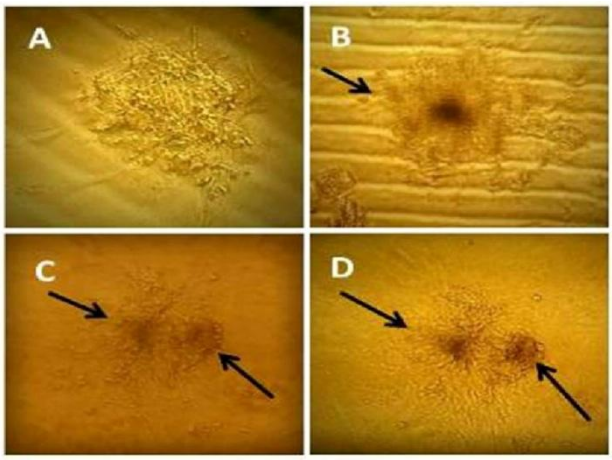

Figure 3. Effect of increasing amounts of Aflatoxin B1 on the bovine mammary epithelial cells. A) Epithelial cells without necrosis in the control group. B) Epithelial cells treated with 15 µL of Aflatoxin B1 (necrosis only in the center of the cell mass). C) Epithelial cells treated with 25 µL of Aflatoxin B1 (necrosis approximately in the half of cells). D) Epithelial cells treated with 35 µL of Aflatoxin B1 (necrosis in the more than half of cells).

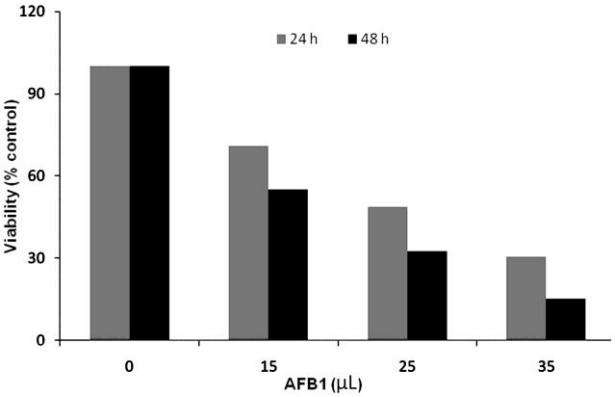

Figure 4. MTT survival assay in bovine mammary epithelial cells treated with Aflatoxin B1 after 24 and 48 h incubation.

Discussion

Due to the need of early identification of chemicals, which could be carcinogenic in humans, the early molecular events of chemical carcinogenesis need to be clarified [4]. Aflatoxins increase DNA fragmentation and cause mutations and chromosomal abnormalities. AFB1 can cause cell necrosis and apoptosis.[14-16] Necrosis of the bovine mammary epithelial cells lead to cell dysfunction and abnormalities as well as cancer.[8] It seems that with increasing concentrations of aflatoxin levels, cell mutations rate and necrosis increase.

In comparison with the traditional 2D cell culture which has been known as the simplest model without most *in vivo* characteristics, the 3D cell culture is going to be the most accepted as a more physiologically relevant and predictive model. Correspondingly, the co-culture 3D system should provide a better *in vitro* culture model for the evaluation of efficacy of anticancer drugs and cytotoxicity.[11-13] In this study, we demonstrated the incubation of primary monolayer cell culture and Matrigel-embedded 3D cultures of bovine mammary epithelial cells with various concentration of AFB1 cause cytotoxicity and necrosis of the AFB1-exposed cells compared to the control cells with dose and time dependent manner. MTT assay revealed significant reduction in cell viability in bovine mammary epithelial cells after 24 and 48 h of exposure. Therefore, AFB1 can result in blocking of cell divisions in mammary epithelial cells.

It should be noted that AFB1 can transfer to bovine mammary gland epithelial cells through digestive systems and it has capability of milk contamination. Since, it can be easily transferred to human being by drinking the contaminated milk.[5] Therefore, contaminated milk should be taken into the consideration for accurate analysis.

Conclusion

Herein, we aimed to study the cellular toxicity of AFB1 on the mammary epithelial monolayer and 3D cell cultures. The results obtained in the present study indicate that the bovine mammary epithelial cells showed necrosis when being treated with AFB1. Thus, it can be considered as a potential *in vitro* model for studying cytotoxicity of AFB1 on bovine mammary gland. Moreover, even though additional studies are required to characterize the mechanism of its toxicity effects, it seems that AFB1 can be entered to human food chain through the contaminated milk. Therefore, it may have the same effects on human health and it is worthy to make a comprehensive analysis on AFB1-contaminated diets.

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Conflict of Interest

There is no conflict of interest in this study.

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