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Evaluation of the Immunomodulatory Effects of Aflatoxin-B1 and Aged Garlic Extract in Tumor Balb/c Mice

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Abstract

Background: AFB1, a secondary metabolite of Aspergillus flavus, is a hepatocarcinogen in humans and can invade tumor cells. If humans receive a little dosage of AFB1daily in a long time, it will be carcinoma. Among all types of cancers, breast cancer has a top position in the woman's death. The increasing speed of cancer research cannot catch up with breast cancer growth and many people suffer from it, for this reason, cancer control is a major health issue. There are several cancer therapies, but all of them has a specific problem, therefore it is necessary to find a new method for cancer therapy that at first kill tumor cells in a specific manner. Garlic has a wide range of biological activities that have been verified *in vitro* and *in vivo*. Our previous studies demonstrated that Aged garlic has enriched immunostimulator fractions and reduced immunosuppressor fractions. Therefore, in this study, we used from aged garlic extract instead of fresh garlic extract.

Method: First of all, garlic extracted by the Mantis method and AFB1 separated of Aspergillus flavus (PTCC 5004) by HPLC and DTH test was carried out on normal female mice sensitized by sheep RBC to specify suitable AGE dosage, which can be stimulated the immune system. Subsequent experiments were carried out on tumor-bearing Balb/c mice to estimate the effects of AGE and AFB1 on number Treg cell and to determine the ratio of IL-4 and IF-γ. Briefly, 10µ/kg/day of AFB1and AGE diluents were administered for 4 consecutive days to group 1: AFB1, 2: control of tumor, 3: AGE+AFB1and 4: AGE via Intra Peritoneal (IP) route, respectively. Mice were sacrificed and splenocytes harvested and the percentage of splenic T-reg cells were measured by flow cytometry analysis. So the ratio of IL-4 and IF-γ determined to ELISA methods.

Result: According to the findings, tumor size increase in the group receiving AFB1 and decrease in the group receiving AGE. So mouse treated with an AGE increased the level of IFN-γ and decreased the level of IL-4, but AFB1 works the opposite of AGE to the control group. (P-value<0.05) AFB1 could increase the Treg cells percentage in the spleen (p-value<0.05).

Conclusion: In general, these results introduce some antitumor properties of AGE and tumorogenic properties of AFB1 *in vivo* that may open up new insights into the development of more effective antitumor agents.

Keywords: AFB1; Aged garlic extract; Tumor; Immunotherapy

Introduction

AFB11 is considered the most toxic and is produced by both *Aspergillus flavus* and *Aspergillus parasi icus* [1-3]. It is also highly carcinogen in mammalian species and its exposure can give rise to different types of tumors, especially in the liver [4-7]. If aflatoxin is given daily by humans at a lower dosage it can affect the number of T reg cells2 in the human body [8]. AFB1 has unique chemical structures, which can cause harm by reacting with the chemicals in living organisms.

Garlic (*Allium sa ivum*, *Liliaceae*) is used by many people around the world as a digestive stimulant, diuretic, and antispasmodic. It has recently been reported to have antibiotic properties and benefits including antifungal, anti-atherosclerosis, anti-carcinogenesis, and antibacterial activities [9,10].

Moreover, Garlic is reported to have hypolipidemic properties and the ability to modulate immune responses [11]. On the other, it can enhance NKc3activity and T-lymphocyte proliferation [12,13]. AGE4 and its protein fraction were shown to augment the oxidative burst in peritoneal macrophages of Balb/c mice [14].

Ghazanfari et al.[15] showed that garlic extract induces a shift in cytokine patterns in Balb/c mouse with a major infection of Leishmania and an upshot in the immune response about Th15 (IFN-γ6, IL-47).

At the same time, a unique garlic preparation called AGE has been reported to have a series of pharmacological effects, including immunomodulation to inhibit the development of chemically induced tumors in the bladder, mammary glands, colon, esophagus, lung, skin stomach in a rodent [16]. In a recent investigation, the immunomodulatory effect of AGE and AFB1 on the immune system of tumors mice is examined.

Materials and Methods

The samples

The groups of inbred female BALB/c mice (n=20; 8 weeks old; 18-20 g) were purchased from the Pasteur Institute of Iran. Four groups (five mice in each group) were housed in a standard polypropylene cage. Animals were housed at 22 ± 5°C in a 12 h light/dark cycle and fed rodent chow and water freely. Animal care and treatment were conducted in conformity by the

Institution of Animal Care and Use Committee of Tarbiat Modares University and in compliance with the Guide for the Care and Use of Laboratory Animals [DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205].

Preparation of the model

Briefly, four groups of five 4-6 week old female Balb/c mice were subcutaneously transplanted with mouse mammary tumor were collected from the Pasteur Institute of Iran [15,17]. The tumor-bearing mouse was treated intraperitoneally with AFB1 and AGE for seven consecutive days.

The first group in this experimental study was treated with 0.1 ml of AFB1 at a daily dose of 10 µl/kg via the IntraPeritoneal route (IP). This dose by Larypoor, et al. [8] had already been defined by the Delayed-Type Hypersensitivity test (DTH) as the optimal immunostimulatory dose that [8,18]. The second group (negative control) also received the same volume of diluents PBS via the IP route. The third group was treated with 0.1 ml of AFB1 (10 µl/kg/day) and 0.1 ml of AGE (20 mg/kg/day) via the IP route and the fourth group with 0.1 ml of AGE (20 mg/kg/day) via the same route [2,19]. The treatments were applied once. Tumor size was measured using a digital vernier caliper (accuracy of 0.01). The mice were sacrificed and the percentage of tumor-infiltrating Treg cells was obtained by using flow cytometry (BD, USA) in blood and spleen. Production of cytokine was obtained using Enzyme-Linked Immunosorbent Measurement (ELISA) assay (Roche) [15,18].

AFB1 preparation

Toxigenic *Aspergillus flavus* (PTCC 5004) was purchased from the Iranian Research Organization for Science and Technology (IROST). Then it was cultured in aflatoxin production medium to generate AFB-1. After the preparation of Aspergillus flavus microbial bank on the specific culture medium of aflatoxin production, the culture medium was extracted and the aflatoxin produced by methanol was separated and then AFB1 was separated from culture extract by HPLC method [2,4].

AGE preparation

Fresh garlic bulbs (*Allium sativum* L.) were collected from a famous city in western Iran called Hamedan. Dry garlic bulbs were peeled and preserved in the freezer (-20˚C) for six months. AGE was prepared by using the mantis method and based on the research method of Larypoor et al. [8,15].

Garlic bulbs were homogenized with two parts of distilled water in a varying blender. The homogenized blend was filtered under vacuum through Whatman paper (No.1) and the filtrate was centrifuged at 3400 g for 30 minutes. Then the clear supernatant was collected. Twenty-seven grams of ammonium sulfate salt was added to one liter of the supernatant and centrifuged at 3400 g for 30 minutes. The residue was re-suspended in saline and dialyzed against buffer saline. AGE samples were then run on G 50 gel chromatography. For measuring protein Bradford assay was used and it was evaluated by Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) [8,20].

Protein extraction for SDS-PAGE

Proteins were separated by SDS-PAGE according to Laemmli [21]. A 12% (weight/volume) polyacrylamide gel was utilized to judge the purity of molecules and to estimate the molecular mass compacted with proteins. After electrophoresis, the gel was fixated with methanol and acetic acid, formaldehyde for 60 minutes and protein subunit bands were stained with coomassie blue R-250 [21]. The protein marker from Sigma was used. The molecular weight of standard protein as follows: 94, 43, 30, 20, 14.4 and 10 kDa.

Delayed-type hypersensitivity test

Evaluate the appropriate dose for subsequent tests was performed Based on Delayed-Type Hypersensitivity (DTH) response test in normal mice. 20 female mice were randomly assigned into four groups of five 0.1 ml of a solution containing 1 × 108 sheep red blood cells (RBCs, Razi Institute, Tehran, Iran) suspended in PBS were subcutaneously injected in the back of all mouse. Three groups received three doses (30, 15, 10 µl/kg/day) of AFB-1 (0.1 ml) via the intraperitoneal route (IP) for five days. The remaining group (control group) received diluent [a solution of ethanol /PBS (40:60)] via the same route, dosage and time interval. On the fifth day, the sensitized animals were subcutaneously challenged with 1 × 108 RBCs in the left hind footpads. The increase in the foot-pad thickness was measured with a vernier caliper (Mitutoyo, Japan) during one, two and three days after the booster injection of RBCs. The results were calculated according to the following formula (8,18,22)

Increased DTH =(increase in Left foot-pad challenged by the sRBC−increase in right foot-pad) × 100 Right foot-pad

Separation of splenic mononuclear cells (MNC

The treated and control mice were sacrificed by cervical dislocation on the 13th day. The spleen of Balb/c mice was isolated under sterile conditions and transferred to a sterile PBS solution. The splenic cell suspension was RBC-lysed with 0.75% NH_4Cl, and Tris buffer (0.02%) (pH~7.4). The cells were washed and the single-cell suspension was prepared in RPMI-1640 (Gibco, 51800-035, Stey cell Technology Company) containing stable glutamine (Cytogen, USA) and 10% heat-inactivated fetal calf serum (Gibco, England).

For describing the density and viability of cells in the suspension was used by the Trypan blue dye exclusion method.

The Cells were counted by the used of hemocytometer in light microscopy. The viability of the splenocytes was generally above 95%. After washing addition, the suspension was adjusted to 4 × 106 cells per milliliters in RPMI-1640 supplemented with 10% FCS, 100 IU/ml penicillin (complete RPMI) and 100 µg/ml streptomycin and for the next steps kept at 4˚C [8, 23,24].

Three-color immunostaining and low cytometry analysis

Following treatment of tumor mice with AGE and AFB1 over a seven-day period mentioned 2.1, the MNCs purified from the spleens were immunostained with the FITC anti-mouse CD4, PE-Cy5 anti-mouse CD25 (BD, eBioscience, UK), and subsequently with PE-Cy5 anti-mouse FoxP3+, according to the eBioscience methods, mouse T reg cells were stained.

Three samples were taken from each mouse and each was analyzed in triplicate. The samples were analyzed by using a FACSCalibur flow cytometer at Tarbiat Modarres University and the results were analyzed by WinMDI 2/8 software [25].

Measurement of splenocyte cytokine production

The isolated spleen mononuclear cells were cultured in 24-well plates (Nunc, Denmark) in a final concentration of 2 × 106 cells/ml. Three samples were taken from each mouse in each group and the experiments were repeated in triplicate.

To stimulate the cells, twenty microliters of purified tumor antigen and PHA (in full first time) were added separately to each one. After 3 days of incubation at 5% CO_2 and 37˚C, the supernatant was collected and frozen at -70˚C then analyzed by ELISA. IL-4 and IFNγ concentrations were measured using the R&D American DuoSet ELISA Development kit [26].

Statistical analysis

In this study, the data were analyzed using SPSS software version 16 and the results are expressed as measures of central tendency and dispersion (mean, SE, etc.) and experiments were performed in triplicate and one-way analysis of variance (ANOVA) or Mann-Whitney non-parametric tests were used to determine the statistical significance ($p<0.05$) between values in the experimental and control groups.

Results

SDS–PAGE electrophoresis and Bradford assay

The results of the gel electrophoresis showed that the most important protein in the AGE was 10 kDa. The protein band isolated on the gel is shown in **Figure 1**. The Bradford evaluation showed that the quantity of effective protein in AGE is 0.27 g/ml.

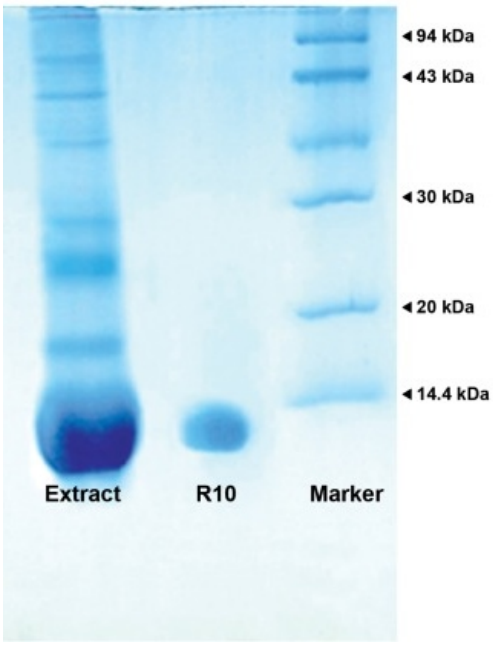

Figure 1: Gel electrophoresis show protein bound in Aged Garlic Extract.

Effect of AFB1 on DTH test

There was a significant difference in mouse treated with a dose of 10 µg/kg/day compared to control mouse two and three days after the footpad challenge. The steady increase in the pad swelling two and three days after injection (p-value=0.015 and 0.021 respectively) showed that a dose of 10 µl/kg/day of AFB1 significantly contributed to a greater DTH response every one day after the footpad challenge compared to controls (p-value=0.01). This increase was not seen in other groups. As a consequence, the optimum dose of 10 µl/kg/day of AFB1 was used for the rest of the investigations. Our results are similar to Ghazanfari studies. The results were shown in **Figure 2** [18].

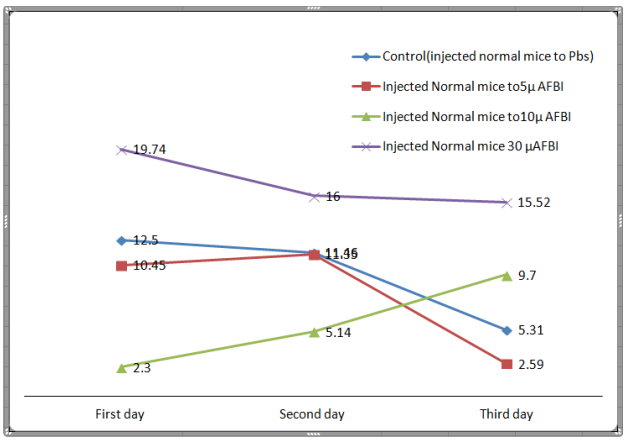

Figure 2: Delayed type hypersensitivity (DTH) graph show a significant increase in the degree of swelling in the group treated with 10 µg of AFB1. (P-value=0. 015 and 0.021 at the second and third days respectively).

Effect of AFBI and AGE on tumor size

Caliper Verniye measured tumor size over 10 days (**Figure 3**). According to the results, tumor size increase in the group receiving AFB1 and decrease in the group receiving AGE and also in the group which receiving AFB1 and AGE to gather than the control group. The result has been shown in **Figure 4**.

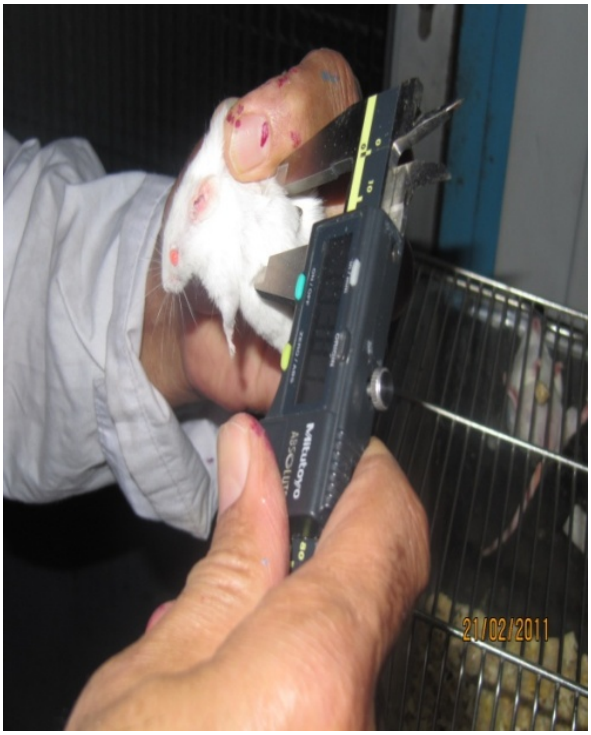

Figure 3: Method for measuring tumor size.

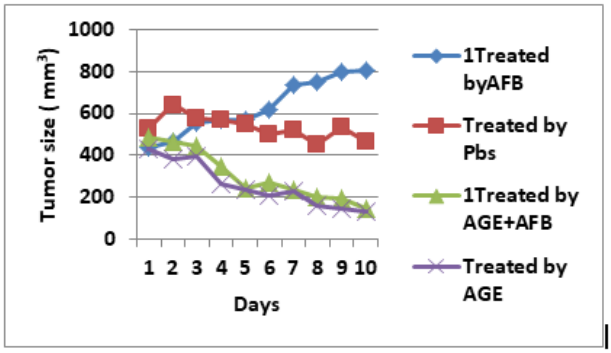

Figure 4: The results of measuring tumor size.

Effect of AFBI and AGE on the concentration of IFN-γ and IL-4

The concentration of IFNγ and IL-4, typical cytokines for Th1 and Th2 pattern, in treated and untreated tumor mice was evaluated by the ELISA technique. The results show that mice treated with AGE showed an increased level of IFN-γ and a decreased level of IL-4, but in the case of a mouse treated with AFB1, the results demonstrated an increased level of IL-4 and a decreased level of IFNγ compared to the control group. The ratio of cytokines secreted Stimulated with tumor-specific antigen was higher than the ratio of cytokines secreted Stimulated with the phytohemagglutinin- specific antigen in all thr ee treated groups.

On the other hand, the rate of cytokines secreted in the AGE recipient group was higher in the other groups and lower in the AFB1 recipient group than in the other groups. These differences were statistically signi icant (p-value<0.05). The results in the IFNγ and IL-4 concentrations are shown in **Figures 5 and 6**.

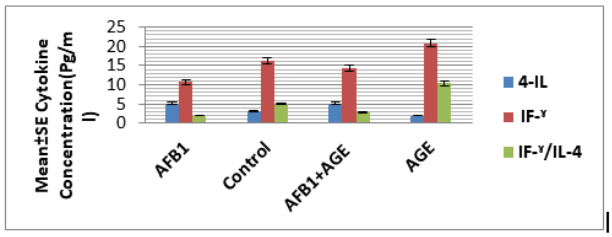

Figure 5: Results of ELISA assessment showing the level of IFNγ and IL-4 cytokines produced from splenocytes stimu-lated by tumor-specific antigen. Results show a statistically significant difference between the AGE-treated group and AFB1-treated group (p-value<0.05).

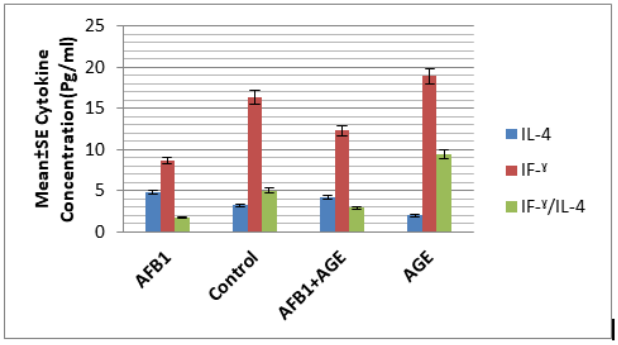

Figure 6: Results of ELISA assessment showing the level of IFNγ and IL-4 cytokines produced bysplenocytes stimu-lated by phytohemagglutinin- speci ic antigen. Results show a statistically signi icant difference between the AGE-treated group and AFB1-treated group. (p-value<0.05).

Effect of AFBI and AGE on splenic T reg cells

The low cytometry technique was used to de ine the percentage of splenic T reg in tumor mice. A control isotype was prepared to determine the percentage of Treg cells for each sample in each treatment group. Then the results were read with a low cytometer using WinMDI 2/8 so tware. First, isotype control samples gate and four areas were identi ied so, then based on its gate and quadrant sample analysis was performed.

As shown in Figure 7 the results indicate a statistically significant difference between the percentage of splenic T reg cells in the three treated groups compared to the control group. The percentage of splenic T reg cells in the AGE and AFB1 treated group was significantly lower than the other three groups. The percentage of splenic T reg cells in the AGE-treated

group was lower than the AFB1-treated group (p-value=0. 049). The results of the comparison of the percentage of splenic T reg cells in the treatment groups with the control group are shown in **Figure 7**.

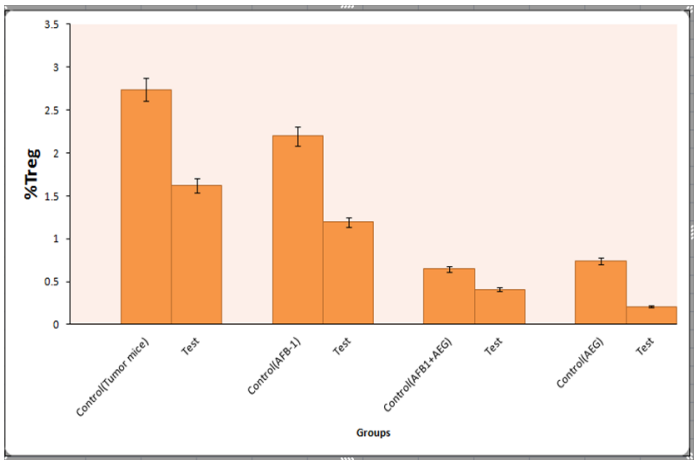

Figure 7: Mean ± SE spleen %Treg cells in the treated and control groups in tumoric mice (n=5 for each group)(P-value=0.049).

Discussion

AFB1 is a highly toxogenic difuranocoumarin compound produced by the fungi Aspergillus flavus and found in different foods such as nuts, maize, peanuts, wheat, and milk. Its mutagenic effects have been well demonstrated in several *in vitro* and *in vivo* models [8].

AFB1has low molecular weight and cannot activate mechanisms of the immune system. Therefore, there aren't symptoms of its existence in the human body. Moreover, due to the carbohydrate structure of mycotoxin, after entering the liver, it does not disappear, but gets impressed by liver enzymes to tumor metabolites and remain in the liver. If humans in the long run daily eat these mycotoxins, it can be a cause of cancer [20,27].

On the other hand, the body's natural immune system incompetency has shown that is a predisposing factor in tumorogenic, which can be cured by medicinal plants.

Studies have shown that garlic has anti-tumor activity as well as derivatives such as di-aLyl disulfide and allicin in vitro condition. For this reason, garlic could be a good resource for the prevention and treatment of many diseases such as cancer.

Nowadays oncologists are trying to develop drugs that target and specials, in tumor cells and don't harm the other healthy cells. Naturally, there is 1% allicin in fresh garlic that increases eight percent in AGE. This process is done via subsequent freezing of garlic and the occurrence of hydrolysis and oxidation reaction.

The final product is glutamylcysteine Elaine, which the effect of enzyme alynaz, becomes an effective compound.

Therefore, the inflammatory properties of AGE have reduced to a large extent. Consequently, the use of AGE is more useful than fresh garlic. During the treatment period, tumor growth was measured in all groups and compared.

In the group receiving AGE, the tumor size significantly decreased and in the group receiving AFB1, the tumor size increased ($p<0.05$). Garlic as an adjuvant is very useful in reducing tumor growth and in groups treated with AFB1, reduced invasiveness of cancer cells and reduce tumor size [27]. However, tumor progression is not eliminated, but the improvement of public tumors mice was affected. The results of cytokine production have been reported based on the concentration and ratio of IFNγ/IL-4.

This shows the status of the activation of T helper cells. Based on the results obtained in this study, the protective Th1 response against the tumor to the production of IFNγ was supported in the presence of garlic and the supportive Th2 response, which is a synergy tumor, to the production of IL-4 was supported in the presence of AFB1.

The results are similar to our previous study [28]. According to the results, in the group receiving AGE the mean ratio of IFNγ/ IL-4 was (0.97) in comparison with the control group (0.63), which shows the increase is remarkable. This significant increase and comparison show the effectiveness of AGE to the pattern of cytokine production from spleen cells, which increases the amount of specific cytokine production. IFNγ decreased the level of IL-4 and support Th1 cell responses and improving the treatment and improve the poor prognosis of cancer patients. Similarly, in the group receiving AFB1, the mean ratio (0.54) compared to the control group (0.63) was reduced.

A significant decrease in the comparison means the direct effect of AFB1 on the pattern of cytokine production from spleen cells, which the total amount of cytokine dropped and our investigations showed, increase levels of production of IL-4 and reduced levels of production of IFNγ [15,28]. Therefore, invasive tumors can suppress the protective response.

According to the flow cytometric results, AGE is reduced T reg cells in the spleen. Possible mechanisms for this reduction in the proliferation of lymphocytes kill tumor cells and decline the proliferation. AFB1, as well as T reg cells in the spleen, has increased, which could be due to the increase in lymphocyte proliferation.

This finding is confirmed by Noori et al. Which showed that AGE can reduce T reg cells and inhibit tumor growth *in vivo* but AFB1 can increase Treg cells and stimulate tumor growth *in vivo*. Therefore, AFB1 is the factor that is more invasive of the tumor.

Conclusion

Thus, eliminating T reg cells, we can improve the efficacy of adoptive immunotherapy of cancer in an effective drug that could reduce T reg cells within the tumor is desired. The results have shown that AGE has this ability and was known as a native and effective drug in preventing cancer.

Acknowledgments

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Upon the contractions continued, 20 ml of 0.5% Marcaine was applied in 5 ml each. The patient was placed in a normal supine position. When the patient's hemodynamics and general condition remained stable, 20 mg of intravenous anesthetic propofol was administered for sedation. After the necessary controls and tests were carried out, the surgical procedure was allowed to begin.

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