Süleyman Demirel Üniversitesi Fen Edebiyat Fakültesi Fen Dergisi
Süleyman Demirel University Faculty of Arts and Sciences Journal of Science
2022, 17(1): 96-110
DOI: 10.29233/sdufeffd.940806
Atıf için / For Citation: M. Ihlamur, H. Başarı, Y. Zengin, E.Ş.Abamor, "Evaluation Of Immunostimulant/Cytotoxic Activity Of Human Breast Cancer Prepared By Different Antigen Preparation Methods With Adjuvants Combination", *Süleyman Demirel Üniversitesi Fen Edebiyat Fakültesi Fen Dergisi*, 17(1), 96-110, 2022.

Evaluation Of Immunostimulant/Cytotoxic Activity Of Human Breast Cancer Prepared By Different Antigen Preparation Methods With Adjuvants Combination

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(Alınış / Received: 25.05.2021, Kabul / Accepted: 07.03.2022, Yayımlanma / Published: 27.05.2022)

Abstract: In last decades, immunotherapies became more and more significant as a cancer treatment method. Many in vivo and in vitro studies have been conducted in the literature in order to determine the appropriate diagnostic and treatment methods for cancer. During the development of therapeutic vaccines, determination of vaccine concentration which is the beginning step of in vivo studies, is critical for the healthy progression of the studies. Breast cancer which occurs by uncontrolled proliferation as a result of exposure of healthy cells in breast tissue to factors such as DNA damage, UV, radiation, carcinogens and, various chemicals has a huge portion among the cancer types. In this study, for the first time, antigens generated by two different antigen preparation methods (autoclaving, freezing-thawing) using AU-565 / MCF-7 cell lines. Then, antigens derived from human breast cancer cell lines were applied alone and with different adjuvants (Polyoxidonium, Freund's) to the J774 macrophage cell culture system and the L929 fibroblast cell line in order to investigate immunostimulant activity and cytotoxicity via Griess reagent and MTT test respectively. At the end of the 48th hour, the cell viability well will be examined and the most appropriate antigen-adjuvant combination and its concentration for vaccine preparation were determined. Among the antigen preparation methods, it was determined that the freeze-thaw method was more effective than the autoclave method, and the antigens prepared by the freeze-thaw method from the AU-565 and MCF-7 cell lines showed the highest immunostimulatory activity at a concentration of 160 µg/ml. Among the adjuvants, the immunostimulant effect of Freund's adjuvant was found to be higher. In the light of these results, the optimal vaccine concentration against breast cancer and the viability analysis of these concentrations were determined. All in all, it is thought that antibodies obtained from in vivo studies using the most appropriate antigen preparation method and the most appropriate vaccine concentration can be used in the diagnosis and treatment of breast cancer**.**

Keywords*:* Breast Cancer, Vaccine, AU-565, MCF-7, Antigen

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Farklı Antijen Hazırlama Metotları İle Hazırlanan İnsan Meme Kanseri Hücre Lizatlarının Adjuvan Kombinasyonları İle İmmünostimülan/Sitotoksik Aktivitelerinin İncelenmesi

Öz: Meme kanseri, meme dokusundaki sağlıklı hücrelerin DNA hasarları, genetik mutasyonlar, UV, radyasyon, kanserojenler ve çeşitli kimyasal maddeler gibi faktörlere maruz kalması sonucunda kontrolsüz çoğalarak oluşmaktadır. Uygun teşhiş ve tedavi yöntemlerini belirlemek amacıyla literatürde birçok in vivo ve in vitro çalışma gerçekleştirilmiştir. In vivo çalışmaların başlangıç basamağı olan aşı konsantrasyonlarının belirlenmesi, çalışmaların sağlıklı ilerleyebilmesi için kritik bir aşamadır. Bu çalışmada ilk kez AU-565 / MCF-7 hücre hatları kullanılarak iki farklı antijen hazırlama yöntemi (otoklavlama, dondurma-çözdürme) ile antijenler oluşturulmuştur. Daha sonra insan meme kanseri hücre dizilerinden elde edilen antijenler, sırasıyla Griess reaktifi ve MTT testi ile immünostimülan aktivite ve sitotoksisiteyi araştırmak için J774 makrofaj hücre kültür sistemine ve L929 fibroblast hücre hattına tek başına ve farklı adjuvanlarla (Polyoxidonium, Freund's) uygulandı. 48. saatin sonunda her kuyudaki hücre canlılık oranı incelenip bu sonuçlar ışığında meme kanserine karşı oluşturulan en uygun aşı konsantrasyonu ve bu konsantrasyonların canlılık analizleri belirlenmiştir. Antijen hazırlama metodları arasında dondurma-çözdürme metodunun otoklavlama metoduna göre daha etkili olduğu, AU-565 ve MCF-7 hücre hattından dondurma-çözdürme metodu ile hazırlanan antijenlerin 160 µg/ml konsantrasyonda en yüksek immünostimülan etkinlik gösterdiği belirlenmiştir. Adjuvanlar arasında ise Freund’s adjuvanının immünostimülan etkisinin daha yüksek olduğu tespit edilmiştir. Bu sonuçlar doğrultusunda en uygun antijen hazırlama metodu ve belirlenen en uygun aşı konsantrasyonu kullanılarak yapılan in vivo çalışmalardan elde edilecek antikorların meme kanserine karşı tanı ve tedavide kullanılmasının mümkün olabileceği düşünülmektedir.

***Anahtar kelimeler**:* Meme Kanseri, Aşı, AU-565, MCF-7, Antijen

1. Introduction

Cancer term is first defined at 460-370 BC. In recent years, although the cancer disease enlightened from many perspectives with recent technological development, it is still second the most deathly disease [1]. Cancer is caused by the uncontrolled proliferation of normal cells as a result of DNA damage, genetic mutations, UV, radiation, carcinogens, and various chemicals. As a result of the deterioration of the regulation in the cell cycle and subsequent rapid proliferation, masses called tumors are formed. Tumor masses can be classified as malignant tumors that can develop in their environment, spread to organs or tissues, or benign tumors that can develop in their environment but cannot spread to surrounding tissues and organs [2].

Breast cancer is a type of cancer that arises from the uncontrolled division of cells located in breast tissue. Breast cancer is the most common type of cancer among women and ranks first among the causes of death. According to the 2020 International Cancer Agency (GLOBOCAN) data, there are 2.3 million breast cancer cases. According to these data, there are approximately 24 thousand breast cancer cases in our country. The incidence of breast cancer is increasing in our country, as in the world. Despite the increase in breast cancer cases, the development of early diagnosis and treatment methods seriously decreases deaths due to breast cancer [3].

There are 85 different breast cancer cell lines [4]. MCF-7, one of the cell lines used in cancer research, has a high level of hormone sensitivity due to estrogen receptor expression. It is the most widely used breast cancer cell line in the world as a model for studying hormone response. There are hormone receptors in the MCF-7 cell line. Human epidermal growth factor receptor 2 (HER2) does not exist in MCF-7. It is in the

Luminal A molecular class and has a cancer stem cell profile. It is possible to say that Luminal A-type breast cancer growing occurs slowly. Also, the survival rate of individual who has Luminal A-type breast cancer is higher compared to other types [5].

AU-565, one of the cell lines used in cancer research, has high HER-2 sensitivity due to human epidermal growth factor receptor expression. AU-565 breast cancer cell line has no hormone receptors but it has HER-2. This cell line belongs to the HER-2 molecular class. The AU-565 cell line amplifies the HER-2/neu oncogene. It overexpresses this gene. HER2-enriched breast cancer comprises approximately 11 to 30% of all breast cancers. The somatic genetic mutation that occurs in the HER-2 gene, leads to overexpression of HER-2. Consequently, cell cycle equilibrium is disrupted and cells divide uncontrollably. It is proven that this type of breast cancer does not inherit [6].

Cells are also used as antigen sources in antigen preparation methods. Antigens obtained from breast cancer cells can also be used in vaccine studies against breast cancer. In hormone receptor positive breast cancer studies, MCF-7 cell line is generally used and antigen production is carried out based on this cell line. Thus, MCF-7 cell line is used as a model cell line in hormone receptor positive breast cancer and shows breast cancer stem cell properties. The AU-565 cell line can be used as a model cell line in HER-2 positive breast cancer [7]. Autoclaving, freezing-thawing are generally used techniques in the studies. In the freeze-thaw method, it is possible to break the membrane proteins and cellular proteins in lysates with ice crystals. Although the randomness of these breaks is a disadvantage of the technique, it also reveals other epitope regions [8]. Autoclaving technique breaks down the cellular structure by denaturing peptide molecules. The conformation of the molecules changes during the protein denaturation, but no splicing or rupture occurs in the proteins. Compared to freezing-thawing methods, it provides more regular cell lysis, and the disruption of the spatial arrangement of proteins can also reveal the regions inside the molecule in a 3-dimensional structure. Because of this effect, it can increase the antigenicity of some molecules with low antigenicity [9].

When using the prepared antigens as vaccine formulation, their effectiveness is increased with adjuvants. In this way, a higher immune response can be obtained against the antigens used [10]. Adjuvants; It is non-immunogenic, does not produce antibody response when used alone, but provides a strong immune response by increasing immunogenicity in the organism it is applied to mineral salts (alumina salt, aluminum hydroxide, aluminum phosphate, calcium salt) oil emulsions, immune-stimulating complex (ISCOM), bacterial derivatives, carbohydrates, liposomes, cytokines, virus-like particles, and polymeric microparticles [11]. Oily adjuvants include water-in-oil or oil-in-water emulsions. These adjuvants are stored at the injection site, providing slow release of antigen and stimulating antibody production in plasma cells. Oily adjuvants are divided into two groups as complete Freund's adjuvant and incomplete Freund's adjuvant. Freund's adjuvants provide slow and continuous release of antigen, reaching tissues such as lymph node and spleen via lymph. One of the disadvantages of Freund's adjuvant is that it can form granulomas at the injection site and cannot be administered intravenously. By mixing the killed Mycobacteria with paraffin oil, complete Freund's (FCA) and incomplete Freund's (FIA) adjuvant were created using only paraffin oil. While the FIA form is used in vaccines such as influenza and lethal polio, the FCA form is also a stimulant for TH1 (T helper-1) and TH2 (T helper-2). Polymeric adjuvants realize the controlled release of nanometer or millimeter antigens [12]. One of the most common is the Polylactide co-glycolide (PLG)

microparticle. This particle absorbs antigen surfaces and transports them to antigen presenting cells (APC). The inability of bacterial or viral antigens to produce an adequate immune response has led to the development of synthetic polyelectrolyte-based adjuvants. A non-toxic triple copolymer of 1,4-ethylenepiperazine, 1,4ethylenepiperazine-N-oxide and (N-carboxymethylene)-1,4-ethylenepiperazine, Polyoxidonium™ has been developed. Although the polyamines in its structure are toxic, Polyoxidonium's being less toxic reduces the toxicity as it enables the N-oxide groups to reduce the linear density of their positive charges [13].
The immunostimulant effect of the prepared vaccine formulations should be examined before proceeding with in vivo studies. Vaccine administration concentrations are determined by assessing the amount of nitric oxide (NO) generated via the application of vaccine formulations in macrophages, generally using the macrophage cell culture system. The Griess method used for the indirect determination of NO includes spectrophotometric measurement of the stable degradation products NO^{-3} and NO^{-2}. The aim of this method is to firstly reduce NO^{-3} to NO^{-2} and to determine NO^{-2} by Griess reaction [14].

For the first time in this study, antigens generated from human breast cancer cell lines (AU-565 /MCF-7) using 2 antigen preparation methods were used alone and with different adjuvants (Polyoxidonium, Freund's) to be used in the production of antibodies against human breast cancer. Immunostimulant activity and cytotoxicity in the J774 macrophage cell culture system and the L929 fibroblast cell line were investigated.

2. Material and Method

In this study L929 mouse fibroblast cell line, J774 mouse macrophage cell line, AU-565, MCF-7 human breast cancer cell lines were utilized. Cells were taken from cryobank and seeding of cryopreserved cells was enabled. The culture of cells was maintained in 25 cm^2 (Polystyrene surface, NEST) flasks. Also, the culture of cells is achieved through suitable growth mediums. RPMI-10% FBS for culture of AU-565, DMEM-10% FBS for culture of MCF-7, RPMI-1640-10%FBS for culture of J774, DMEM-10%10 FBS for culture of L929 were used. While AU-565 and MCF-7 breast cancer cell line culture were carried out to acquire antigen, J774 macrophage and L929 cell lines were handled to perform seeding.

2.1 Cell Culture

During the experimental process, the maintenance of cell culture was accomplished under 37 °C, 95% humidity, and 5% CO_2 incubation conditions. After reaching 80-90% confluency, AU-565, MCF-7, and L929 cells were detached enzymatically, and the J774 macrophage cells were detached physically. Then, they were centrifuged at 25 °C, 1000 rpm for 5 minutes. After centrifugation, the supernatant was removed and 1 ml of appropriate growth medium was added to the pellet of the L929 and J774 cell lines. After cell counting with the hemocytometer, Cell inoculation was carried out in 96-well plates at $1x10^4$ cells per well. Plates were incubated in 37 °C, 95% humidity, and 5% CO_2 incubation conditions Cells planted in the formed culture media were kept under incubation conditions for 24 hours. After that, the continuation of J774 macrophage cell and L929 fibroblast cell culture was enabled. On the other hand, after the removal of supernatant of MCF-7 and AU565 cells, 1 ml PBS was added to pellets in order to prepare antigen. Then, they kept at -40 °C.

2.2 Antigen Preparation

After passage, the autoclaving method [15] and freeze-thaw method [16] were utilized to prepare AU-565 and MCF-7 cell lysates. Firstly, in the autoclaving method, the suspended cells in 1 ml of PBS in a 15 ml falcon were placed into a sterile Pasteur bottle for lysate preparation. The Pasteur bottle was placed into the autoclave device that operated at 121 °C for 20 minutes. After the autoclave, the Pasteur bottle was taken into the laminar flow cabinet. The liquid in the Pasteur bottle was homogenized by pipetting and transferred to a 15 ml flask. It was centrifuged at 10,000 rpm for 3 minutes and the supernatant was taken. Subsequently, measurements were taken at 280 and 260 nm wavelengths using the Warburg-Christian method [17] in UV spectrometer for the determination of the amount of protein in the lysate.

Lastly, in the freeze-thaw method, a 37 °C water bath was prepared before the replacement of the suspended cells in 1 ml of PBS in a 15 ml falcon. The cells in 15 ml falcon were placed in a liquid nitrogen container so that they were covered with liquid nitrogen. Cells were allowed to freeze for 15 minutes. Falcon, which was kept in liquid nitrogen for 15 minutes, was placed in a 37 °C water bath. The liquid in the falcon was thawed for 15 minutes. Freezing of the liquid in the falcon with liquid nitrogen and thawing in a water bath was repeated 5 times. It was centrifuged at 10,000 rpm for 3 minutes, and the supernatant was taken. In the end, measurements were taken at 280 and 260 nm wavelengths using the Warburg-Christian method in UV spectrometer to determine the amount of protein in the lysate.

2.3 Nitric Oxide

The amount of nitric oxide (NO) produced by macrophages was determined by the Griess method [18] in order to determine the immunostimulant activity of different lysate concentrations alone and in combination with adjuvants in the macrophage cell culture system. After incubation of macrophage cells in a 37 °C incubator containing 5% CO_2 for 24 hours, various concentrations of prepared antigen formulations (10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml, 100 µg/ml, 120 µg/ml, 160 µg/ml) and adjuvant combinations (Freund's (10µg/µl) and Polyoxidonium (10µg/µl)) were added to macrophage seeded 96 well plates through adjusting with the medium.

The nitric oxide experiment plan was shown in Figure 1. After 48 hours of incubation, supernatants were collected and reacted with Griess reagent. Griess reagent was prepared by adding 2.5 ml phosphoric acid, 0.1 g N- (1- Naphthyl) Ethylenediamine and 1 g Sulfanilamide to 100 ml distilled water. 50 µl of the culture medium to be measured for NO was added to the 96-well plates. Then, 50 µl of Griess reagent was added to the samples and it was incubated at room temperature for 10 minutes. Absorbance values were measured at 540 nm in a 96 well plate reader.

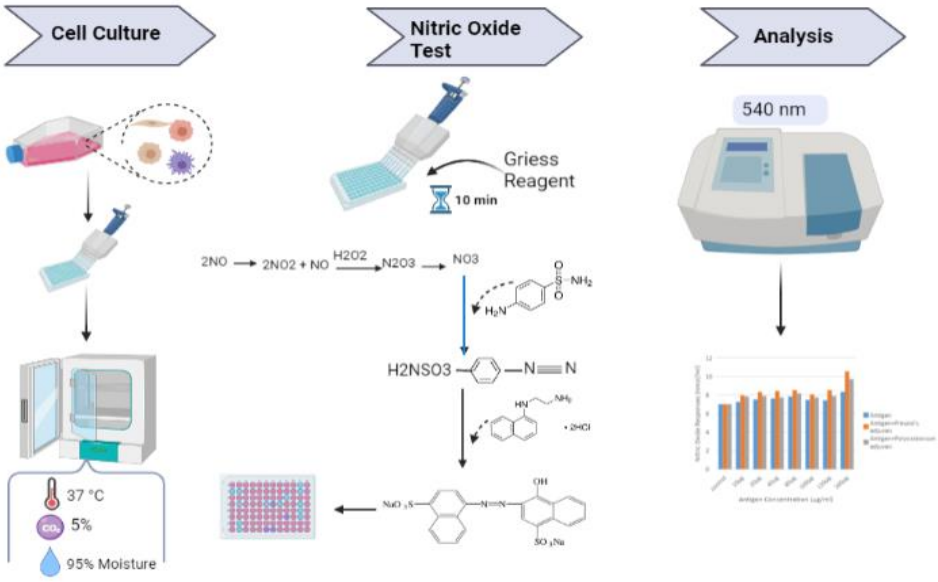

Figure 1. Representation of nitric oxide experiment

2.4 MTT Analysis

MTT analysis was represented in Figure 2. J774 macrophage cells, and L929 fibroblast cells that were incubated for 24 hours, and 48 hours under 37 °C temperature, 95% humidity, and 5% CO_2 incubation conditions were applied MTT test to carry out cell viability analysis. Cell viability rates were evaluated in cell lines with MTT containing 3- (4,5-dimethylthiazol-2-yl) -2,5-Diphenyltatrazyllium bromide. 10 µl of MTT solution was added to each well on the 96 well plates. Cells in the well plates were incubated for 3 hours at 37 °C in a dark environment. After the incubation, the liquids containing MTT solution were removed from the environment by aspirating.

Then, the addition of 100 µl of dimethylsulfoxide (DMSO) to each well was carried out at room temperature, and well plates were kept in the dark for 30 minutes. Once the formazan crystals were completely dissolved, the plate was placed in the ELISA reader. Cell viability analysis was performed by measuring the optical density in the samples/well plates at a wavelength of 570 nm. Each experimental group was repeated three times and the results were averaged. Cell viability analysis data were obtained using equation 1 and data graphs were created.

$$Cell\ Viability\ (\%) = \left(\frac{Absorbance\ of\ Sample}{Absorbance\ of\ Control}\right) * 100 \quad (1)$$

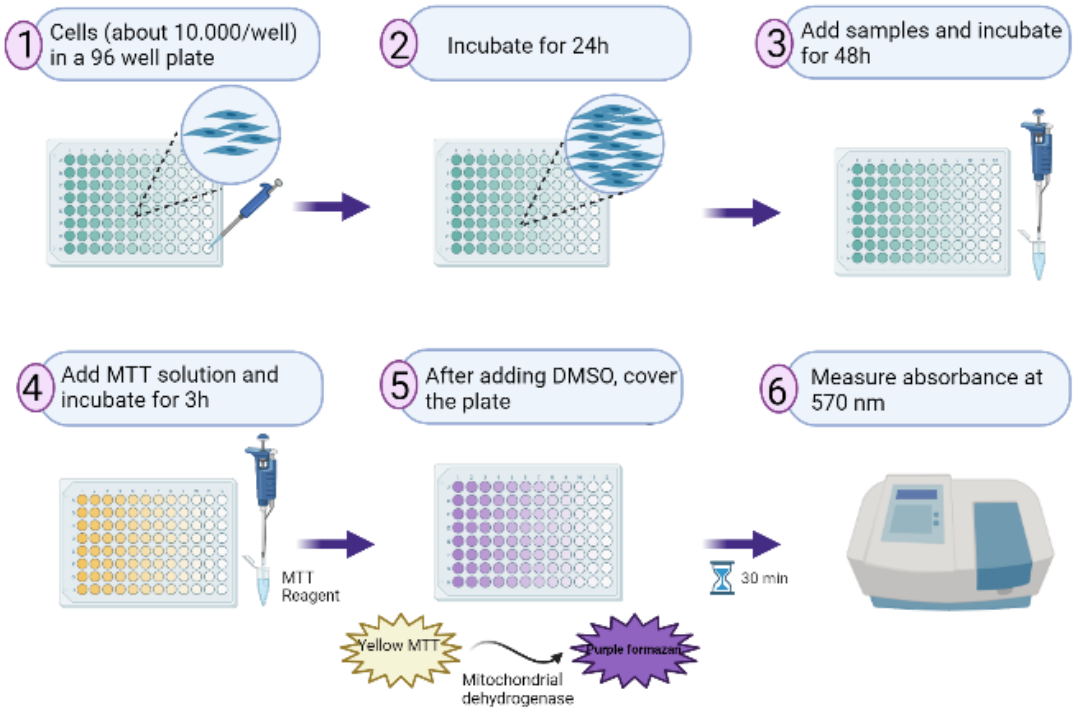

Figure 2. Illustration of cell culture and MTT analysis

2.5 Statistical Analysis

The data obtained from the study were analyzed in the IBM SPSS 25.0 (IBM Corporation, Armonk, NY, USA) package program. Comparisons between groups were made with one-way analysis of variance One-Way ANOVA test. Results were given as mean ± standard deviation (Mean±SD) and statistical significance was accepted as $p<0.05$. It is concluded that null hypothesis was rejected and there is a significant different between pox and freund adjuvants.

3. Result

This study is aimed to define optimum vaccine administration concentrations before in-vivo experiments. To do this, AU565 and MCF-7 breast cancer cell lines were utilized. The passage numbers of the cell lines used in the study ranged from the 10th to the 15th. Antigens were prepared from these two breast cancer cell lines via autoclaving and freeze-thaw technique.

The immunostimulant activity of the J774 macrophage cell culture system was determined and its cytotoxic effect was examined in order to determine the usability of the antigens prepared alone or in combination with adjuvants in vaccine studies, In addition, their cytotoxicity was investigated on L929 fibroblast cell culture. Viability analyzes were shown in Figure 3 depending on the immunostimulant activity and cytotoxicity of macrophage and fibroblast cell lines treated with antigens prepared by autoclaving from the AU-565 breast cancer cell line. Viability analysis of the AU-565 breast cancer cell line, depending on the immunostimulant activity and cytotoxicity of the macrophage and fibroblast cell lines treated with antigens prepared by the freeze-thaw method, were shown in Figure 4. Viability analyzes of macrophage and fibroblast

cell lines treated with antigens prepared by autoclaving method from MCF-7 breast cancer cell line were shown in Figure 5, depending on their immunostimulant activity and cytotoxicity. Viability analyzes of macrophage and fibroblast cell lines treated with antigens prepared by freeze-thaw method from MCF-7 breast cancer cell line were shown in Figure 6, depending on their immunostimulant activity and cytotoxicity. In the study, 48-hour cell data were used because it was desired to examine the long-term effects of vaccine formulations.

The viability percentages of the cells shown in the figures are the % value obtained by simple ratio calculation in other groups when the value of the positive control group is accepted as 100% and indicates the living cell ratio. According to MTT test results, cell viability was evaluated by % cell viability among all groups at the end of the 48th hour.

The immunostimulant activity and cytotoxic activity of the antigens obtained from the AU-565 cell line by the autoclaving method in the macrophage cell culture system and fibroblast cell culture are shown in Figure 3. According to analysis tests, lysates obtained from the AU-565 breast cancer cell line showed the highest immunostimulant activity at a concentration of 80 μg/ml. The highest immunostimulant activity obtained from the treatment of lysates obtained from AU-565 breast cancer cell line with macrophages at a concentration of 80 μg/ml is 8,217 nmol/ml. The immunostimulant activity of Freund's and Polyoxidonium adjuvants in combination with antigens was also investigated in the macrophage cell culture system. The highest efficiency obtained from the treatment of lysate at a concentration of 80 μg/ml with Freund's adjuvant with macrophages is 9,419 nmol/ml ($p<0,05$). The highest efficiency obtained from the treatment of lysate at a concentration of 80 μg/ml with the Polyoxidonium adjuvant with macrophages is 8,876 nmol/ml ($p<0,05$). Also, 69.21% vitality was detected in macrophages, while this rate was 86.39% in fibroblasts in the cytotoxicity analysis of lysate at a concentration of 80 μg/ml.

The immunostimulant activity and cytotoxic activity of antigens obtained from AU-565 breast cancer cell line by the freeze-thaw method in macrophage cell culture system and fibroblast cell culture are shown in Figure 4. Lysates obtained from the AU-565 breast cancer cell line showed the highest immunostimulant activity at 160 μg/ml concentration. The highest immunostimulant activity obtained from the treatment of lysates obtained from AU-565 breast cancer cell line with macrophages at a concentration of 160 μg/ml is 9,147 nmol/ml. The immunostimulant activity of Freund's and Polyoxidonium adjuvants in combination with antigens was also investigated in the macrophage cell culture system. The highest efficiency obtained from the treatment of lysate at a concentration of 160 μg/ml with Freund's adjuvant with macrophages was 10,659 nmol/ml ($p<0,05$). The highest efficiency obtained from the treatment of the combination of lysate with a polyoxidonium adjuvant at a concentration of 160 μg/ml with macrophages is 10,194 nmol/ml ($p<0,05$). 65.39% vitality was detected in macrophages, while this rate was 84.36% in fibroblasts in the cytotoxicity analysis of lysate at 160 μg/ml concentration.

The immunostimulant activity and cytotoxic activity of antigens obtained from MCF-7 breast cancer cell line by the autoclaving method in macrophage cell culture system and fibroblast cell culture are shown in Figure 5. In the study conducted, lysates obtained from the MCF-7 breast cancer cell line showed the highest immunostimulant activity at 160 μg/ml concentration. The highest immunostimulant activity obtained from the treatment of lysates obtained from MCF-7 breast cancer cell line with macrophages at a concentration of 160 μg/ml is 8,256 nmol/ml. The immunostimulant activity of Freund's

and Polyoxidonium adjuvants in combination with antigens was also investigated in the macrophage cell culture system. The highest efficiency obtained from the treatment of lysate at a concentration of 160 µg/ml with Freund's adjuvant with macrophages is 9,845 nmol/ml ($p<0,05$). The highest efficiency obtained from the treatment of the lysate at a concentration of 160 µg/ml with the Polyoxidonium adjuvant with macrophages is 9,186 nmol/ml ($p<0,05$). 71.52% vitality was detected in macrophages, while this rate was 80.89% in fibroblasts in the cytotoxicity analysis of lysate at 160 µg/ml concentration.

The immunostimulant activity and cytotoxic activity of antigens obtained from MCF-7 breast cancer cell line by the freeze-thaw method in macrophage cell culture system and fibroblast cell culture are shown in Figure 6. Lysates obtained from the MCF-7 breast cancer cell line showed the highest immunostimulant activity at 160 µg/ml concentration. The highest immunostimulant activity obtained from the treatment of lysates obtained from the MCF-7 breast cancer cell line with macrophages at a concentration of 160 µg/ml is 8,333 nmol/ml. The immunostimulant activity of Freund's and Polyoxidonium adjuvants in combination with antigens was also investigated in the macrophage cell culture system. The highest efficiency obtained from the treatment of lysate at 160 µg/ml concentration with Freund's adjuvant with macrophages is 10,543 nmol/ml ($p<0,05$). The highest efficiency obtained from the treatment of the combination of lysate with a polyoxidonium adjuvant at a concentration of 160 µg/ml with macrophages is 9.69 nmol/ml ($p<0,05$). 66.81% vitality was detected in macrophages, while this rate was 92.95% in fibroblasts in the cytotoxicity analysis of lysate at 160 µg/ml concentration.

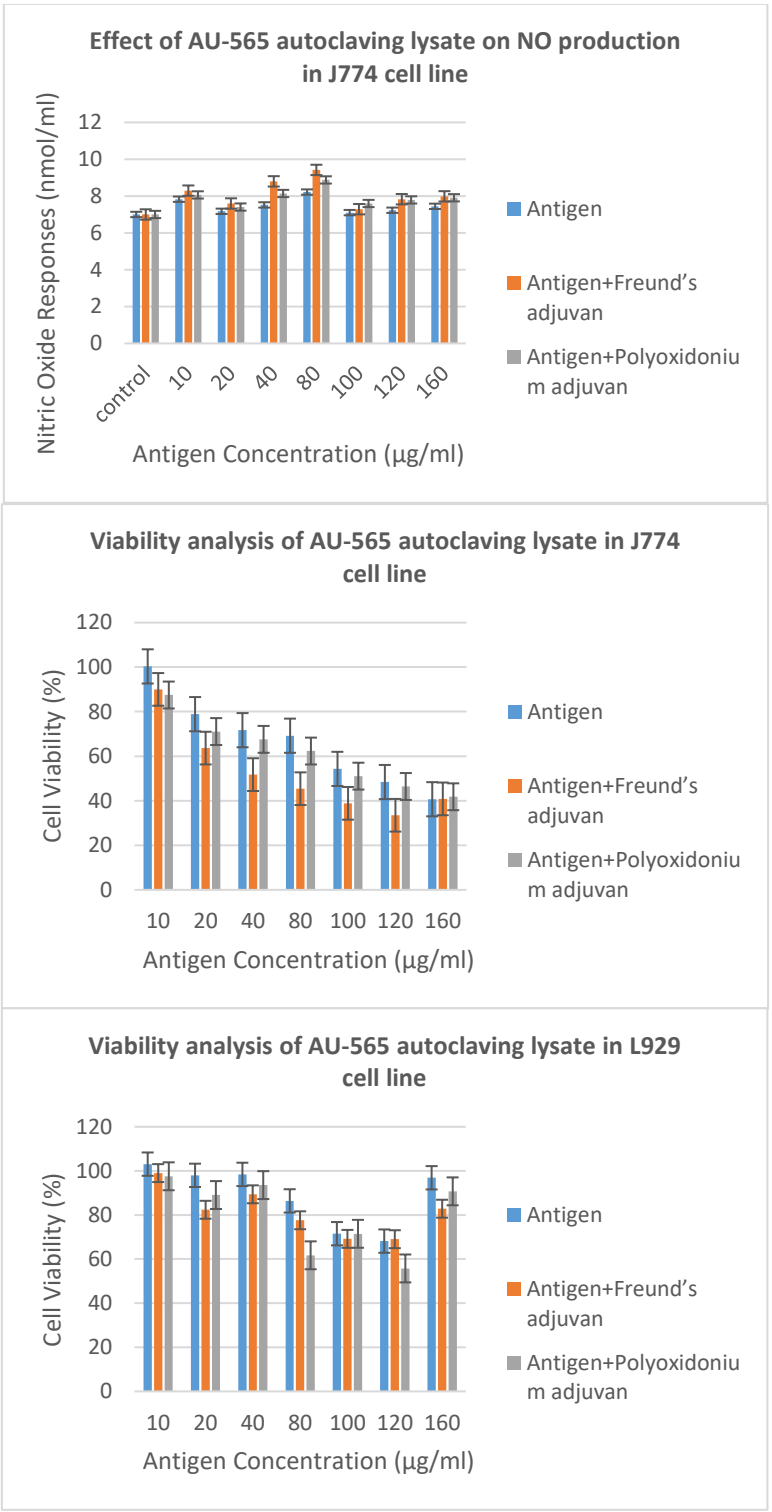

Figure 3. Viability analysis based on immunostimulant activity and cytotoxicity of macrophage and fibroblast cell lines treated with antigens prepared by autoclaving from AU-565 breast cancer cell line

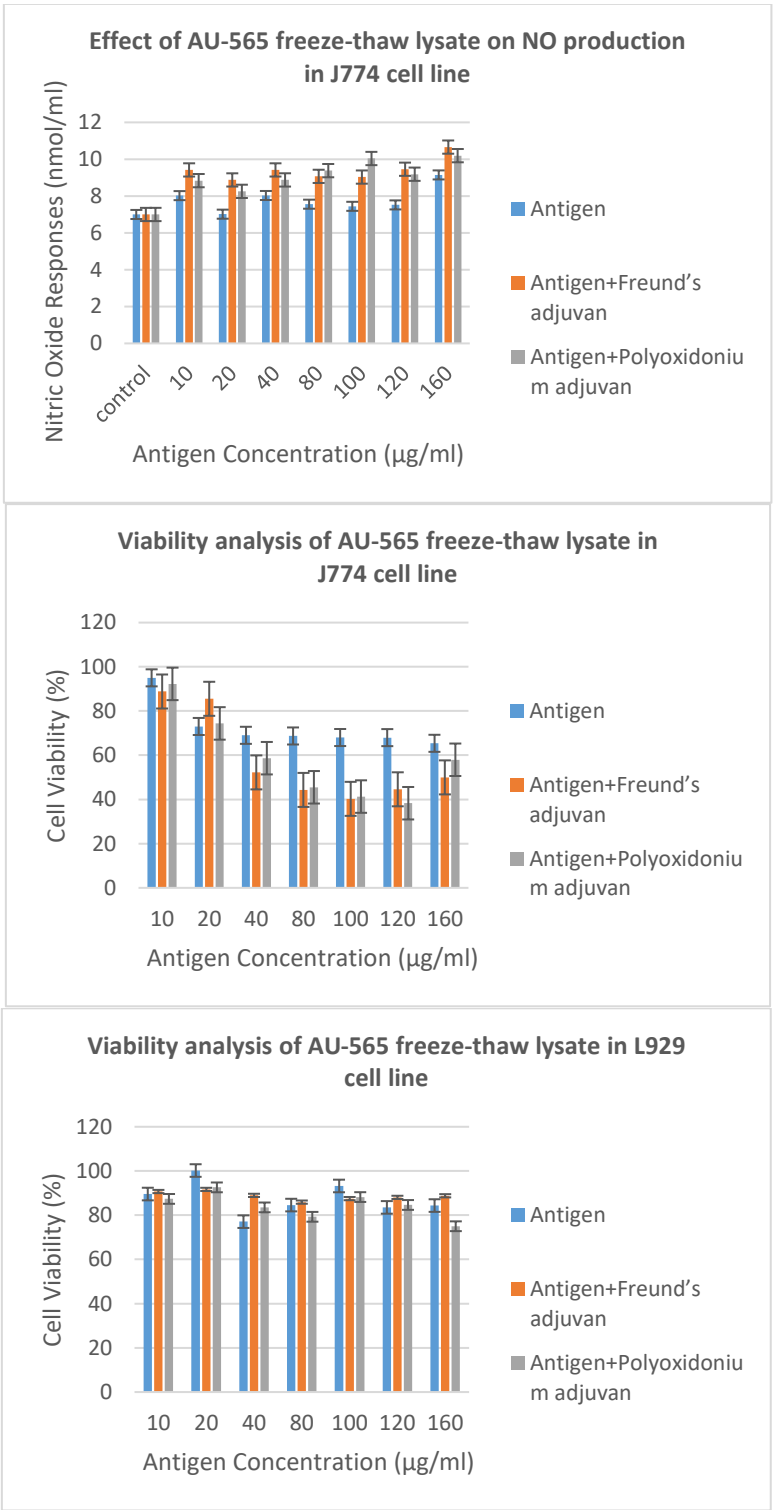

Figure 4. Viability analysis based on immunostimulant activity and cytotoxicity of macrophage and fibroblast cell lines treated with antigens prepared by freeze-thawing method from AU-565 breast cancer cell line

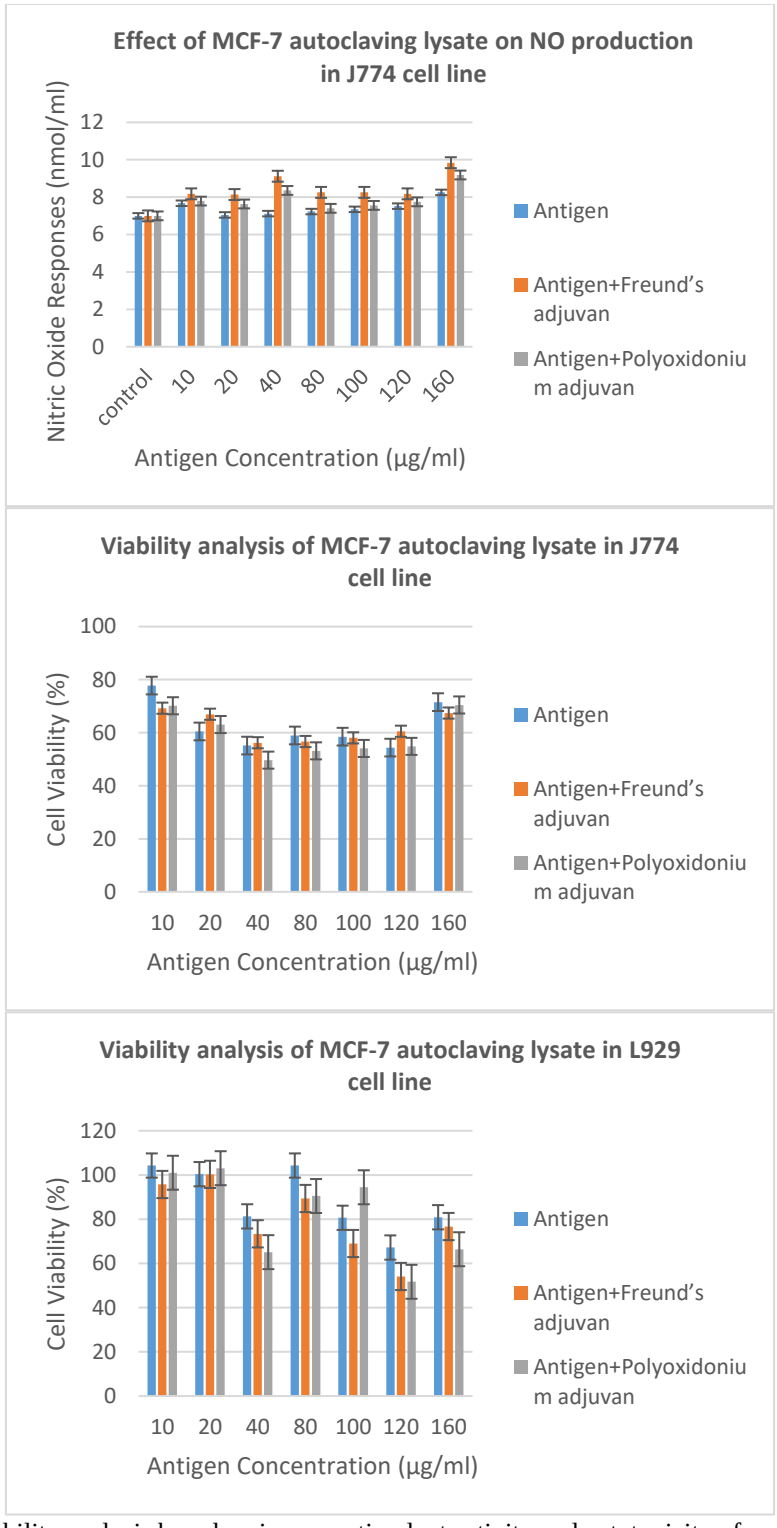

Figure 5. Viability analysis based on immunostimulant activity and cytotoxicity of macrophage and fibroblast cell lines treated with antigens prepared by autoclaving from MCF-7 breast cancer cell line

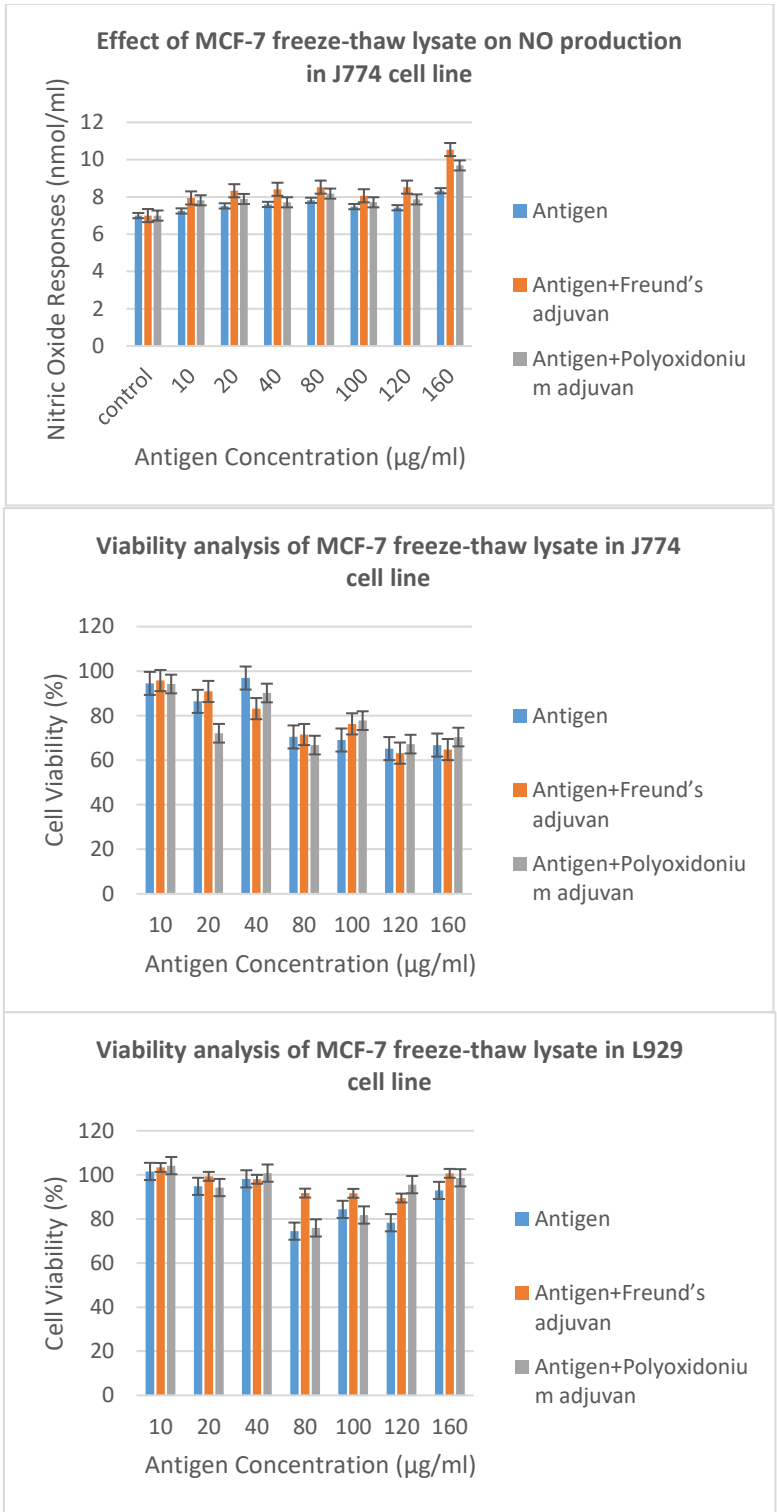

Figure 6. Viability analysis based on immunostimulant activity and cytotoxicity of macrophage and fibroblast cell lines treated with antigens prepared from MCF-7 breast cancer cell line by the freeze-thaw method

4. Conclusion and Comment

The purpose of the study is to use the antigens prepared by autoclaving and freeze-thawing methods using AU-565 and MCF-7 breast cancer lines alone and in combination with Freund's and Polyoxidonium adjuvants in vaccine studies.

In the literature, there are antigenic structures prepared for vaccine formulation by freeze-thaw and autoclaving methods. These antigen preparation methods are not only developed for cancer vaccines, but they are also used in vaccine studies for different organisms such as leishmania**.** In studies using pure antigen, nitric oxide production is lower than control groups. However, when used with adjuvant combinations, the nitric oxide production efficiency of the prepared formulations is high. In the light of this information, the results obtained in this study are consistent with the literature [19].

With this study, the most appropriate antigen preparation method for AU-565 and MCF-7 breast cancer lines and the most appropriate antigen concentration for vaccination can be determined. Moreover, the vaccine formulations combined with the adjuvant to be used in in vivo studies can be determined.

As a result, it was determined that antigens prepared from the AU-565 cell line by freeze-thaw method showed the highest immunostimulant activity at 160 µg/ml concentration. Also, it is proven that antigens prepared from the MCF-7 cell line by freeze-thaw method showed the highest immunostimulant activity at 160 µg/ml concentration. In addition, it has been found that Freund's adjuvant has a higher immunostimulant effect compared to Polyoxidonium. The results show that combinations of lysates isolated from AU-565 and MCF-7 lines with Freund's and Polyoxidonium adjuvants may elicit a higher immune response than lysate alone. It is thought that antibodies to be obtained from in vivo studies using these methods can be used in the diagnosis and treatment of breast cancer.

Author Statement

Murat IHLAMUR: Investigation, Original Draft Writing
Hümeyra BAŞARI: Investigation, Visualization, Review, and Editing
Yağmur ZENGİN: Investigation, Visualization, Review, and Editing
Emrah Şefik ABAMOR: Validation, Supervision

Acknowledgment

As the authors of this study, we declare that we do not have any support and thank you statement.

Conflict of Interest

As the authors of this study, we declare that we do not have any conflict of interest statement.

Ethics Committee Approval and Informed Consent

As the authors of this study, we declare that we do not have any ethics committee approval and/or informed consent statement.

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