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In-vitro Evaluation of the Antibacterial and Cytotoxicity Activity of the PAD4 Antigen of Bacillus anthracis as a vaccine candidate

Mohsen Parsa [1], Jamil Zargan [1*], Hossein Honari [1], Ashkan Hajinourmohammadi [1], Seyyed Mohsen Mousavi [1], Hani Keshavarz Alikhani [2]

1. *The center of biological science and technology, Imam Hussein University, Tehran, Iran*
2. *Biology Department, Razi University, Kermanshah, Iran*

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ABSTRACT

Introduction: Infectious diseases are one of the main causes of death worldwide. This has driven scientists to invest in extraction and identification of antimicrobial agents from natural toxins and presentation of novel antibiotics and vaccines. The aim of the current study is to investigate the antibacterial and cytotoxicity effects of the protective antigen domain 4 (PAD4) from Bacillus anthracis as a strong immunogen and vaccine candidate for B. anthracis.

Matherial and Methods: In this study, the antibacterial effect of the antigen was evaluated in concentrations of 0.28-4.5µg/ml using MTT reduction and MIC assays and the anticancer effect of the recombinant PAD4 on MCF-7 cell line was examined in concentrations of 0.5-2µg/ml via MTT, neutral red uptake, and comet assays. NO, GSH and catalase determination assays following the treatment with PAD4 was also evaluated.

Results: According to the antibacterial results, PAD4 did not show any antibacterial effect against S. aureus, but very little inhibition on E. coli cells' growth was recorded. The results of MTT and neutral red assays showed that this antigen has a significant inhibiting effect on cancer cell growth. Comet assay results showed that PAD4 can cause death of breast cancer cells by apoptosis induction. NO, GSH and catalase determination assays did not show any significant fluctuations following the treatment with PAD4.

Conclusion: Our results showed that this antigen does not have any antibacterial effect but it can inhibit the proliferation of breast cancer cells, making PAD4 a candidate for producing antitumor drugs.

Corresponding Information: *Jamil Zargan, The center of biological science and technology, Imam Hussein University, Tehran, Iran.*
E-mail: kpahaji@ihu.ac.ir

Abbreviations

PAD4, protective antigen domain 4; EF, edema factor; PA, protective antigen; LF, lethal factor; NO, Nitrite oxide; GSH assay, Reduced glutathione assay; GPx, Glutathione peroxidase; GSSG, Oxidized glutathione

Introduction

Bacillus anthracis is the cause of anthrax, a zoonotic disease which is transmitted to humans via direct or indirect contact with grazing herbivores, such as cattle, sheep, etc (1). This bacterium has a circular DNA of 4.5 megabase pairs which has been sequenced (2). Besides the circular DNA, the bacterium also has two very significant plasmids which contain the genes for virulence and capsid production (3).

A pathogenic B. anthracis must contain two large plasmids: pXO1 which holds the genes coding the toxins and pXO2 inclosing the capsule producing genes. Removal of either plasmid results in the deletion of the genes coding the toxin or the capsule proteins, which in turn causes the loss of pathogenicity. The toxin is produced by the plasmid PBA1 or pXO1 which hold coding genes for three proteins: the edema factor (EF), the protective antigen (4) and the lethal factor (5). The protective antigen is coded by the *pag* gene enriched in A/T (69%) and lacks cysteine.

The molecular weight of the protein is 83kDa, consisting of 735 amino acids which result in a flat and tall protein. This protein enables the entrance of the EF and LF toxins to the cytosol (6).

A study in 2001 on some domains of PA in A/J mice showed that the immune response to these domains is similar to that caused by the complete recombinant protein (7). So PA is the major component of the current vaccine against anthrax infection (4).

The cell binding domain of protective antigen (PAD4) has high immunogenicity potential and always was considered as a vaccine candidate against anthrax (8, 9). For this reason, PAD4 has been considered for vaccine design against B. anthracis as PAD4 preparation is more cost-efficient than PA preparation. It should be mentioned that for assurance of the lack of side effects and toxicity of the antigen in vitro, the antibacterial properties of PAD4 on prokaryotic cell (E. coli and S. aureus) were analyzed using MIC and MTT assays andalso cytotoxic effects of this protein on MCF7 cell line was evaluated.

Materials and method

Recombinant PAD4 was provided by Biology Research Center of Imam Hossain University (10).

- Microorganisms and cell lines:

The Gram-negative bacteria *E. coli* (ATCC: 25922) and a pathogenic strain of the Gram-positive bacteria *S. aureus* (ATCC: 25923) were purchased from the Persian Type Culture Collection (PTCC) and the MCF7 breast cancer cell line was provided from the cellular banks of Iran's Biological Research Center.

- Antigen solution preparation

The buffers needed for antibacterial tests were passed through a 0.2 µm filter. The PAD4 antigen was dissolved in PBS buffer (pH=7.4) and to prevent any contamination during the cellular assays, a 1% solution of antibiotics-antimycotics (Invitrogen, USA) was added and stored overnight at 4°C prior to use. It should be mentioned that the Bradford method was used for protein assays.

- Antibacterial assays

I. Antibacterial assessment of the recombinant PAD4 via MTT assay:

The MTT assay was used for assessment of antibacterial properties of recombinant PAD4 (11).

The bacterial strains were cultured in a Muller-Hinton Broth (MHB) medium and incubated for 5 hours in 37°C, was reached, 5µl of each bacterial solution (0.5 McFarland (0.08 – 0.1 OD in 600nm)) was transferred to sterile 96-well plates. The recombinant PAD4 solution was added to the wells in concentrations of 0.28, 0.56, 1.12, 2.25 and 4.5µg/ml. MHB was added to each well to reach the 100µl standard. For positive and negative controls, 50µg/ml tetracycline solution and a suspension of the bacteria and the culture medium were used, respectively. A culture medium with no bacteria was used as a blank solution. Each plate was incubated for 23 hours at 37°C. Then 5µl MTT (concentration of 5µg/ml) was added to each plate. The plates were then incubated at 37°C for 1 hour in darkness condition. After formation of Formazan crystals, 100µl DMSO (Sigma-Aldrich, USA) was added to each well and incubated 2 hours, the ODs were measured using a plate reader (Biotek, USA). This assay was performed 3 times, so 3 repeats would be obtained for each concentration. The viability of the bacteria after incubated with different concentrations of the recombinant PAD4 was calculated with the formula given below:

$$Bacteria\ Vitality\ \% = \frac{OD_{sample} - OD_{Blank}}{OD_{negative\ control} - OD_{Blank}}\ x\ 100$$

II. Antibacterial assessment of the recombinant PAD4 via MIC assay:

In this study, the antibacterial activity of recombinant PAD4 was assessed *in vitro*. The steps for the MIC (minimum inhibitory concentration) assay (12) were similar to that of the MTT assay, but after incubation of the bacteria at 37°C for 24 hours, without adding any other reagents, the OD of each well was measured at 605nm using a plate reader (Biotek, USA). This assay was also repeated 3 times for each concentration. The inhibition percentage of the recombinant PAD4 was calculated via the below formula:

$$Inhibition\ \% = \left(1 - \frac{OD_{sample} - OD_{Blank}}{OD_{negative\ control} - OD_{Blank}}\right) x\ 100$$

- Toxicity and anticancer evaluation of the recombinant PAD4

I. Cell culture:

For MCF7 culture, DMEM-F12 containing 10% FBS (Gibco, USA) was used as a culture medium.

The cells were cultured in 25-50m^3 culture flasks and were stored at 37°C and 5% CO_2.

The culture medium was replaced every 2-3 days.

II. Toxicity evaluation of the recombinant PAD4 via MTT reduction:

This assay was done according to the method used by Zargan et al. In this method, 3×10^4 cells were cultured in 96-well plates. The cells were incubated overnight at 37°C with 5% CO_2 and 80% humidity. Thus, the old culture medium was evacuated and new culture medium containing concentrations of 0.5, 1 and 2µg/ml PAD4 was added to the wells and the plates were incubated for 23 hours at the stated conditions. After that, 5µl MTT solution (5µg/ml) was added to each well and incubated for 2 hours in order to formation of formazon crystals. Then each well was washed with PBS and 100µl DMSO was added. The culture medium and medium containing cells were used as positive and negative controls in this assay, respectively. The OD for each plate was determined using a plate reader (Biotek, USA) at 570nm. This assay was also repeated 3 times. The viability percent of cells after incubation with the recombinant PAD4 was evaluated according to the formula given below:

$$Cell\ Vitality\ \% = \frac{OD_{treated\ cells} - OD_{Blank}}{OD_{negative\ control} - OD_{Blank}}\ x\ 100$$

III. Toxicity evaluation of the recombinant PAD4 via neutral red uptake assay:

The procedure for this assay (13) are similar to that of the MTT assay mentioned above, but after incubation with the recombinant PAD4 for 23 hours, instead of MTT solution, 1µl neutral red solution (5µg/ml) was added to each well. Thus, the cells were incubated at darkness conditions at 37°C with 5% CO_2 and 80% humidity until the formation of neutral red crystals (upon the linkage of neutral red with the cell lysozyme). The solution in each well was evacuated and after washing each cell with PBS, 100µl fixing buffer (37% formaldehyde, 10% calcium chloride) was added and incubated for 1 min. Then100µl of the solvent buffer (0.5% acetic acid) was added. The plates were then placed on a shaker for 20 minutes in darkness conditions and room temperature. Thus, the ODs were assessed at 540nm via a plate reader (Biotek, USA). This assay was also repeated 3 times.

The growth inhibition percent of different concentrations of recombinant PAD4 was evaluated according to the below formula:

$$Cell\ Inhibition\ \% = \left(1 - \frac{OD_{treated\ cells} - OD_{Blank}}{OD_{negative\ control} - OD_{Blank}}\right) x\ 100$$

- Assessment of apoptosis induction by recombinant PAD4 by Comet assay:

Comet assay or single-cell gel Electrophoresis is one of the best methods for DNA fragmentation analysis (14). 12×10^4 cells were cultured in 24 well plateand incubated overnight at 37°C, 5% CO_2 and 80% humidity. The culture medium and culture medium containing cells were used as the blank and control, respectively. After that, the primary culture medium was evacuated from each well and 300µl of new free serum medium and 0.5, 1 and 2µg/ml of the recombinant PAD4 was added. The plates were incubated for 24 hours at 37°C, 80% humidity and 5% CO_2. After 24 hours, the cells from each well were collected using trypsin-EDTA solution and gathered in a 1.5ml tube. The microtubes were centrifuged at 1500rpm for 5 minutes at 4°C and the supernatant was discarded. The cells were washed with PBS (pH=7.4). Thus, 200µl PBS was added to each microtube and with a sampler and insulin syringe, the cells were separated from each other to become singular. The required slides were covered with normal melting point agarose (1% NMA) suspension. The cell suspension was mixed with a low melting point agarose (1% LMA) by a 1 to 2 ratio and the solution was transferred to the slides. In order to create one layer of cells, a covering glass was placed on each slide. In order to achive cell lysate, all the slides were placed in cold, fresh lysis buffer (2.5M NaCl, 100mM EDTA, 10mM Tris, 9,2M NaOH, 1% Triton X-100, pH=10) for16-18 hours in 4°C. Then, the slides were washed for 20 minutes with an electrophoresis buffer two times (300mM NaOH, 1mM EDTA, pH>13) and in order to separate the double-stranded DNA, the slides were placed in a cold electrophoresis buffer and were incubated in the refrigerator for 40 minutes. Subsequently, the slides were placed in an electrophoresis tank containing buffer and electrophoresis was done in 4°C for 45 minutes in 25V and 300mA. The samples were placed in a neutralizing buffer (100mM Tris, pH=7.5) for 10 minutes so the basic environment would be balanced.

For staining, 100µl of ethidium bromide was added to each slide and the slides were placed in room temperature for 10 minutes. The slides were then washed for 10 minutes with doubled distilled water and examined under a fluorescent microscope.

For each sample, images of at least 100 cells were gathered and the results were analyzed statistically.

- Cellular NO assessment via reactive nitrogen species assay:

NO assay was done according to the Griess reaction assay (15). The cells were cultured in 96-well plate; 2×10^4 cells per each well. The plate then incubated for overnight. After that, the culture medium was emptied and the cells, along with fresh medium, were exposed to different concentrations of the recombinant PAD4 (0.5, 1 and 2µg/ml). After 24 hours, the culture medium was emptied and the contents of each well were transferred to a vial. After centrifugation under 500g for 5 minutes in 4°C, 100µl of the supernatant was transferred to a 96-well plate and mixed with equal amounts of Griess reagent (0.04g/ml in PBS, pH=7.4Sigma aldrich USA). Thus, the plates incubated in room temperature for 10 minutes. The ODs were read in 540nm with a microplate reader (Bio-Rad, USA). Nitrite concentrations in control cells and treated cells were calculated using the NO standard graph and presented as µM/ml.

- GH evaluation in breast cancer cells

Reduced glutathione (GSH) evaluation was determined according the Owen JB *et al*. method (16). 5×10^5 cells were cultured in 24-well plate in. After incubation for an overnight, a fresh culture medium with different concentrations of recombinant PAD4 (0.5, 1 and 2 µg/ml) was added to each well and the plates were again incubated for 24 hours. The cells were then removed from the plates, washed with PBS (pH=7.4) and centrifuged for 5 minutes under 5000g in 4°C. Thus, they were incubated in -20°C for 30 minutes. 100µl of cold lysis buffer, containing 10mM Tris-HCL, 150mM NaCL, 1mM EDTA and 1% Triton X-100 (pH=7.5), was added and the plates were then incubated in room temperature for 30 minutes. The cell extract was sonicated for 10-15 minutes and then centrifuged for 10 minutes under 2000g and the supernatant was gathered. The protein concentration was evaluated via the Bradford method. After that, equal amounts of 10% TCA were added to the supernatant and the solution was stored at 4°C for 2 hours. The contents were then centrifuged for 15 minutes under 5000g and the supernatant was gathered. Thus, 20µl of the sample was mixed with 75µl lysis buffer, 55µl Tris buffer containing 0.02M EDTA (pH=8.5), and 25µl DTNB (5,5/ dithiol bis (2-N benzoic acid)).

The ODs of the chromogen (yellow) were assessed in 412nm with a microplate reader. The result was expressed as µg GSH/mg protein using molar extinction coefficient of 13600.

$$GSH\ estimation = \frac{OD\ x\ 1.998}{Sample\ protein\ (mg)}$$

- Catalase activity evaluation

This assay was done according to the method proposed by Montavon *et al*. (17) and the extracellular catalase activity was evaluated. In a 24-well plate, 5×10^5 cells were cultured in each well. After overnight incubation, the fresh culture medium was replaced and different concentrations (0.5, 1 and 2µg/ml) of PAD4 recombinant protein was added. The plates were incubated for 24 hours. Cells then trypsinized and harvested in fresh 1.5 ml tubes and centrifuged at 1500 rpm for 5 min at 4°C and washed with PBS (pH 7.4) two times and incubated at -20°C for 30 min. 200 µl chilled lysis buffer (NaCl 2.5 M, EDTA 100 mM, Tris 10 mM, NaOH 0.2 M, Triton X-100 %1 and pH 10) was added and incubated for 30 min at room temperature. The lysate was sonicated for 10-15 minutes and then, centrifuged under 2000g for 10 minutes and the supernatant was gathered. After protein assessment via the Bradford method, 5µl of the sample was transferred to an Eppendorf tube and was mixed with 50µl of the lysis buffer, 20µl double distilled water and 25µl H_2O_2 (15%). After the mixtures were shaken, they were incubated for 2 minutes at 37°C. They were then mixed with 100µl dichromate acid reagent (0.1 M potassium dichromate in glacial acetic acid) and placed in a water bath of 100°C for 10–15 minutes so the sample color would change to a greenish/faint color. Then, 200µl of the sample was poured into the wells of a 96-well plate and the ODs were assessed with a plate reader at 570nm. The results were converted into activity using molar extinction of catalase as 43.6 and expressed as µm of hydrogen peroxide consumed/min/mg protein.

$$Catalase\ estimation = \frac{OD\ x\ 36.49}{Sample\ protein\ (mg)}$$

- Statistical analysis

The results of each test were presented as Mean±SD values and the acquired data were analyzed statically using the GraphPad InStat software.

The results from the different protein concentrations were surveyed in comparison to the control group and also, to the other concentrations via the One-way Anova and the tukey test. The significance level was set to $p<0.05$. Microsoft Excel (2013) was used to draw the graphs for this study.

Results

I. Antibacterial assessment of the recombinant PAD4 via MTT assay:

The results from the MTT assay showed that at concentrations of 0.28, 0.56, 1.12, 2.25 and 4.5µg/ml, there is significant inhibition of growth when compared to the control. But the inhibition for each concentration is not considered significant when compared to other concentrations. Viability percentage for the bacteria at concentrations of 0.28, 0.56, 1.12, 2.25 and 4.5µg/ml is 80.16, 82.6, 87, 81 and 83.6, respectively.

The recombinant protein did not show any significant inhibition of growth on the S. aureus at concentrations of 0.28, 0.56, 1.12, 2.25 and 4.5µg/ml. The viability percentage of the bacteria at concentrations of 0.28, 0.56, 1.12 and 2.25µg/ml was 100 and at 4.5µg/ml, 93.

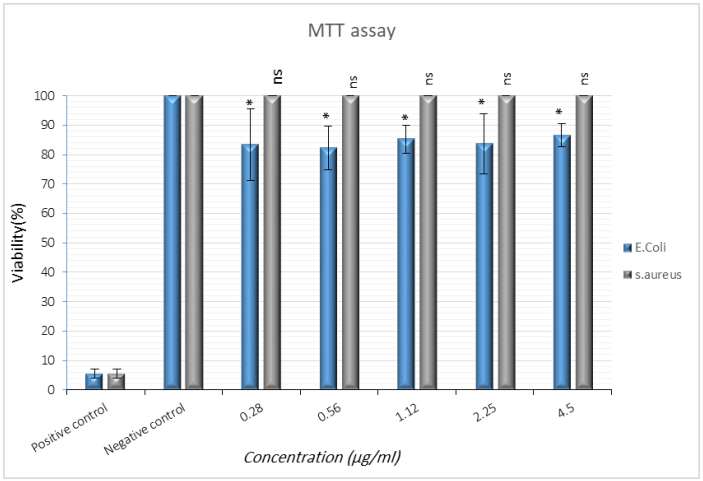

Figure 1. Viability percentage of E. coli and S. aureus after exposure to certain concentrations of the recombinant PAD4 based on MTT assay. The results for each concentration were assessed with respect to the control group. (: p<0.05), (**: p<0.01), (***: p<0.001), (ns: non-significant).*

II. MIC assay results for the recombinant PAD4:

In vitro antibacterial assessment of the recombinant PAD4 via the MIC assay showed that:

1. The viability percentages of E. coli at concentrations of 0.28, 0.56, 1.12, 2.25 and 4.5µg/ml of the recombinant PAD4 were 20, 28.9, 23.7, 23.7 and 39.3, respectively. The results for the mentioned concentrations, when compared to the negative control, were significant.

2. The viability percentages of S. aureus at concentrations of 0.28, 0.56, 1.12, 2.25 and 4.5µg/ml of the recombinant PAD4 were 15.43, 16.93, 23.95, 15.56 and 19.6, respectively. The results for the mentioned concentrations, when compared to the negative control, were significant.

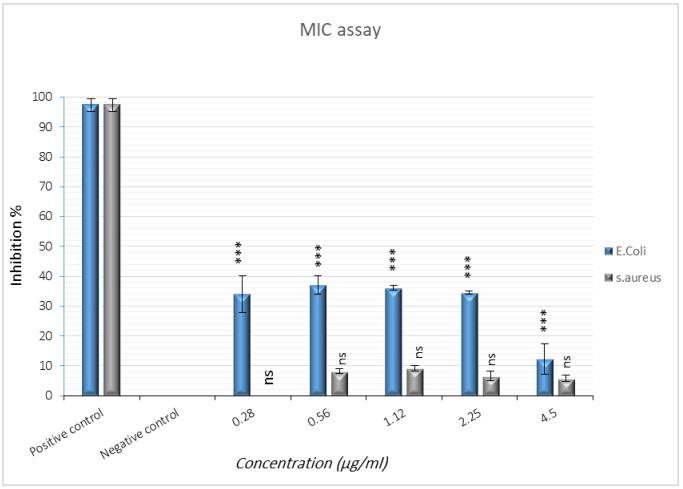

Figure 2. Viability percentage of E. coli and S. aureus after exposure to certain concentrations of the recombinant PAD4 based on the MIC assay. The results for each concentration were assessed with respect to the control group. (: p<0.05), (**: p<0.01)), (***: p<0.001), (ns: non-significant).*

III. Cytotoxicity evaluation results of the recombinant PAD4

- MTT assay results for the effect of the recombinant PAD4 on MCF7 cell growth:

In this study, the MTT assay showed that PAD4 recombinant ptotein has cytotoxic effect on MCF7 cell line. Viability percentage of the cells under the influence of PAD4 in concentrations of 0.5, 1 and 2µg/ml was 89, 61 and 46.3, respectively. The recombinant protein proved to have significant inhibition in the mentioned concentrations with respect to the control (MCF7 cells and culture medium).

- Neutral red uptake results for the effect of the recombinant PAD4 on MCF7 cell growth:

Inhibition percentage of PAD4 in concentrations of 0.5, 1 and 2µg/ml on MCF7 cells was 30.2, 40 and 50.56, respectively.

The PAD4 recombinant protein showed to have significant inhibition of the cells in the mentioned concentrations in respect to the control (MCF7 cells and culture medium).

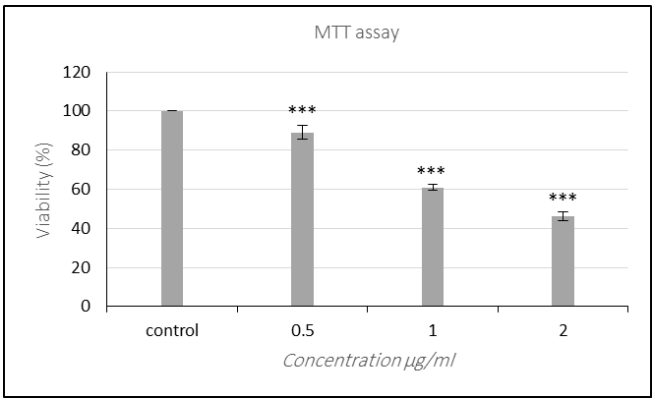

*Figure 3. Evaluation of different concentrations of the recombinant PAD4 on breast cancer cells via the MTT assay. The concentrations were assessed with regard to the control (***: p<0.001).*

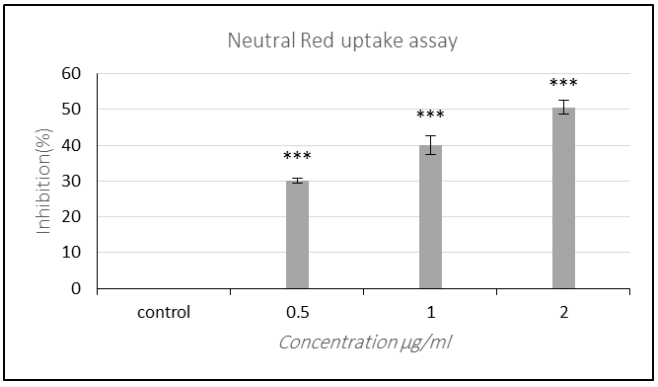

*Figure 4. Evaluation of different concentrations of the recombinant PAD4 on breast cancer cells via the neutral red uptake assay. The concentrations were assessed in regard to the control (***: p<0.001).*

• Comet assay for cytotoxic evaluation of the recombinant PAD4 on MCF7 cell growth:

The comet assay results showed that apoptosis induction by concentrations of 0.5, 1 and 5µg/ml of the recombinant PAD4 was 18.64%, 19.63%, and 29.69%, respectively, which were statistically significant in regard to the control.

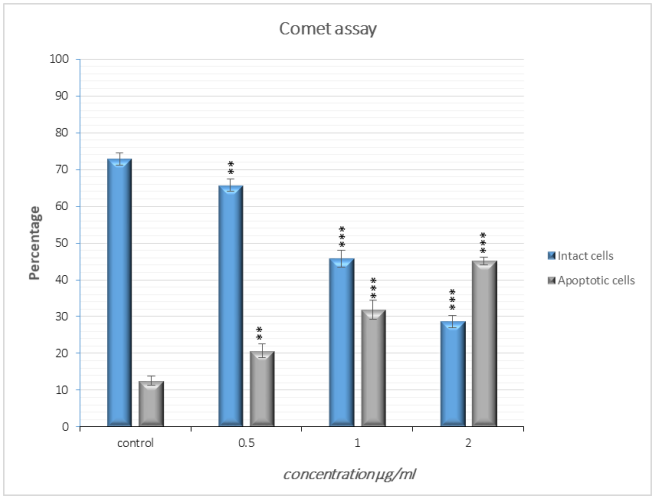

Figure 5. Percentage of apoptosis induction by the recombinant PAD4 in breast cancer cells assessed by the Coet assay. The results from each concentration were evaluated with respect to the control group. (: p<0.05), (**: p<0.01), (***: p<0.001).*

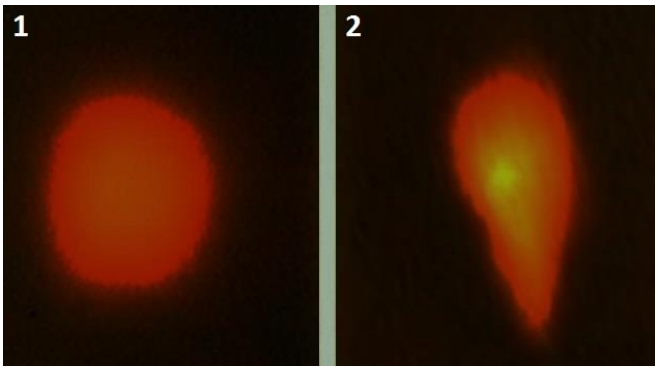

Figure 6. Images gathered from the Comet assay; 1) DNA from intact cells, 2) apoptotic cells; 40x.

IV. Reactive oxygen intermediated

The amount of nitric oxide in cells treated with concentrations of 0.5, 1 and 2µg/ml of the recombinant PAD4 was 39.61, 40.05 and 40.23, respectively, which were not significant when compared to the control.

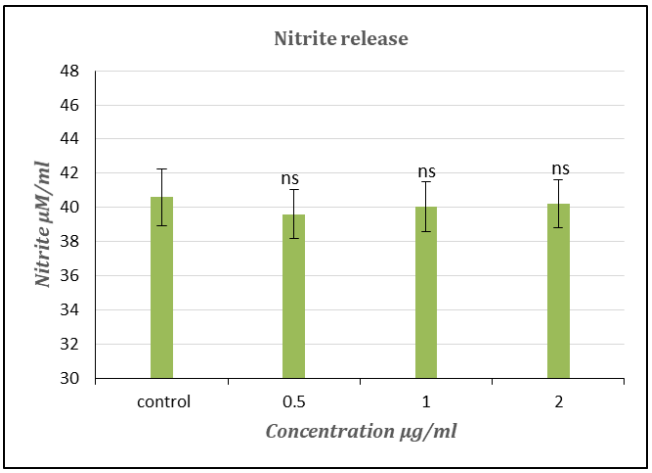

Figure 7. The amount of NO relased from MCF7 cells into the media measured via the reactive nitrogen intermediate assay. The results for each concentration were assessed in regard to the control. (ns: non-significant).

V. Reduced glutathione

In this study, the possible variations in glutathione levels in cells treated with the recombinant PAD4 in concentrations of 0.5, 1 and 2µg/ml were assessedThe amount of glutathione under the influence of 0.5, 1 and 2µg/ml of the recombinant PAD4 were 10.97, 10.8 and 10.34, respectively. These results were not significant when compared to control (10.85).

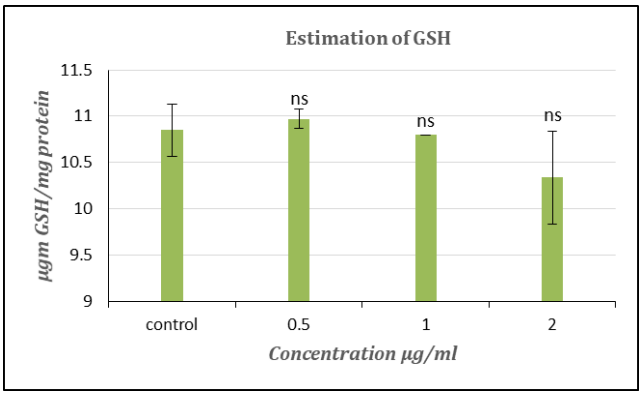

Figure 8.The amount of glutathione produced under the effect of the recombinant PAD4 assessed via the reduced glutathione (GSH) assay. The results for each concentration were evaluated with respect to the control. (ns: non-significant).

VI. Catalase activity test results

Catalase levels in cells treated with the recombinant PAD4 in concentrations of 0.5, 1 and 2µg/ml were also evaluated. The amount of catalase in cells under treatment with the mentioned concentrations was 744.45, 743.46 and 663.485, respectively. The reduction of catalase for concentrations of 0.5 and 1µg/ml was significant in comparison with the control.

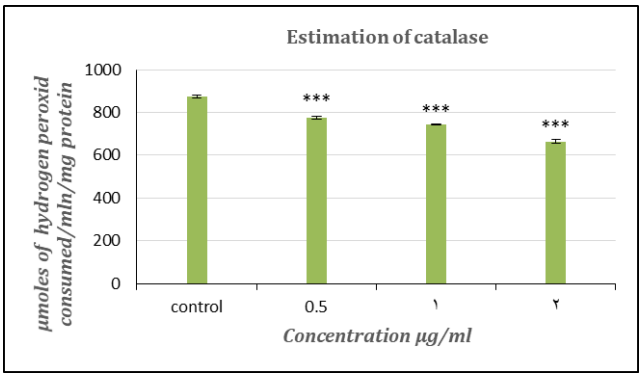

*Figure 9. The amount of catalase produced in response to the recombinant PAD4 in breast cancer cells assessed via the catalase activity assay. The results for each concentration were evaluated with respect to the control. (**: p<0.01) and (ns: non-significant).*

Discussion

PA is one of the recognized antigens for the induction of supportive antibodies against Anthrax. The PA protein is one of the main vaccine candidates considered in researches. When PA is produced in the absence of LF and EF as a purified protein, it has proven to be effective as a recombinant or weakened vaccine (18).

In 2001, Flick-Smith et al. conducted a study on the different domains of PA along with aluminum hydroxide (1.3%) as an adjuvant on A/J mice. They showed that the immune response induced after immunization with incomplete regions of the recombinant PA against STI spores of B. anthracis is similar to that induced by the complete, recombinant PA. After more research, in this study, it was proven that all the groups consisting of the domain 4 in their composition were completely preserved (19). Brossier et al. in another study showed that there is a reduction in immunity of mice immunized with a mutant strain of B. anthracis without the PA domain 4 (20). On another hand, it was shown that the monoclonal antibodies produced against PA, have the most interactional with PAD4, which further validates the importance of this region (21). All the evidence confirms that PAD4 is a principal immunodominant, which can be employed to design an engineered vaccine. For the first time, the antibacterial activity of the recombinant PAD4 was investigated in this study. The MTT reduction assay and MIC assay were employed for this matter. The results showed that the PAD4 antigen has very little inhibitory effect on E. coli and no effect on S. aureus.

Also, in this study, the cytotoxic effect of the recombinant protein was assessed in vitro via the MTT assay, neutral red uptake assay and single-cell gel electrophoresis (Comet assay). The effect of the protein on the production of NO, GSH, and catalase in cells was also examined.

Results of the MTT assay and neutral red uptake assay showed that the recombinant PAD4 has significant inhibitory effects in concentrations of 0.5, 1 and 2µg/ml on the growth of breast cancer cells. The Comet assay results also proved that the protein significantly induces apoptosis in cells in the mentioned concentrations. Evaluation of the results from the MTT assay, neutral red assay, and comet assay showed that the PAD4 causes death in a cancer cell by necrosis and apoptosis induction.

The results from this study are consistent with the results from previous studies which proved that the fatal toxin from B. anthracis can destroy macrophages by necrosis or apoptosis, depending on two factors: toxin concentration and phosphatase activity (22). Rogers et al. showed that wild-type PA inhibits both vascular endothelial growth factor–induced and basic fibroblast growth factor–induced angiogenesis at moderate but statistically significant levels. Structure-activity studies identified a PA mutant that exhibited markedly enhanced inhibition of angiogenesis and also inhibited tumor growth in vivo (23).

Leppla and coworkers have constructed mutated PA proteins in which the furin protease cleavage site has been replaced by sequences selectively cleaved by MMPs 2, 9, and urokinase plasminogen activator. These mutated PA proteins selectively translocate toxin into MMP-over expressing tumor cells while sparing non-tumorigenic cells when these were grown together in a co-culture model (24).

The investigation also shows that the PAD4 does not have a significant effect on the amount of cellular NO and glutathione in treated cells compared to the control, but in the case of the amount of catalase it's not like that. These results prove that NO and GSH do not have a role in apoptosis induction by PAD4 in breast cancer cells. Cui et al. showed that continuous anthrax lethal toxin infusion is associated with circulatory shock but not inflammatory cytokine or nitric oxide release in rats (25).

Conclusion

Overall, this study shows that the PAD4 antigen significantly inhibits the proliferation of breast cancer cells in vitro, which is very important from a pharmacologic prospect and introduces this antigen as a valuable anticancer molecule. However, no significant antibacterial activity was recorded for PAD4.

Declaration

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Conflicts of interest/Competing interests

The authors declare no conflict of interest.

Authors' contributions

M.P. involved in methodology, and writing the original draft, J.Z. and H.H. involved in conceptualization and methodology, A.H.N., M.M. involved in methodology, H.K.A. involved in methodology, and writing the original draft.

Ethics approval

Not applicable.

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