Original Article

In Vitro Study of the Toxicity Effects of *Bacillus anthracis* Protective Antigen

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Abstract

Anthrax, a common disease of human and cattle, is caused by *Bacillus anthracis* infection. Protective antigen (PA) from Bacillus anthracis is a potent immunogen, which has been of interest in the development of new candidate vaccines against the disease. In this study, the toxicity effects of this antigen on prokaryotic (*Escherichia coli* and *Staphylococcus aureus*) and eukaryotic (MCF-7) cells were investigated. Antibacterial effects of the recombinant PA were analyzed using MTT and MIC (Minimum Inhibitory Concentration) assays. Cytotoxicity effect of the recombinant protein (in concentrations ranging from 0.5 to 2 µg/ml) on MCF-7 cell line was analyzed using MTT, Neutral red uptake, and comet assays. MCF-7 cells' oxidative stress following the treatment with PA (0.5-2 µg/ml) was analyzed by NO assay, reduced glutathione assay (GSH), and catalase activity assay. MTT and MIC assays showed that PA has a low inhibitory effect on *Escherichia coli* and no inhibitory effect on *Staphylococcus aureus*. Cell cytotoxicity assays indicated that the antigen significantly inhibits the growth of MCF-7 cells. Comet assay also showed that the antigen induces apoptosis in MCF-7 cells. According to nitrite oxide, reduced glutathione, and catalase activity assays, PA has not a significant effect on MCF-7 cells in comparison to the control ($P<0.001$). Protective antigen has no significant inhibitory effect on the growth of bacterial cells. However, it significantly inhibits the growth of the breast cancer cells ($P<0.001$). The effect of PA on breast cancer cells is pharmacologically important so that the antigen can be considered as a candidate anticancer molecule.

Keywords: *Bacillus anthracis*, Protective Antigen, Antibacterial, Anticancer, Cytotoxicity

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Introduction

Anthrax is a common disease of humans and chattels which is caused by *Bacillus anthracis* infection. Humans are infected through the direct contact to sick chattels or their products such as kips, hairs, or wools, so, people working with chattels, such as veterinarians, ranchers, microbiologists, tillers, pastors, and labors working in the slaughterhouses should be immunized against the disease. Okinaka and Brey in different studies demonstrated *pXO1* (toxins coding gene) and *pXO2* (capsule coding gene) plasmids are needed for *Bacillus anthracis* pathogenicity [1, 2]. To Dixon and Dai, have shown three proteins (Edema Factor (EF), Protective Antigen (PA), and Lethal Factor (LF)) are encoded by *pXO1* or *PBA1* plasmids [3, 4]. Protective antigen, which is coded by *Pag* gene, is a long and flat protein with no cysteine residues. This protein is responsible for the transfer of LF and EF proteins into the cytosol [5]. In 2001, demonstrated that the immunogenicity of a portion of PA following administration with aluminum hydroxide adjuvant to A/J mice was comparable to the immunogenicity level of the whole recombinant PA [6]. Another study by Yogendra *et al*., in 2001 showed that mice immunized with a mutant strain of *Bacillus anthracis* lacked the domain 4 of PA, has lower immunity level, so, PA is immunodominant antigen and can be used for the recombinant vaccine design [7]. To demonstrate toxicity of antigen in prokaryotic and eukaryotic cells and investigating the safety of the antigen as a candidate vaccine, in the present study the toxicity effects of the recombinant PA on two prokaryotic cells (*Escherichia coli*, *Staphylococcus aureus*) and one eukaryotic cell line (MCF-7) were analyzed.

Materials and Methods

PA protein and cells

Recombinant PA was obtained from Imam Hossain University, investigated by Mehr-Azin *et al*., 2011 [8]. *Escherichia coli* (ATCC 25922) as Gram-negative bacteria and *Staphylococcus aureus* (ATCC 25923) as Gram-positive bacteria were purchased from Persian Type Culture Collection (PTCC) and MCF-7 cell line was purchased from Iranian Biological Resource Center.

Antigen preparation

Phosphate buffer in which the antigen was dissolved passed through a 0.2 micron filter before doing the toxicity tests. To remove possible contaminants in PBS (pH 7.4) before doing the tests, Antibiotic-Antimycotic 1% (Invitrogen, USA) was added to the buffer and stored one night at 4°C. In this study, protein assay was performed using the Bradford method.

Determination of the antibacterial effects of PA using MTT assay

MTT assay is colorimetric method based on the reduction and breakdown of tetrazolium yellow crystals by dehydro-

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genases in the cytoplasm and make them insoluble purple crystals. DMSO was used to solve the crystals [9]. Accordingly, bacterial cells were cultured in Mueller-Hinton broth (Sigma, USA) for 5 hours and when the concentration reached to the concentration equivalent to 0.5 McFarland (the absorbance at 600 nm was 0.08-0.1), 5µl of the bacterial culture was added to each well of a 96-well plate [10]. Recombinant PA (0.28, 0.56, 1.12, 2.25, and 4.5 µg/ml) was added to wells and the final volume for each well was set to 100 µl by addition of appropriate volume of Mueller-Hinton broth. Tetracycline antibiotic (50 µg/ml) was used as positive control and bacterial suspension was used as negative control, also Mueller-Hinton broth was used as the blank. All samples were incubated at 37°C for 23 hours. Then, 5 µl MTT (5 mg/ml) (Sigma, USA) was added to each well and incubated at 37°C for an hour and then 100 µl DMSO was added and incubated for additional 2 hours. The absorbance was measured at 595 nm and the assay was repeated three times for each concentration. Bacterial cells viability was calculated using below equation [10]:

Viability = (OD sample-OD blank)/(OD negative control-OD blank) ×100 (1)

Determination of the antibacterial effects of PA using MIC assay

According to Lu *et al*., (2002) MIC assay protocol was similar to MTT assay [11], but after incubation of bacteria with different concentrations of PA at 37°C for 24 hours, the absorbance was measured at 600 nm using a plate reader (Biotek, USA). The assay was repeated three times for each concentration and inhibitory effect of PA on bacterial cells was calculated using equation 2 [12]:

MIC = (1- (OD sample-OD blank)/(OD negative control (without antibiotic)- OD blank)) × 100 (2)

Assessment of the cytotoxicity effects of PA using MTT assay

DMEM-F12 media (Gibco, USA) containing 10% fetal bovin serum (FBS) (Gibco, USA) was used for MCF-7 cells culture. Cells were cultured in 25T flasks and incubated at 37°C and 5% CO_2. The media was changed every 2-3 days and hemocytometer was used for counting the cell growth.

For cytotoxicity effects of PA, MTT assay was done using the standard protocol [13]. Briefly, 3×10^4 cells were seeded in 96-well plate containing 100 µl media (without serum) and incubated overnight in 5% CO_2 and 80% humidity at 37°C. Then, the media was discarded and fresh media containing 0.5, 1, and 2 µg/ml of recombinant PA was added and the plate was incubated for 24 hours at 37°C. 5 µl MTT dye (5 mg/ml) was added and incubated for 1 hour in dark condition at 37°C. Then, 100 µl DMSO was added to every well and incubated 2 hours in the same condition. Media was used as the blank and untreated cells were used as the negative control. The absorbance was measured at 570 nm.

The assay was repeated three times for every concentration. Bacterial cells viability was calculated using equation 3 [14]:

Viability = (OD sample-OD blank)/(OD negative control-OD blank) × 100 (3)

Assessment of the cytotoxicity effects of PA using neutral red uptake assay

Neutral red uptake assay protocol was similar to MTT assay [13, 15], but after incubation of cells with recombinant PA for 23 hours, 1 µl neutral red dye (5 µg/ml) was added to every well and incubated at 37°C. After the formation of red crystals, the supernatant was discarded and the pellet washed by PBS two times. About 100 µl fixation buffer (formaldehyde 37%, $CaCl_2$ 10%, water) was added to every well and incubated for 1 minute and then 100 µl solubilizing buffer (acetic acid 5%) was added and incubated for 20 min in dark condition in a shaker incubator and finally, the absorbance was read at 570 nm. The mortality rate was measured as equation 4:

Mortality = (1- (OD sample-OD blank)/(OD control- OD blank) × 100 (4)

Assessment of apoptosis effect of the recombinant PA using alkaline comet assay

Alkaline comet assay, also called single-cell gel electrophoresis assay, is a good method for DNA fragmentation analysis in cells. The assay was performed according to Sajad *et al*., [16]. Briefly, 12×10^4 cells were seeded in a 24-well plate containing 300 µl media (without serum) and incubated overnight in 5% CO_2 and 80% humidity at 37°C. Then, the media was discarded and 300 µl of fresh media containing 0.5, 1, and 2 µg/ml of recombinant PA were added and incubated for 24 hours at 37°C. 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. 200 µl PBS was added to tubes and cells were separated by a needle. Slides were covered by normal melting agarose (1%) and incubated for 10 min at 4°C. Cell suspensions were mixed with low melting agarose 1% (1:2 ratios) and were added to the slides.

To form one cell layer, a glass lamel was placed on every slide. In order to lyse the cells and distract the nuclei, all slides were incubated for 16-18 hours in fresh and cold lysis buffer (NaCl 2.5 M, EDTA 100 mM, Tris 10 mM, NaOH 0.2 M, Triton X-100 %1, and pH 10) at 4°C. Then, slides were washed two times with electrophoresis buffer for 20 min and incubated in fresh electrophoresis buffer for 40 min at 4°C. The electrophoresis was done in 25 V and 300 mA for 45 min at 4°C. For neutralization, slides were incubated for 10 min in neutralizing buffer (Tris 0.04M pH7.5). Then slides were incubated in 100 µl ethidium bromide (20 µg/ml) for 10 min at room temperature. Slides were washed two times (10 min each) with water and analyzed by fluorescent microscope. For each sample at least 100 pictures were captured and pictures were analyzed by one way ANOVA test.

Reactive nitrogen species assay

To perform NO assay 2×10^4 cells were seeded in 96-well plate containing 100 µl media (without serum) and incubated overnight in 5% CO_2, 80% humidity, and 37°C. Then, old media was discarded and fresh media containing 0.5, 1, and 2 µg/ml of recombinant PA were added and incubated for 24 hours at 37°C. Then, media was transferred to fresh tubes and centrifuged at 500 g for 5 min at 4°C. 100 µl media was transferred to a fresh 96-well plate and mixed with equal volume of Griess reagent (0.04 g/ml PBS pH 7.4) and incubated for 10 min at room temperature. Absorbance was measured at 540 nm by a microplate reader (Biorad, USA). Nitrite oxide concentration (µM/ml) in treated and untreated cells was calculated using nitrite oxide standard curve.

Reduced glutathione (GSH) assay

For doing GSH assay, 5×10^5 cells were seeded in a 24-well plate containing 300 µl media (without serum) and incubated overnight in 5% CO_2, 80% humidity, and 37°C. Then, old media was discarded and 300 µl fresh media containing 0.5, 1, and 2 µg/ml of recombinant PA were added and incubated for 24 hours at 37°C. Cells then trypsinized and harvested in fresh 1.5ml tubes and centrifuged at 1500 rpm for 5 min at 4°C and washed with PBS (pH 7.4) two times and incubated in -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. After 10-15 min of sonication, cells suspension was centrifuged at 2000 g for 10 min and supernatant was transferred to fresh tubes. Protein concentration was measured using Bradford method and equal volume of TCA (10%) was added and stored at 4°C for 2 hours and then centrifuged and supernatant was transferred to fresh tubes. 20 µl of the supernatant was mixed with 75 µl of lysis buffer, 55 µl of Tris buffer (pH 8.5) containing 0.02 M EDTA and 25 µl DTNB (5,5-dithio-bis-(2-nitrobenzoic acid)). The absorbance of yellow chromogen was measured using microplate reader. The result was expressed as µgGSH/mg protein using molar extinction coefficient of 13600 (equation 5).

$$GSH = (OD\times 1.998)/(\text{mg protein of sample}) \quad (5)$$

Catalase activity assay

Catalase activity assay was performed as following: 5×10^5 cells were seeded in 24-well plate containing 300 µl media (without serum) and incubated overnight in 5% CO_2, 80% humidity, and 37°C. Then, old media was discarded and 300 µl of fresh media containing 0.5, 1, and 2 µg/ml of recombinant PA protein were added and incubated for 24 hours at 37°C. Cells then trypsinized and harvested in fresh 1.5 ml tubes and centrifuged at 1500 rpm for 5 min at 4°C and washed two times with PBS (pH 7.4) 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. After sonication for 10-15 min, cells suspension was centrifuged at 2000 g for 10 min and the supernatant was transferred to fresh tubes. Protein concentration was measured using Bradford method. Then, 5 µl of samples were mixed with 50 µl of lysis buffer, 20 µl DDW, and 25 µl H_2O_2 (15%). Samples were incubated at 37°C for 2 min and were mixed with 100 µl dichromate acid reagent (0.1 M potassium dichromate in glacial acetic acid) and incubated in boiling water for 10-15 min until greenish or faint greenish color appeared. 200 µl of samples were transferred to a flat 96-well plate and the absorbance was measured at 570 nm using a plate reader. The results were converted into activity using the equation 6.

$$\text{Catalase activity} = (OD \times 36.49)/(\text{mg protein of sample}) \quad (6)$$

Statistical analysis

Results were reported as Mean ± SD and data were analyzed using GraphPad InStat software (version 3, 2003). Treated cells and controls were analyzed using One-way ANOVA and tukey tests. $P<0.05$ was considered as significant. Microsoft Excel software (2013 version) was used for plotting the curves.

Results

Antibacterial effects of the recombinant PA using MTT assay

Antibacterial effects of different concentrations of PA (0.28, 0.56, 1.12, 2.25, and 4.5 µg/ml) on *E. coli* and *S. aureus* were analyzed *in vitro*. Different concentrations of PA had significant inhibitory effects on *E. coli* cells' viability in comparison to the control. However, the viability rate between different concentrations was not significant (Fig. 1).

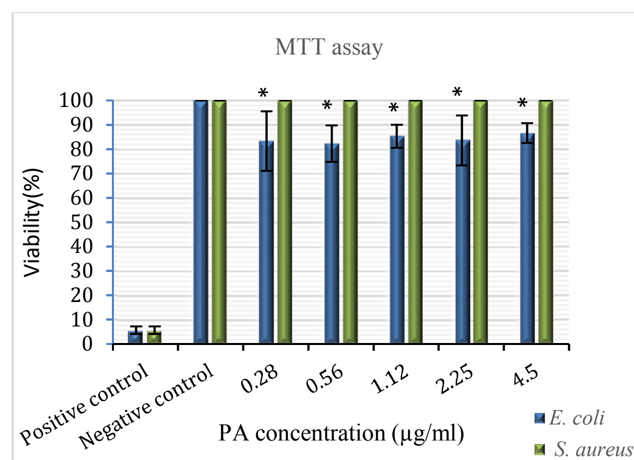

Figure 1. Viability of *E. coli* and *S. aureus* after treatment with different concentrations of the recombinant PA using MTT assay. Results were compared with the negative control. (*$p<0.05$)

Determination of the antibacterial effects of PA using MIC assay

MIC assay was used to analyze the antibacterial effects of recombinant PA *in vitro*. 0.28, 0.56, 1, 12, 2.25, and 4.5 µg/ml of recombinant PA had 12.3, 34.3, 36.1, 37, and 34 percent inhibitory effects on *E. coli* growth, respectively. It indicates that PA significantly inhibits the growth of *E. coli* cells. For *S. aureus*, 0.28, 0.56, 1.12, 2.25 and 4.5 µg/ml of recombinant PA had 5.8, 6.6, 9.16, 8.16, and 0

percent inhibitory effect, respectively. Which indicates that PA did not significantly inhibit the growth of *S. aureus* as compared with the control (Fig. 2).

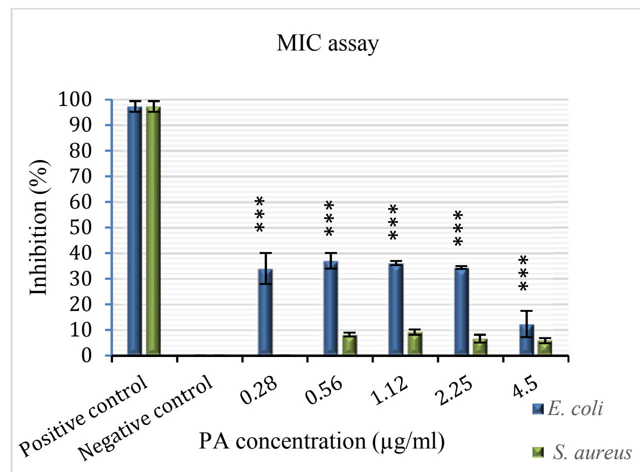

Figure 2. Determination of the inhibitory effect of recombinant PA on *E. coli* and *S. aureus* using MIC assay. Results were compared with the negative control. (*p<0.05) (**p<0.01), (***p<0.001)

Cytotoxicity effects of the recombinant PA on MCF-7 cells using MTT assay

In this study, the cytotoxicity effects of different concentrations of recombinant PA (0.5, 1, and 2 µg/ml) on MCF-7 cell line were analyzed. The viability of MCF-7 cells was 62.3, 33.3, and 20.5 percent in the presence of 0.5, 1, and 2 µg/ml of recombinant PA, respectively. The results indicates that the recombinant PA significantly inhibits the growth of MCF-7 cells (Fig. 3).

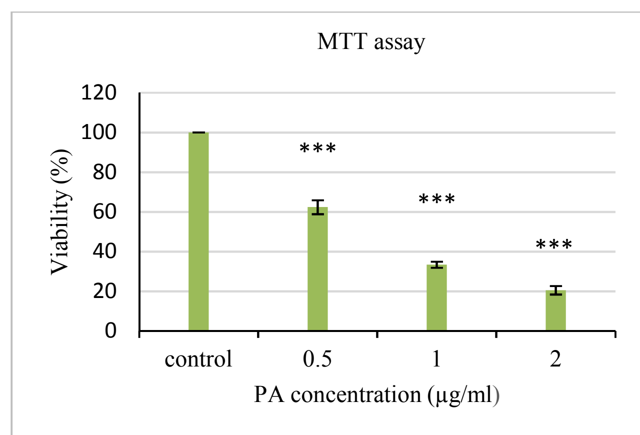

Figure 3. The inhibitory effects of different concentrations of the recombinant PA on MCF-7 cells using MTT assay. Results were compared with the negative control. (***p<0.001)

Cytotoxicity effects of the recombinant PA antigen on MCF-7 cells using neutral red uptake assay

Growth inhibition of MCF-7 cells following addition of 0.5, 1, and 2 µg/ml of recombinant PA to the media was obtained and the results showed that the recombinant PA protein significantly inhibited the growth of MCF-7 cells, as compared with control (Fig. 4).

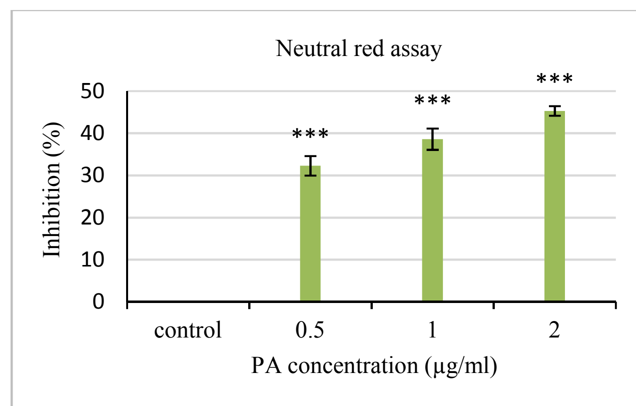

Figure 4. The inhibitory effects of different concentrations of recombinant PA on MCF-7 cells using neutral red uptake assay. Results are compared with the negative control. (***p<0.001)

Apoptosis effects of the recombinant PA using alkaline comet assay

Alkaline comet assay was used for analyzing apoptosis induction by recombinant PA. So the results demonstrate that PA significantly induced the apoptosis of MCF-7 cells as compared with the control (Figure 5 and 6).

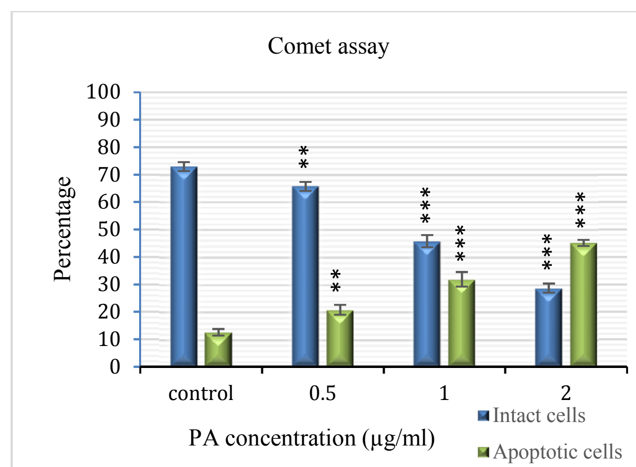

Figure 5. Analysis of Apoptosis effect of the recombinant PA on MCF-7 cells using alkaline comet assay. Results are compared with the negative control. (*p<0.05), (**p<0.01), (***p<0.001)

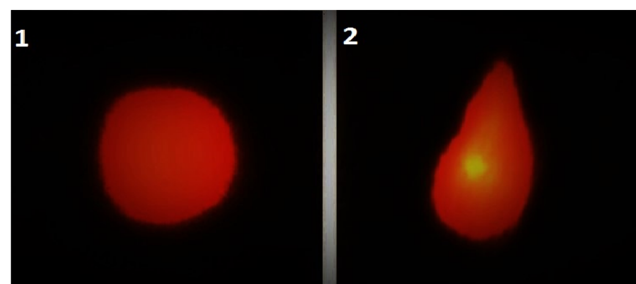

Figure 6. Alkaline comet assay pictures. 1) Intact cell; 2) Apoptotic cell (Magnitude×40)

Reactive nitrogen species assay

The amount of nitrite oxide released from MCF-7 cells treated with different concentrations of the recombinant PA, 0.5, 1, and 2 µg/ml, was 39.12, 38.57, and 36.17

μM/ml, respectively. In comparison to the control, the recombinant PA did not significantly induced the release of NO from MCF-7 cells (Fig. 7).

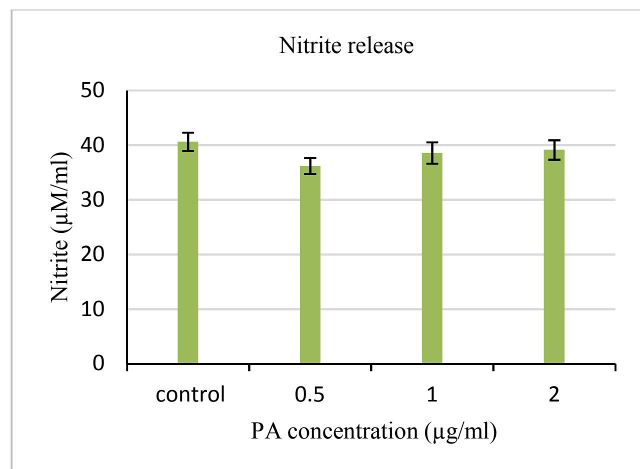

Figure 7. Nitrite oxide release from MCF-7 cells following recombinant PA treatment using reactive nitrogen species assay. Results are compared with the negative control.

Assessment of Reduced glutathione (GSH)

GSH content of MCF-7 cells after the treatment with 0.5, 1, and 2 μg/ml of recombinant PA were 10.45, 9.98, and 9.98 μg, respectively. The released GSH from treated cells was not significant as compared with the control (10.85 μg GSH) (Fig. 8).

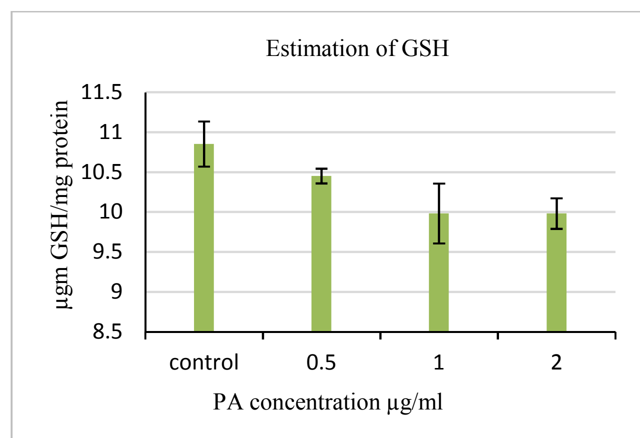

Figure 8. GSH release from MCF-7 cells using reduced glutathione (GSH) assay. Results are compared with the control.

Catalase enzyme activity assay

Catalase enzyme activity following treatment of cells with 0.5, 1, and 2 μg/ml of recombinant PA was assayed. The enzyme activity in MCF-7 cells was decreased after 24 hours treatment, in a dose dependent manner. Catalase activity was 839.265, 830.14, and 784.53 μM of hydrogen peroxide consumed/min/mg protein for 0.5, 1, and 2μg/ml of recombinant PA antigen and not significant as compared with the control (860 μM of hydrogen peroxide consumed/min/mg protein) (Fig. 9).

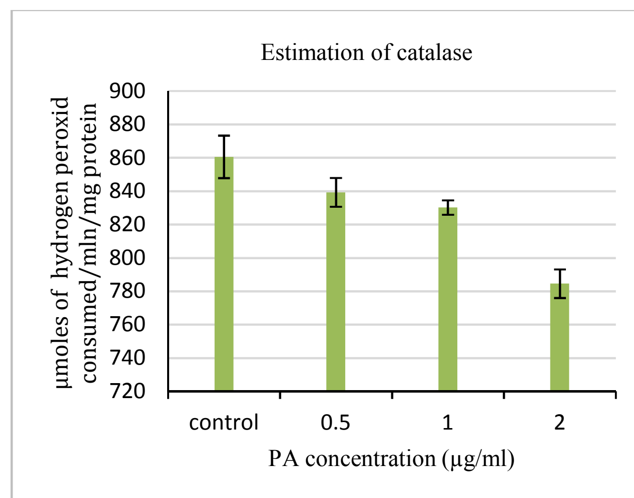

Figure 9. Catalase activity following recombinant PA treatment. Results are compared with the control (ns: not significant).

Discussion

According to our results, PA has significant inhibitory effects on MCF-7 cell line. This antigen also has partial inhibitory effects on Escherichia coli and no inhibitory effect on Staphylococcus aureus. In the present study, the toxicity effects of PA were analyzed on *E. coli*, *S. aureus*, and MCF-7 cells. Antibacterial effects of the protein were analyzed for the first time using MTT reduction and MIC assays. Results showed that PA has a partial inhibitory effect on *E. coli* and no inhibitory effect on *S. aureus*, while it was cytotoxic on MCF-7 cells. Results of MTT and neutral red uptake assays demonstrated that different concentrations of recombinant PA (0.5, 1, and 2 μg/ml) significantly inhibited the growth of the breast cancer (MCF-7) cells. Also, the results of alkaline comet assay showed that the protein significantly induced the apoptosis in MCF-7 cells. The comparison of MTT, neutral red uptake, and comet assay results indicated that PA induces apoptosis and necrosis in these cells. The results of NO, GSH, and catalase assays showed that PA could not change the oxidation and reduction potential of MCF-7 cells, so nitrite oxide has no role in the induction of cell apoptosis.

The results of this study were compatible with a study by Ching-Gong *et al*., (1996), which showed that Bacillus anthracis LF toxin can induce apoptosis and necrosis in macrophages in a dose dependent manner [17]. A study by Liu *et al*., (2000) indicated that mutated protective antigen (PA) in which the furin protease cleavage site was replaced by specific sequences, which are selectively cleaved by MMPs, can induce toxicity in MMP-overexpressing cells [18]. A study by Park *et al*., (2002) showed that *B. anthracis* lethal factor (LF) selectively induces apoptosis in activated macrophages, which is in agreement with our results [19]. Rogers *et al*., represent that PA can inhibits angiogenesis and tumor growth [20].

Liu *et al*., (2017) demonstrated that PA can cause a pore in the cancer cells' membrane and LF can enter the cytosol and induces the apoptosis, so the use of PA and LF proteins simultaneously have potent anti-tumor activity [21].

Also, in another study Liu *et al*., showed that recombinant and engineered tumor-targeted anthrax lethal toxin proteins strongly suppress the growth of solid tumors in mice. In this regard, native toxin receptors tumor endothelium marker-8 (TEM8) and capillary morphogenesis protein-2 (CMG2) act as the receptors for the PA domain of these toxins. [22]. Compatible with our results, Khandia *et al*., (2017) demonstrated that recombinant lethal toxin of *Bacillus anthracis* had anti-proliferative activity on primary mammary ductal carcinoma cells, so this toxin has therapeutic potential [23].

Conclusion

The results of this study demonstrated that the *Bacillus anthracis*' protective antigen has a significant cytotoxic effect on cancer cells, so it can be considered as a candidate antitumor drug. However, more investigations are required. On the other hand, since this protein is considered as a candidate vaccine against *B. anthracis*, the safety of the protein should be noticed. Indeed, no significant growth inhibition was observed against bacterial samples so it seems the protein could not be important in developing of antibacterial drugs.

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