Staphylococcus aureus enterotoxin type B (SEB) and alpha-toxin induced apoptosis in KB cell line

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A R T I C L E   I N F O

A B S T R A C T

Introduction: According to global cancer statistics, oral cancer is the 11th most prevalent cancer worldwide. Despite the availability of numerous modern treatments for oral cancer, a complete reduction of mortality rates has not been achieved. Bacterial toxins have potential applications in inducing apoptosis or targeting tumor cells for destruction. The present study aimed to investigate the impact of *Staphylococcus aureus seb* and *α-toxin* genes on apoptotic-related gene mRNA expression, as well as apoptosis induction in KB cell lines, focusing on *BAX*, *RB*, *BCL-2*, and *BAG-1* genes. **Methods**: The transfection of KB cells was performed using Lipofectamine 2000 to introduce pcDNA3.1 (+)-*seb*, pcDNA3.1 (+)-*α-toxin*, or an empty pcDNA3.1 (+) plasmid. The cells were cultured in DMEM supplemented with 10% FBS and 800 mg/L of G418 to select cells containing the plasmids. Subsequently, real-time RT-PCR was performed to measure the mRNA expression levels of *BAX*, *RB*, *BCL-2*, and *BAG-1* genes. Cell apoptosis was assessed using Annexin V/PI staining and flow cytometry. **Results**: The *seb* and *α-toxin* significantly alter the expression of apoptotic-related genes in the KB cell line. In transfected KB cells, there was a significant increase in the mRNA expression of *BAX* and *RB* genes and a substantial decrease in the mRNA expression of *BCL-2* and *BAG-1* compared to the control group. Annexin test and flow cytometry analysis revealed that *seb* was more effective than *α-toxin* in inducing apoptosis. Conclusions: The *seb* and *α-toxin* genes of *S. aureus* exhibit an inhibitory effect on the growth, proliferation, and invasion of oral cancer cells by modulating gene expression in the apoptotic pathway. Hence, these toxins are promising for controlling and treating human oral cancer.

INTRODUCTION

Cancer represents a significant global health burden, with oral cancer constituting a prevalent malignancy that affects the oral cavity [1]. While oropharyngeal cancer incidence is higher in developed countries, developing nations are also experiencing an increase in the prevalence of the disease [2]. Oral cancer risk factors include tobacco and alcohol use, exposure to UV radiation, viral infections, poor oral hygiene, genetic predisposition, and male sex [3]. Radiation therapy is the most commonly used treatment, but there is an urgent need for new cancer medications [4].

Bacterial infections have been reported to possess anti-tumor properties and may serve as potential anticancer agents due to their immunogenicity, cytotoxicity, and survival ability in anoxic tumor environments [5]. Bacterial toxins have been used in cancer treatment for almost a century, and researchers have discovered that bacterial infection may inadvertently suppress certain cancers [6]. Toxins produced by bacteria effectively induce tumor death and promote cancer vaccination, as they can cause cell death and alter biological processes such as proliferation, differentiation, and apoptosis [7-12].

S. aureus, a highly virulent human pathogen, has been found to produce toxins with potential anticancer properties [13]. Toxins produced by *S. aureus* include pore-forming, exfoliative, and superantigen toxins, and most clinical *S. aureus* strains produce alpha-toxin. This protein monomer becomes lethal after oligomerizing into a heptameric form on the host cell's plasma membrane [14]. The alpha toxin has been shown to induce apoptosis in both endothelial cells and T-cells isolated from

peripheral blood and in Jurkat T cells by releasing cytochrome C and increasing caspase activity [15]. Staphylococcal enterotoxins, known for causing food poisoning and diarrhea, have also been found to induce apoptosis and potentially possess anticancer effects [16].

Oral squamous cell carcinoma (OSCC) is the most prevalent malignant neoplasm of the oral cavity, and the KB cell line was established from an epidermoid carcinoma of the mouth in an adult Caucasian male [17]. This study examined the impact of *S. aureus seb* and *alpha-toxin* genes on apoptosis-related genes, including *BAX*, *BCL-2*, *BAG-1*, and *RB*, in the KB cell line.

MATERIAL AND METHODS

Preparation of recombinant vectors. The *seb* (Accession: AM158256.1) and *α-toxin* genes (Accession: MZ398129.1) from *S. aureus* were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), and codon optimization was performed using Gene Runner version 6.5.52 for expression of these genes in human cells. Restriction sites for *Bam*HI and *Eco*RI were inserted at the N- and C-terminals of the genes, respectively. GenRay Biotechnology (Dongjing Town Songjiang, Shanghai, China) constructed two recombinant vectors, pcDNA3.1(+)-*seb* and pcDNA3.1(+)-*α-toxin*. TOP10F chemically competent cells were used to amplify the recombinant plasmids and the empty pcDNA3.1(+) vector. A commercial kit (YTA, Yekta Tajhiz Azma, Iran) was used to extract the plasmids. Confirmation of the recombinant plasmids was performed using PCR, double enzymatic digestion, and sequencing.

Cell lines transfection. The KB cell line (CCL-17), a type of human mouth epidermal carcinoma, was obtained from the National Cell Bank of Pasteur Institute of Iran. The KB cell line (CCL-17) was maintained in Dulbecco's modified Eagle's medium (DMEM; Bio-IDEA-Iran) supplemented with 10% heat-inactivated fetal bovine serum (hi-FBS; Bio-IDEA-Iran) and 100 U/mL penicillin-streptomycin (Bio-IDEA-Iran). The KB cells were incubated in a 37 °C humidified atmosphere containing 5% CO_2 using a CO_2 incubator to maintain steady-state conditions. Each well of a 6-well plate (SPL Life Sciences; South Korea) was seeded with 300,000 cells. The KB cells were transfected with Lipofectamine 2000 reagent (Invitrogen, USA) when they reached 85% confluency.

The first and second wells were transfected with recombinant plasmids, pcDNA3.1(+)-*seb* and pcDNA3.1(+)-*α-toxin*, respectively. In addition, the third well was lipofected with an empty pcDNA3.1(+) vector (control plasmid), while the fourth was left untransfected. Each transfected well was supplemented with 2 µg of plasmid and 6 µL of Lipofectamine 2000 reagent per well in serum-free DMEM medium containing 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Four hours after transfection, the medium was replaced with a fresh medium containing 800

mg/L G418 and 10% FBS, and the cells were selected for stable cell line generation over three weeks.

RNA extraction and cDNA synthesis. Total RNA was extracted from KB cells transfected with recombinant or control plasmids using RNX-Plus reagent (SinaClon, Iran) following the manufacturer's instructions. The concentration and quality of the extracted RNA were assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) at wavelengths of 260 nm and 280 nm. The Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was used to synthesize cDNA from 1000 ng of RNA per sample, following the manufacturer's instructions.

Detection of *seb* and *α-toxin* mRNAs by RT-PCR. PCR reactions were carried out in a 20 µL volume consisting of 1 µL cDNA, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 units of Taq DNA polymerase (SinaClon), and 0.5 µM of each forward and reverse primer (*seb*-F/R and *α-toxin*-F/R primers, respectively, as listed in Table 1). The reaction mixture was subjected to an initial denaturation step at 95 °C for 3 min. PCR amplification was carried out using an Eppendorf Mastercycler gradient (Eppendorf, Germany) with the following cycling conditions: initial denaturation at 94 °C for 1 min, annealing at the temperatures specified in Table 1 for 1 min, extension at 72 °C for 1 min for 30 cycles, and a final extension step at 72 °C for 5 min. The PCR products were subjected to electrophoresis on a 1.5% agarose gel for analysis.

Real-time RT-PCR. Real-time PCR was performed using a Rotor-Gene 6000 machine (Corbett Life Science, Concorde, NSW, Australia) and SYBR® Premix Ex TaqTM II kit (TaKaRa, Japan) with specific primers as listed in Table 1. Amplification for each gene was performed in duplicate for samples in a 20 µL reaction volume. No template controls (NTCs) were also included in each run. The amplification conditions for quantification were as follows: initial denaturation at 95 ⁰C for 4 min, followed by 40 cycles of denaturation at 95 ⁰C for 15 s, annealing at the temperatures specified in Table 1 for 20 s, and extension at 72 ⁰C for 20 s. After amplification, a melting curve analysis was performed to confirm the specificity of the PCR product. *GAPDH* was used as the internal reference gene for all experimental quantification. Relative quantification analysis was performed using the comparative C_T method ($2^{-\Delta\Delta Ct}$) [24]. The qPCR products were analyzed by electrophoresis on a 2% agarose gel to confirm the expected product sizes.

Flow cytometry. Apoptotic cell death induced by recombinant vectors (pcDNA3.1(+)-*seb* and pcDNA3.1(+)-*α-toxin*) and empty pcDNA3.1(+) was quantified using the FITC Annexin V Apoptosis Detection Kit I (556547, BD Biosciences Pharmingen, USA) and flow cytometry. All experiments were performed in duplicate. Cell lines (KB) were harvested and washed twice with ice-cold phosphate-buffered saline (PBS). The cells were then resuspended in 100 µL of 1X

binding buffer (provided in the kit) and stained with 5 µL of FITC-conjugated Annexin-V (10 mg/mL) and 10 µL of propidium iodide (50 mg/mL) in a flow cytometry micro-tube. After incubation at room temperature (25 °C) in the

dark for 15 min, 400 µL of binding buffer was added to the cells, and the samples were analyzed using a flow cytometry instrument (FACSCalibur, Becton Dickinson).

Table 1. Primers used for real-time PCR amplification of genes in KB cells.

Gene	Primer's name	Sequence	Ta (ºC)	Product length (bp)
GAPDH	F	5'- GCCAAAAGGGTCATCATCTCTGC -3'	65	183
	R	5'-GGTCACGAGTCCTTCCACGATAC-3'		
Seb	F	5'- AGGACACAAAGCTGGGCAACTAC-3'	65	171
	R	5'-TACGCTTGTCTGTCTGGTGGGAG-3'		
α-toxin	F	5'-ACACTGCTGCTCGGCTCCATTC-3'	67	227
	R	5'-ATGGTGCCCTTTGTGCGAATC-3'		
BAG-1	F	5'-ACGACCTTCATGTTACCTCCCAG-3'	64	227
	R	5'-TAGTTCAACCTCTTCCTGTGGACTG-3'		
BCL-2	F	5'-GACGACTTCTCCCGCCGCTAC-3'	65	245
	R	5'-CGGTTCAGGTACTCAGTCATCCAC-3'		
RB	F	5'-GCAAATTGGAAAGGACATGTGAAC -3'	65	220
	R	5'-TTGAGCAACATGGGAGGTGAGAG -3'		
BAX	F	5'-AGGTCTTTTTCCGAGTGGCAG-3'	65	234
	R	5'-GCGTCCCAAAGTAGGAGAGGAG-3'		

Statistical analysis. The KB cell transfection was performed in duplicate using the corresponding plasmids. The real-time PCR, data, and flow cytometry assays were performed, and the results were presented as mean ± standard deviation (SD). The results were analyzed using one-way analysis, and the significance of the differences between sample means was determined. Statistical significance was defined as $P \leq 0.05$. Statistically significant differences were not considered for duplicate samples.

RESULTS

Confirmation of recombinant plasmids. The pcDNA3.1(+)-*seb* and pcDNA3.1(+)-*α-toxin* recombinant plasmids were confirmed via PCR, followed by double-enzyme digestion, and sequencing. The *seb* and *α-toxin* genes were amplified using specific primers (Table 1), resulting in PCR products of 171-bp and 227-bp for *seb* and *α-toxin*, respectively (data not shown). Furthermore, the pcDNA3.1(+)-*seb* recombinant plasmid was confirmed via restriction enzyme analysis using *Bam*HI and *Eco*RV, resulting in an 813 bp fragment for the full-length *seb* gene on agarose gel electrophoresis. In addition, digestion of pcDNA3.1(+)-*α-toxin* with *Bam*HI/*Eco*RV and subsequent electrophoresis revealed a 963-bp fragment for α-toxin (Fig. 1A and B). Furthermore, DNA sequencing was performed to confirm recombinant plasmids (data not shown).

Eukaryotic expression of *S. aureus* toxin genes. KB cells were transfected with pcDNA3.1(+)-*seb* and pcDNA3.1(+)-*α-toxin* expression plasmids and were harvested three weeks after transfection. RT-PCR

analysis revealed successful amplification of 171 bp and 227 bp fragments for seb and α-toxin genes, respectively, indicating successful transfection of the recombinant plasmids into KB cells (Fig. 1A).

seb effects on gene expression in KB cells. Real-time PCR analysis demonstrated significantly lower expression levels of *BAG-1* and *BCL-2* in KB cells transfected with the recombinant pcDNA3.1(+)-*seb* plasmid compared to the control group (KB cells transfected with empty plasmid) ($P < 0.042$; Fig. 2). Moreover, real-time PCR analysis demonstrated significant upregulation of *RB* and *BAX* in KB cells transfected with pcDNA3.1(+)-*seb* compared to control cells ($P < 0.036$; Fig. 2). These results indicate successful transfection of pcDNA3.1(+)-*seb* and subsequent expression of *seb* in KB cells, resulting in altered apoptotic gene expression.

α-toxin effects on gene expression in KB cells. Analysis of apoptosis-related gene expression in KB cells transfected with pcDNA3.1(+)-*α-toxin* demonstrated statistically significant changes in the expression levels of *BAX*, *BCL-2*, *RB*, and *BAG-1* genes compared to control cells ($P < 0.049$; Fig. 3). Our findings indicate upregulation of the pro-apoptotic *BAX* and *RB* genes by approximately 2.5- and 7-fold, respectively, in α-toxin-treated KB cells compared to the control group (KB cells transfected with empty plasmid). Moreover, the expression levels of *BCL-2* and *BAG-1* genes showed statistically significant differences between the α-toxin-treated and control groups. The levels of *BCL-2* and *BAG-1* in treated KB cells decreased significantly compared to the control group ($P < 0.033$; Fig. 3)
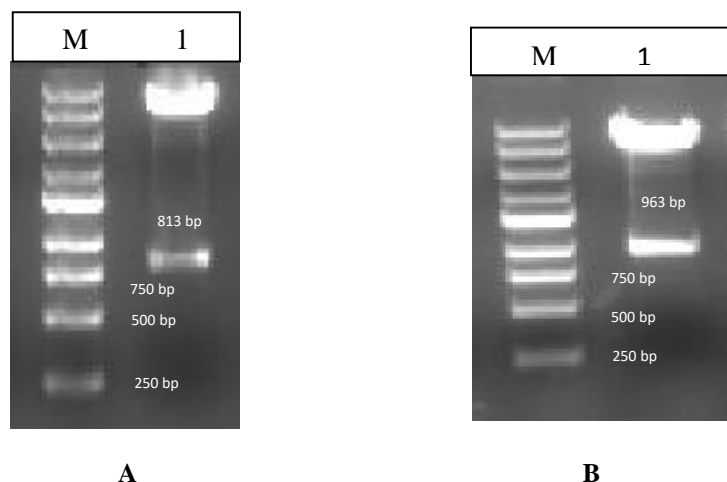
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A   **B**

Fig 1. Characterization of recombinant plasmids via restriction enzyme digestion. A) Agarose gel electrophoresis of *Bam*HI/*Eco*RV-digested pcDNA3.1(+)-*seb*, with 1Kb DNA ladder as a size marker. Lane 1 corresponds to the digested pcDNA3.1(+)-*seb* plasmid. B) Agarose gel electrophoresis of *Bam*HI/*Eco*RV-digested pcDNA3.1(+)-*α-toxin*, with 1Kb DNA ladder as a size marker. Lane 1 corresponds to the digested pcDNA3.1(+)-*α-toxin* plasmid.
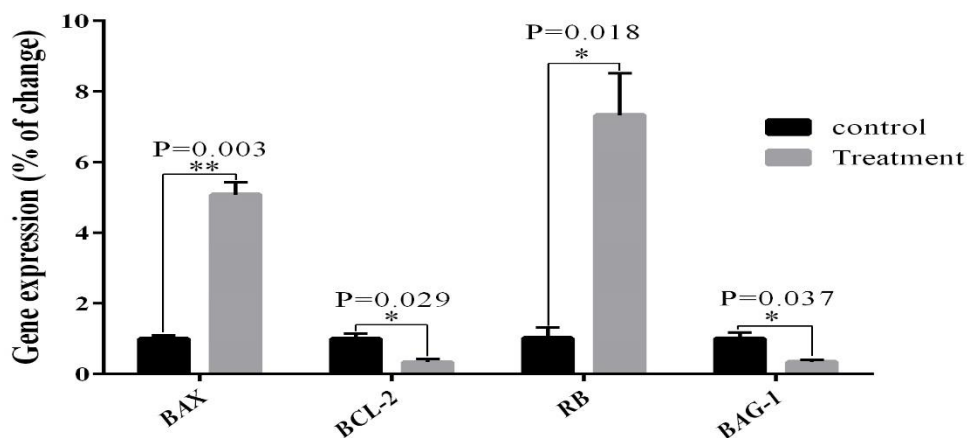

Fig 2. Statistical analysis of mRNA expression levels of *BAX*, *BCL-2*, *RB*, and *BAG-1* genes in KB cells treated with *seb* (treatment group) and control group (empty vector). All changes in gene expression were statistically significant.

Induction of apoptotic death in KB cells by *S. aureus* toxins. In the present study, Annexin V/PI staining and flow cytometry were utilized to analyze the proportion of cells undergoing early apoptosis, late apoptosis, and cell death. The apoptotic rates of KB cells transfected with pcDNA3.1(+)-*seb* were significantly higher than those of untreated control cells. The percentages of cells undergoing early and late apoptosis in *seb*-treated cells were 61.36% and 14.40%, respectively (Fig. 4A). Cell death, attributed to both apoptosis and necrosis, occurred in 76.00% of treated cells. The mortality rate in the control group was significantly lower (6.46%) compared to the treated KB cells (Fig. 4B). Additionally, as demonstrated in Fig. 4A, the percentage of viable cells in the *seb*-treated group decreased significantly (24.00%) ($P<0.0065$).

Moreover, following the transfection of KB cells with pcDNA3.1(+)-*α-toxin*, there was a significant increase in the cell death percentage of KB cells. The rate of viable cells in *α-toxin*-treated KB cells and control cells were 40.90% and 93.54%, respectively (Fig. 4B and 4C). Our findings show early and late apoptosis occurred in 58.54% of *α-toxin* treated cells. Thus, the apoptosis difference between treated and control cells was statistically significant ($P<0.029$).
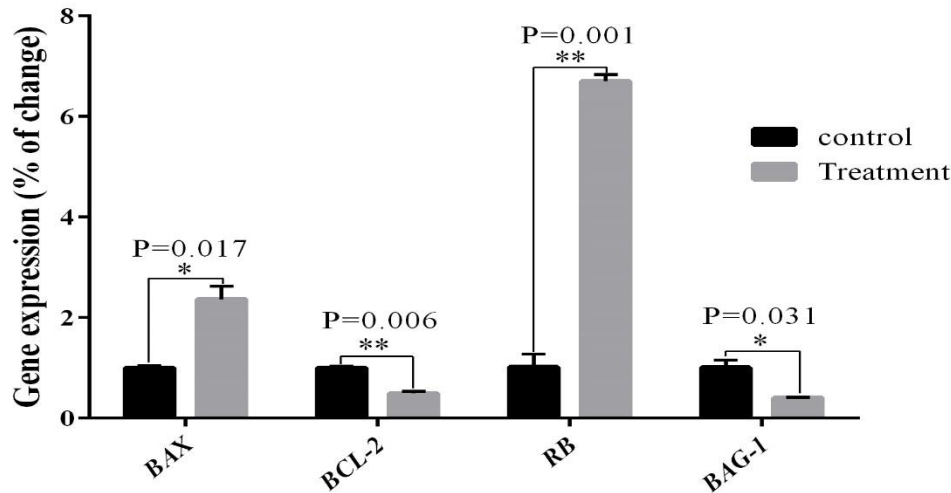
Fig 3. mRNA expression levels of *BAX*, *BCL-2*, *RB*, and *BAG-1* genes in *α-toxin*-treated KB cells (treatment group) and control group (empty vector).
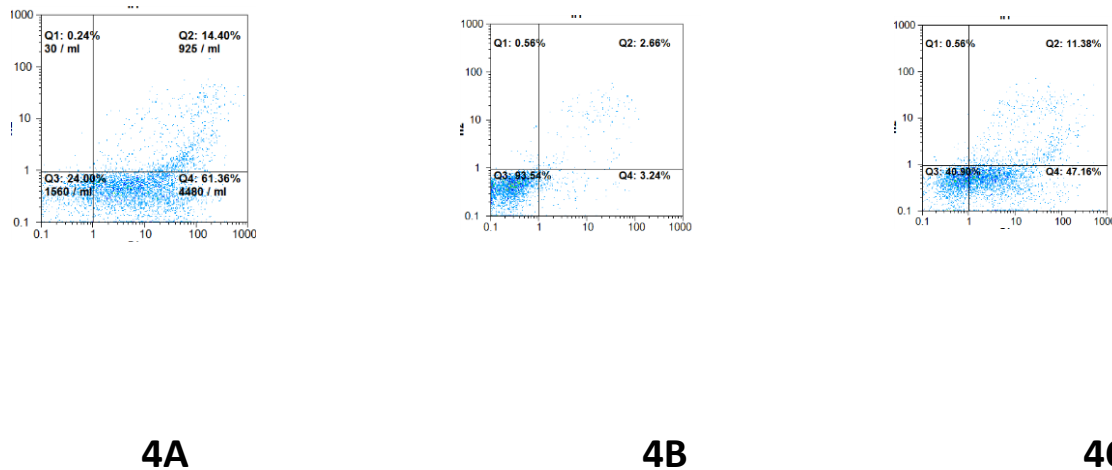

4A   4B   4C

Fig 4. The *S. aureus* toxins-induced apoptotic death of KB cell lines. 4A) 4B) 4C) statistically significant (*P*<0.047).

DISCUSSION

Cancer is a leading cause of death worldwide, affecting individuals across all age groups. Although current cancer therapies, including chemotherapy, radiation, and surgery, are widely available, they have not yet successfully eliminated cancer-related deaths [25]. Bacterial toxins have been studied to a limited extent for cancer therapy. Bacterial toxins can either destroy cells or, at low concentrations, alter cellular processes that regulate proliferation, differentiation, and apoptosis [26]. *S. aureus* bacteria produces approximately 20 types of staphylococcal enterotoxins, commonly called SEs [27]. In addition, *S. aureus* produces other types of enterotoxins, including SEK, SEL, SEM, SEN, SEO, and SEU, which are classified into antigenic groups known as staphylococcal enterotoxin type A to J (SEA to SEJ) [27].

Moreover, *α-toxin* is released by over 95% of clinically tested *S. aureus* strains. This water-soluble toxin can form beta-barrels in the body. The toxicity of the poison is achieved through oligomerization of the toxin on the host cell membrane, creating a toxic heptameric structure. This toxin affects various human cell types, including endothelial cells, epithelial cells, T cells, macrophages, and monocytes [28].

Genes involved in cell proliferation and differentiation include *BAX*, *RB*, *BCL-2*, and *BAG-1*. The involvement of these genes in either activating or preventing apoptosis is highly probable [29-30].

For this investigation, KB cells were transfected with either pcDNA3.1(+)-toxin or pcDNA3.1(+)-*seb* recombinant vectors, and the mRNA expression levels of *BAX*, *RB*, *BCL-2*, and *BAG-1* genes were assessed using

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real-time reverse transcription polymerase chain reaction (PCR) technique. Furthermore, Annexin V/PI labeling and flow cytometry were employed to determine cell viability. Our study confirms the practical expression of *α-toxin* or *seb* genes introduced into the pcDNA3.1(+) vector in transfected KB cells. Quantitative analysis revealed a significant increase of *BAX* and *RB* pro-apoptotic gene mRNAs in transfected KB cells than in control cells transfected with an empty pcDNA3.1(+) plasmid. KB cells transfected with pcDNA3.1(+)-sea or *α-toxin* recombinant vectors showed a statistically significant decrease in *BCL-2* and *BAG-1* expression compared to the control group.

Numerous studies have been conducted to investigate the impact of *S. aureus* on the expression of apoptotic genes. According to a study by Zhang et al. (2017), *S. aureus* toxins can impact the expression of genes such as *BAX* and *BCL-2* [22]. This review suggests that overexpression of *BCL-2* may mitigate the apoptotic effects of *S. aureus*-produced α-toxin. Our results were consistent with these findings, as we observed the downregulation of the *BCL-2* gene and increased apoptosis in KB cell lines in response to α-toxin. Busbee *et al.* (215) conducted a study examining the effects of *seb* injection in rats [31]. Elevated levels of aspartate transaminase enzyme have been linked to heightened activity of inflammatory cytokines and the induction of apoptosis through the caspase-2-dependent apoptotic pathway. Research into the expression of genes involved in apoptosis indicated that *seb* could reduce *BCL-2* expression in liver cells, leading to an increase in apoptosis. Our survey findings are consistent with prior research, as we observed the downregulation of *BCL-2* in KB cell lines of oral cancer in response to *seb*. Sakagami reported that paclitaxel, an antimitotic chemotherapeutic drug, induced apoptosis in human oral cancer cells by downregulating *BCL-2* expression and increasing *BAX* expression [32]. Our study suggests that *seb* and *S. aureus*-produced *α-toxins* may induce apoptosis in an oral cancer cell line similarly. Another study published in 2009 indicates that in malignancies where *RB* is not irreversibly inactivated, it may be possible to restore *RB* activity to halt further carcinogenesis [33]. Our investigation revealed that treatment with seb and S. aureus-produced α-toxin positively impacted the expression of the *RB* gene in treated cells. However, it is worth noting that the *S. aureus*-produced *α-toxin* appears more effective than the other two toxins, as it induces up-regulation of the *RB* gene. Subsequently, *seb* and *S. aureus*-produced *α-toxin* treatments increased apoptosis in the KB cell lines.

Shindoh *et al.* (2000) reported increased *BAG-1* expression in tumor tissue compared to the adjacent normal epithelium in 60-80% of the samples in a review of oral squamous cell carcinomas [34]. Our findings not only corroborated the results of their study but also demonstrated that treatment with *S. aureus*-produced α-

toxin and *seb* can reduce *BAG-1* expression in oral cancer KB cell lines, resulting in increased apoptosis.

Furthermore, we conducted flow cytometry assays on cells exposed to *seb* or *S. aureus*-produced *α-toxin*. The percentage of total apoptosis was 75.76% and 58.54% in cells treated with *seb* and *S. aureus*-produced *α-toxin*, respectively. The viable cells were also 24% and 40.90% in the *seb*-treated and *S. aureus*-produced *α-toxin*-treated groups, respectively.

The flow cytometry experiment results indicate that the percentage of apoptosis induced by *seb* is higher than that induced by *S. aureus*-produced *α-toxin*. However, treatment with *S. aureus*-produced *α-toxin* resulted in a more significant increase in *RB* gene expression than treatment with *seb*. The up-regulation of *BAX* and down-regulation of *BCL-2* and *BAG-1* induced by both *seb* and *S. aureus*-produced *α-toxin* had similar effects .

Our findings demonstrate that *seb* and *S. aureus*-produced *α-toxin* up-regulate the *RB* and *BAX* genes and down-regulate *BCL-2* and *BAG-1* in oral cancer KB cell lines. Consequently, our data imply that these two *S. aureus* toxins may hold promise as *in vivo* inhibitors of cancer cell development and as a potential therapy for oral cancer. In conclusion, further investigation of additional oral cancer cell lines and animal tumor models is warranted based on our findings.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

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