Recombinant Staphylococcal Enterotoxin Type A Stimulate Antitumoral Cytokines

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Abstract
Background: About 20 different types of staphylococcal enterotoxins are produced by Staphylococcus aureus, in which type A is more common in food poisoning syndrome. Also staphylococcal enterotoxin A superantigen is a potent inducer of cytotoxic T lymphocyte activity and cytokine production and could stimulate T cells containing T-cell receptor beta chain domains when binding to major histocompatibility complex class II molecules. Hence, it is an important reagent in cancer immunotherapy.

Methods: For the construction of pET-21a/entA cassette, the staphylococcal enterotoxin type A gene was isolated from S aureus strain HN2, cloned into pET-21a, and introduced into Escherichia coli strain BL-21(DE3). Consequently, Western blot analysis showed pET-21a/entA cassette expression inserted entA gene successfully. It is the first prompt using a pET-21a as a cloning vector for entA gene and expression of construct in BL-21(DE3). In addition, this study examined the ability of standard staphylococcal enterotoxin A and cloned staphylococcal enterotoxin A to activate T cells in vitro. Lymphocyte cells derived from lymph node BALB/c mice were exposed to standard staphylococcal enterotoxin A and cloned staphylococcal enterotoxin (1.10, 102,103, and 104 ng/mL) in order to evaluate the magnitude of proliferation, activation, and apoptosis of lymphocyte cells based on MTT and apoptosis assays, respectively.

Results: Our investigation showed that the function of cloned staphylococcal enterotoxin A was same as standard staphylococcal enterotoxin A, and the optimal concentration for the activation of lymphocyte cells and induction of apoptosis was 100 ng/mL and 1000 ng/mL (P < .05), respectively. Quantification of cytokines clearly showed that lymphocyte cells exposed to standard staphylococcal enterotoxin A and cloned staphylococcal enterotoxin A significantly secreted higher interferon γ and tumor necrosis factor α compared to control.

Conclusion: According to our results, the biological activity of standard staphylococcal enterotoxin A and cloned staphylococcal enterotoxin A is identical; therefore, these procedures may be approved as an efficient method to express and purify this protein in a large scale.

Keywords
SEA, Staphylococcus aureus, cloning, expression, bioactivity

Abbreviations
C-SEA, cloned-SEA; CTL, cytotoxic T lymphocyte; DAB, 3,3'-Diaminobenzidine; EGF, epidermal growth factor; FBS, fetal bovine serum; IFN-γ, interferon γ; IL-4, interleukin 4; LAL, Limulus amebocyte lysate; LB, Lysogeny broth; LPS, Lipopolysaccarid; L3, third loop; mAbs, monoclonal antibodies; MHC, major histocompatibility complex; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; Sag, superantigen; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Ses, staphylococcal enterotoxins; SEA, staphylococcal enterotoxin type A; SEB, staphylococcal enterotoxin type B; SI, stimulation index; S-SEA, standard-SEA; TM, transmembrane; TNF-α, tumor necrosis factor α.

Introduction
Staphylococcus aureus is the principal cause of the food poisoning syndrome worldwide. Staphylococcal enterotoxins (SEs) are the known cause of intoxication staphylococcal food poisoning syndrome; the greatest risk of SEs is still remaining.
and improper hygienic activities during the preparation of food is a major possibility of contamination. The Staphylococcus aureus has several enterotoxins that are classified into the major antigenic types that have been recognized as staphylococcal enterotoxin type A (SEA) to staphylococcal enterotoxin type J (SEJ) and also in recent times, several enterotoxins such as SEK, SEL, SEM, SEN, SEO, and SEU have been identified. Another classification on the basis of sequence similarity was divided into 3 groups and SEA is in the same group with SED and SEE. Among these SEs, SEA is the most important food poisoning enterotoxin and is associated with about 70% of the verified cases of staphylococcal food poisoning (European Commission, 2007). The entA gene is composed of 771 base pairs (bps) and encodes an enterotoxin and a precursor of 257 amino acid residues. A 24-residue N-terminal hydrophobic leader sequence is apparently processed, yielding the mature form of SEA.

Unlike conventional antigens, superantigens (SAgs) bind to certain regions of major histocompatibility complex (MHC) class II molecules on antigen-presenting cells outside the classical antigen-binding groove and concomitantly bind in their native form of T cells at specific motifs of the variable region of the beta chain of the T-cell receptor. This interaction triggers the proliferation of the T lymphocytes and leads to the in vivo or in vitro release of large amounts of various antitumoral cytokines and other effectors by immune cells. Moreover, though SEs are the cause of intoxication, they are powerful inducers of cytotoxic T-cell activity and cytokine production in vivo; concentrations of <0.1 μg/mL of bacterial SAgs are sufficient to stimulate T lymphocytes. Therefore, SEA as SAg, trigged the activation of the targeted T lymphocytes and leads to the in vivo or in vitro release of large amounts of various cytokines and other effectors by immune cells. It has been useful when used as SEA fused with other proteins like single chain Fv (SEA-scFv) and could be an important reagent for cloning and subcloning of entA gene polymerase chain reaction (PCR) products, pJET1.2 (Fermentas, Germany) and pET-21a (Invitrogen, USA) vectors were used, respectively, Escherichia coli strains Top10F’ and BL-21(DE3; Invitrogen) were used as the host strains for the recombinant plasmids. Bacteria were cultured in LB broth or on LB agar (Merck, Germany) with or without 30 μg of kanamycin/mL (Sigma, USA). pET-21a vector was used as the expression vector.

DNA Isolation and Purification

The genomic DNA from S. aureus strain HN2 was extracted using an AccuPrep Genomic DNA Extraction Kit (KB-1041; Bioneer, Korea). The plasmids were extracted using Gene JET Plasmid Miniprep Kit (Fermentas). All the kits were used according to the manufacturer’s instructions. Plasmid DNA concentrations were determined by spectrophotometer (NanoDrop-1000, Wilmington, Delaware). All DNA preparations were stored at −20°C until used.

Cloning of the entA Gene

The specific primers were designed according to the entA sequences of S. aureus strain HN2 from National Center for Biotechnology Information (GenBank accession no: c2052229-2051) and from the gel using High Pure PCR Purification Kit (Roche, Germany) (Table 1). The amplified target fragment (1080 bp; Figure 1) was ligated into a linearized pJET1.2 (Fermentas) vector. After ligation and transformation, plasmids were introduced into E. coli strain Top10F’ by a chemical method (CaCl2). A recombinant clone (pJET/entA) was confirmed by sequencing.
Subcloning and the Construction of Expression Plasmid

For subcloning of the entA amplified, the vector was digested by NdeI and XhoI restriction enzymes. The entA was ligated to the pET-21a vector at 4 °C for 12 hours. Then the ligation product was analyzed on agarose gel for its integrity and transformed into competent Escherichia coli strain Top10F0 by a chemical method (CaCl2); finally, bacteria transformation was selected on LB agar plates containing 30 mg kanamycin/mL. Then plasmid was isolated using the Gene JET Plasmid Mini-prep Kit (Fermentas). The selected clones were further evaluated by restriction enzymes and PCR and as a final point sequenced by a commercial facility using universal forward and reverse T7-promoter.

Expression of entA Gene in E coli Strain BL-21(DE3)

For expression, the recombinant plasmid, pET-21a/entA, was transformed into competent E coli strain BL-21(DE3). Escherichia coli cells containing expression vector pET-21a/entA were grown in LB medium supplemented with kanamycin (30 μg/mL) and at 37°C to an optical density (OD600) of 0.8. For induction, isopropyl-β-D-thiogalactopyranoside (Sigma) was added to a final concentration of 1 mM, and the culture was incubated at 37°C for 4 hours. The cells were subsequently harvested and suspended in lysis buffer (20 mM sodium phosphate, 10 mM EDTA, 1% [v/v] Triton X-100, pH 7.5) followed by freezing, thawing, and sonication on ice in the presence of PMSF (1 mM) as a protease inhibitor. To chelate the EDTA, 10 mM MgSO4 was added followed by DNase (0.01 mg/mL) at room temperature for 20 minutes. Later, centrifugation was performed. The pellet was thoroughly resuspended in 0.1 culture volume of the same buffer without Triton X-100. The presence of enterotoxin protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Purifications and Detoxifying of C-SEA Protein

To purify the protein, ToxinEraserTM Endotoxin Removal Kit (GenScript, China) was used according to the manufacturer’s instructions. This kit enables to remove any LPS from the purified protein. Moreover, the LAL test was carried out for further evaluation of the presence of LPS.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

The protein was analyzed by SDS-PAGE on 12.5% polyacrylamide gels and stained with Coomassie brilliant blue R-250. For Western blot analysis, 20 μL of cultured cells were electrophoresed and transferred to the nitrocellulose membranes (Hi-bond Amersham Biosciences, USA) by using a semidry blotting system (Bio-Rad, Hercules, California) in tris/glycine buffer with pH 8.4, containing 20% (v/v) methanol. The membrane was blocked by 0/1% (w/v) Tween 20 according to the standard procedures. The monoclonal anti-his6 (Roche, Cat No: 04905270001), diluted with phosphate-buffered saline (PBS) 0.1% (v/v) Tween 20 and a final anti-his6 concentration of 0.4 μg/mL was added and membrane was incubated for 3 hours at 4°C with shaking. The block membranes were washed with PBS-Tween 20 and incubated with sheep antimouse horseradish peroxidase conjugate antibody (Bio-Rad), at a 1/5000 dilution in PBS-Tween 20. Membranes were then washed 3 times with PBS-Tween 20 and were developed using DAB solution (Sigma, St Louis, Missouri).

Mice and Injection Program

Female inbred BALB/c mice (6- to 7-week old) were purchased from the Pasteur Institute (Tehran, Iran). A total of 18 mice were divided into 3 six-mice groups designated as the following—group PBS, intravenous injection of PBS (negative control); group S-SEA, intravenous injection of 100 ng S-SEA; and group C-SEA, intravenous injection of 100 ng C-SEA.15 These challenges were performed every 72 hours for 2 weeks by methods described previously.10 Animal experiments in this study were done in compliance with Baqiyatallah University of Medical Sciences guidelines.

Table 1. Primers Used in This Study.a

| Gene    | Forward Primer | Reverse Primer         |
|---------|----------------|------------------------|
| entA    | TGGGAAGTGTGT   | CACCATTATCCACTTTCGC    |
|         | GTTAATACCT     | CATTTACATCCACTTTCGC    |
| pET-21a/entA | TATAAACCATCG   | ATATGCTTTCATTTCGC    |
|         | AAATACCGTA     | TAAATATATATCAAT        |

*aItalic text indicates restriction site of Ndel and Xhol on forward and reverse primers, respectively.

Figure 1. Results of polymerase chain reaction (PCR) on genomic DNA, Staphylococcus aureus strain HN2. Lane 1 is the negative control. Amplicon size is 1080 bp (lanes 2-5). Lane 6 is 1-kb DNA ladder and arrow indicates 1-kb band.
Cell Preparation and Cell Culture Conditions

The lymphocytes were isolated from axillary and inguinal lymph nodes of BALB/c mice. Isolated lymph nodes were cut into small pieces, rinsed twice with PBS, and minced with forceps and scalpels. The suspensions were passed through a 100-μm stainless steel mesh to obtain a single-cell suspension, and erythrocytes were lysed at room temperature using ACK lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM Na₂-EDTA). The cells were washed and resuspended in RPMI-1640 (Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and incubated at 37°C with appropriate humidity.

Cell Proliferation Analyses by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

The 1 x 10⁵ cells/100 μL of cells were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS (Invitrogen), penicillin (100 U/mL), and streptomycin (100 μg/mL; Sigma-Aldrich, Munich, Germany) with 1, 10, 10², 10³, 10⁴ ng/μL of either S-SEA or C-SEA and incubated at 37°C in the 5% CO₂ with appropriate humidity (Figure 2). Stimulation of mice lymphocytes was measured by MTT assay. After incubation, an aliquot of 100 μL of MTT reagent (0.5 mg/mL final concentration) was added to each well and incubated for another 4 hours. Then the OD was measured at 540 nm using a UV microplate reader (Tecan Austria GmbH, Austria). The stimulation index (SI) was calculated according to the following formula:

\[ SI = \frac{OD(\text{test})}{OD(\text{negative control})} \]

\[ \text{OD, optical density} \sqrt{b^2 - 4ac} \]

Enzyme-Linked Immunosorbent Assay for Measuring Cytokines

Mice splenocytes were isolated by the same methods used for isolation of lymphocytes from lymph nodes. Cells at a concentration of 1 x 10⁵ cells/100 μL were cultured with S-SEA and C-SEA at a concentration of 100 ng/μL. After 3 days of incubation, the supernatants were collected, and IFN-γ and interleukin 4 (IL-4) were measured by enzyme-linked immunosorbent assay kits (R&D, Minneapolis, Minnesota) according to the manufacturer’s instructions.

Statistical Analysis

All experiments were performed 4 times, and the mean ± standard deviation was calculated. Statistical analyses were performed using the 2-tailed Mann-Whitney nonparametric test and a P value of <.05 was considered statistically significant. All statistical analyses were conducted with SPSS 13.0 software (SPSS Inc, Chicago, Illinois).

Results

Cloning of entA Gene

Specific primers were designed to amplify the entA gene from the S. aureus strain HN2. The expected size of the PCR product, about 1080 bp, was obtained (Figure 1). The amplified entA gene was blunt-end fragment, purified from gel and inserted into linearized pJET cloning vector yielding pJET/entA.
Construction of the pET-21a/entA

After confirmation of pJET/entA via sequencing, it was used as a template for amplifying exact gene with NdeI and XhoI restriction sites. Then, 788 bp entA gene was double digested and subcloned into the pET-21a as an expression vector. The pET-21a/entA was detected by digestion (Figure 2), and finally, the identity and orientation of entA in the construct were confirmed by DNA sequencing.

Expression of entA gene and Western Blot Analysis

The E.coli BL21(DE3) was transformed with the pET-21a/entA and accumulated large amounts of a protein migrating in SDS-PAGE with an apparent molecular weight of approximately 30 kDa (Figure 3A, lane 5). Both supernatant and the pellet of cell lysates were tested for the presence of recombinant proteins. The majority of the expressed protein was detected in the pellet. The SEA protein was purified, and the major band was observed in SDS-PAGE (30 kDa). Then Western blot analysis was done with monoclonal anti-his6 and specific band was detected with DAB (Figure 3B).

Cell Proliferation Assay (MTT Assay)

For the assay of lymphocytes cell proliferation, cells were cultured in RPMI-1640 containing graded concentrations of mitogens S-SEA and C-SEA for 48 hours and were examined by the MTT assay. The results were shown only in 1 \times 10^2 \text{ng/mL} concentration of S-SEA and C-SEA; there is significant difference between S-SEA and C-SEA in comparison to the PBS (negative control). Therefore, we set the concentration at 10^2 \text{ng/mL} for proper activation of lymphocytes (Figure 4).

In Vitro Apoptosis Assay

For in vitro apoptosis assay, Hoechst 33258 staining was used. Typical examples are shown in the Supplemental Figure. As shown in Figure 5, the results obtained suggested that concentrations of 10^3 \text{ng/mL} of S-SEA and C-SEA seemed optimal for stimulating apoptosis. However, a statistically significant difference between S-SEA and C-SEA in comparison to the PBS was observed (Figure 6A).
Level of IFN-γ and IL-4 After the S-SEA and C-SEA Injection

After spleen was challenged with S-SEA and C-SEA, their ability to produce IFN-γ and IL-4 was evaluated. Results are shown in Figure 6B. Although no significant differences in the IL-4-producing ability were observed, S-SEA and C-SEA showed significant higher levels of IFN-γ-producing ability in comparison to the negative control PBS ($P < .01$). These results showed that C-SEA has biological activity same as that of S-SEA and suggest that the concentration of $10^2 \text{ng/µL}$ level can be a good candidate for tumor therapy because it can induce IFN-γ by utilizing the response of immune system.

Discussion

Conventionally, preparation of native SEA for in vitro studies is time-consuming; so large amounts of bacterial culture, complicated technical instrument, and the process of several purification steps that generally lead to a low yield are required. Hence, developing a method to overcome the conventional approaches to obtain high yields is a benefit. In the present study, recombinant DNA technology was applied to obtain SEA. The entA gene from a library of $S$ aureus strain HN2 was cloned into $E$ coli strain TOP10F' via pJET; then entA gene was expressed when inserted into the vector pET-21a. Cloned toxin was provided by transforming recombinant vector to $E$ coli and recombinant protein was purified with 6 histidine residues.

pET-21a vector carries 6 histidine residues in the C-terminal of the fusion protein. The SEA protein was soluble but non-secreted and contains His tag; then for the purification of protein the Ni–NTA His.Bind system was used.

Although SEA is the cause of most food poisoning outbreaks by $S$ aureus, it is well known as a very potent activator of T cells that can elicit strong immune responses both in vitro and in vivo. Ma and colleagues showed that cloning the entA gene in the pET-28a. This gene was fused with TM sequence form a c-erb-B2 gene derived from human ovarian cancer. In another study, Lu and colleagues found that the construct obtained from fusing cloned-SEA into pLXSN with
linker-CD80TM can be used as an antitumor agent. The studies of Yue et al and Xu et al used pET-42b (+) and pET-22b (+) as expression vectors for cloning of SEA, respectively; they also used BL21(DE3) as an expression host, whereas in our study, entA gene was cloned into the pET21a vector and we believe that, this is the first report of expression of entA gene into pET-21a as an expression plasmid; also in our study, BL21(DE3) was used as an expression host like the other study mentioned above.

In this research, the forward primer, containing NdeI restriction site, had 1 ATG codon as a part of NdeI restriction site, which we use from this ATG as start codon in entA gene. With the use of NdeI restriction site, other start codon in start site of gene was not required.

Finally, according to the Western blot result, we confirmed that entA gene inserted into pET-21a vector was expressed in BL21(DE3) successfully. Extremely purified recombinant protein was attained after purification. The achievement of cloning and expression of SEA in E coli supplies basis for studying the preparation of diagnostic reagent and monoclonal antibody. In this research, we cloned, expressed, and purified the recombinant SEA into pET-21a as an expression plasmid, which has not been reported before. In addition, cytotoxicity and apoptosis assay demonstrated that S-SEA and C-SEA have same bioactivity effects. Quantification of cytokines clearly showed that lymphocyte cells were exposed to S-SEA and C-SEA significantly and secreted higher IFN-γ and TNF-α compared to control. The SEA protein approach represents a novel approach for anchoring immunostimulatory proteins into living tumor cells. We hypothesize that SEA can induce an augmentation of immunity resulting in enhanced antitumor response. The antitumor effects of the cell-based tumor vaccine SEA in vivo are currently being investigated.

**Conclusion**

According to our results, the biological activity of S-SEA and C-SEA is identical, therefore, these procedures may be approved as an efficient method to express and purify this protein in a large scale. Also, production of these recombinant proteins in our country could improve cancer therapy and create new way in cancer therapy. Furthermore, it is appropriate for studying its action mechanisms and therapeutic applications.

**Declaration of Conflicting Interests**

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**Supplemental Material**

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**References**

1. Dinges MM, Orwin PM, Schliewert PM. Exotoxins of *Staphylococcus aureus*. Clin Microbiol Rev. 2000;13(1):16-34.
2. Letertre C, Perelle S, Dilasser F, Fach P. Detection and genotyping by real-time PCR of the staphylococcal enterotoxin genes SEA to SEJ. Mol Cell Probes. 2003;17(4):139-147.
3. Akineden O, Hassan AA, Schneider E, Uisleber E. Enterotoxigenic properties of *Staphylococcus aureus* isolated from goats’ milk cheese. Int J Food Microbiol. 2008;124(2):211-216.
4. Orwin PM, Leung DY, Tripp TJ, et al. Characterization of a novel staphylococcal enterotoxin-like superantigen, a member of the group V subfamily of pyrogenic toxins. Biochemistry. 2002;41(47):14033-14040.
5. Tamarapu S, McKillip JL, Drake M. Development of a multiplex polymerase chain reaction assay for detection and differentiation of *Staphylococcus aureus* in dairy products. *J Food Prot*. 2001;64(5):664-668.
6. Betley MJ, Borst DW, Regassa LB. Staphylococcal enterotoxins, toxic shock syndrome toxin and streptococcal pyrogenic exotoxins: a comparative study of their molecular biology. *Chem Immunol*. 1992;55:1-35.
7. Bergdoll MS. Enterotoxins. In: Easmon CSF, Adlam C, eds. *Staphylococci and Staphylococcal Infections. The Organism in Vivo and In Vitro*. London, UK: Academic Press; 1983:2:559-598.
8. Huang IV, Bergdoll MS. The primary structure of staphylococcal enterotoxin B. 3. The cyanoamide bridge peptides of reduced and aminooethylated enterotoxin B, and the complete amino acid sequence. *J Biol Chem*. 1970;245(14):3518-3525.
9. Ma W, Yu H, Wang Q, Bao J, Yan J, Jin H. In vitro biological activities of transmembrane superantigen staphylococcal enterotoxin A fusion protein. *Cancer Immunol Immunother*. 2004;53(2):118-124.
10. Imani Fooladi AA, Sattari M, Hassan ZM, Mahdavi M, Azizi T, Horii A. In vivo induction of necrosis in mice fibrosarcoma via intravenous injection of type B staphylococcal enterotoxin. *Bio-technol Lett*. 2008;30(12):2053-2059.
11. Imani Fooladi AA, Sattari M, Reza Nourani M. Synergistic effects between staphylococcal enterotoxin type B and monophosphoryl lipid A against mouse fibrosarcoma. *J BUON*. 2010;15(2):340-347.
12. Sakurai N, Kudo T, Suzuki M, et al. SEA-scFv as a bifunctional antibody: construction of a bacterial expression system and its functional analysis. *Biochem Biophys Res Commun*. 1999;256(1):223-230.
13. Si SY, Hu PZ, Huang YY, et al. Tumor cells with B7.1 and transmembrane anchored staphylococcal enterotoxin A generate effective antitumor immunity. *Biochem Biophys Res Commun*. 2006;347(1):208-214.
14. Sun J, Zhao L, Teng L, et al. Solid tumor-targeted infiltrating cytotoxic T lymphocytes retained by a superantigen fusion protein. *PLoS One*. 2011;6(2):78-84.
15. Mondal TK, Bhatta D, Biswas S, Pal P. Superantigen-induced apoptotic death of tumor cells is mediated by cytotoxic
lymphocytes, cytokines, and nitric oxide. *Biochem Biophys Res Commun.* 2002;290(4):1336-1342.

16. Rajapakse N, Mendis E, Kim MM, Kim SK. Sulfated glucosamine inhibits MMP-2 and MMP-9 expressions in human fibrosarcoma cells. *Bioorg Med Chem.* 2007;15(14):4891-4896.

17. Fooladi AAI, Sattari M, Nourani MR. Study of T-cell stimulation and cytokine release induced by staphylococcal enterotoxin type B and monophosphoryl lipid A. *Arch Med Sci.* 2009(3):335-341.

18. Sinha P, Sengupta J, Ray PK. A minimized Fc binding peptide from protein A induces immunocyte proliferation and evokes Th1-type response in mice. *Biochem Biophys Res Commun.* 1999;258(1):141-147.

19. Catt SL, Sakkas D, Bizzaro D, Bianchi PG, Maxwell WM, Evans G. Hoechst staining and exposure to UV laser during flow cytometric sorting does not affect the frequency of detected endogenous DNA nicks in abnormal and normal human spermatozoa. *Mol Hum Reprod.* 1997;3(9):821-825.

20. Sadraie SH, Saito H, Kaneko T, Saito T, Hiroi M. Effects of aging on ovarian fecundity in terms of the incidence of apoptotic granulosa cells. *J Assist Reprod Genet.* 2000;17(3):168-173.

21. Lee YD, Moon BY, Park JH, Chang HI, Kim WJ. Expression of enterotoxin genes in *Staphylococcus aureus* isolates based on mRNA analysis. *J Microbiol Biotechnol.* 2007;17(3):461-467.

22. Halpin-Dohnalek MI, Marth EH. *Staphylococcus aureus*: production of extracellular compounds and behaviour in foods. A review. *J Food Prot.* 1989;52:267-282.

23. Holmberg SD, Blake PA. Staphylococcal food poisoning in the United States. New facts and old misconceptions. *JAMA.* 1984;251(4):487-489.

24. Nielsen SE, Zeuthen J, Lund B, Persson B, Alenfall J, Hansen HH. Phase I study of single, escalating doses of a superantigen-antibody fusion protein (PNU-214565) in patients with advanced colorectal or pancreatic carcinoma. *J Immunother.* 2000;23(1):146-153.

25. Cheng JD, Babb JS, Langer C, et al. Individualized patient dosing in phase I clinical trials: the role of escalation with overdose control in PNU-214936. *J Clin Oncol.* 2004;22(4):602-609.

26. Lu SY, Sui YF, Li ZS, et al. Superantigen-SEA gene modified tumor vaccine for hepatocellular carcinoma: an in vitro study. *World J Gastroenterol.* 2004;10(1):53-57.

27. Xu QB, Liu CX, Ma QJ. Gene cloning, soluble expression and activity analysis of rSEA [In Chinese]. *Sheng Wu Gong Cheng Xue Bao.* 2003;19(4):402-406.

28. Yue CP, Jiemin WU. The cloning and expression of staphylococcal enterotoxin A gene from *Staphylococcus aureus*. *J Lab Med.* 2006;24(9):206-212.