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Computer Simulation and Additive-Based Refolding Process of Cysteine-Rich Proteins: VEGF-A as a Model

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Abstract Refolding of cysteine-rich protein for establishing native conformation and a biologically active form is the most challenging step in recombinant protein synthesis. In this study, expressed vascular endothelial growth factor-A (VEGF-A), as a cysteine-rich protein, in a prokaryotic expression cell was refolded based on computer simulation technique and multiple chemical additive-based buffers to recover its biologically active form. For this purpose, cloned and expressed VEGF-A in *Escherichia coli* BL21 (DE3) was purified and dialyzed by a basic buffer containing nine diverse chemical additives. In parallel with the evaluations of the applied additives, professional computer simulation software was also used. The activity of refolded protein was evaluated in differentiation of mesenchymal stem cells (MSCs) to the endothelial cells (ECs). The results showed that dialyzing the produced recombinant VEGF-A in chemical additive-based buffers containing cysteine, 1, 4-dithiothreitol (DTT), arginine, and Triton X-100 led to efficient VEGF-A refolding. The results of flowcytometry analysis indicated that CD31 and CD144 as the specific ECs markers in VEGF-A treated MSCs were 31 and 73%, respectively. Protein refolding method using chemical additive-based buffers containing cysteine, DTT, arginine and Triton X-100 was the best accessible technique for refolding cysteine-rich recombinant VEGF-A.

Keywords Chemical additive · Computer simulation · Protein dialysis · VEGF-A

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Introduction

Multiple expression systems, including prokaryotic and eukaryotic cells, are the most practical and accessible systems to produce specific recombinant proteins. Recombinant protein expression in *E.coli* is cost-effective, fast, and practical; however, this system lacks the post-translational modification potency which is extant in most recombinant products deposited in insoluble form called inclusion bodies (Tsumoto et al. 2003; Yamaguchi and Miyazaki 2014; Ueda et al. 2016). Hence, optimized refolding methods are needed for obtaining native or correctly-folded proteins from inclusion bodies.

In the case of cysteine-rich proteins, in which stable structures are formed via inter- and intra-molecular disulfide

bonds, designing an approach for acquiring actively refolded structures are required (Clark 1998; Wingfield et al. 2001; Jungbauer and Kaar 2007). The vascular endothelial growth factor-A (VEGF-A), as a dimeric and multipotent protein, is one class of cysteine-rich proteins that plays vital roles as an endothelial cell-specific mitogen and permeability growth factor (Claffey et al. 1995a, b; Jingjing et al. 1999; Lee et al. 2008). Moreover, VEGF-A can be used as a potential alternative therapeutic agent in excisional wound healing and repair (Keswani et al. 2013; Nauta et al. 2013; Khaki et al. 2017).

To refold an inactive protein to native form several procedures have been used such as dilution, dialysis, and chromatography procedures. Furthermore, different systems including reversed micelle, zeolite absorbing, and natural chaperone have also been used (Zhi et al. 1992; Fischer et al. 1993; Middelberg 2002; Li et al. 2004; Sakono et al. 2004). Recently, chemical additive-based methods have been applied for refolding recombinant proteins. Several studies have reported that chemical agents including urea, guanidinium chloride (GdnHCl), or ionic detergents can decrease the non-covalent interactions and suppress the formation of inclusion bodies (Zhi et al. 1992; Clark 2001; Li et al. 2004; Sakono et al. 2004; Eiberle and Jungbauer 2010; Prasad et al. 2011).The above-mentioned additives have been widely used for protein refolding by the reduction of aggregation formation.

For comparative protein structure modeling, in addition to optimizing a process for protein refolding by using chemical additives, computational modeling known as computer simulation is needed. Computer-based simulation prior to laboratory procedures for protein refolding can not only approve the experimental results, but also minimize the time and cost of the project through the elimination of unnecessary laboratory steps (Dubchak et al. 1993; Huang et al. 2012; Vempati et al. 2014; Pitman et al. 2015).

In this study, recombinant VEGF-A refolding was performed by using of several compositions of chemical additives in dialysis buffer in parallel with professional simulation software to predict the additive-recombinant protein interaction. Finally, activity of refolded-dialyzed protein was evaluated through their impact on mesenchymal stem cell differentiation into the endothelial cell.

Materials and Methods

Recombinant Protein Cloning and Expression

The VEGF-A protein encoding region was obtained from NCBI (National Center for Biotechnology Information) gene bank database. (Accession No: NM_001204384). Gene construct was optimized for expression in *E. coli* K12 DH5α and synthesized by Biomatic Company (Biomatic Corporation, Cambridge, Ontario). Plasmid construct containing VEGF-A gene was transformed into DH5α (Stratagene, La Jolla, Calif.) as a replicative host cell. Plasmid purified by using QIAprep Spin Miniprep kit following the manufacturer's instruction (Qiagen, Hilden, Germany). Target gene and pET32a (Novagen, Madison, USA) were double digested by restriction enzymes (BamH1, Xho1; Roche, Penzberg, German) and transformed into *E. coli* BL21 (DE3) and BL21 (DE3) pLysS (National Institute for Genetic Engineering and Biotechnology, Tehran, Iran) as the expression host cells. The protein induction was performed using IPTG (Thermo Scientific, Waltham, USA) to a final concentration of 0.1 µg/ml and cultured for more 8 h. After induction of protein expression, the bacteria were harvested and lysed by 8M urea solution. The lysed solution was centrifuged at 9000×*g* for 10 min at 4 °C; the supernatant was collected for the protein purification (Molaee et al. 2017).

Protein Purification and Immunoblotting Assay

Recombinant VEGF-A was purified by Ni-NTA column (Qiagen, Hilden, Germany) according to manufacturer's instructions. The quality of the purified recombinant protein was assessed by 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE; Mini-Protean II Cell, Bio-Rad, USA). Protein concentration was evaluated by measuring optical density according to the following formula:

Protein concentration (mg/ml) = $(1.55 \times OD280) - (0.76 \times OD260)$. Also, protein concentration was approved by using Bradford protein assay. Immunoblot assay was performed on polyvinylidene difluoride membrane (Roche, Mannheim, Germany) using primary specific anti-VEGF (abcam, Cambridge, UK) followed by treating with conjugated anti-human IgG horseradish peroxidase (HRP: Abcam, Cambridge, UK). Reaction was developed by diaminobenzidine solution (Abcam, Cambridge, UK) in dark condition. (Sadoogh Abbasian et al. 2015). Vacuolating cytotoxin A (VacA A) as non VEGF-A and histidine-tagged protein was used as negative control (Hasanzadeh et al. 2013).

Molecular Dynamics Simulation

Using the online ExPASy server (http://web.expasy.org/protparam), isoelectric pH (PI) of VEGF-A was determined. Prone areas in protein aggregation were predicted by Aggrescan server (http://bioinf.uab.es/aggrescan/). Crystallographic structure of the VEGF-A (resolution: 2.4 Å, code: 1BJ1) were obtained from the PDB protein data bank (http://www.rcsb.org). Protein structure was optimized by using CymeraPhoto Editor software (Pettersen et al. 2004). Three-dimensional structures of cysteine, (CID of pubchem: 5862), proline (CID of pubchem: 145,742), glycine (CID

of pubchem: 750), and arginine (CID of pubchem: 6322), as chemical additives were applied from PubChem site (https://pubchem.ncbi.nlm.nih.gov/) and energy optimized by Hyperchem (Caffery, Dobosh et al. 1998). AutoDock Software was used for prediction of docking ligand process to VEGF-A ligands attachment area and subsequently to simulate additive connection with the protein binding sites. Finally, LigPlot software was applied for prediction of hydrogenic and hydrophobic VEGF-A-ligand complex interactions and evaluation of the length of hydrogen bonds.

Dialysis Procedure

The dialysis membranes were embedded in boiling buffer A (0.7 g EDTA, 2 g NA2CO3 in 200 ml DW) for 10 min, followed by washing with distilled water (DW) and were put in boiling buffer B (0.14 g EDTA in 400 ml DW).

The additives compounds were prepared in phosphate buffer solution (PBS) according to the Table 1. Samples (1–2 ml) were loaded into the dialysis membrane followed by embedding into the chambers containing 300 ml external dialysis buffer with gentle agitation at 4 °C. Dialysis buffer was changed after 2 h and dialyzed for 24 h. Finally, dialyzed protein samples were maintained at 4 °C for the next stages of the project.

Biological Activity of the Recombinant VEGF-A in Cell Differentiation

Differentiation of MSCs into endothelial cells by VEGF-A were evaluated for assessment of VEGF-A activity. Cell culture with 50% confluency was performed in 24-well plates (Nunc, Nuncolon, Denmark) in Dulbecco's modified eagle's medium (DMEM: Invitrogen, UK) supplemented with 10% fetal bovine serum (FBS: Gibco, NY, USA) at 37 °C under 95% humidity and 5% CO2. For stimulation

Table 2 The VEGF-A aggregation prone area

MNFLLSWVHWSLALLLYLHHAKWSQAA
PMAEGGGQNHHE**VVKFMDV**YQRSYCHP**I** **ETLVDIFQE**YPDEI**EYIFK**PSCVP**LMRCG**G CCNDEGLECVPTEESNI**TMQIMRI**KPHQGQ HIG**EMSFL**QHNKCECRPKKDRARQEKKSV RGKGKGQKRKRKKSRYKSWSVCDKPRR

Underlined bold letters are prone area of protein aggregation

of the cell differentiation, MSCs were treated in duplicates with 50 ng/ml of dialyzed VEGF-A samples, according to the nine above-mentioned programs, along with 50 ng/ml commercial VEGF-A (abcam, MA, USA) as positive control and PBS as negative control with changing culture media every 48 h for 10 days. The cells were dissociated by mechanical procedure at the end of the differentiation time. The separated cells were analyzed by flowcytometry (BD Biosciences, CA, and USA, 10,000 events) using characteristic markers of endothelial cells including fluorescein isothiocyanate (FITC) anti-CD31 and phosphatidylethanolamine (PE)-conjugated anti-CD144 and characteristic markers of MSCs including FITC anti-CD73 and PE anti-CD105. Appropriate PE and FITC isotypes were used as negative controls.

Results

Molecular Analysis, Modeling, and Docking of VEGF-A

According to the data obtained from ExPASy server, pI of VEGF-A was 9.2. Based on the aggrescan server, protein aggregation prone area is shown in underlined bold letter (Table 2). It is more important to identify these areas,

Table 1 The composition of additive supplements

Add: Pro	NaCl 200 mM	EDTA 1 mM	Arg 150 mM	Cys 150 mM	Gly 150 mM	Pro 150 mM	DTT 1 mM	Glu 0.1 mM	Tri 0.5%
1	+	+	+	−	+	−	−	−	−
2	+	+	−	+	−	−	−	−	−
3	+	+	−	−	−	+	−	−	−
4	+	+	+	+	−	−	+	−	+
5	+	+	−	+	−	−	+	−	−
6	+	−	−	+	−	−	−	+	−
7	+	−	+	+	−	−	+	−	−
8	+	−	−	+	−	−	+	−	−
9	+	+	+	+	−	−	+	−	−

EDTA ethylene diamine tetraacetic acid, *DTT* dithiothreitol, *Cys* cysteine, *Pro* proline, *Arg* arginine, *Gly* glycine, *GLU* glucose, *Tri* TritonX-100, *Add* additives, *Pro* program, + yes, − no

especially in the case of effective proteins in therapeutic interventions. The non-covalent contacts in these areas, enhance the stability of the native structures of refolded protein (Jahn and Radford 2008; Zambrano et al. 2015). Results of LigPlot software in selective amino acids additives interactions showed weak hydrogenic bonds between cysteine stabilizer and VEGF-A (Fig. 1a–c).

Approved Purified Proteins

According to the measurement of protein concentration and SDS-PAGE analysis, concentration of produced recombinant VEGF-A in *E. coli* BL21 (DE3) was 0.8 mg/ml in comparing 0.1 mg/ml concentration in pLysS (Fig. 2a). Western blotting results confirmed a similar protein band with an approximate molecular weight of 45 kDa (Fig. 2b).

Characteristic Evaluation of the Differentiated Cells

Data of flowcytometry is shown in the Fig. 3 in which refolding procedures including four, five and eight programs showed higher amounts of CD31 and CD144. Flowcytometry analysis showed that specific ECs markers including CD144 and CD31counts of 4th dialyzed protein treated cells were 23 and 15% respectively in compare with 21 and 10% for 8th dialyzed product. Counts of blast cell and MSCs markers CD34, CD 90, CD73, CD44 and CD105 for 4th procedure was 16, 39, 25, 57 and 43%, respectively. These markers for 8th procedure was 13, 30, 22, 52 and 33% respectively. For 5th program, count of CD144 and CD31 were 19 and 15% respectively. Blast cell and MSCs markers CD34, CD 90, CD73, CD44 and CD105 for the 5th procedure was 16, 35, 24, 39 and 38%, respectively. The expression levels of ECs CD markers of other procedures were too low to be noticeable (Table 3).

Discussions

In this study, recombinant VEGF-A refolding process, as a model of the cysteine-rich proteins, was conducted based on the usage of different buffers and diversified chemical additives in parallel with computer modeling simulation.

Plasmid (pET32a) construct containing VEGF-A gene construct was optimized for expression in *E. coli* K12 DH5α as a replicative host cell. Since, DH5α strain lacks endonuclease, and any internal plasmid, so it has a strong tendency to receive and reproduce foreign plasmids and genes. pET32a by adding His-tag to a protein N-terminal provides protein purification by Ni–NTA resin.

T7 RNA polymerase is controlled by a plasmid T7 lysozyme in DE3, while controlling of T7 RNA polymerase is done via a T7 lysozyme encoded by the cell genome in
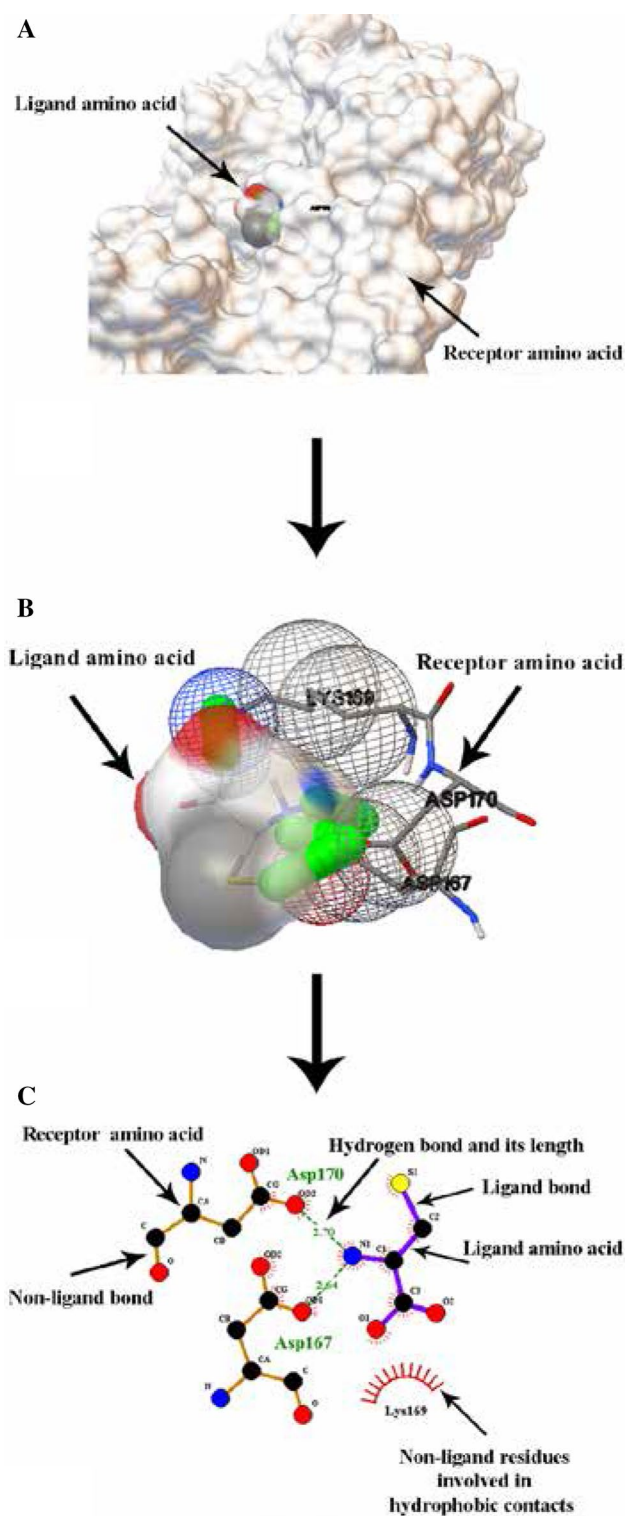

Fig. 1 View of hydrogenic and hydrophobic bonds between the VEGF-A and cysteine presented by LigPlot software with increasing magnification from **a** to **c**

Fig. 2 a Purified VEGF-A proteins in *E. coli* BL21 (DE3) and in *E. coli* BL21 (DE3) pLysS. (Line 1: Marker, Line 2: pLysS, Line 3: DE3). **b** Western-blot analysis of purified VEGF-A proteins (Line 1: Marker, Line 2: Recombinant VEGF in *E. coli* DE3, Line 3: Commercial VEGF = Positive control, Line4: VacA: Negative control)
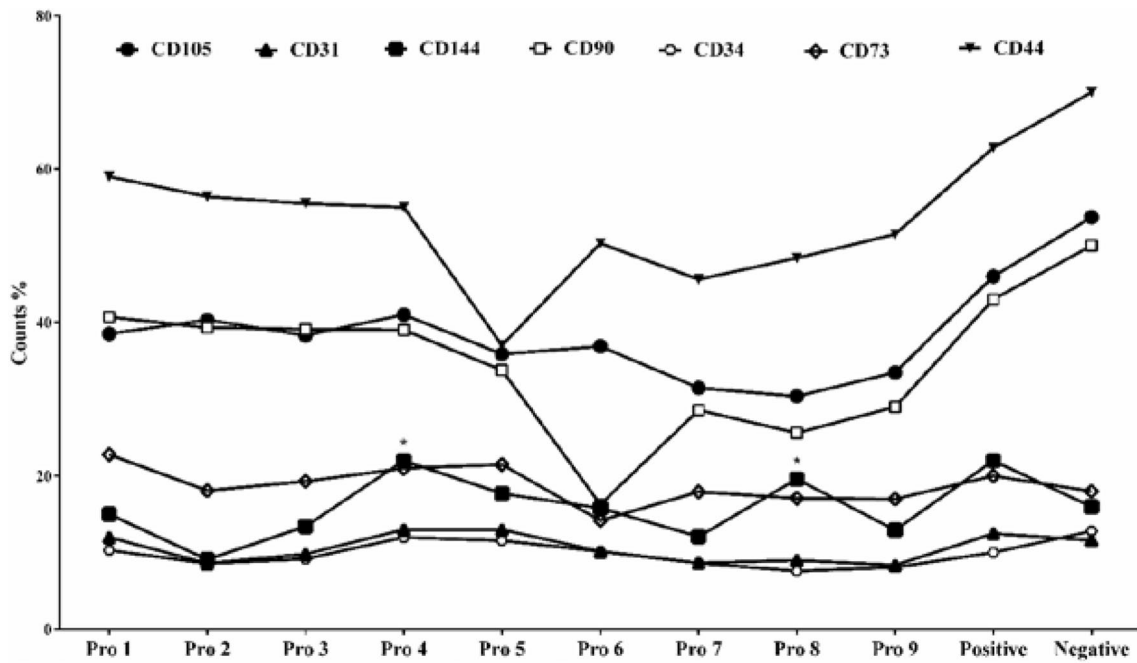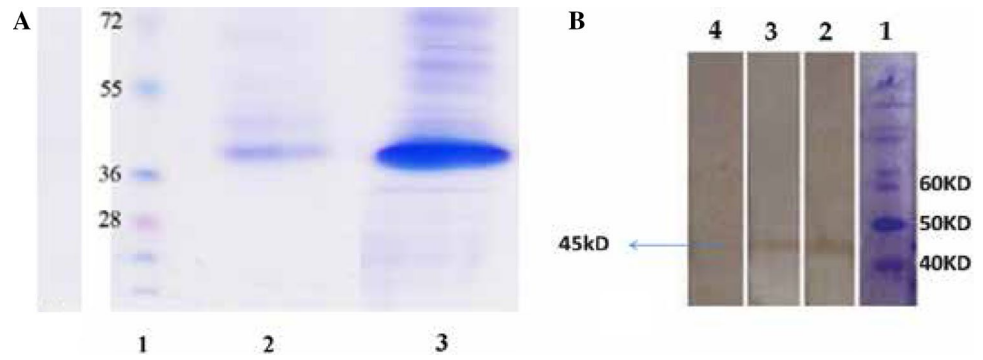

Fig. 3 Flowcytometry analysis chart of nine dialyzed proteins for evaluation of CD markers

Table 3 The effects of the selected programs with different additives on the cell specific markers

Prog	Additives	CD144 (%)	CD31 (%)	CD34 (%)	CD90 (%)	CD73 (%)	CD44 (%)	CD105 (%)
4	Cys-DTT Arg-EDTA-Tri. X100	23	15	16	39	25	57	43
5	Cys-DTT EDTA	19	15	16	16	24	39	38
8	Cys-DTT	21	10	13	30	22	52	33

pLysS. Therefore, a large amount of the energy in pLysS is spent on the production of T7 lysozyme that results in lower recombinant protein production (Hasanzadeh et al. 2013; Seyedarabi et al. 2013; Anton and Raleigh 2016).

Recombinant proteins expressed in *E. coli* BL21 (DE3) usually form insoluble inclusion bodies. To restore the maximum biological activity, the recombinant product must be converted into soluble and natural structure by an ideal in-vitro protein refolding like chemical additive-based methods. The process of recombinant protein production and protein refolding is time-consuming and costly. Therefore, the use of software and virtual models to simulate protein refolding plan, before running the laboratory procedures, is affordable.

Our results of the 4th and 8th programs of different refolding buffers were almost in line with the commercial VEGF-A that were suitable for high yield recovering of recombinant human VEGF-A; however, the 8th program was better than other programs, especially because of the lower amount of CD34 as specific blast cell marker.

According to the results, compared with other additives, cysteine was the best chemical additive for the rearrangement of the VEGF-A. Considering the multiplicity of VEGF-A disulfide bonds, application of cysteine as an effective amino acid could improve refolding buffer composition for improvement of VEGF-A refolding (Walker et al. 1996). Cysteine facilitates shuffling of disulfide bonds because it can interact with target protein via strong bonds instead of weaker intra disulfide bonds in the unfolded protein (Walker et al. 1996).

Protein docking is a computational technique that predicts the bound conformation of target protein to another material such as chemical additives. In this algorithm, compounds with the least amount of binding energy with VEGF and the most effects on target protein rearrangement were selected. (Atilgan and Hu 2011).

LigPlot is a completely general bioinformatics program that automatically generates schematic 2D conformation of protein–ligand complexes. This program can also be used to show other types of interaction in proteins and additives (Wallace et al. 1995).

Noteworthy, results of protein docking and LigPlot software about additive interactions have showed that cysteine, as a stabilizer, has weaker hydrogenic bonds with VEGF-A, as a cystein rich protein, than with the other amino acids. Therefore, Virtual simulation findings are consistent with the experimental results and this confirms that the applied simulation program was a reasonable estimation program for the interaction of VEGF-A with the applied chemical additives (Claffey et al. 1995a, b; Walker et al. 1996; Cothran et al. 2011; Moghadam et al. 2015; Yoshizawa et al. 2016).

Arginine as the other applied additive, acts as a strong chaotropic reagent with increasing the protein solubility via its guanidinium group (Gdn). On the other hand, amino and carboxyl groups of arginine interact with the denatured protein and H_2O by weaker bonds such as hydrogen ones while Gdn group of arginine interacts with hydrophobic amino acid of the protein surface by stronger bonds such as cation, electrostatic and hydrophobic ones. Therefore, arginine binds to the aggregated protein moderately and can act as an aggregation inhibitor of the target protein (Buchner and Rudolph 1991; Reddy et al. 2005; Kudou et al. 2011; Inoue et al. 2014).

Several previous studies were coordinated with our study and showed that arginine is useful for refolding several proteins such as Fab antibody fragments, casein kinase, single-chain immunotoxin, growth hormone etc. (Buchner and Rudolph 1991; Buchner et al. 1992; Lin and Traugh 1993; Arora and Khanna 1996) while, some authors have argued that arginine may act as a protein-denaturant. Xia et al. showed that arginine can disturb aminoacylase properties and Yancey et al. concluded that arginine may agitate protein stability and hence it acts as protein destabilizer (Yancey et al. 1982; Xia et al. 2007).

Based on the results, the additives used in successful refolding programs (4, 5 and 8) were cysteine and DTT; however, 4th and 5th programs involved EDTA. EDTA as a chemical chelator factor and metalloprotease inhibitor reduces oxidation reactions and increases the protein solubility. DTT is an effective agent in protein solubilization by keeping reduced form of cysteine and controlling disulfide bonding. DTT has a binary function as a redox (reduction–oxidation) system. The reduction of common disulfide bond is pushed forward by two sequential thiol-disulfide exchange reactions that do not stop at the mixed disulfide species. The other DTT thiol closes the ring, forming oxidized DTT and leaving behind reduced disulfide bond. The DDT reducing power is present in the alkali conditions like those of this study (Lilie et al. 1998; Yamaguchi and Miyazaki 2014). The performance of arginine in recombinant VEGF refolding process was less than in cysteine and DTT but in general the presence of arginine in the VEGF rearrangement process was positive.

Results showed that proline, glucose, and glycine had a preventive role in the process of recombinant protein refolding. Glycine and glucose could enhance protein misfolding and aggregation by collapsing and compacting protein structure (Bourot et al. 2000). Glucose as low molecular weight additive agent could be effective in refolding of recombinant protein from inclusion bodies (Alibolandi and Mirzahoseini 2011; Yamaguchi et al. 2013). Proline could inhibit protein aggregation by binding to some folded intermediates; it could also cover the hydrophobic areas of the target protein and consequently inhibit protein aggregation (Yamaguchi and Miyazaki 2014). More importantly, the results of these cases would probably refer to the unique structure of VEGF, including the large number of disulfide bonds and intramolecular hydrophobic interactions. Moghadam et al. concluded that cysteine-rich chitinase protein can be refolded using additive-based refolding buffer containing sucrose, Triton X-100, DTT, guanidinium chloride, and glycerol (Moghadam et al. 2015). Due to the presence of multiple disulfide bonds in chitinase and VEGF-A, the use of Triton X-100 and DTT are common points of that study and the present one. On the contrary, great diversities of hydrogenic and hydrophobic bonds in the structure of chitinase, sucrose and guanidinium were used instead of cysteine and arginine. Triton X-100 is a nonionic surfactant and an effective detergent in lysis and refolding buffers. It does these through suppressing the protein aggregation that

can increase the amount of refolded protein. Triton X-100 can alter the protein structure (Lee et al. 2006; Thomson et al. 2012; Ke and Berkmen 2014). In parallel with the present study, Lee et al. concluded that the ability of Triton X-100 and other similar agents to induce the solubility of oligomeric proteins was more than their impact on the monomeric structure (Lee et al. 2006).

Data of this study showed efficient refolding of the recombinant VEGF-A with the selected chemical additives, including cysteine and DDT that were common between the proposed dialysis buffers. Differentiation of MSCs into ECs was affected by recovered VEGF-A; this confirms the success of laboratory and software-based protein dialysis method. Since previous experimental data, showed VEGF-A in wound healing, the refolded protein can be used as a potential alternative therapeutic agent in excisional wound healing and repair treatment after more evaluation.

Compliance with Ethical Standards

Conflict of interest Authors declare that no conflict of interest in this study.

Ethical Approval This article does not contain any studies with human and animals performed by any of the authors (Ethical code R, ARAKMU. REC.1394.199, Arak University of Medical Sciences, Arak, Iran).

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