Iranian Journal of Basic Medical Sciences
ijbms.mums.ac.ir

Decrescent role of recombinant HSP60 antibody against atherosclerosis in high-cholesterol diet immunized rabbits

Saghar Zonnar [1], Said Abdul Ghafour Saeedy [2], Fahimeh Nemati [3], Mohammad Javad Motamedi [4], Hamidreza Raeespour [5], Jafar Amani [6*]

[1] Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2] Department of Paraclinic, School of Medicine, Herat University, Herat, Afghanistan
[3] Department of Biotechnology, Faculty of Advanced Science and Technology, Tehran Medical Sciences, Islamic Azad University (IAUPS), Tehran, Iran
[4] Department of Plant Bioproducts, Institute of Agricultural Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
[5] Department of Genetics, Islamic Azad University, Varamin Pishva Branch, Tehran, Iran
[6] Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article type:
Original

Article history:
Received: Mar 14, 2021
Accepted: Oct 26, 2021

Keywords:
Atherosclerosis
Coronary artery disease
Heat- shock proteins
Plasmids
Recombinant protein

ABSTRACT

Objective(s): Atherosclerosis is the main cause of cardiovascular disease (CVD) which has a key role in the development of coronary artery disease (CAD). Based on clinical studies, HSP60 is the only HSP that can cause atherosclerosis. In this paper, the expression level of HSP60 and the pathogenic degree of its cloned part was investigated in atherosclerosis condition.

Materials and Methods: After the designation of the specific primers for HSP60, PCR was done by the Pfu enzyme. Subsequently, the PCR products were cloned into a prokaryotic expression vector pET-28a. The resultant recombinant vector was transferred in BL21 and purified. Purification of protein was done by the Nickel affinity column. After confirmation of Western blotting and HSP60 protein purification, purified protein concentration was measured by the Bradford method, and purity was analyzed by SDS PAGE 12%. New Zealand rabbits were tested as an animal model. At the next step, the recombinant protein was injected into the animal model that was on a fatty diet.

Results: The prokaryotic expression plasmid pET28a-hps60 was successfully constructed, the HSP60 protein was expressed and purified in *Escherichia coli* BL21 (DE3). We found that the rabbit that was receiving the recombinant vaccine with the fatty diet showed a lower amount of fat deposition at the media endothelial level than the rabbit which received only the fatty diet.

Conclusion: Taking recombinant protein concomitant with a fatty diet, causes betterment of atherosclerosis via decreasing aggregation of cholesterol and thickness of the endothelial media.

▶ Please cite this article as:
Zonnar S, Saeedy SAG, Nemati F, Motamedi MJ, Raeespour HR, Amani J. Decrescent role of recombinant HSP60 antibody against atherosclerosis in high-cholesterol diet immunized rabbits. Iran J Basic Med Sci 2022; 25:32-38. doi: https://dx.doi.org/10.22038/IJBMS.2021.56382.12580

Introduction

Atherosclerosis is a condition in which fat builds up in the walls of the arteries, causing narrowing of the lumen and impeding blood flow through them. This condition can cause blockage of the heart arteries, resulting in chest pain or a heart attack (1). In terms of grading, through the intima-to-media thickness, atherosclerosis is divided into 4 grades: Grade 1, the thickness of the atherosclerotic lesions in the intima is less than half the thickness of the media, with some impairment of the endothelium and the presence of macrophages within the endothelium. Grade 2, the thickness of the atherosclerotic lesions is about half the thickness of the media, with the accumulation of the lipid cells, macrophages, and smooth muscle cells. Grade 3, the thickness of the atherosclerotic lesions is as media's thickness, with greater amounts of macrophages, smooth muscle cells, and connective tissue. Grade 4, the thickness of the atherosclerotic lesions is greater than the thickness of the media, with the appearance of large amounts of lipid-stacked cell centers in endothelial-like forms and infiltration of inflammatory cells such as macrophages and calcification of lipid centers.

Although this disease had been considered as a bland lipid storage disease, recent decades' studies have revealed the role of inflammation in atherogenesis (2). Inflammation has been recognized as a major factor in atherogenesis through its influence on lipoprotein metabolism in the arterial wall, which leads to the involvement of the innate (monocyte-derived macrophages) and the acquired immune system (T cells) (3). Interleukins, macrophage-associated cytokines such as interferon (IFN), tumor necrosis factor (TNF)-α, colony-stimulating factors (CSFs), and macrophage migration inhibitory factor (MIF) have been identified as key players in atherosclerosis pathogenesis (4, 5).

Atherosclerosis can lead to diseases such as ischemic heart disease (IHD), ischemic stroke, and peripheral arterial disease (PAD). In high-income countries, the incidence and mortality arising from atherosclerosis have been significantly reduced since the mid-20th century. However, the Global Burden of Disease study has reported that the

*Corresponding author: Jafar Amani. Applied Microbiology Research Center, System Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Vanak Sq. Molasadra St. Tehran, Iran. Tel: +98-21-82482592; Fax: +98-21-88068924; Email: jafar.amani@gmail.com

share of deaths due to cardiovascular diseases increased in Iran from 31.9% in 1990 to 46.8% in 2010 (6).

Epidemiological studies have shown that in addition to factors such as age, sex, hypercholesterolemia, smoking, hypertension, obesity, and diabetes, genetic factors are also associated with atherosclerosis development. According to experimental and clinical studies, so far, HSP60 is the only protein from the heat shock proteins family which has been detected to induce atherosclerosis (7). These proteins are also induced through activation of heat shock transcription factors in pathological conditions and environmental stress response. When these proteins are overexpressed in stress response, they are translocated on the cell surface and act as auto-antigens that can lead to innate or acquired immune response (8). HSP60 protein is highly expressed in stem cells and germline cells and is essential for the formation and maintenance of blastoma (9). Besides, HSP60 is involved in the development of tumor cells, cell survival, and apoptosis signaling pathways. Therefore, its expression level may also play a key role in the pathogenesis of cancer. Furthermore, it plays an important role in the transport and maintenance of mitochondrial proteins and transfer and replication of mitochondrial DNA (10, 11). This is evident that the abnormal expression level of HSP60 protein is effective in causing inflammatory diseases, and also the role of this protein has been proven in atherosclerosis development (12).

Some successful efforts were done on the E. coli platform to increase the microbial survival ratio at 50 °C. It was demonstrated that overexpression of HSP20 can sustain cells with high stability of the enzymes in high temperature and low pH and can increase the concentration of metals such as Ga^{2+}, Zn^{2+}, Mn^{2+} Fe^{3} in cells (13). It has been demonstrated that the membrane fraction of E. coli contains more HSPs than its other parts (14).

Therefore, the current study aims to evaluate the expression level of HSP60 protein in E. coli and immunologically study the function of HSP60 in decreasing the aggregation of lesions in the endothelium of the aortas in a well-characterized animal model of atherosclerosis.

Materials and Methods

Bacterial strains, plasmids, and antibiotics

E. coli DH5α and BL21 (DE3) strains were obtained from the National Institute of Genetic Engineering (Tehran, Iran). The pET-28a Plasmid was prepared for expression in the prokaryotic host from the National Institute of Genetics (Tehran, Iran), and pBSK plasmid for cloning was purchased from the BioMATIK Company (Ontario, Canada). Ampicillin and kanamycin antibiotics were purchased from the Merck Company (New Jersey, United States).

Primer design, transformation, and cloning

The protein sequence of HSP60 has been retrieved from the Uniport database, with accession No. P10809. Multiple sequence alignments were carried out using the National Centre for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih/blast (Karkhah *et al.* 2016). The Forward primer (5'-AGGCGAATTCATGACCGTGATTATTGAAC-3') site with the EcoRI site and the Reverse primer (5'-ATATAAGCTTGCCCTTCAGCAGCATG-3') with HindIII were used for HSP60 gene amplification. Then, PCR products were initially cloned into a pBSK vector (BioMATIK, Canada) and subsequently sub-cloned into a pET-28a expression vector. Then, the transformation of plasmid DNA into E. coli cells were done by a heat shock method and the plasmid was purified in a plasmid extraction kit (Genet Bio, Korea).

Expression and purification of HSP60 protein

E. coli strain (BL21DE3) carrying the recombinant plasmid of pET-28a was grown at 37 °C to an OD600 of 0.6. The culture was induced by 1mM isopropyl-β-D-galactopyranoside (IPTG, Sigma) and incubated at 37 °C for further 4 hr. Centrifugation was used for cell gathering (5000 ×g, 10 min, 25 °C), and after that cells were resuspended in the lysis buffer (100 mM NaH_2PO_4, 10 Mm Tris-Cl, 8 M urea, pH 8.0). sonication was done with high power 3 times and each time for 10 seconds. subsequently, we did the centrifugation of the lysate at 4 °C temperature for 15 min and 10000 ×g, and the supernatant was put on a Ni-NTA chromatography column (Qiagen, USA). The recombinant protein appeared in inclusion bodies after analysis on 12% SDS-PAGE. The denaturing condition was used for the purification of the recombinant. Then, the protein concentration was determined by a spectrophotometer at 595 nm using the Bradford method. Dialysis was performed and then reiterated two times versus PBS buffer (pH: 7.4) inclosing 10% glycerol and subsequently glycerol free to counteract protein aggregation. Ultimately, the purified form of HSP60 protein was stored at -70 ° C.

Animal immunization

Each experimental group consisted of 1 New Zealand juvenile, white, female rabbit, weighing about 2 Kg, either fed a normal chow diet or a diet containing 2% (w⁄w) cholesterol. The cholesterol content of the diet was individualized for each group. The rabbits and diet were obtained from Razi Vaccine and Serum Research Institute (Karaj, Iran), and rabbits were individually housed under conventional conditions. The studied rabbits were divided into 3 main groups: the control group (group 1), (n=1), received no vaccine or specific diet; the second group (n=1) was treated by the fatty diet; and the third group (n-1), received the fatty diet after immunization with the recombinant HSP60 protein. Every 14 days, the third group received the recombinant protein and was immunized with Freund's complete adjuvant (FCA) with 5 mg/mL Mycobacterium tuberculosis. Adjuvant was combined with 300 µg recombinant protein and injected subcutaneously. After that, feeding with high cholesterol diet was launched for 2 weeks to make a reaction with recombinant protein (Table 1). Then blood samples were collected from the central ear artery of rabbits. The sera were obtained by centrifugation at 10000 rpm for 5 min. Biochemical elements such as HDL and LDL values were measured using the laboratory methods by Cobus device using cholesterol test kit (Biosystem, Germany). Next, animals were killed by ether anesthesia and the arches of the aorta were carefully removed and prepared across the section. Tissue fragments were fixed in 4% phosphate-buffered (pH 7.2) formaldehyde 10%, embedded in paraffin to process for conventional histology examination. Hematoxylin and Eosin (H&E) staining was used for histologic examination. It is apparent that H&E staining is one of the principal tissue stains used in histology it is the most widely used stain in medical

Table 1. Grouping, treatment, diet, and immunization state of the rabbits used in the study

Groups	Sample size	Treatment	Immunization	Cholesterol feeding period
Normal control	N=1	Control (normal chow diet)	None	None
Fat control	N=1	Diet containing 2% cholesterol without recombinant HSP60 protein	None	2 weeks
Immunized	N=1	Diet containing 2% cholesterol + recombinant HSP60 protein	Every 14 days (2 times)	2 weeks

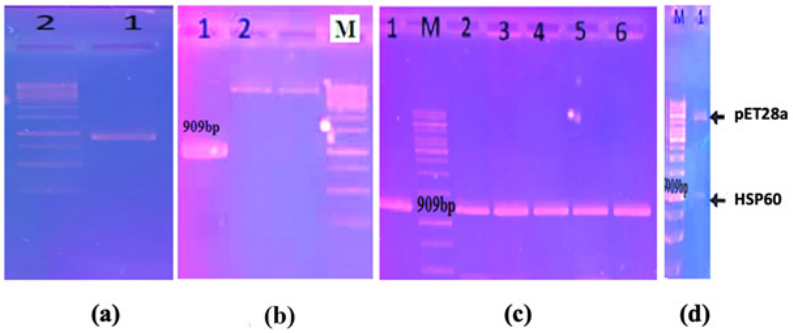

Figure 1. Results show confirmation of HSP60 (909bp) gene cloning by PCR and enzymatic digestion. Columns 1, 2, and M are related to size markers. (a) Column 1 is related to the linear product of pET28a plasmid enzymatic digestion of HSP60 with HindIII and EcoRI. (b) Columns 1 and 2 are related to the recovered gene and pET28a vector. (c) PCR cloning analysis of recombinant plasmids in BL21 (DE3) by T7 Primers. Column 1 is related to positive control and others are related to samples cultured on master plate. (d) Confirmation of enzymatic digestion in recombinant plasmids containing PET28a-HSP60 in BL21. Column 1 is related to HSP60 gene (909bp) digest

diagnosis, and is often the gold standard. In the H&E staining, the cell nuclei will be stained blue by hematoxylin, and the extracellular matrix and cytoplasm of the cell will be colored pink by eosin. Other structures of the cell will take diverse shades and combinations of these mentioned colors.

Western blotting

The recombinant proteins (HSP60), in denature phase, were separated by 12% SDS-PAGE and transferred onto the PVDF membrane. Membrane blocking was done with non-fat skim milk (5%) in 100 ml TBS buffer (50 mM Tris-Cl, 150 mM NaCl, pH 7.5) containing 0.05% Tween 20 at 37 °C for one hour and washed with TBS (1X). The anti-His tag antibody was prepared (1:2000; Sigma, Germany) in TBS buffer (1X) and incubated at 37 °C for one hour. Finally, the membrane was soaked in 3, 3'-Diaminobenzidine tablets (DAB Reagents; Sigma and H_2O_2 30%) for signal development. Finally, the reaction was inhibited with distilled water.

ELISA analysis of the recombinant proteins

The sera of the rabbits were prepared for revealing antibodies by indirect ELISA test. The antigen was quoted in the wells. Then the antibodies of rabbits were added to the wells (100 µl). The purified HSP60 (500 ng per well) in 100 µl bicarbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$) was used to coat Maxisorb plates (Nunc, Denmark) overnight at 4 °C. The wells were blocked for an additional two hours at 37 °C by covering the well with 200 µl of 5% (w/v) non-fat skim milk in PBST (PBS containing 0.05% Tween-20). After washing with PBST (3 times), the wells were exposed to the diluted immunized serum in a triplicate manner at 100 µl per well for one hour at 37 °C. The bound antibodies were detected with the goat anti-rabbit IgG conjugated with HRP (Sigma, Germany) in 1:5000 dilutions for one hour and washed with PBST (3 times). The response was improved with O-phenylenediamine (OPD) as a medium for HRP (Sigma, Germany) for 15 min in a dark environment and at ambient temperature. Then, we added 2.5 M of sulfuric acid to the reaction and measured the absorbance in each well at 492 nm.

Statistical analyses

To reveal the significance of differences in rabbits among the experimental groups and calculation of the P-values, we used Student's t-test. *P*-values <0.05 were determined as significance.

Results

Cloning procedures

The specific primers were designed to amplify HSP60 in the pET28a plasmid as a template. First of all, we had to do Gradient PCR for obtaining the appropriate temperature for running PCR by pfu. PCR done by the pfu enzyme has proofreading and a slower polymerization process. Because of the non-specific band in electrophoresis, we had to recover our gene from electrophorese gel by a GenoBiot kit. Following the use of EcoRI and HindIII enzymes for enzymatic digestion in pET28a plasmid, the linear product was obtained from enzymatic digestion of the plasmid containing the HSP60 gene (909 bp), which is visible in Figure 1a. The PCR product of HSP60 (909bp) was digested and cloned in pET28a which was digested with the same enzymes. The proper cloning procedure was confirmed by colony PCR and restriction enzyme digestion (EcoRI and HindIII, Figure 1b).

Transformation

The recombinant plasmid was transformed into TOP 10-competent cells and confirmed by colony-PCR. (Figure 1c).

The expression and purification of recombinant protein

The expression of recombinant proteins was analyzed on

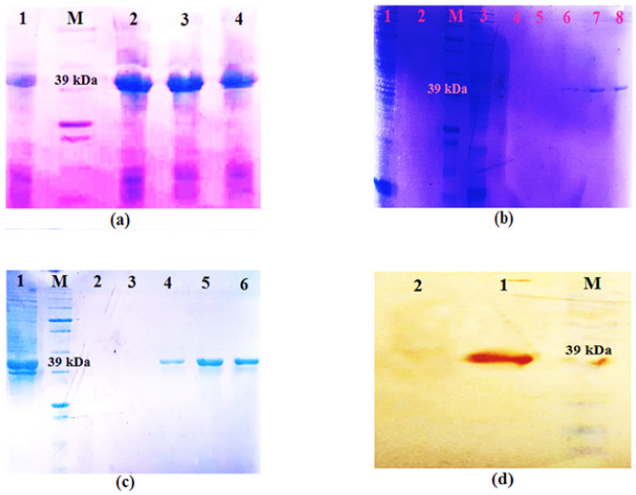

Figure 2. Protein evaluation on SDS-PAGE gel. (a) Validity of *HSP60* protein expression in both denatured and native state by SDS-PAGE technique. Column 1 is related to before induction by IPTG and columns 2 to 4 are related to samples (Hsp60 was induced by IPTG). (b) Verification of *HSP60* protein expression by Native technique. Column 1, sample before induction; Column 2, flow; Column 3, sample washed; Column 4 to7, output samples with Elution; and column 8, extracted sample with MES buffer (this buffer can separate any Hsp60 protein remaining). (c) Verification of *HSP60* protein (39 KDa) expression by denature technique. Column 1, flow; columns 2 to 5, output samples with elution; and column 6, extracted sample with MES buffer. (d) Immunoblotting of the recombinant proteins. Recombinant proteins verified by Western blotting using Anti-His Tag antibody. Columns 1 and 2 are related to recombinant proteins and negative control, respectively

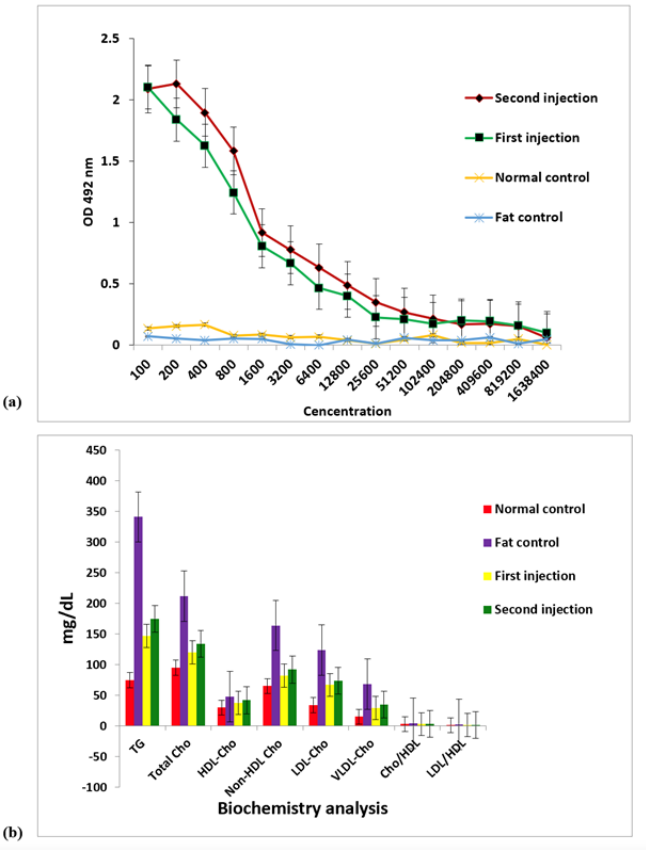

Figure 3. Antibody titration and biochemistry analysis from blood samples. (a) and (b) are related to antibody titration and biochemistry analysis, respectively. Diagram (a) shows that in the second injection immunogenicity is increased and gets in the same endpoint with the first injection. It means that there is a significant statistical correlation, and sufficient immunogenicity was created by recombinant protein, in (b) the fat control rabbit has the highest triglycerides (TG) and non-HDL cholesterol (a golden factor for patients at very high risk of heart disease) is increased significantly (P<0.05). Conversely, the rabbit that was immunized after every injection had lower cholesterol and triglycerides (TG)

12% SDS-PAGE and one fragment was observed (39kDa) in the fusion with 6 amino acids His-tag (N-terminal). This sequence is essential for gaining our protein more specific in affinity chromatography. Figures 2a and 2b illustrate SDS-PAGE, Native, and Denature methods, respectively. Hsp60 had more expression in denature phase and we used this type of protein for the rest of the experiment. After recombinant plasmid extraction from DH5α host, transfer was conducted to E. coli BL21 (DE3) competent cells and following that, PCR cloning was performed to confirm the recombinant vector transformation. Figure 1d demonstrates the result of PCR cloning of transferred BL21 (DE3) clones using primer T7. Also, the enzymatic digestion of recombinant plasmids containing PET28a-HSP60 in BL21 was performed.

Western blot method

The recombinant protein (HSP60) was separated by 12% SDS-PAGE and transferred onto the PVDF membrane (Roche, Germany). Membrane blocking was done with non-fat skim milk (this skim milk can fill the outlet to prevent losing protein) (5%) in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.05% Tween 20 at 37 °C for 2 hr. The membrane was further incubated with an anti-His tag at the N terminal of HSP60 (1:1000 dilution) and hyper-immune serum specific to Newcastle B1 strain (1:500), separately. The HRP-conjugated goat anti-rabbit IgG (1:2000; Sigma, Germany) was used as the secondary antibody. Finally, the membrane was soaked in 3, 3'-Diaminobenzidine tablets (DAB Reagents; Sigma) for signal development. This procedure was repeated with an HRP-conjugated anti-His tag (1:500, Roche) for recombinant HSP60 protein. Furthermore, to verify the cross-reactivity of the immune sera, the Newcastle B1 strain was blotted onto the PVDF and incubated with antisera from HSP60.

ELISA analysis

According to the antibody titration diagram given in Figure 3a, the immunogenicity is higher in the second step, both steps interrupted each other at two different points, which demonstrates a significant correlation (P<0.05). It means that sufficient immunogenicity was created by recombinant Hsp60 protein. Regarding Figure 3b, in the immunized group, after the second injection, in addition to decrease in the levels of total cholesterol (TC) and triglycerides (TG) that had occurred after the first injection, levels of Low-density lipoprotein cholesterol (LDL-C), and non-high-density lipoprotein cholesterol (non-HDL-C) had decreased significantly in contrast to fat in a control group that had not received any injection (P<0.05). The mean TC level of the immunized rabbit was 100–150 mg/dl (acceptable ≤ 200 mg/dl), and the TC of the fat control rabbit was nearly 250 mg/dl. Non-HDL-C is a gold factor for patients at very high risk of heart disease. This factor in fat

control rabbit has been increased sharply and nearly to 200 mg/dl (poor control ≥ 130 mg/dl) and the TG in fat control has been elevated up to 350 mg/dl (clinical interpretation according to the recommendation of the European Atherosclerosis Society, acceptable range in the fasting state is ≤ 200 mg/dl). In normal control, all the biochemical factors were in the normal range and it was concluded that the rabbit was healthy and without high cholesterol. The other factors such as, LDL-cholesterol (acceptable value is ≤ 100 mg/dl), very low-density lipoprotein cholesterol (VLDL-C) which its acceptable range between 8–38 mg/dl, and an atherogenic factor which refers to LDL/HDL (normal ratio between 1-5), were investigated in all 3 groups ($P<0.05$). According to Figure 3a, in the immunized group, as expected, after the first and second injections, antibody and measurement of optical density (OD) were increasing and got to the endpoint which demonstrates a significant statistical correlation ($P<0.05$).

Histological assay

Figure 4a illustrates the status of the immunized rabbit that received a fatty diet. Also, Figure 4b demonstrates the status of the rabbit in the fatty diet without a recombinant protein (HSP60). Considering the thickness of the fat aggregations at the media endothelial surface, it was revealed that this thickness is much higher in a rabbit that has not received the recombinant protein than in an immunized rabbit, which could be attributed to grade 4 or the most acute step. The normal control rabbit is shown in Figure 4c, which has not received the fatty diet and the recombinant protein. So, the fatty lesions have not precipitated on media endothelial. As mentioned, in the fat control rabbit, fat aggregation at the media endothelial surface could be attributed to Grade 4. It means that the penetrance of lesions, macrophages, and calcification in the endothelium of aortas in this animal occurred. In other words, in the immunized rabbit which received recombinant protein and fatty diet, the aggregation of lesions was much lower (nearly Grade 2) than fat control rabbit ($P<0.05$).

Discussion

Atherosclerosis is a chronic inflammatory disorder of the artery wall, and both innate and adaptive immunity play important roles in the pathogenesis of this disease. In several experiments, it has been shown that the first pathogenic event in atherogenesis is intimal infiltration of T cells at predilection sites. These T cells react to heat shock protein 60 (HSP60), which is a ubiquitous self-antigen expressed on the surface of endothelial cells (ECs) together with adhesion molecules in response to classical risk factors for atherosclerosis(15). The current study leads to measuring the expression level of HSP60 protein in E. coli and immunologically study the function of HSP60 in decreasing the aggregation of lesions in the endothelium of the aortas in a well-characterized animal model of atherosclerosis and result in decreasing the expression of its protein Hsp60 by the immune system. It can be proven that this experiment can help decrease levels of TG and cholesterol in the blood.

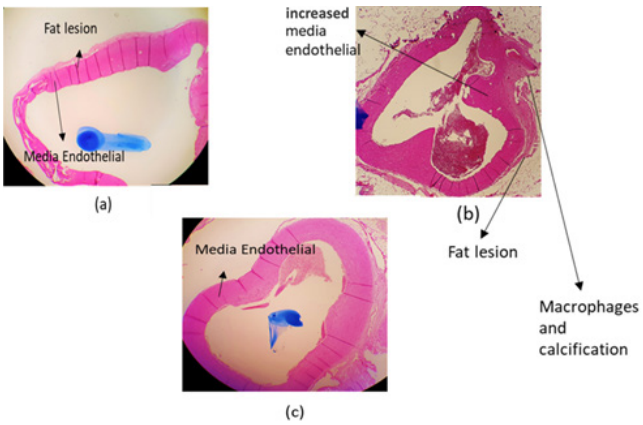

Figure 4. Immunized Rabbit by recombinant protein in histological assay across the fragments. (a) Belongs to the immunized rabbit with the amount of fat aggregation and macrophage accumulation (Grade 2(15) b) Is related to rabbit in the fatty diet without recombinant Hsp60 which demonstrates the penetration of fat lesions into the media endothelial and the calcification process (Grade 4(15)c) is related to a normal control rabbit with a healthy endothelial wall

After designing the plasmid containing the HSP60 gene fragment, we cloned it with the expression plasmid PET28a into DH5α. In another experiment, the genes of Tm-HSP60 and Tm-p36 were cloned in Taenia multiceps tapeworm (its larval form) and molecular characterization was analyzed by bioinformatics tools. The validity and immunogenicity of rTm-HSP60 and rTm-p36 were confirmed by Western blotting.

Our target was about setting the His6-tag at the end of the protein, in order to purify Hsp60 more specifically in Ni column affinity chromatography. BL21(DE3) has a strong promoter to overexpression Hsps, and protease enzyme was declined to not denaturation the specific protein.

In another experiment, as we did in this experiment, HSP27, HSP104, and HDJ1 were inserted in pET28-a with N-terminal His6-tag. All HSPs were expressed in BL21 (DE3). In our project, the purpose was to inhibit the expression of the Hsp60 gene by the immune system but at first, the rabbit should be immunized by recombinant protein, then fed a high-cholesterol diet in order to get infected in atherosclerosis.

HSP27 with overexpression can inhibit amyloid aggregation of α-syn even at a molar ratio as low as 50:1 (α-syn/HSP27), demonstrating that HSP27 exhibits potent chaperone activity for α-syn (16).

Xu Huang1 in 2017, truncated HSP110, as a tumor vaccine to hinder *in vivo* tumor growth, the tHSP110 has the same capacity to bind macromolecular antigens and initiate tumor immune reactions as full-length HSP110 in mice (17). Similarly, we have tried to increase the antibody titration to inhibit the expression of Hsp60 by the immune system automatically. As mentioned above, we studied the HSP protein for the treatment of atherosclerosis in an animal model and stimulating the immune system after cloning and monitored the disease process.

Based on the numerous studies that we mentioned, we tried to somehow show the important roles of this protein in rabbits. To this end, we tested the recombinant protein on a number of rabbits that were affected by certain diets and observed that the recombinant protein has prevented excessive deposition of fat lesions on the media endothelial surface. According to the thickness formed by the fat lesions at the media endothelial surface, the thickness of the fat lesions was about half the level of the endothelial thickness, which is related to the second grade of classification criteria for atherosclerosis

In our experiment, each of the cross-sections that were

obtained from the extracted heart was stained and observed by a professional pathologist. It was expected that the rabbits that received a fatty diet and recombinant protein, have a narrower thickness in their endothelial intima (Grade 2) and the normal control have a normal thickness endothelial media. In addition, fatty control has calcification and a thicker endothelial layer with the aggregation of macrophage and fat lesions (Grade 4).

The results of our study are in line with a study done by Zhang *et al.* who showed that raised HSP60 levels are related to an augmented threat for CHD and HSP60 and anti-HSP60 antibody levels combine to surge this risk (18). Comparable results can be seen when animals are immunized with peptides of the corresponding HSP60. For example, immunizations with mbHSP65 peptide (91–105 amino acids) lead to enhanced atherosclerosis in rabbits and aortic EC injury in mice. Surprisingly, HSPs include large proteins that, when processed, provide a multitude of potential epitopes; only a few of these, however, are atherogenic (19).

We realized that the group of rabbits had not received the recombinant protein and fed with high-cholesterol chew, it has a thickness lesion of atherosclerosis and the calcification is observed. Our purpose has been directed in the direction of recently done studies, such as the study that was done by Ghayour *et al.* in 2007 and showed that the von Willebrand factor and antibodies to HSP60, 65, and 70 all rise rapidly during early atherogenesis, and are associated with the extent of atherosclerotic lesion formation in a rabbit model (20). In the study of Khera *et al.*, a rabbit on a fatty diet with 2.0% cholesterol was vaccinated with CETP (cholesteryl ester transfer protein). In the rabbit that received recombinant protein, HDL range was accelerated sharply and the fat aggregation in their aorta arch endothelial was not decreased significantly. Already, it was proven that the HDL function can be effective in preventing atherosclerosis lesions (21). In this study, finally, we measured Chol, HDL, LDL and the other parameters related to the fatty lesion and it can be concluded that the group that received the recombinant Hsp60, has a low level of Chol and Triglyceride and Non-HDL Chol (directly related to coronary disease) decreased sharply. In a similar study that has evaluated the function of CETP (cholesteryl ester transfer protein) by the recombinant vaccine, they found that it was effective in attenuating atherosclerosis in a rabbit model. Rabbits were fed a high cholesterol diet until atherosclerosis lesions formed in the aorta. The result showed that after vaccination with anti-CETP, the fraction of plasma HDL cholesterol increased and LDL decreased. The average size of aorta atherosclerosis plaque in the rabbit treated with rAnsB-TTP-CETP was 42.3% less than control (22). In our experiment, we tried to demonstrate the increasing IgG after two injections and collecting blood samples two weeks after each injection. The sera were specific experimented by ELISA. Significantly, the anti-Hsp60 antibody titration (IgG) was increased, and both IgG titration meet each other in the graph. Therefore, there is a significant correlation between the two results. (according to the Figure 3a). Similarly, in a study by Bacillus *et al.* mice were immunized subcutaneously by HSP65, and the heart and spleen were removed. Experimental data demonstrated that IgG anti-HSP65 antibodies could accelerate fatty streaks in recipient mice. IgG HSP65 antibodies can be formed in response to infections and react with self-HSP60 in humans. The level of anti HSP65 antibodies correlates with carotid atherosclerosis and their sustained presence appears to predict mortality among atherosclerosis patients. (23). Another study showed the roles of HSP65 and 70 in CHD. They observed that anti HSP65 significantly increased in a rabbit that was fed a high cholesterol diet. High antibody titers to HSP65 were found to be associated with coronary calcification (24). In another study, rabbits were immunized with BCG (Bacillus Calmette-Guerin) vaccine and control with saline. They were fed a 0.25–1.0% cholesterol diet for 10 weeks. After immunization, they found out that there is a correlation between antiHSP60 and aortic lesion area. They indicate that there is a correlation between infection and atherosclerosis, and immunization with HSPs containing vaccine exacerbates thermogenesis in a high cholesterol-fed rabbit model (25).

The target is prevention of atherosclerosis in families that are more susceptible to this disorder. Because this disease is chronic and can last for undefined times, it should be prevented as soon as possible in these families. Soluble HSP60 (sHSP60) and/or anti-hHSP60 antibody concentrations may be used as prognostic biomarkers for the risk of developing cardiovascular disease (CVD). On one hand, the limitation in atherosclerosis is that the different epitopes from the same HSP may therefore have a very different functional effect on the immune response. On other hand, the limitation of this study is about the number of rabbits and the statistical society but the most significant achievement is just the performance of this protein and release of the antibodies and the measurement of it. Also, this thesis can be experimented in other animal models such as rats or those who are similar to the human heart and its mechanism.

According to the other thesis, atherosclerotic lesions can be induced in normocholesterolemic rabbits by immunization with recombinant mycobacterial HSP65. Levels of serum antibodies to HSP65 were sharply increased in clinically healthy human subjects with carotid atherosclerosis compared with those without lesions. It can be suggested that the other subset of Hsps can improve the lesions. The other advantage of this study is that it can be detected sooner in the early grades of coronary disease and prevent malignancy. It is essential to follow up on this hypothesis and do some professional experiments to achieve effective discovery.

Conclusion

The results that are obtained from this study confirm the ultimate aim of designing and cloning HSP60. This protein has several epitopes that are proving to create atherosclerosis. The antigenicity of the purified HSP60 protein has been proven to be satisfactory and this protein was injected into the rabbit model. In the rabbit which received recombinant protein, the aggregation of atherosclerosis lesions was decreased significantly and the results of biochemical factors such as LDL-C and Non-HDL-C proved this ($P<0.05$). On the one hand, the fat control rabbit that was fed with a fatty diet without any recombinant protein had high levels of LDL-C and VLDL-C. On the other hand, the layer of the aortic arch was covered by calcification and aggregation of macrophages and atherosclerosis lesions. This protein can create and exacerbate CHD in various ways but atherosclerosis is one of the main diseases that should be

detected as soon as it is possible.

Acknowledgment

The present research is part of the Master's thesis for the field of genetics approved by the Islamic Azad University of Science and Research.

Authors' Contributions

SZ is the student; HR, FN, and MJM are experimental assistants; and SS was responsible for writing and editing the manuscript. JA is the supervisor of this research and final approval is done by him.

Funding

No funding.

Ethical Authorization and Informed Agreement

All techniques performed in studies including human participants were in conformity with the ethical principles of the institutional and national research committee and with the 1964 Helsinki declaration and its following modifications.

Conflicts of Interest

The authors of this article state that they have no conflicts of interest.

References

1. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002; 105:1135-1143.
2. Hansson GK, Libby P. The immune response in atherosclerosis: A double-edged sword. Nat Rev Immunol 2006; 6:508-519.
3. Bernhagen J, Krohn R, Lue H, Gregory JL, Zernecke A, Koenen RR, *et al*. MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. Nat Med 2007; 13:587-596.
4. Harvey EJ, Ramji DP. Interferon-γ and atherosclerosis: pro-or anti-atherogenic? Cardiovasc Res 2005; 67:11-20.
5. Kleemann R, Zadelaar S, Kooistra T. Cytokines and atherosclerosis: A comprehensive review of studies in mice. Cardiovasc Res 2008; 79:360-376.
6. Sepanlou SG, Newson RB, Poustchi H, Malekzadeh MM, Asl PR, Etemadi A, *et al*. Cardiovascular disease deaths and years of life lost attributable to non-optimal systolic blood pressure and hypertension in Northeastern Iran. Arch Iran Med 2015; 18:144.
7. van Puijvelde GH, van Es T, Van Wanrooij E, Habets K, De Vos P, Van Der Zee R, *et al*. Induction of oral tolerance to HSP60 or an HSP60-peptide activates T cell regulation and reduces atherosclerosis. Arterioscler Thromb Vasc Biol 2007; 27:2677-2683.
8. Shatilina ZM, Riss HW, Protopopova MV, Trippe M, Meyer EI, Pavlichenko VV, *et al*. The role of the heat shock proteins (HSP70 and sHSP) in the thermotolerance of freshwater amphipods from contrasting habitats. J Therm Biol 2011; 36:142-149.
9. Lu X, Kakkar V. The role of heat shock protein (HSP) in atherosclerosis: Pathophysiology and clinical opportunities. Curr Med Chem 2010; 17:957-973.
10. Kaufman BA, Kolesar JA, Perlman PS, Butow RA. Studies on mitochondrial DNA nucleoids in Saccharomyces cerevisiae: Identification of bifunctional proteins. J Cell Biol 2003; 163:457-461.
11. Ghosh JC, Dohi T, Kang BH, Altieri DC. Hsp60 regulation of tumor cell apoptosis. J Biol Chem 2008; 283:5188-5194.
12. Zininga T, Ramatsui L, Shonhai A. Heat shock proteins as immunomodulants. Molecules 2018; 23:2846.
13. Kim DS, Ko YJ, Lee MW, Park HJ, Park YJ, Kim D-I, *et al*. Effect of low oxygen tension on the biological characteristics of human bone marrow mesenchymal stem cells. Cell Stress Chaperones 2016; 21:1089-1099.
14. Urban-Chmiel R, Dec M, Puchalski A, Wernicki A. Characterization of heat-shock proteins in Escherichia coli strains under thermal stress *in vitro*. J Med microbiol 2013; 62:1897-1901.
15. Wick C. Tolerization against atherosclerosis using heat shock protein 60. Cell Stress Chaperones 2016; 21:201-211.
16. Jia C, Ma X, Liu Z, Gu J, Zhang X, Li D, *et al*. Different Heat Shock Proteins Bind α-Synuclein With Distinct Mechanisms and Synergistically Prevent Its Amyloid Aggregation. Front Neurosci 2019; 13.
17. Huang X, Dong H, Ting Z. Cloning, expression of a truncated HSP110 protein that augments the activities of tumor antigen-specific cytotoxic and apoptosis via tHSP110-peptide complex vaccines. Int J Clin Exp Pathol 2017; 10:10304-10314.
18. Zhang X, He M, Cheng L, Chen Y, Zhou L, Zeng H, *et al*. Elevated heat shock protein 60 levels are associated with higher risk of coronary heart disease in Chinese. Circulation 2008; 118:2687-2693.
19. Zhang Y, Xiong Q, Hu X, Sun Y, Tan X, Zhang H, *et al*. A novel atherogenic epitope from Mycobacterium tuberculosis heat shock protein 65 enhances atherosclerosis in rabbit and LDL receptor-deficient mice. Heart Vessels 2012; 27:411-418.
20. Ghayour-Mobarhan M, Lamb DJ, Tavallaie S, Ferns GA. Relationship between plasma cholesterol, von Willebrand factor concentrations, extent of atherosclerosis and antibody titres to heat shock proteins-60, -65 and -70 in cholesterol-fed rabbits. Int J Exp Pathol 2007; 88:249-255.
21. Khera AV, Cuchel M, De La Llera-Moya M, Rodrigues A, Burke MF, Jafri K, *et al*. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. N Engl J Med 2011; 364:127-135.
22. Gaofu Q, Jun L, Xiuyun Z, Wentao L, Jie W, Jingjing L. Antibody against cholesteryl ester transfer protein (CETP) elicited by a recombinant chimeric enzyme vaccine attenuated atherosclerosis in a rabbit model. Life Sci 2005; 77:2690-2702.
23. George J, Afek A, Gilburd B, Shoenfeld Y, Harats D. Cellular and humoral immune responses to heat shock protein 65 are both involved in promoting fatty-streak formation in LDL-receptor deficient mice. J Am Coll of Cardiol 2001; 38:900-905.
24. Ghayour-Mobarhan M, Rahsepar A, Tavallaie S, Rahsepar S, Ferns G. The potential role of heat shock proteins in cardiovascular disease: evidence from *in vitro* and *in vivo* studies. Adv Clin Chem 2009; 48:27-72.
25. Lamb DJ, Ferns GA. The magnitude of the immune response to heat shock protein-65 following BCG immunisation is associated with the extent of experimental atherosclerosis. Atherosclerosis 2002; 165:231-240.