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Evaluating the effect of the antiPCSK9 vaccine on systemic inflammation and oxidative stress in an experimental mouse model

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Abstract

Background: *To investigate whether the antiPCSK9 vaccine can affect the CRP and oxidative stress (OS) during acute systemic inflammation.*

Methods: *Male albino mice were randomly divided into three groups: non-treated mice (the sham group), treated with a nonspecific stimulator of the immune response — Freund's complete adjuvant (CFA; the CFA group), and vaccinated mice treated with CFA (the vaccine group). The vaccine group was subcutaneously immunized with the antiPCSK9 formulation, 4 × in bi-weekly intervals. To induce inflammation, all mice were subjected to the CFA challenge after the vaccination plan. The hsCRP level and OS status were evaluated by a mouse CRP assay kit and the pro-oxidant antioxidant balance (PAB) assay, respectively.*

Results: *The vaccine induced a high-titer IgG antiPCSK9 antibody, which was accompanied with a significant PCSK9 reduction (–24.7% and –28.5% compared with the sham and CFA group, respectively), and the inhibition of PCSK9/LDLR interaction (–27.8% and –29.4%, respectively). hsCRP was significantly increased in the vaccine and CFA groups by 225% and 274%, respectively, when compared with the sham group; however, it was non-significantly decreased (–18%; $p = 0.520$) in the vaccine group in comparison with the CFA group. The PAB values indicated that OS was significantly increased in the CFA group (by 72.7%) and the vaccine group (by 76%) when compared to the sham group; however, there was no significant difference in the PAB values between the vaccine and CFA groups.*

Conclusions: *The antiPCSK9 vaccine failed to significantly reduce the serum hs-CRP and OS induced in the CFA-challenged albino mice.* (Cardiol J 2025; 32, 1: 73–82)

Keywords: C-reactive protein, inflammation, oxidative stress, PCSK9 vaccine

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Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a well-known regulator of cholesterol homeostasis, which acts via the binding to the hepatocyte low-density lipoprotein (LDL) receptor (LDLR) that will be consequently targeted to the lysosomal degradation [1–4]. Immediately after the discovery of PCSK9 protein and its function, growing evidence from genetic association studies showed PCSK9 inhibition as a potential lipid-lowering target [1–5]. Currently there are several types of PCSK9 inhibitors such as the FDA-approved PCSK9 monoclonal antibodies (mAbs) alirocumab and evolocumab [6–9] and small interference RNA against mRNA PCSK9 [10]. Additionally, there are under-investigation oral PCSK9 inhibitors (e.g., macrocyclic peptide MK-0616) [11–14]. Finally, there are antiPCSK9 vaccines [15–21], which have emerged as effective therapeutics for ameliorating hypercholesterolemia and atherosclerosis in preclinical studies.

Besides the role in cholesterol metabolism, there is also experimental and clinical evidence showing that PCSK9 can act as a pro-inflammatory mediator, however, there are contradictory reports regarding the effect of PCSK9 inhibitors on inflammation [22]. Inflammation contributes to the initiation and progression of atherosclerosis up to plaque rupture and erosion, causing atherosclerotic cardiovascular disease (ASCVD) [23].

High-sensitivity C-reactive protein (hs-CRP) is an acute-phase mediator mainly produced by the hepatocytes, which is considered as a sensitive but non-specific biomarker of systemic inflammation [24]. Hs-CRP has been known as a risk marker/risk enhancer and potential risk factor for atherosclerosis [25] as well as a strong cardiovascular risk predictor [26], however the casual association between hs-CRP and CVD events has not been confirmed [27]. Mechanistically, hs-CRP can elevate the LDL uptake by macrophages and consequently accelerate foam cell formation, which has a direct role in the initiation of atherosclerotic plaque formation [28]. Several epidemiological studies have indicated a positive and strong association between plasma levels of PCSK9, hs-CRP, and acute-phase inflammation in patients with coronary artery disease (CAD) [29–31]. Nevertheless, despite the aforementioned association between PCSK9 and hs-CRP, there is evidence indicating no association between the treatment with mAbs-based PCSK9 inhibitors and changes in hs-CRP levels in CVD patients [32–36].

Other important inflammation modulators are reactive oxygen species (ROS) and related enzymes responded to oxidative stress, such as myeloperoxidase (MPO), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase. Oxidative stress is observed when there is an imbalance between the ROS generation and elimination, due to the impaired antioxidant defence system and/or the exacerbated activity of pro-oxidant enzymes [37]. It has been found that there is a link between PCSK9 production and oxidative stress [38–41], mediated predominantly by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent ROS generation [42, 43]. There are reports that demonstrated the up-regulatory effect of ROS on the PCSK9 expression and *vice versa* in vascular cells, leading to destructive inflammatory responses within atherosclerotic plaques [42–44]. Notably, the PCSK9 inhibition, by either the gene manipulation or anti-PCSK9 monoclonal antibodies, has been found to significantly attenuate ROS-mediated oxidative damage in the *in vitro* cellular model [45, 46] and various animal models [47, 48].

To the best of our knowledge, the antiPCSK9 vaccines on the hs-CRP level and the oxidative stress in an experimental inflammation model is understudied. During the recent few years, we have developed an antiPCSK9 vaccine [21] that could effectively induce the safe and long-lasting generation of the functional antiPCSK9 antibodies, which was accompanied with significant therapeutic [49, 50] and preventive [51, 52] effects against hypercholesterolemia and atherosclerosis in mouse and primate models. In the present study, we aimed to find whether the PCSK9 inhibition using the antiPCSK9 vaccine can affect the hs-CRP level and the oxidative stress during systemic inflammation.

Methods

The vaccine preparation

An immunogenic peptide construct containing PCSK9 and tetanus epitopes was designed using AFFITOME® technology [21, 53]. The peptide sequence (Table 1) with a purity grade of > 95% was synthesized and high-performance liquid chromatography (HPLC)-purified by ChinaPeptides Co., Ltd. (Shanghai, China). The peptide was adsorbed to 0.4% Alum adjuvant (Sigma-Aldrich) at the 1:1 (v:v) ratio and used for *in vivo* studies on mice.

Table 1. Sequences of the immunogenic peptides used in the current study

Peptide name	Sequence	Immunogenicity
PCSK9	S-I-P-W-N-L-E-R-I-T-P-V-R	B cell epitope
Tetanus	A-Q-Y-I-K-A-N-S-K-F-I-G-I-T-E-L	T cell epitope
PCSK9 peptide vaccine	SIPWNLERITPVRkkAQYIKANSKFIGITEL	

*A 2 lysine-spacer sequence (kk) as the target sequence of cathepsin protease involved antigen processing

Animals

8–10 weeks old albino mice (28 ± 3 g) were purchased from the laboratory animal research centre of Razi Vaccine and Serum Research Institute, Mashhad, Iran. All animal handling procedures were carried out in strict accordance with the Animal Welfare guidelines approved by the Institutional Ethics Committee and Research Advisory Committee of the Mashhad University of Medical Sciences, Iran (code: IR.MUMS.PHARMACY.REC.1400.010). The animals were housed in an air-conditioned room at a constant temperature of 22 ± 2°C with 12:12 h light/dark cycle and fed a standard rodent diet and water ad libitum. At the end of the study, all animals were euthanized by intravenous injection (30 mg/kg) of thiopental sodium.

Vaccination plan

Thirty male albino mice were randomly divided into three groups, including non-treated mice (the sham group), the mice treated with the Freund's complete adjuvant (CFA; the CFA group), and the vaccinated mice treated with CFA (the vaccine group). The vaccine group was subcutaneously immunized with the antiPCSK9 formulation (15 μg/mouse), four times in bi-weekly intervals, while the sham and CFA groups received the phosphate buffer by a similar route. After the vaccination plan, all mice were subjected to the CFA challenge to evaluate the effect of the antiPCSK9 vaccine on inflammation and oxidative stress status.

Developing CFA-challenged mice

To develop an animal model with acute inflammation and oxidative stress, the method of Fehrenbacher et al. [54] with some changes was used. In brief, CFA emulsion (0.5 mg/ mL) was prepared via mixing 0.5 mL of CFA (1 mg/mL of *Mycobacterium tuberculosis*, heat-killed and dried; Sigma-Aldrich, St. Louis, MO, USA) in 0.5 mL of sterile 0.9% saline buffer. The CFA group and the vaccine group were treated with 50 μL of freshly prepared homogeneous CFA emulsion (0.5 mg/mL) by subcutaneous injection into the left hind paw, while the sham group received 50 μL of the saline buffer by a similar route. According to our previous evaluation of CRP's kinetic [55], the serum hs-CRP reaches the highest level in CFA-challenged albino mice after 16-24 h; thus, a point in time was selected to evaluate the effect of the antiPCSK9 vaccine on inflammation and oxidative stress status. Mice were anesthetized and blood was withdrawn by cardiac drainage into a dry tube. Serum was separated by centrifugation at 1800 g for 10 min and kept at −20°C prior to analysis.

Evaluating the serum hs-CRP level and oxidative stress

To find the effect of the antiPCSK9 vaccine on acute inflammation, serum concentrations of hs-CRP were measured using a mouse CRP ELISA kit (Abcam; ab157712). To determine oxidative stress status, the pro-oxidant antioxidant balance (PAB) in the serum samples was assayed according to the previously described method [56]. In brief, a mix of 10 μL of each serum sample or standard solution and 200 μL of fresh working solution [containing TMB/ /DMSO solution, 0.05 M acetate buffer (pH 4.5), 100 mM chloramine T fresh solution, and 25 U of peroxidase enzyme solution] was loaded into a 96-well plate and incubated in a dark place for 12 minutes at 37°C. Then, 100 μL of 2 N HCL was added to each well and the OD was measured at 450 nm, with a reference wavelength of 620 nm or 570 nm. A standard curve was prepared using standard solutions with different proportions (0–100%) of hydrogen peroxide (250 μM) and uric acid (3 mM in 10 mM NaOH). Finally, the samples' PAB values were measured according to the prepared standard curve. The values of the PAB assay were expressed in an arbitrary HK (Hamidi-Koliakos) unit based on the percentage of hydrogen peroxide detected in the standard solution.

Evaluating the vaccine efficacy

To determine the efficacy of the antiPCSK9 vaccine in mice, plasma antiPCSK9 antibody titer, plasma PCSK9 concentration, and antibody inhibited PCSK9/LDLR interaction were measured as described in the following subsections.

Measuring plasma antiPCSK9 antibodies

The ELISA method was employed to evaluate the titer of plasma antiPCSK9 antibodies in vaccinated mice. In brief, 100 μL of serially diluted plasma samples (1:400 × 1:4) were loaded and incubated for 1h at 37°C in a 96-well Nunc-MaxiSorp plate coated with PCSK9 peptide. To detect attached antiPCSK9 antibodies, HRP-conjugated anti-mouse IgG (H + L) (Sigma Aldrich; dilution 1:1000) was added and incubated for 1 h at 37°C followed by the addition of the substrate TMB (3,3',5,5'-tetramethylbenzidine, Sigma-Aldrich) for 10 min at room temperature (RT). The optical density (OD) at 450 nm was measured with a microwell plate reader (Sunrise, Tecan, Switzerland) and the titers were defined as the dilution factor referring to 50% of the maximal optical density ($OD_{max}/2$). The results were presented as the mean titers ± SD of all animals per group.

Measuring plasma PCSK9 concentration

The concentration of plasma PCSK9 protein in vaccinated mice was measured by a PCSK9 ELISA kit (CircuLexTM, Cy-8078, MBL, Woburn, MA) in accordance with the manufacturer's manual. In brief, 100 μL of the diluted plasma samples (1:100) was incubated on a 96-well microplate for 1 h at RT. An HRP-conjugated anti-PCSK9 antibody was incubated for 1 h followed by adding the substrate reagent and stop solution, all at RT. The microwell plate reader was used to detect the OD at 450 nm. Eventually, a standard curve provided by the supplier was defined to measure the plasma concentration of PCSK9.

Evaluating the effect of plasma antiPCSK9 antibodies on the PCSK9-LDLR interaction

The potential of vaccine-induced antibodies to inhibit the interaction of PCSK9/LDLR was assayed by using a PCSK9-LDLR *in vitro* binding assay kit (CircuLex™, Cy-8150, MBL, Woburn, MA) in accordance with the manufacturer's manual. In brief, 100 μL of vehicle control or the plasma samples of vaccinated mice were loaded in a 96-well microplate pre-coated with the recombinant EGF-A domain of LDLR containing the binding site for PCSK9. Thereafter, the reaction was immediately initiated by adding a "His-tagged PCSK9 wiled type" solution incubated for 2 h followed by adding a biotinylated anti-His-tag monoclonal antibody for 1 h at RT. Subsequently, HRP-conjugated streptavidin was incubated for 1 h at RT followed by adding the substrate reagent and stop solution. The OD at 450 nm was measured with the microwell plate reader. Notably, the higher ELISA OD indicates a higher amount of PCSK9-LDLR interaction, while in the presence of plasma containing antiPCSK9 antibodies the interaction is inhibited and consequently a reduced ELISA OD will be detected.

Statistical analysis

One-way ANOVA and Tukey-Kramer post-hoc multiple comparison tests were carried out to measure the significance of the difference among groups, (Graph Pad Prism Software, version 9.0, San Diego, CA). Data were reported as mean ± SD. Data with $p < 0.05$ were regarded to be statistically significant.

Results

The antiPCSK9 vaccine induced the functional antibodies in albino mice

Upon three boosters, the antiPCSK9 vaccine was found to significantly promote a high-titer IgG antibody against the PCSK9 peptide in albino mice — the antiPCSK9 antibody titer (ODmax/2) was 12,925 ± 929 in the vaccinated mice, two weeks after the last immunization (Fig. 1A). The plasma concentration of free PCSK9 was found to be significantly ($p \leq 0.001$) reduced by –24.7% and –28.5% in the vaccine group when compared to the sham and CFA group, respectively (Fig. 1B). Moreover, to determine whether the vaccine-induced antiPCSK9 antibodies can inhibit PCSK9 function, CircuLex PCSK9-LDLR *in vitro* binding assay kit was employed. In the presence of the vaccinated mice's plasma samples, *in vitro* binding of murine PCSK9 and LDLR in the vaccinated group was significantly ($p < 0.05$) reduced by –27.8% and –29.4% when compared to the plasma samples of the sham group and the CFA group, respectively (Fig. 1C).

The antiPCSK9 vaccine and acute inflammation in CFA-challenged mice

It was shown that the antiPCSK9 vaccine could not significantly affect the increased level of serum hs-CRP in the CFA-challenged albino mice — the serum levels of hs-CRP in the vaccine, CFA, and sham groups were 14.65 ± 4.66 mg/L,

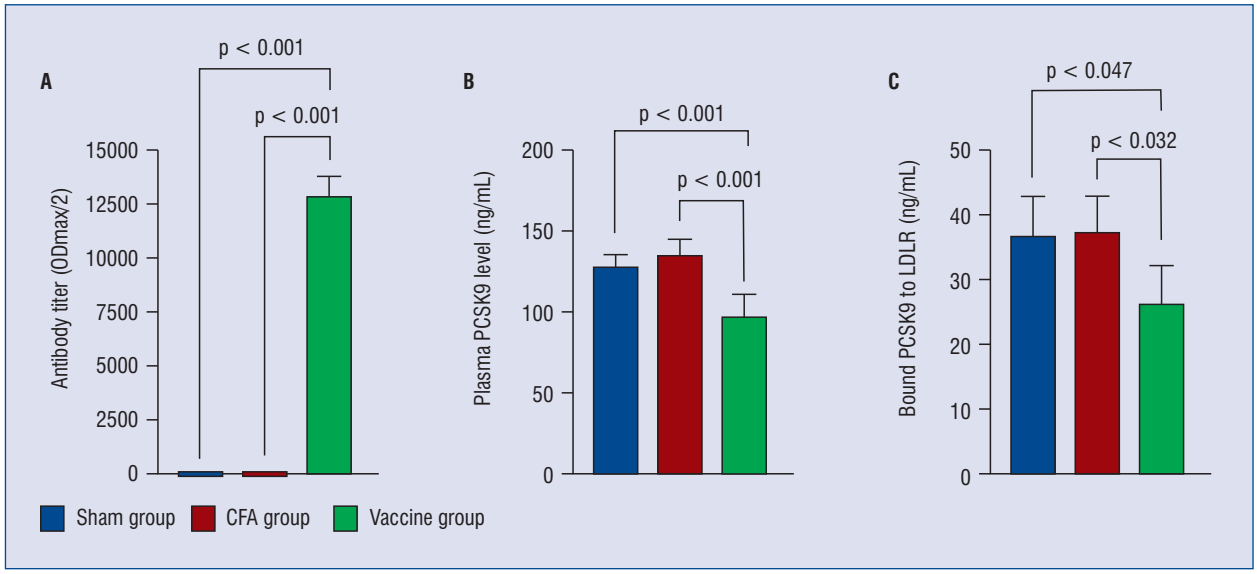

Figure 1. The efficacy of the antiPCSK9 vaccine in albino mice, two weeks after the last immunization. The sham group involved non-treated mice, the CFA group involved the CFA-treated mice, and the vaccine group involved mice who after vaccination were treated with the CFA. **A** — The antiPCSK9 antibody titer (ODmax/2) in the vaccinated and non-vaccinated albino mice. **B** — The plasma concentrations of the free PCSK9 in the vaccine, CFA, and sham groups were 97.4 ± 13.8 ng/mL, 136.2 ± 9.8 ng/mL, and 129.4 ± 7.8 ng/mL, respectively. **C** — *In vitro* PCSK9/LDLR binding assay. The levels of bound PCSK9 to LDLR in assays using the plasma samples of the vaccine, CFA, and sham groups, were 26.4 ± 5.4 ng/mL, 37.4 ± 5.6 ng/mL, and 36.6 ± 6.4 ng/mL, respectively. Data are expressed as mean ± SD (10 mice per group). Pooling of samples was performed to obtain sufficient sample volume for assay, when needed. Statistical differences at a p-value less than 0.05 were considered to be significant

17.84 ± 5.37 mg/L, 6.5 ± 2.02 mg/L, respectively (Fig. 2). The statistical analysis indicated that the level of hs-CRP was significantly increased in the vaccine and CFA groups by 225% ($p = 0.037$) and 274% ($p = 0.004$), respectively when compared with the sham group. It was non-significantly decreased in the vaccine group in comparison with the CFA group (by 18%, $p = 0.520$).

The antiPCSK9 vaccine and the oxidative stress in CFA-challenged mice

To determine the effect of the antiPCSK9 vaccine on oxidative stress, the balance between the plasma pro-oxidant load and antioxidant capacity was evaluated using the PAB assay. It was shown that the antiPCSK9 vaccine could not significantly affect the serum pro-oxidant/antioxidant status in CFA-challenged albino mice. The PAB values in the vaccine, CFA, and sham groups were 54.22 ± 10.93 HK, 53.19 ± 9.8 HK, and 30.8 ± 6.7 HK, respectively (Fig. 3). The PAB value (oxidative stress) was significantly increased in the CFA group (by 72.7%, $p < 0.001$) and the vaccine group (by 76%, $p < 0.001$) when compared with the sham group with no significant difference between the vaccine and CFA groups.

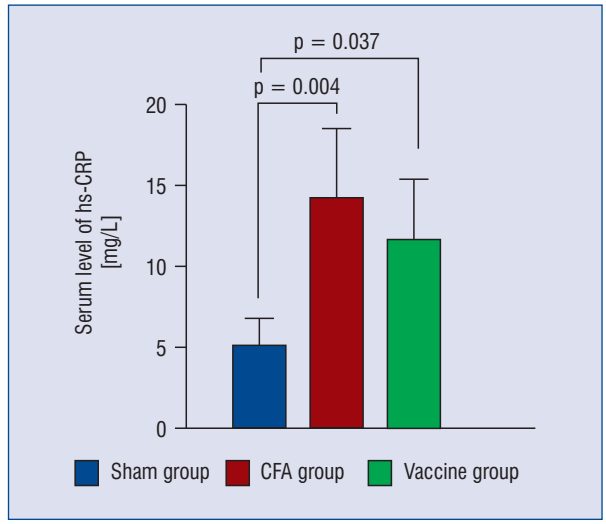

Figure 2. The effect of the antiPCSK9 vaccine on the serum level of hs-CRP in albino mice. The sham group involved non-treated mice, the CFA group involved the CFA-treated mice, and the vaccine group involved mice who after vaccination were treated with the CFA. Pooling of samples was performed to obtain sufficient sample volume for assay, when needed. Data are expressed as mean ± SD (10 mice per group). Statistical differences at a p-value less than 0.05 were considered to be significant

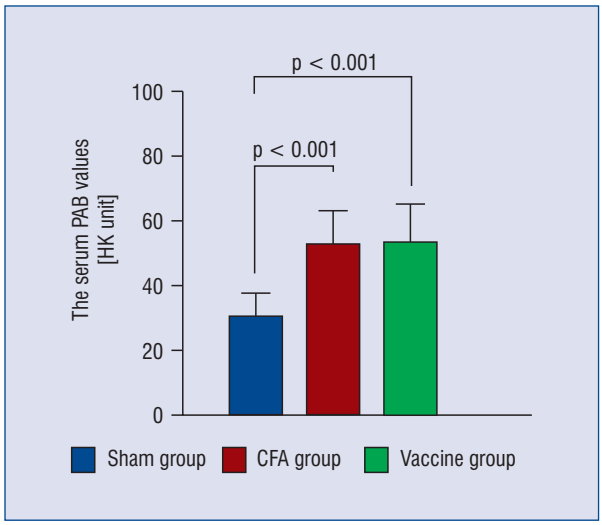

Figure 3. The effect of the antiPCSK9 vaccine on the serum PAB (pro-oxidant antioxidant balance) in albino mice. The PAB values were expressed in an arbitrary HK (Hamidi-Koliakos) unit. The sham group involved non-treated mice, the CFA group involved the CFA-treated mice, and the vaccine group involved mice who after vaccination were treated with the CFA. Pooling of samples was performed to obtain sufficient sample volume for assay, when needed. Data are presented as mean ± SD (10 mice per group). Statistical differences at a p-value less than 0.05 were considered to be significant

Discussion

The present study indicated that, despite inducing the production of the functional antiPCSK9 antibodies, the antiPCSK9 vaccine failed, despite numerical reduction, to significantly reduce the serum hs-CRP in the CFA-challenged albino mice. It was also observed that there was a lack of any reducing effect on the oxidative stress in this model. The production of the functional antiPCSK9 antibodies bounding blood circulating PCSK9 protein and consequently reducing the plasma level of PCSK9 and its interaction with the live LDLR has also been detected in previous studies, supporting the efficacy of this antiPCSK9 vaccine [21, 49–52].

Of note, there has been a paucity of studies evaluating the association of an antiPCSK9 vaccine and the serum hs-CRP levels in the inflammatory condition. A recent study [57] indicated that the present antiPCSK9 vaccine did not change the serum level of hs-CRP in healthy rhesus macaque monkeys. There have also been several clinical trials that investigated the effect of mAb-based PCSK9 inhibitors on inflammatory markers, especially on the hs-CRP levels, in patients with CVD, supporting the present results [31–36]. Data of the EQUATOR study, a randomized, multicenter, double-blind, and placebo-controlled phase II trial, demonstrated that 6 months of treatment with the antiPCSK9 mAb RG7652 did not change levels of the serum hs-CRP and pro-inflammatory cytokines IL-6 and TNF-α in patients at high risk for or with established CAD [31]. Similarly, a study in patients with stable CAD after premature myocardial infarction and very high lipoprotein(a) levels showed that plasma levels of hs-CRP were not altered after 6 months of treatment with the PCSK9 inhibitors alirocumab or evolocumab [32]. Consistently, no association between baseline levels of hs-CRP and efficacy of evolocumab in reducing adverse cardiovascular outcomes was also found in the FOURIER trial [33]. On the other hand, the larger efficacy of PCSK9 inhibitors in the reduction of CVD events was observed in the very high-risk patients with high baseline levels of hs-CRP [34, 58, 59]. These findings can be further supported by two independent meta-analyses of randomized controlled trials that failed to find a significant effect of antiPCSK9 mAbs on serum/plasma levels of hs-CRP [34, 35]. Therefore, the aforementioned findings imply that hs-CRP is not a response mediator to PCSK9 inhibitors, contrary to other lipid lowering drugs, especially statins, and more recently bempedoic acid (*via* AMP-activated kinase pathway activation) [60, 61].

Moreover, oxidative stress is an important inflammation modulator, and the current results indicated that the antiPCSK9 vaccine does not change the CFA-induced oxidative stress in albino mice. Similarly, a clinical trial showed that the administration of evolocumab had no impact on the activity of key antioxidant enzymes including catalase, SOD, and GSH-Px in erythrocytes of patients with CAD [62]. However, there are several reports showing the protective effect of PCSK9 inhibition against oxidative stress, by reducing the pro-oxidant load. An *in vitro* study showed that evolocumab could significantly reduce the concentration of malondialdehyde (MDA) and the ROS-mediated oxidative damage in human umbilical vein endothelial cells [45]. Another PCSK9 inhibitor, alirocumab, was found to decrease oxidative stress reactions in a rat model of alcoholic liver disease by reducing lipid peroxidation, the MPO activity, and frequency of infiltrating MPO-generating cells in the liver [48]. These findings suggest that although inhibition of the circulating PCSK9 does not affect the blood antioxidant capacity, it can reduce the pro-oxidant load through oxidative stress conditions. Of note,

since the present result was based on the PAB assay that shows the general changes of both pro-oxidants and antioxidants simultaneously in the serum/plasma samples, the lack of the effect of the antiPCSK9 vaccine on the PAB values may be due to a high load of the blood pro-oxidants in the CFA-challenged albino mice [63–71].

There are some limitations deserving acknowledgment. Firstly, despite the fact that a widely used inflammation model was used in this study, every experimental model of inflammation has limitations, and the results of this study may not be applicable to other types of inflammation, especially chronic inflammation such as that found in atherosclerosis. Secondly, there are many differences in the inflammation process between humans and rodents that should be considered when interpreting the results. Another noteworthy point is that in this study, the PCSK9 peptide vaccine was used in pure form without any delivery system, while the previous reports of the current group have mainly focused on the nanoliposomal form of the vaccine. Therefore, the comparison of liposomal and non-liposomal forms of peptide vaccine in terms of the effect on inflammatory and oxidative indicators can be investigated in future studies. Finally, according to the observed decrease in serum CRP levels in the vaccine group, conducting additional studies in this regard is suggested.

Conclusions

The results of the present study indicate that the antiPCSK9 vaccine, despite its significant efficacy in inhibiting PCSK9 function, could not protect against the CFA-induced acute systemic inflammation and oxidative stress in mice.

Acknowledgment: The study is reported in accordance with the ARRIVE guidelines (PLoS Bio 8(6), e1000412,2010).

Conflict of interests: M.B.: speakers bureau: Amgen, Daichii Sankyo, KRKA, Polpharma, Novartis, Sanofi-Aventis, Teva, Zentiva; consultant to Adamed, Amgen, Daichii Sankyo, Esperion, NewAmsterdam, Novartis, Sanofi-Aventis; Grants from Amgen, Daichii Sankyo, Mylan/Viatris, Novartis, and Sanofi; all other authors have no conflict of interest.

Data availability: The datasets generated during and/or analysed during the current study are available from the corresponding authors on reasonable request.

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