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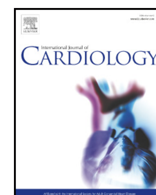
IJCA-26302; No of Pages 7

International Journal of Cardiology xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard



APOA-1Milano muteins, orally delivered *via* genetically modified rice, show anti-atherogenic and anti-inflammatory properties *in vitro* and in *Apoe*$^{-/-}$ atherosclerotic mice☆

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ARTICLE INFO

Article history:
Received 10 October 2017
Received in revised form 30 November 2017
Accepted 5 April 2018
Available online xxxx

Keywords:
Atherosclerosis
Inflammation
Nutraceutic
Apolipoprotein A-1

ABSTRACT

Background: Atherosclerosis is a slowly progressing, chronic multifactorial disease characterized by the accumulation of lipids, inflammatory cells, and fibrous tissue that drives to the formation of asymmetric focal thickenings in the *tunica intima* of large and mid-sized arteries. Despite the high therapeutic potential of ApoA-1 proteins, the purification and delivery into the disordered organisms of these drugs is still limited by low efficiency in these processes.

Methods and results: We report here a novel production and delivery system of anti-atherogenic APOA-1Milano muteins (APOA-1M) by means of genetically modified rice plants. APOA-1M, delivered as protein extracts from transgenic rice seeds, significantly reduced macrophage activation and foam cell formation *in vitro* in oxLDL-loaded THP-1 model. The APOA-1M delivery method and therapeutic efficacy was tested in healthy mice and in *Apoe*$^{-/-}$ mice fed with high cholesterol diet (Western Diet, WD). APOA-1M rice milk significantly reduced atherosclerotic plaque size and lipids composition in aortic sinus and aortic arch of WD-fed *Apoe*$^{-/-}$ mice as compared to wild type rice milk-treated, WD-fed *Apoe*$^{-/-}$ mice. APOA-1M rice milk also significantly reduced macrophage number in liver of WD-fed *Apoe*$^{-/-}$ mice as compared to WT rice milk treated mice.

Translational impact: The delivery of therapeutic APOA-1M full length proteins *via* oral administration of rice seeds protein extracts (the 'rice milk') to the disordered organism, without any need of purification, might overcome the main APOA1-based therapies' limitations and improve the use of this molecules as therapeutic agents for cardiovascular patients.

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1. Introduction

The most common underlying cause of cardiovascular diseases (CVD) is atherosclerosis, a slowly progressing, chronic inflammatory disease in which lesions called plaques are formed in focal areas of large and mid-size arteries [1]. The atherogenic process starts as a complex result of activation of endothelial cells, that, once exposed to injurious stimuli (such as dyslipidemias and pro-inflammatory mediators), change their permeability [2] triggering subendothelial retention of cholesterol-containing plasma lipoproteins [3] and recruiting innate immunity cells that ultimately lead to intraplaque inflammation and towards a pro-thrombotic state [4]. Increased blood total cholesterol (TC)

Abbreviations: CVD, cardiovascular diseases; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoproteins cholesterol; gDNA, genomic DNA; WT, wild type; oxLDL, oxidized LDL; MCP-1, monocyte chemoattractant protein-1; WD, Western diet.

☆ Acknowledgement of grant support. This work was supported by the University of Milano-Bicocca [2015-ATE-0277, 2016-ATE-0083 to RG]. The funding sources had no involvement in study design, in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the article for publication.

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[1] This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

https://doi.org/10.1016/j.ijcard.2018.04.029
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and low density lipoprotein cholesterol (LDL-C) as well as the so called atherogenic lipid triad (increased very low density lipoprotein, VDLD; increased small dense low density lipoproteins; reduced high density lipoproteins cholesterol, HDL-C) appear to be relevant for cardiovascular diseases [5]. The early identification and management of modifiable risk factors, primarily those contributing to dyslipidaemias, is the first prevention line for cardiovascular diseases [6]. Since plasma levels of blood HDL-C inversely correlated with risk of coronary heart disease [7], the anti-inflammatory and atheroprotective effects of HDL-C have been deeply investigated [8]. Among the protein components of HDLs, there have been interest in the therapeutic potential of the ApoA-1, which is able to stimulate reverse cholesterol transport, thus facilitating the removal of free cholesterol from peripheral tissues, especially from arterial walls resulting in an anti-atherogenic effect [9,10]. Moreover, the importance of ApoA-1 in atherosclerotic process has also been demonstrated by the observation that anti-ApoA-1 antibodies increased plaque vulnerability in *Apoe*$^{-/-}$ mice *via* a TLR2 and TLR4 pathways [11,12] and serum levels of anti-ApoA-1 auto-antibodies correlate with more vulnerable plaques in humans [11]. Although several studies on animal models of atherosclerosis demonstrated the potential of using HDLs as therapeutic strategy, clinical trials have substantially failed to show significant reduction in atheroma volume when recombinant or mimetic HDLs were administered by infusion [13]. APOA-1Milano is a naturally occurring mutation of human ApoA-1 which results in reduced HDL-cholesterol levels, also in APOA-1_{Milano} transgenic mice [14], with a concomitant low prevalence of CVD [15]. The APOA-1Milano protein has been demonstrated to be effective in rapidly reducing atherosclerotic plaques in mice, rabbit and porcine models [10,16–18] and in clinical trials [19]. Nevertheless, the therapeutic use of these lipoproteins has not been fully exploited yet probably because of the very low efficiency of production and/or purification of these lipoproteins.

We report here the effects, evaluated in appropriate experimental *in vitro* and *in vivo* models of dyslipidemia and atherosclerosis, of a novel drug delivery system, without any need of purification, of anti-atherogenic ApoA-1Milano molecules, by means of their synthesis in seeds of genetically modified rice plants administered to the disordered organism by oral gavage in form of seed extract, the “APO milk”.

2. Materials and methods

Standard methods are detailed in the online Supplementary methods.

2.1. Genetically modified rice plants and rice protein extract

Genetically modified rice milk (APOA-1M) was produced as indicated in patent no PCT/IB2006/054948. The rice milk was provided as lyophilized powder by GRG Gene Technology SA (Minusio, Switzerland). Non-genetically modified rice milk of the same variety (Rosa-Marchetti) was used as a control. For *in vitro* experiments rice milk was handled in sterile conditions, resuspended at a concentration of 2,5 g/ml in Phosphate Buffer Saline (PBS) and additioned with Zell-Shield (Minerva Biolabs). For *in vivo* experiments, rice milk was resuspended at a concentration of 2,5 g/ml in sterile water.

2.2. Genetic modification and molecular analysis of transgenic rice plants

The engineered plasmids were introduced in *Agrobacterium tumefaciens* strain EHA 105 by electroporation. *Oryza sativa* ssp. Japonica Rosa Marchetti was transformed as described previously [20]. Putatively, transformed plants (Hygromycin Resistant) were potted in a greenhouse together with controls (untransformed, wild type rice). Total genomic DNA was isolated from leaves of putative transgenic and wild-type rice plants [21] and analyzed by PCR using specific primers for the human *ApoA-I* sequence. PCR reaction was performed by using the following cycle conditions: 94°Cx2′; 94°Cx45″, 55°Cx45″, 72°Cx45″ for 30 cycles; 72°Cx5′.

2.3. Rice milk production and ApoA-I protein quantification

Rice seeds from wild type or transgenic plants were grinded in a fine powder. 100 g of the obtained flour were liquefied at 90 °C for 30 min in a solution of alpha-amylase (GAMALPHA SPEZIAL, Barentz) in protease-free aqueous medium (0,02% W/V NaCl) (100 ml Gamalpha spezial/t starch). An indirect competitive ELISA (IC-ELISA) was developed to detect ApoA-I protein in rice milk. Recombinant hApoA-I (Apolipoprotein A-I from human plasma, A0722, Sigma Aldrich) was coated onto micro-well plate overnight at 4 °C. Then the plate was washed three times with 0,01 M PBS (pH 7) and blocked with 200 μl of 5% (W/V) BSA for 2 h at 37 °C. After washing the plate with 0,01 M PBS added with 0,05% (v/v) Tween 20 (PBST), 100 μl of the primary antibody (1:6000, goat polyclonal anti-ApoA1 antibody, ACRIS R1029P) solution were added to 100 μl of different dilution of rice milk. 100 μl of this mixture were added to each well and the plate was incubated at 37 °C for 1 h. After washing the plate with PBST, 100 μl of anti-goat IgG-HRP antibody solution (1:10000) were added to each well and the plate was incubated at 37 °C for 1 h. The plate was washed with PBST and a 50 μl of TMB were added to each well and the plate was incubated at 37 °C for 15 min. In order to stop the reaction, 150 μl of 0,4 N hydrochloric acid (HCl) were added to each well, and absorbance was measured at 450 nm by using an ELISA plate reader (BIORAD model 680). Each experiment has been performed in triplicates. In order to prepare a standard curve for the IC-ELISA, various parameters such as concentrations of coating antigen, primary and secondary antibodies, incubation time and temperatures were optimized [22]. Finally, on the basis of optimal conditions for IC-ELISA, the standard curve using recombinant hAPOAI protein was elaborated.

2.4. In vivo studies

All experiments on animals were performed in accordance to the Italian Law and the European guidelines, following a protocol approved by the Institutional Committee for Animal Health (08/2014) and by the Ministry of Health (N. 202/2015-PR). For the tolerability study, 8–10 weeks old B6 male mice (Charles River, Calco, LC, Italy) were used. At day 0, mice were randomized in two groups (n = 10 each group) and orally administered with WT or APOA-1M rice milk (10 ml/kg, 5 d a week) for 3 weeks. At the end of the treatments, blood samples were collected for each animal. Hematological analyses were performed at the mouse facility of the University of Milano-Bicocca. For the efficacy study, 8–10 weeks old B6.129P2-*Apoe*^{tm1Unc}/J (*Apoe*$^{-/-}$) male mice were fed with Western Diet (Mucedola Srl, Settimo Milanese (MI), Italy) for 56 days *ad libitum*. After 56 days, mice were randomized in two groups (n = 8 each group) and administered with APOA-1M or WT rice milk for 15 days by oral gavage. Western Diet was maintained for the whole period of the experiments. At the end of the treatments, animals were perfused and hearts, entire aortas and livers were harvested and processed for histology and immunohistochemistry analyses.

3. Results

3.1. APOA-1(Milano) muteins produced in seeds of genetically modified rice plants

In order to overcome relevant purification issues that limited the potential use of APOA-1Milano (APOA-1M) proteins as a therapeutic agent, and since other groups clearly demonstrated that HDL mimetics can be efficiently delivered to disordered organisms by means of oral administration in the diet [23], we genetically engineered rice plants to express the full length APOA-1M in their seeds [24]. To this extent, the APOA-1M-expressing plasmid pPLT501 (Fig. 1A) was introduced in *A. tumefaciens* EHA105 strain and rice plants (Rosa Marchetti variety) were then transformed [20]. The presence of the transgene was verified by PCR on genomic DNA (gDNA) and the band of the expected size (732 bp) corresponding to the APOA-1M amplicon was observed in gDNA samples corresponding to lanes 1 to 9 and lanes 11 and 12 (Fig. 1B).

To test if seeds from genetically modified rice plants did express APOA-1M protein properly, western blot analysis were performed on wild type and transgenic rice seed protein extracts. As shown in Fig. 1C, in non-denaturing condition a band corresponding to 56 kDa was detected in transgenic rice lines corresponding to lanes 3, 6 and 7. A less intense band of 28 kDa was detected in the same samples, suggesting that these transgenic lines expressed APOA-1M protein primarily in the dimeric form. No signal was detected in wild type (WT) and transgenic lines 4 and 5 protein extracts (Fig. 1C). The genetically modified rice plant strain 7 was selected for further experiments. Western blotting carried out on seed pulps and seeds protein extract processed as ‘rice milk’ showed the same pattern as observed in transgenic and wild type protein extracts (Supplementary Fig. 1A). No expression of APOA-1M was detected in leaves, stems, roots of the transgenic rice *via* western blotting analyses (data not shown), suggesting the tissue-specific expression of exogenous APOA-1M proteins. Interestingly, no degradation products were observed in any of the transformed sample, even 10 days after rice milk preparation (Supplementary Fig. 1B), demonstrating a substantial protein stability over time. The amount of APOA-1M present in the rice seeds and in the rice milk was then

Please cite this article as: G. Romano, et al., APOA-1Milano muteins, orally delivered *via* genetically modified rice, show anti-atherogenic and anti-inflammatory properties *in vi*..., Int J Cardiol (2018), https://doi.org/10.1016/j.ijcard.2018.04.029

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