Letter

JVR JOURNAL OF VASCULAR RESEARCH

J Vasc Res 2019;56:267–270
DOI: 10.1159/000502692

Received: May 30, 2019
Accepted after revision: August 13, 2019
Published online: September 24, 2019

Low Dose of Dipotassium Glycyrrhizate Counteracts Atherosclerosis Progression in *Apoe–/–* Female Mice

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Dear Editor,

Recently, Ding et al. [1] reported that administration of glycyrrhizin (50 mg/kg/day), a glycoconjugated triterpene produced by the licorice plant *Glycyrrhiza glabra*, reduced high-fat diet-induced atherosclerosis in *Apoe*$^{-/-}$ mice by significantly decreasing serum high-mobility group box protein 1 (HMGB1) and lipid levels and by increasing the Treg/Th17 ratio.

Several natural compounds, such as krill oil, vitamin D, and dipotassium glycyrrhizate (DPG), a salt of glycyrrhizin, have been extensively investigated in our laboratory as therapeutic options for the treatment of intestinal inflammation, showing reliable anti-inflammatory properties [2–4]. Currently, atherosclerosis is thought as a chronic disease in which systemic inflammation underlies the accumulation of plaques in the arterial intima. Thus, we also considered intriguing possibility to use DPG, a natural compound with known anti-inflammatory properties and scarce or negligible side effects [5], as a good strategy to reduce atherosclerotic lesions.

Francesca Palone and Emanuela Pasquali contributed equally to the paper.

Cardiovascular disease (CVD) remains the most common cause of death in both males and females; however, in the last decades, female CVD deaths exceeded those of males [6–8]. Furthermore, most of the risk factors for CVD (hypertension, high blood cholesterol level, lack of physical activity, and obesity) are similar for males and females, but smoking has a greater negative effect in females [9]. Notably, the influence of gender on atherosclerosis development has also been described in animal models. Indeed, female *ApoE*$^{-/-}$ mice develop significantly increased aortic atherosclerotic lesion and intima/media thickness compared to male mice [10]. Despite these findings, most in vivo studies have been carried out on males. To overcome this gap, here, we used female *ApoE*$^{-/-}$ mice.

For our purposes, 14 week-old *ApoE*$^{-/-}$ female mice (n = 8/group), fed a standard diet, were treated daily with 8 mg/kg/day DPG or vehicle by oral gavage for 100 days (approx. 14 weeks). Since effects on blood pressure, mediated by cortisol accumulation [11], have previously been attributed to DPG [12], the cortisol level was monthly analyzed by ELISA (MyBiosource, San Diego, CA, USA) in the total serum. Serum cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) concentrations were also evaluated at the same time points by Mouse Cholesterol ELISA Kit (MyBioSource and EMELCA Bioscience, Breda, The Netherlands) and by HDK and LDL/VLDL Quantitation Kit (Sigma-Aldrich), respectively. At the end of treatment, hearts and descending thoracic aortas (DTA) were collected and analyzed as described in our previous work [13]. Specifically, DTA (n = 8/group) were cut longitudinally, pinned en face, and stained with Oil Red O stain. Plaque density (number of plaques/mm^2) was quantified on digital images of the aorta (from the aortic arch down to the diaphragm) captured with a Leica digital camera and analyzed by the software NIS-Elements BR4.00.05 (Nikon Instruments S.p.A., Florence, Italy). Heart sections were cut in a plane perpendicular to the aorta axis and, once the aortic root was identified by the appearance of aortic valve leaflets, serial sections were collected and stained with Masson's trichrome or underwent morphometric analyses and immunohistochemistry to assess CD68 and α-SMA expression.

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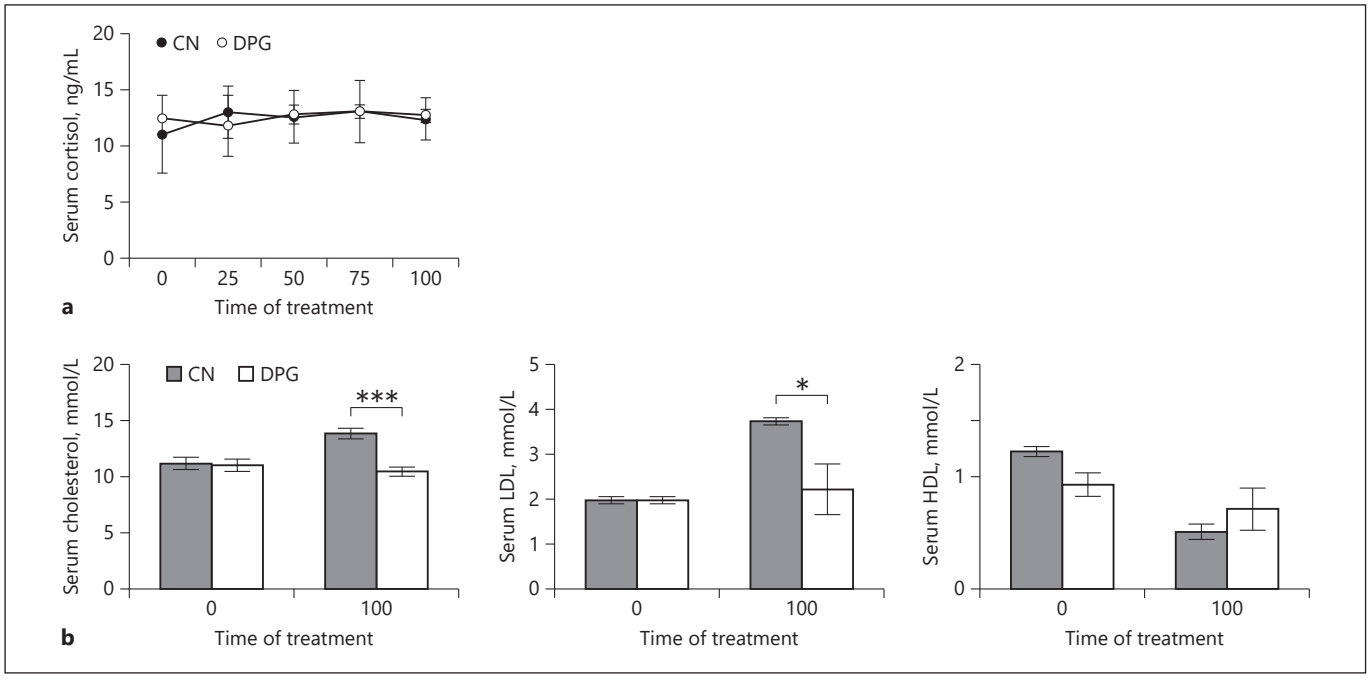


Fig. 1. Effect of DPG on serum cortisol and cholesterol levels in female *ApoE*$^{-/-}$ mice and age-matched controls (CN) 14 weeks after treatment. Serum cortisol (**a**), cholesterol, LDL, and HDL concentrations (**b**) were analyzed by ELISA. Data are reported as mean ± SEM. Differences were tested with Student's *t* test. *** $p < 0.001$, * $p < 0.05$. The concentration of cortisol is given as ng/mL. For cholesterol determination, murine sera were diluted (1:5), and the concentration is given as mmol/L. For cortisol and cholesterol, ELISA changes in the optical density were measured at 450 nm; for LDL and HDL, the concentration is given as mmol/L, and changes in the optical density were measured at 560 nm. Each sample (8 samples per group) was analyzed in duplicate.

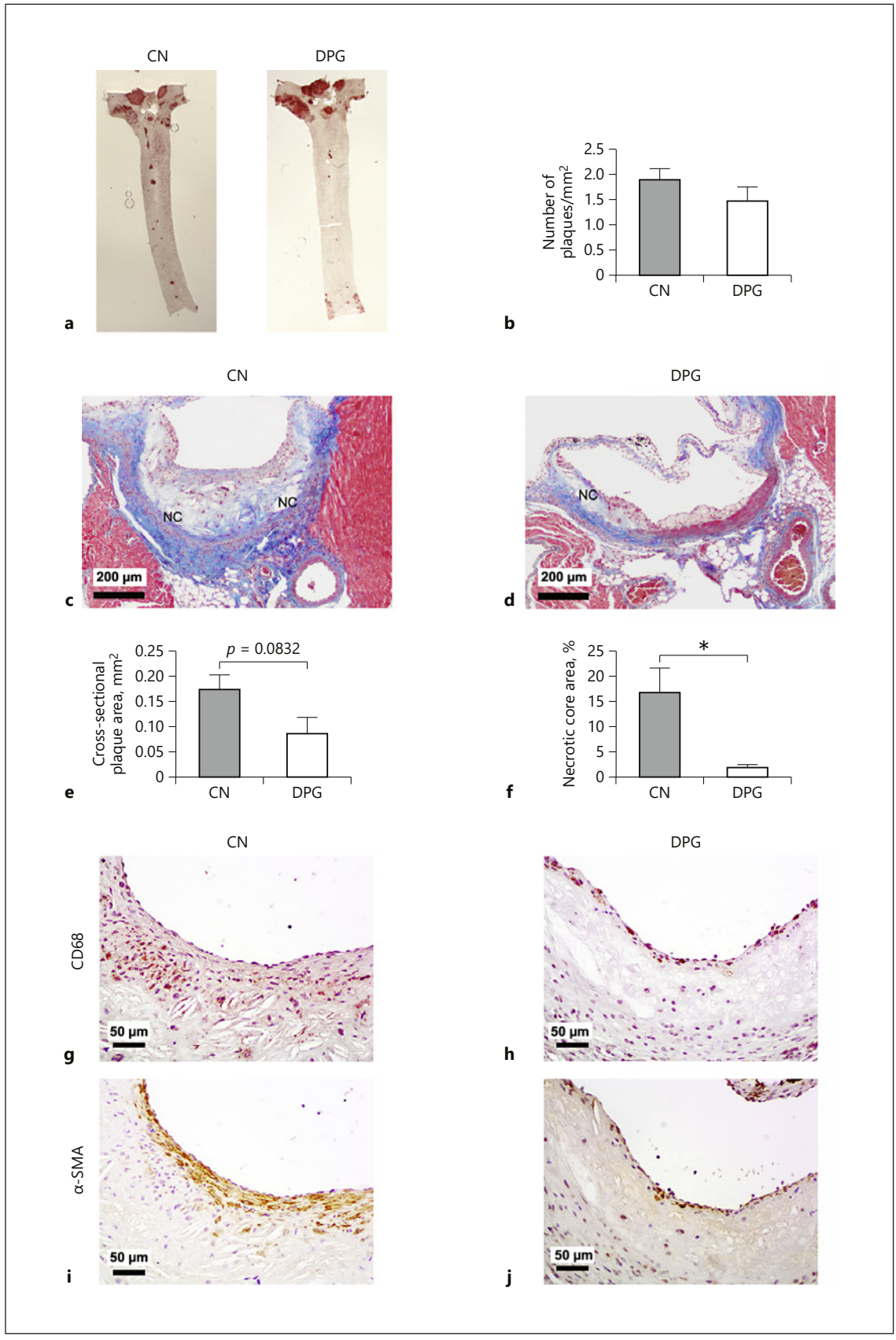
Macroscopically, we did not observe any heart changes (i.e., cardiac hypertrophy) in DPG-treated *ApoE*$^{-/-}$ mice compared with controls. In support of our results, other authors reported that in *ApoE*$^{-/-}$ mice, heart changes were related to and/or aggravated by aging and Western diet [14]. Our results showed that the cortisol level is not modulated by DPG treatment (serum cortisol: matched controls [CN]: 12.37 ng/mL ± 1.86; DPG 12.70 ng/mL ± 0.59, $p = 0.25$; Fig. 1a), suggesting that at this concentration, DPG does not raise blood pressure, an important side effect of licorice consumption. Furthermore, DPG-treated mice showed significantly reduced levels of serum cholesterol and LDL compared with the age-matched control group (serum cholesterol: CN: 13.96 mmol/L ± 0.45; DPG 10.50 mmol/L ± 0.42, $p < 0.001$; serum LDL: CN: 3.76 mmol/L ± 0,06; DPG 2.24 mmol/L ± 0.56, $p < 0.05$; Fig. 1b). No change in HDL concentration was observed after DPG treatment (Fig. 1b).

The density of the atherosclerotic lesion (number of plaques/mm^2) in the DTA region was not significantly affected by DPG treatment in our experimental conditions (Fig. 2a, b). Conversely, cross-sectional analysis of the aortic root clearly demonstrated that DPG treatment induced a decrease in the plaque area, approaching borderline statistical significance (Fig. 2c–e). Together with the decreased size, DPG-treated mice showed plaques with a statistically significant lower necrotic core, one of the defining characteristics of a vulnerable plaque, compared with control mice (Fig. 2f). The more vulnerable status of plaques in untreated mice is consistent with the higher percentage of foam cells (CD68+; Fig. 2g) and smooth muscle cells (α-SMA+; Fig. 2i) migrating into the plaque from the underlying media compared with plaques from DPG-treated mice (Fig. 2h, j).

HMGB1, a known inflammatory mediator, is overexpressed in atherosclerotic lesions and exerts proatherogenic effects by stimulating macrophage migration, induc-

Fig. 2. Effects of DPG on atherosclerosis. Representative en face preparations of DTA and aortic root sections from female *ApoE*$^{-/-}$ mice 14 weeks after treatment with 8 mg/kg/day DPG or from age-matched controls. Plaque density in the DTA: representative images (**a**) and histogram (**b**) of all samples analyzed ($n = 8$/group). Aortic root sections (Masson's trichrome stain) from CN (**c**) and DPG-treated mice (**d**). Graphic representation of the plaque area (**e**) and necrotic core (**f**) measured on aortic root cross-sections of all samples analyzed ($n = 8$/group). Sections of atherosclerotic plaques immunostained with antibodies against CD68 (**g**, **h**) and α-SMA (**i**, **j**) from CN- and DPG-treated mice. Data are shown as mean ± SEM. Differences were tested with Student's *t* test. * $p = 0.0168$.

(For figure see next page.)



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ing proinflammatory cytokines and promoting the accumulation of immune and smooth muscle cells [15]. Thus, the selectively targeting of HMGB1 by DPG, a small inhibitor of HMGB1 [16], could decrease HMGB1-mediated local inflammation, inducing improvement in atherosclerotic plaque progression.

Although our data are in agreement with those reported by Ding et al. [1] regarding the beneficial effect of licorice on the atherosclerosis progression, important additional information can be extrapolated from our study. Indeed, in agreement with the current evidence of a progressive increase in atherosclerosis incidence among female, our study was carried out in female mice, during standard diet and using a 5-fold lower concentration of DPG compared to the study by Ding et al. [1].

In conclusion, our study highlights that a low dosage of DPG counteracts atherosclerosis progression occurring during standard diet. This finding opens the possibility to propose DPG administration in combination with balanced diet to predisposed individuals without the concern about the rise in blood pressure, an important parameter to be considered with higher doses.

Acknowledgement

The authors thank the DMG Italia Srl for providing DPG.

Disclosure Statement

The authors have no conflicts of interest to declare.

Funding Sources

Intramural institutional funding was received.

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