Cardiac Protective Effects of *Moringa oleifera* Seeds in Spontaneous Hypertensive Rats

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BACKGROUND
Hypertension is characterized by a maintained high blood pressure leading to cardiac complications such as left ventricular hypertrophy and fibrosis and an increased risk of heart failure and myocardial infarction. This study investigated the cardiac effects of oral administration of *Moringa oleifera* (MOI) seed powder in spontaneous hypertensive rats (SHR).

METHODS
SHR received food containing MOI seed powder (750 mg/d, 8 weeks) or normal food. *In vivo* measurement of hemodynamic parameters by telemetry and cardiac structure and function analysis by echocardiography were performed. Histological studies were performed to determine fibrosis and protein expression.

RESULTS
MOI treatment did not modify blood pressure in SHR but reduced nocturnal heart rate and improved cardiac diastolic function (reduction of isovolumetric relaxation time and deceleration time of the E wave, increase of ejection volume and cardiac output compared to non-treated SHR). Left ventricular anterior wall thickness, interseptal thickness on diastole, and relative wall thickness were reduced after MOI treatment. Furthermore, we found a significant reduction of fibrosis in the left ventricle of MOI-treated SHR. This antihypertrophic and antifibrotic effect of MOI was associated with increased expression of peroxisome proliferator-activated receptor (PPAR)-α and δ, reduced cardiac triglyceride level, and enhanced plasmatic prostacyclins.

CONCLUSIONS
Our data show a beneficial effect of MOI on the cardiac structure and function in SHR associated with an upregulation of PPAR-α and δ signaling. This study thus provides scientific rational support for the empirical use of MOI in the traditional Malagasy medicine against cardiac diseases associated with blood pressure overload.

Keywords: blood pressure; cardiac hypertrophy; fibrosis; hypertension.

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High blood pressure induces vascular and cardiac complications such as left ventricular hypertrophy, heart or kidney failure, myocardial infarction, stroke, and cognitive impairment.[1] Hypertension is a multifactorial disease involving both genetic and environmental factors. The causes of the increased blood pressure are generally unknown and numerous animal models have been developed to address the pathogenesis of hypertension. The spontaneous hypertensive rats (SHR), one of the most animal models used for this purpose, are characterized by an increased nervous-dependent noradrenaline release in the circulation, contributing to enhanced blood pressure.[2] These rats gradually develop left ventricular hypertrophy associated with impairment of both systolic and diastolic functions starting at 6 weeks of age.[3] Cardiac hypertrophy is accompanied by myocardial fibrosis that involves, at least in part, an alteration of peroxisome proliferator-activated receptor (PPAR)-α and/or δ signaling, known to participate in fatty acid catabolism in the heart.[4]

Several previous studies reported beneficial effects of natural treatments using medicinal plants in experimental animal models of cardiovascular diseases. Some of these plants possess antihypertensive properties and improve both vascular and heart functions.[5–7] Bio-guided fractionation of these plant extracts suggested that active compounds such as coumarins and/or polyphenols with antioxidant and anti-inflammatory properties contribute to the efficiency of these plants against cardiovascular disorders.[7]

Moringa oleifera (MOI; Moringaceae family) is a little tree used in folk medicine in tropical Africa, America, and Asia. Based on empiric knowledge, different parts of this plant (roots, leaves, and seeds) are used for several therapeutic applications in inflammatory, infectious, gastrointestinal, and cardiovascular diseases.[8–10] MOI seed oil contains phenols, and in particular flavonoids, with free radical scavenging activity.[11]

The aim of this study was thus to assess possible beneficial effects of oral MOI seed treatment to ameliorate cardiac dysfunction and remodeling associated with high blood pressure in SHR. We provide some evidence of a protective role of this plant on the cardiac dysfunction induced by hypertension.

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METHODS

Animals

This study has been performed on male SHR of 16 weeks. Wistar Kyoto (WKY) rats of same age were also used for echography analysis. Animals were housed in acclimatized room (temperature 22 ± 2 °C and hygrometry 55 ± 4%). The circadian cycle was scheduled for 12 hours with light and 12 hours in dark and all the experiments were conducted in accordance with the international guidelines for care and use of laboratory animals. The animal protocols used have been approved by the National Ethical Committee (authorization number: 00909.01).

Hemodynamic parameter measurements

To measure hemodynamic parameters, arterial pressure, and heart rate (HR), a telemetric transmitter (TA 11PA-C40, DSI) was implanted surgically in the abdominal aorta of isoflurane-anaesthetized SHR. The hemodynamic values were measured after 1 week of transmitter implantation for 8 weeks. Rats were distributed in 2 groups: 6 in the control group (SHR CTRL) receiving normal food and 6 rats in the treated group (SHR MOI) receiving MOI seed powder mixed to the food (750 mg/d/rat for 8 weeks). Cardiac function analysis was conducted by echocardiography after the treatment. At the end of the experimental protocol, all the rats were anesthetized by isoflurane inhalation and sacrificed by cervical dislocation to harvest blood samples and hearts.

Cardiac echocardiography

After the treatment, echocardiographic analyses were performed on isoflurane-anaesthetized SHR and WKY rats placed in left lateral decubitus position. A GE Vivid7 (GE Healthcare) apparatus equipped with S10 wave at 9 MHz transducer was used. The cardiac left ventricle (LV) geometry was investigated by measuring wall thickness (anterior, posterior, and interseptal), and shortening and ejection fraction were evaluated in time motion mode following small and large parasternal axis. The HR was continuously monitored and pulsed Doppler was used to assess isovolumetric relaxation time (IVRT).

The relative wall thickness (RWT) was calculated with Reid Hayward and Chia-ying Lien formula as follow: RWT = (LVPWTd + IVSd)/LVDd, where LVPWTd represents LV posterior wall thickness on diastole; IVSd, the interventricular septal thickness on diastole; and LVDd, the LV diameter on diastole. Cardiac output was calculated as follow: $CO = \pi \times D2/4 \times IVTAo$, where D represents the diameter of the aortic LV outflow tract and IVTAo, the velocity–time integral in the LV outflow tract. The LV mass (LV_{mass}) and the LV mass ratio to body weight (LV_{mass}/BW) were also evaluated. LV_{mass} was calculated as follow: $LV_{mass} = 1{,}04((LVDd + LVPWTd + IVSd)^3 - (LVDd)^3)$, according to Hayward and coworkers.[12]

Tissue preparation for histological analysis

The hearts harvested from SHR CTRL and SHR MOI were rinsed with phosphate-buffered saline and fixed for 24 hours in 4% paraformaldehyde at room temperature and then processed routinely in paraffin. Serial 7 µm thick tissue sections from the middle levels of both ventricles were stained with picric acid-Sirius. In bright-field microscopy, sections had a pale pink background and collagen was stained in red. Collagen fibers were detected by polarized light microscopy and type I collagen fibers were stained in orange/red. Sections were observed with ×10 magnification using a Leica DMLB light microscope with cool-snap camera and orange/red collagen brightness level was evaluated using ImageJ software.

In another set of experiments, the sections were used for a semiquantitative assessment of proteins expression and tissue location. Heart sections were deparaffinized in tissue clear and fat was removed with 100% methanol, and then were incubated for 2 hours in a blocking buffer containing 5% bovine serum albumin. After an overnight incubation at 4 °C with a polyclonal rabbit antibody anti-PPARα (1:100, Abcam, France) or anti-PPARδ (1:100, Abcam, France), slides were washed and incubated with a secondary anti-rabbit antibody. After washes, labeling was revealed by 3.3-diaminobenzidine (DAB Kit Substrate, BD Biosciences). Hematoxylin and eosin were used to label nuclei and cytoplasm, respectively, of cells in the tissue. After washing, heart sections were mounted on glass slides and observed with ×60 magnification using a Nikon eclipse E600 light microscope equipped with a Nikon DS-Ri1 camera.

Cardiomyocyte size was also measured in histological preparations (length and wide calculated as the mean of 10 cardiomyocytes/LV of each heart).

ELISA measurements

Diacylglycerol and triglycerides were measured in cardiac LV lysates from SHR CTRL and SHR MOI using ELISA assay kits (MyBiosource and Abcam (France), respectively). Prostacyclin (PGI_2) metabolite was evaluated in rat plasma by ELISA assay kit from BlueGene (China).

Data analysis

A two-way analysis of variance for repeated measures and Bonferroni *post hoc* test were performed for telemetric experiences. A one-way analysis of variance with subsequent Tukey *post hoc* test or the analysis of variance on ranks was performed when 3 means were compared. An unpaired Student *t*-test or a Mann and Whitney test were used for comparison of 2 means. All the statistical analyses were performed with the Statview software (SAS Institute, Cary, NC). $^*P < 0.05$ was considered statistically significant. All values are presented as mean ± SEM, *n* represents the independent experiments or samples.

RESULTS

Effect of MOI treatment on blood pressure and HR

Telemetry records show that MOI treatment for 8 weeks did not change diurnal and nocturnal systolic and diastolic arterial pressures in SHR (Figure 1a–d). In contrast, MOI

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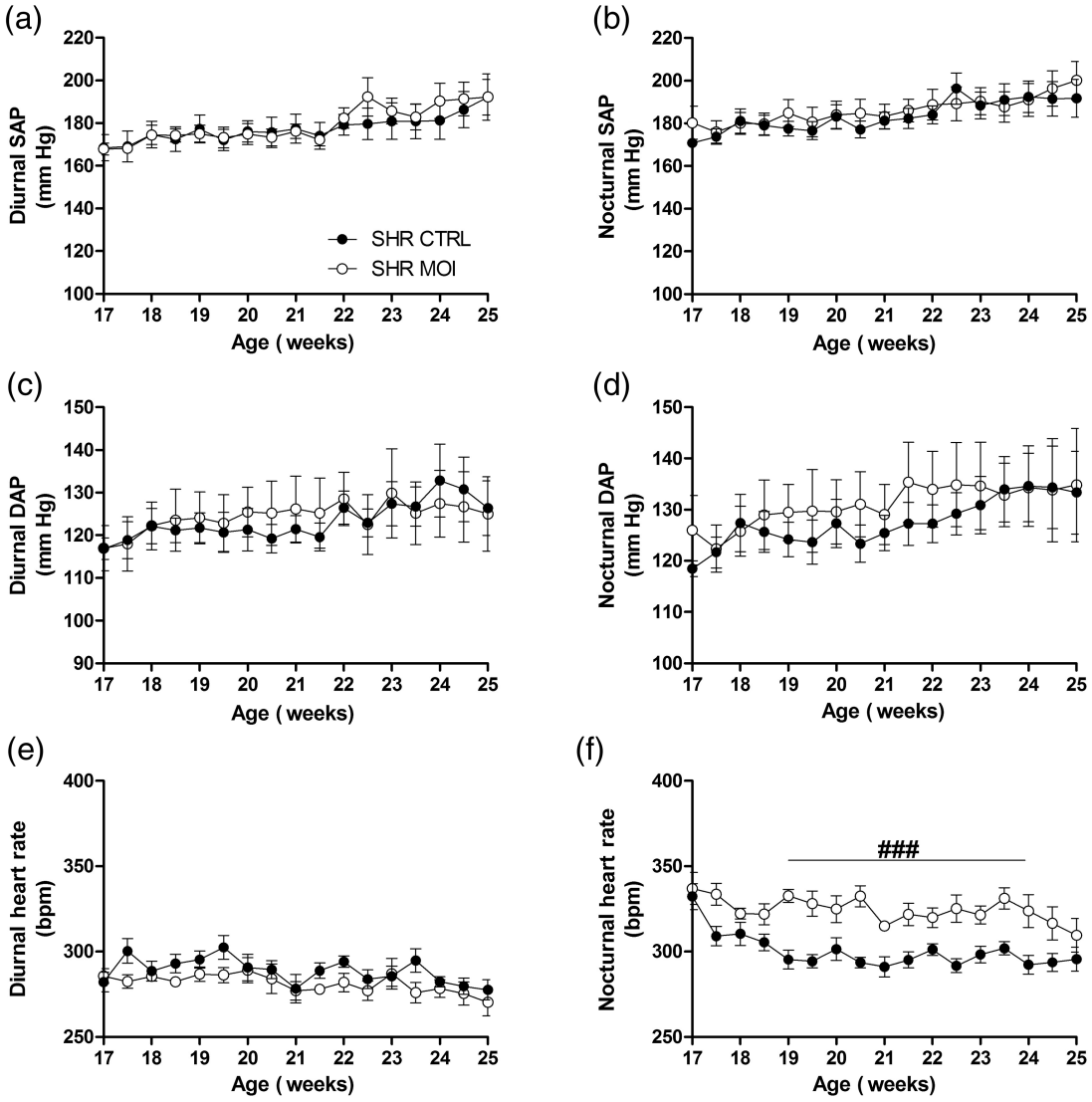


Figure 1. Circadian hemodynamic parameters of SHR treated with MOI seeds compared with SHR control group. (**a**,**b**) Diurnal and nocturnal SAP, respectively. (**c**,**d**) Diurnal and nocturnal DAP, respectively. (**e**,**f**) Diurnal and nocturnal heart rate, respectively (mean ± SEM with $n = 5–6$, $^{###}P < 0.001$ SHR CTRL vs. SHR MOI). MOI treatment started at 17 weeks of age. Abbreviations: CTRL, control; DAP, diastolic arterial pressure; MOI, *Moringa oleifera*; SAP, systolic arterial pressure; SHR, spontaneous hypertensive rat.

treatment induced a reduction of the nocturnal HR when animals were awake and active, without change in the diurnal HR (Figure 1e,f). This effect of MOI started to be significant after ten days of treatment (Figure 1f).

Effect of MOI treatment in cardiac structure and function

To detect potential cardiac structural differences between SHR CTRL and SHR MOI, transthoracic echocardiography was performed using normotensive WKY rats as reference. Increased LV anterior and posterior wall thicknesses on diastole (LVAWTd and LVPWTd) were found in SHR CTRL compared to WKY rats (Figure 2a,b). The IVSd and the RWT also were greater in SHR CTRL than in WKY rats (Figure 2c,d). These data attest the LV hypertrophy installation in SHR CTRL compared to normotensive rats. MOI treatment in SHR significantly reduced LVAWTd, IVSd, and RWT without changing the LVPWTd values (Figure 2a–d).

The LV hypertrophy was confirmed by the significant increase of LV_{mass} and LV_{mass}/BW in both SHR CTRL and SHR MOI compared to WKY rats (LV_{mass}: 678.84 ± 33.16 mg in WKY rats, 1005.02 ± 52.50 mg in SHR CTRL ($P < 0.001$ vs. WKY), and 857.42 ± 39.95 in SHR MOI ($P < 0.01$ vs. WKY); LV_{mass}/BW: 1.54 ± 0.05 mg/g in WKY rats, 2.33 ± 0.13 mg/g in SHR CTRL ($P < 0.001$ vs. WKY), and 1.97 ± 0.076 in SHR MOI ($P < 0.01$ vs. WKY); $n = 6$ in each group of rats). Nevertheless, the significant decrease of LV_{mass} and LV_{mass}/BW in SHR MOI compared to SHR CTRL ($P < 0.05$) suggests an antihypertrophic effect of MOI.

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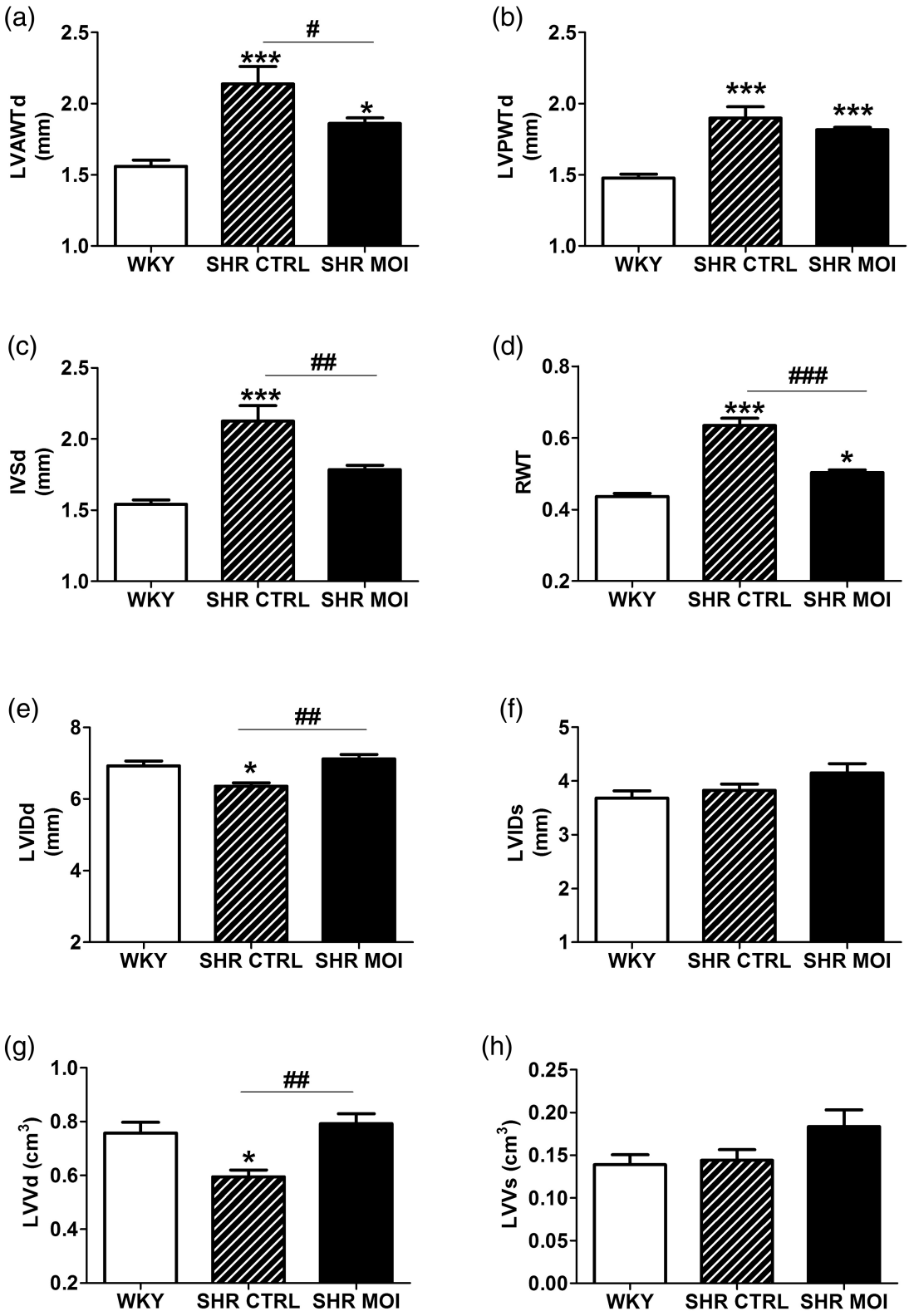


Figure 2. Effects of MOI seeds on left ventricle cardiac hypertrophy in SHR. (**a**) LVAWTd: left ventricular anterior wall thickness on diastole. (**b**) LVPWTd: left ventricle posterior wall thickness on diastole. (**c**) IVSd: interventricular septal thickness on diastole. (**d**) RWT: relative wall thickness. (**e,f**) LVIDd and LVIDs: left ventricular internal diameter on diastole or left ventricular internal diameter on systole. (**g,h**) LVVd and LVVs: left ventricular volume on diastole or left ventricular volume on systole. Results are expressed in mean ± SEM, $n = 5–6$, $^{*}P < 0.05$, $^{***}P < 0.001$ WKY, white bars vs. SHR CTRL or SHR MOI, black bars, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ SHR CTRL vs. SHR MOI. Abbreviations: CTRL, control; MOI, *Moringa oleifera*; SHR, spontaneous hypertensive rat; WKY, Wistar Kyoto.

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The left ventricular internal diastolic diameter (LVIDd) was significantly reduced in SHR CTRL and the treatment with MOI restored this parameter to a level comparable to that of WKY rats (Figure 2e) while the LV internal systolic diameter (LVIDs) was similar in the three groups of rats (Figure 2f). Interestingly, MOI treatment in SHR restored the volume of LV in diastole (LVVd), significantly reduced in SHR CTRL compared to WKY rats (Figure 2g). The LV volume in systole (LVVs) was similar in the 3 groups of rats (Figure 2h).

Concerning systolic ventricular function, the left ventricular fractional shortening was not significantly reduced in SHR CTRL and SHR MOI (Figure 3a). Ejection fraction was significantly decreased in both SHR CTRL and SHR MOI compared to WKY rats, suggesting that MOI treatment was not able to ameliorate systolic ventricular function in SHR (Figure 3b). By contrast, the increased IVRT, attesting an impairment of diastolic function in SHR CTRL compared to WKY rats, was completely reversed by MOI treatment (Figure 3c). This result is in agreement with the measurements of the deceleration time of the E wave that also was increased in SHR CTRL compared to WKY rats and partially normalized in SHR MOI (Figure 3d). Moreover, ejection volume and cardiac output, significantly reduced in SHR CTRL, attesting a decreased cardiac compliance, were restored in SHR MOI to levels similar to those of WKY rats (Figure 3e,f). Altogether these data are consistent with a beneficial effect of MOI on diastolic function of SHR.

HR checked during echography measurements was found similar in the 3 groups of anesthetized rats indicating that all the observed differences did not indirectly result from changes in cardiac rhythm (in beats per minute: 371 ± 10.12 in WKY, 339 ± 17.42 in SHR CTRL, and 358 ± 17.73 in SHR MOI).

MOI reduces cardiac remodeling and fibrosis

MOI treatment also reduced cardiomyocyte size in SHR MOI compared to SHR CTRL hearts (cardiomyocyte length: 158.88 ± 3.22 µm in SHR CTRL vs. 138.64 ± 4.71 µm in SHR

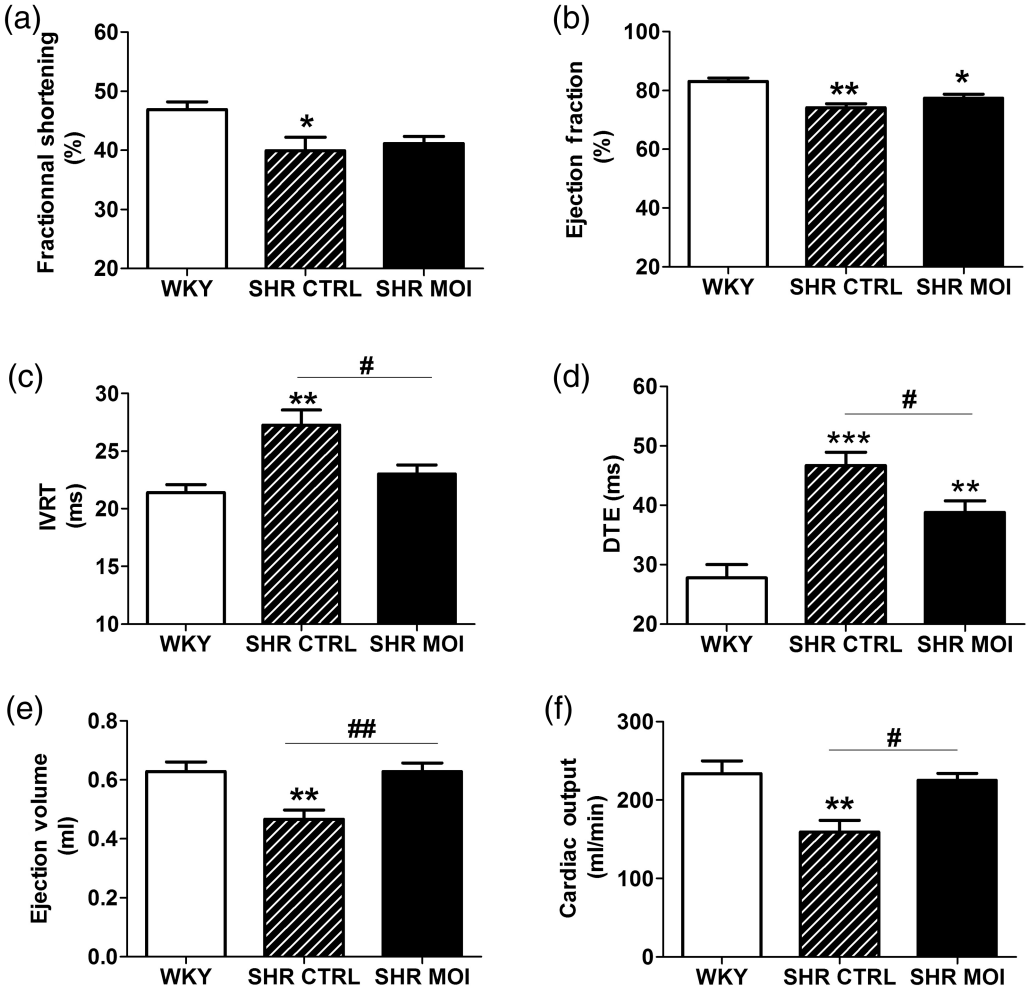


Figure 3. Effects of MOI seeds on cardiac function in SHR. (**a**) Fractional shortening of left ventricle. (**b**) Ejection fraction. (**c**) IVRT: isovolumetric relaxation time. (**d**) DTE: deceleration time of the E wave. (**e**) Ejection volume. (**f**) Cardiac output. Results are expressed in mean ± SEM, $n = 4–6$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, WKY, white bars vs. SHR CTRL or SHR MOI, black bars, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, SHR CTRL vs. SHR MOI. Abbreviations: CTRL, control; MOI, *Moringa oleifera*; SHR, spontaneous hypertensive rat; WKY, Wistar Kyoto.

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MOI ($P < 0.05$); cardiomyocyte wide: 36.44 ± 0.85 µm in SHR CTRL vs. 29.95 ± 2.19 µm in SHR MOI ($P < 0.05$); $n = 6$ hearts in each group), confirming at cell level, the antihypertrophic effect of MOI treatment in SHR observed in the heart of treated SHR.

We also determined the area of myocardial fibrosis in the LV by the collagen circularly polarized light analysis of picrosirius red stained sections. The hearts of SHR CTRL showed a strong red staining in bright-field microscopy that appeared as red brightness in polarized light confirming collagen type I infiltration in cardiac tissue (Figure 4a,b). In contrast, in sections from SHR MOI hearts, red/orange staining in bright-field microscopy and red brightness in polarized light were very weak (Figure 4a,b). We did not find green brightness in polarized light indicating the absence of collagen III infiltration. Histograms obtained from

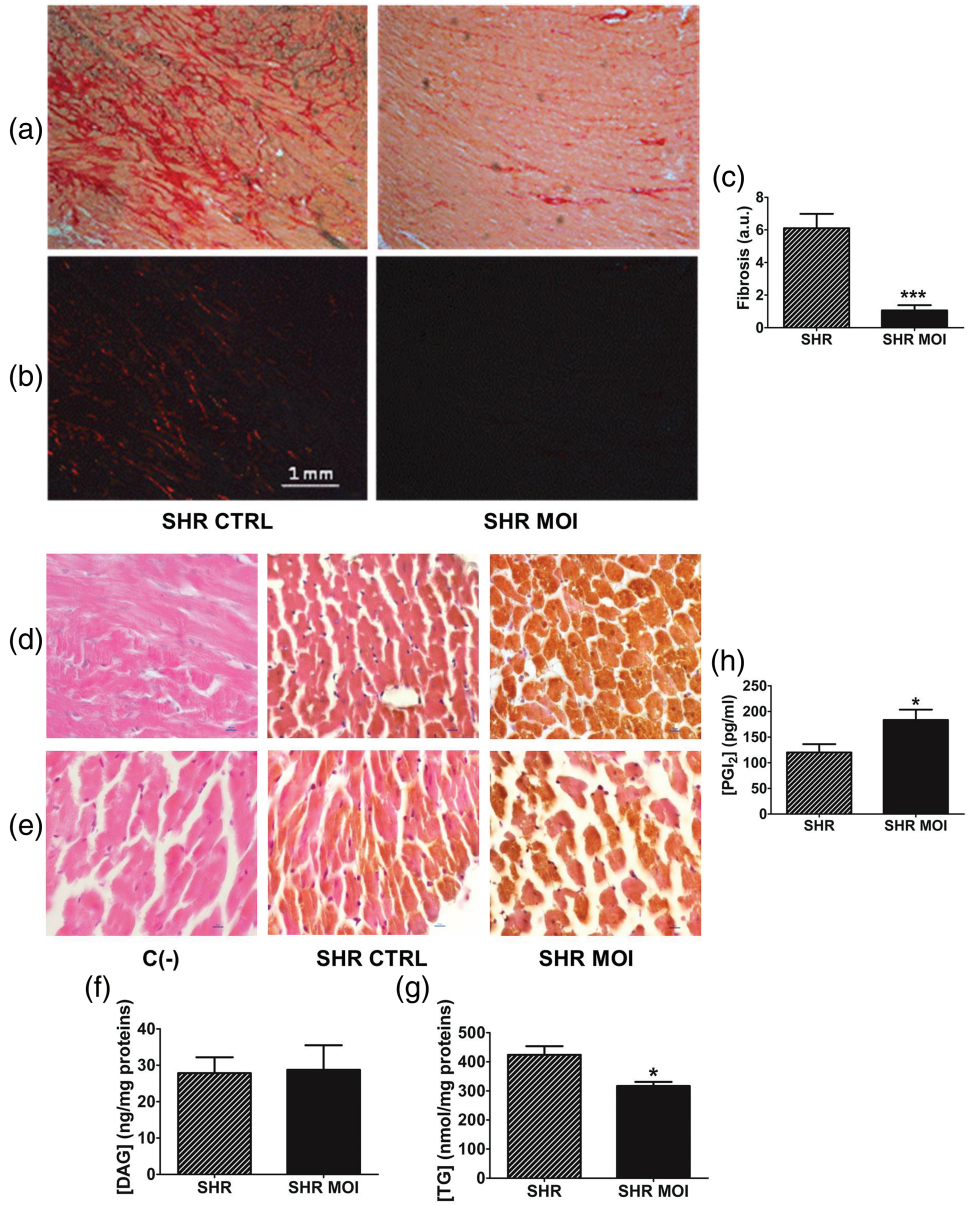


Figure 4. Effect of MOI on cardiac fibrosis and PPARα and PPARδ expression/activation. Histological analysis of Sirius red staining in cardiac tissue. (**a**) In red, bright-field microscopy images of cardiac fibrosis in SHR CTRL and SHR MOI. (**b**) The same images observed with a crossed polarized filter, bar = 1 mm. The red/orange light corresponds to collagen I infiltration. (**c**) In arbitrary units (a.u.), the histograms of fibrosis in SHR CTRL and SHR MOI, values are expressed as mean ± SEM, $n = 6$ experiments, ***$P < 0.001$ SHR CTRL vs. SHR MOI. (**d,e**) Immunohistochemical staining (brown) of PPARα and PPARδ on left ventricular myocardium of SHR CTRL and SHR MOI. Eosin stained in pink the cytoplasm of cardiac cells and hematoxylin stained in violet the nuclei of the cells, $n = 5$–6 experiments for each group of samples. C(–) indicates negative control. Bar = 10 µm. (**f**) Diacylglycerol levels in cardiac left ventricle lysates from SHR CTRL and SHR MOI (values are expressed in ng/mg of cardiac proteins). (**g**) Triglyceride concentration in cardiac left ventricle lysates in SHR CTRL and SHR MOI (values are expressed in nmol/mg of cardiac proteins). (**h**) Plasmatic levels of prostacyclin in SHR CTRL and SHR MOI (values are expressed in pg/ml of plasma). For (f), (g), and (h), values are expressed as mean ± SEM, $n = 5$–6 for each group of samples, *$P < 0.05$, SHR CTRL vs. SHR MOI. Abbreviations: CTRL, control; MOI, *Moringa oleifera*; PPAR, peroxisome proliferator-activated receptor; SHR, spontaneous hypertensive rat.

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measurements of red brightness showed a significant reduction of fibrosis in hearts from SHR MOI compared to SHR CTRL (Figure 4c).

MOI increases cardiac PPARα and PPARδ expression and modifies cardiac lipid content

PPAR signaling has been reported to have beneficial effects on cardiac fibrosis and hypertrophy.[13] To investigate the potential role of PPAR signaling pathways in the protective effect of MOI on cardiac fibrosis in SHR, we assessed the expression of PPARα and PPARδ in cardiac tissue. Staining of PPARα and PPARδ was increased in the cardiac LV from SHR MOI compared to those from SHR CTRL (Figure 4d,e). MOI treatment did not modify diacylglycerol concentration (Figure 4f) but significantly reduces triglyceride levels in LV from SHR (Figure 4g). Interestingly, a significant increase in circulating level of PGI_2, known to activate directly or indirectly PPARs,[14] was found in SHR MOI (Figure 4h).

DISCUSSION

Here, we report experimental evidences of the beneficial effects of orally administrated MOI seeds on hypertension-induced cardiac hypertrophy and fibrosis. MOI is currently used in traditional medicine to treat several disorders and mainly cardiovascular diseases such as hypertension. Our results thus provide scientific basis for the use of this widely distributed plant in cardiovascular disorder treatment.

MOI specifically decreased the HR of SHR during the nocturnal active period without effect on diurnal HR and arterial blood pressure. This is particularly relevant since a decreased HR is described to be associated with vascular and heart protection, with a direct correlation between HR, coronary atherosclerosis, and cardiovascular morbidity.[15–17]

MOI treatment, at the dose used in the present study, did not modify the circulating levels of urea and of transaminases (alanine amino-transferase and aspartate amino-transferase; not shown) in SHR, suggesting no effect of the treatment on renal and hepatic functions.

Chemical analysis of MOI seeds confirmed the presence of flavonoids and alkaloids evidenced in previous studies.[18,19] Indeed, we found alkaloids (2.4 g/100 g MOI seeds) that could participate in the MOI effect on HR by regulating cholinergic function. We also found the presence of the thiocarbamate glycoside, niazimicin, described for its negative chronotropic activities independent of β-adrenoceptor antagonistic effect.[20]

Clinically, sustained hypertrophy induced by hypertension is the initial step of human heart failure and is correlated with increased cardiovascular mortality. This cardiac remodeling is also a risk factor of arrhythmia and sudden cardiac death.[3,21] The present study shows an antihypertrophic role of MOI administration. Indeed, MOI treatment restored to values similar to those recorded in WKY, some morphological parameters associated with LV cardiac hypertrophy induced by pressure overload, such as LVAWTd, IVSd, and RWT that are increased in SHR.[3] Moreover, MOI treatment partially decreased the LV_{mass} and LV_{mass}/BW in SHR and reduced the LV cardiomyocyte size confirming the protective effect of MOI in the overload-dependent LV remodeling. The 8-week treatment was possibly too short to completely rescue all the parameters linked to the cardiac hypertrophy. Furthermore, the cardiac remodeling was already too advanced in 17-week-old SHR when we started the treatment. Indeed, it was previously described that concentric LV hypertrophy was already apparent in 6-week-old SHR compared to WKY rats.[3]

Regarding cardiac functions, LVIDd and LVVd, both reduced in SHR CTRL, were restored in SHR MOI to levels similar to WKY rats. The same parameters during cardiac systole, LVIDs and LVVs, were not different in the 3 groups of rats. Echographic measurements did not allow to detect a clear beneficial effect of MOI on cardiac systolic function. In contrast, concerning diastolic function, MOI improved ejection volume and cardiac output in SHR and this also was associated with the restoration to normal values of IVRT and deceleration time of the E wave in SHR MOI compared to the elevated values found in SHR CTRL. All these results suggested an amelioration of the diastolic function *in vivo*.

Left ventricular end-diastolic pressure (LVEDP) is a commonly used parameter in patients with heart failure.[22] Although it did not reach significance, LVEDP, calculated by a noninvasive method as previously described,[23] was reduced by MOI (in mm Hg: 19.2 ± 1.75 in SHR CTRL vs. 14.9 ± 1.03 in SHR MOI; $n = 6$). Echocardiographic diurnal measurements indicated that the HR was similar in the 3 groups of anesthetized rats suggesting that the differences obtained in cardiac parameters were independent of cardiac rhythm changes or LVEDP.

Cardiac hypertrophy and diastolic dysfunction in SHR are associated with increased fibrosis and collagen infiltration in the LV wall.[24] Activation of PPARs plays a protective role against cardiac hypertrophy and fibrosis because their pivotal role in myocardial fatty acid metabolism.[25–27] Here, we found that the cardiac fibrosis observed in SHR CTRL was significantly reduced in SHR MOI. This structural effect of MOI was associated with an increased expression of the nuclear receptors, PPARα and PPARδ, and an elevated plasmatic level of the PPAR activator prostacyclin. The significant reduction of triglyceride level in cardiac LV of SHR MOI suggests an increased fatty acid oxidation in SHR MOI hearts. This is in line with previous findings showing the association of increased expression/activity of PPARs, and in particular of PPARδ, with the upregulation of oxidative genes implicated in fatty acid oxidation that could be involved in the antihypertrophic protective role of PPARs during hypertension.[28] Our observations are thus consistent with a role of PPARs in the antihypertrophic and antifibrotic effects of MOI treatment. These data are in agreement with other studies that described a cardiac protective role of natural substances or PPAR agonists, improving cardiac function and preventing LV hypertrophy and fibrosis in animal models of pressure overload by PPARα and PPARδ upregulation.[29,30] In addition, plant polyphenols, which are also present in MOI seeds, have been described to reduce triglyceride accumulation by PPAR-dependent mechanisms.[31]

We focused this study on MOI administration effect in the SHR model of hypertension because the plant is empirically used to treat this condition. We did not test MOI seeds in

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normotensive WKY as numerous previous studies demonstrated that diets containing natural cardiovascular active substances in disease models have no effect in nonpathological animals.[32–35] We used a WKY group of rats only for *in vivo* echographic analysis to better show the cardiac remodeling and cardiac functional impairment due to hypertension in SHR.

The clear beneficial effect of MOI in cardiac function described in this study suggests that MOI treatment affected signaling pathways involved in pressure overload-induced LV hypertrophy, in particular calcium handling mechanisms. For instance, the calmodulin-activated serine-threonine protein phosphatase calcineurin pathway could be a potential target of MOI since calcineurin activity has been shown to progressively increase with age in the heart of SHR and the inhibition of calcineurin reduces hypertrophy development.[36–39]

Altogether these data identified a beneficial role of MOI seed against hypertension-induced functional and structural cardiac remodeling and support scientifically the empirical use of this plant in traditional Malagasy medicine to treat cardiac complications due to blood pressure overload. Further studies now deserved to be performed to completely elucidate the mechanisms involved in MOI positive cardiac effect.

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DISCLOSURE

The authors declared no conflict of interest.

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