Carvacrol improves erectile dysfunction in spontaneously hypertensive rats

Tays A. F. Gonçalves, Priscila M. P. Maciel, Larissa I. M. Villanueva, Pablo F. dos Santos, Ismael de L. O. Júnior, Robson C. Veras, Islania G. A. Araújo, Isac A. de Medeiros*

* Correspondence: isac@ltf.ufpb.br; Tel.: +55(083) 3209-8787; Universidade Federal da Paraíba, Campus I, Cidade Universitária, João Pessoa, PB, CEP: 58051-900, Brazil

Abstract: Carvacrol is a monoterpene found in essential oils from various plants. Several pharmacological properties have already been described for carvacrol, including antimicrobial, anti-inflammatory, anticarcinogenic, antioxidant, vasorelaxant and hypotensive activities. The present study evaluated the effect of carvacrol on hypertensive rats with erectile dysfunction. Twelve-week-old spontaneously hypertensive rats (SHR) were treated with vehicle, carvacrol (50 or 100 mg/kg/day) or sildenafil (1.5 mg/kg/day), intragastrically, for four weeks. Wistar Kyoto (WKY) rats were used as the normotensive controls. All substances tested reduced systolic blood pressure during the treatment period. The intracavernosal pressure/mean arterial pressure ratio of the hypertensive rats was improved by carvacrol and sildenafil treatments. In isolated rat corpora cavernosa, the acetylcholine- and SNP-induced relaxation responses were significantly increased by carvacrol or sildenafil treatments. In SHR corpora cavernosa, treatment with carvacrol attenuated the hypercontractility induced by phenylephrine or electrical field stimulation. Phe-induced hypercontractility in the presence of tempol was not altered when compared to the response induced by carvacrol alone. In rat corpus cavernosa fluorescence intensity emitted by the DHE probe was significantly reduced in SHR treated (carvacrol or sildenafil) groups when compared to that emitted in the SHR-CTL. This study showed that carvacrol improves the erectile function of hypertensive rats and reduces endothelial dysfunction, smooth muscle cell hypercontractility and superoxide anion generation.

Keywords: corpus cavernosum, erectile dysfunction, reactive oxygen species, SHR

1. Introduction

Normal erectile function depends on a precise balance between relaxation and contraction of the cavernous smooth muscle, which is regulated by neural and local control mechanisms. Therefore, changes in these mechanisms may lead to erectile dysfunction (ED) [1]. This clinical condition has been defined by the United States National Institutes of Health as the inability to achieve or maintain a penile erection that would enable satisfactory sexual activity [2].

ED is closely associated with chronic diseases, such as diabetes and systemic arterial hypertension [3]. Several studies have reported a greater prevalence of ED in hypertensive patients compared to normotensive individuals [4-6]. In addition, studies have identified ED as a predictive factor for the development of hypertension [7]. In both situations, ED is triggered by deregulation of endothelial factors and increased smooth muscle contraction,
which consequently causes an increase in vascular pressure, poor cavernous perfusion and inadequately intumescence [8].

ED treatment is based on the use of PDE-5 inhibitors, such as sildenafil, vardenafil and tadalafil. However, in recent years, the number of studies seeking to develop new strategies for the treatment of ED has been increasing [9] since treatment with PDE-5 inhibitors is less effective in patients with an impaired nitric oxide (NO) pathway, especially individuals with vascular diseases [10]. Antioxidant compounds have been gaining prominence in these studies as they reduce oxidative damage, improve NO bioavailability and have a protective effect on erectile function [11-13].

Carvacrol is an aromatic monoterpene found in essential oils from various plants, such as *Origanum vulgare* (oregano), *Thymus vulgaris* (thyme) and *Citrus aurantium bergamia* (bergamot) [14], and has been shown to have potent antioxidant activity [15, 16] in addition to antifungal [17], antibacterial [18], antiviral [19], anti-inflammatory [20] and anticarcinogenic [21] activities.

Some studies highlight antioxidant activity induced by carvacrol, with the compound improving not only the activity of antioxidant enzymes but also the activity of non-enzymatic antioxidants [14]. In addition, carvacrol plays a role in the cardiovascular system, acting as a vasodilator through the interaction with voltage-gated calcium (CaV) and transient receptor potential (TRP) channels, which also contribute to a hypotensive effect [22, 23]. Based on the range of pharmacological activities already described for carvacrol and especially its antioxidant activity, this study aimed to evaluate the effect of carvacrol on the erectile dysfunction of SHR.

2. Results

2.1. Carvacrol induces antihypertensive effect in SHR

Treatment with carvacrol at doses of 50 mg/kg (SHR-C50; SBP: 166.3 ± 2.7; n = 6) and 100 mg/kg (SHR-C100; SBP: 159.2 ± 2.4; n = 5) was able to decrease the blood pressure in the SHR (SHR-CTL; PAS: 209.2 ± 4.0 mmHg; n = 5; p < 0.05) in a manner similar to the response produced by treatment with 1.5 mg/kg sildenafil (SHR-S1.5; PAS: 172 ± 3.6; n = 6) (Figure 1).
Figure 1 – SBP (mmHg) weekly changes measured by tail-cuff. Groups: WKY-CTL (n = 6; ●); SHR-CTL (n = 5; ■); SHR-C50 (n = 6; △); SHR-C100 (n = 6; ◀); SHR-S1.5 (n = 6; ◇). Results are expressed as mean ± SEM. Data were analyzed using two-way ANOVA, followed by Bonferroni post-test. *p < 0.05 vs WKY-CTL; #p < 0.05 vs SHR-CTL.

2.2 Carvacrol improve the ICP/MAP ratio in SHR

The ICP/MAP ratio was significantly lower in the SHR-CTL group (1Hz = 0.09 ± 0.02; 2Hz = 0.12 ± 0.02; 4Hz = 0.15 ± 0.02; 8Hz = 0.18 ± 0.02; 12Hz = 0.18 ± 0.02; n = 5) when compared to that in the WKY-CTL group (1Hz = 0.32 ± 0.04; 2Hz = 0.38 ± 0.04; 4Hz = 0.40 ± 0.04; 8Hz = 0.42 ± 0.05; 12Hz = 0.41 ± 0.04; n = 5) (p < 0.05) (Figure 2a, b).

The SHR-C100 treatment (2Hz = 0.27 ± 0.03; 4Hz = 0.32 ± 0.04; 8Hz = 0.36 ± 0.04; 12Hz = 0.34 ± 0.04; n = 5) improved the ICP/MAP ratio of hypertensive animals (SHR-CTL) (Figure 2a, b). Interestingly, the response induced in the SHR-C100 group did not present a significant difference when compared to the responses in the WKY-CTL and SHR1.5 groups (2Hz = 0.27 ± 0.04; 4Hz = 0.29 ± 0.04; 8Hz = 0.33 ± 0.04; 12Hz = 0.33 ± 0.03; n = 6) (Figure 2a, b). The ICP/MAP ratio in the SHR-C50 group (Figure 2a, b) improved at only the 12 Hz frequency (0.30 ± 0.04; n = 5), when compared to the SHR-CTL group (12Hz = 0.18 ± 0.02; n = 5; p < 0.05).
Figure 2 – Effect of carvacrol on ICP/MAP ratio in response to pelvic ganglion stimulations (0.2 – 12 Hz). a) Original tracings of ICP/MAP ratio. b) ICP/MAP ratio curves. Groups: WKY-CTL (n = 5; ●); SHR-CTL (n = 5; ■); SHR-C50 (n = 5; Δ); SHR-C100 (n = 5; △); SHR-S1.5 (n = 6; ◊). Results are expressed as mean ± SEM. Data were analyzed using two-way ANOVA, followed by Bonferroni post-test. *p < 0.05 vs WKY-CTL; †p < 0.05 vs SHR-CTL.

2.3 Carvacrol improves relaxation of the corpora cavernosa when exposed to ACh and SNP in SHR

ACh (0.1 nM - 100 μM) induced significantly lower relaxation in the corpora cavernosa of the SHR-CTL group (E\text{max} = 16.5 ± 3.8%, n = 6) when compared to that in the corpora cavernosa of the WKY-CTL group (E\text{max} = 36.7 ± 6.7%, n = 5, p < 0.05) (Figure 3a).

The SHR-C100 treatment (Figure 3a) improved the ACh-induced relaxation response (E\text{max} = 38.0 ± 6.4%, n = 5, p < 0.05) in hypertensive rats, and this response was similar to the response observed in the WKY-CTL and SHR-S1.5 groups (E\text{max} = 28.6 ± 3.9%, n = 5) (Figure 3a). However, the SHR-C50 group (E\text{max} = 20.4 ± 3.3%, n = 5) did not exhibit an altered ACh-induced relaxation response (Figure 3a).
Figure 3 – Vascular reactivity to ACh (a) and SNP (b) in rat corpora cavernosa. Groups: WKY-CTL (n = 5; ●); SHR-CTL (n = 6; □); SHR-C50 (n = 5; △); SHRC100 (n = 5; ▼); SHR – S1.5 (n = 5; ○). Results are expressed as mean ± SEM. Data were analyzed using two-way ANOVA, followed by Bonferroni post-test. *p < 0.05 vs WKY-CTL; **p < 0.05 vs SHR-CTL.

SNP (10 nM - 300 μM) induced significantly lower relaxation in the corpora cavernosa of the SHR-CTL group (Emax = 72.2 ± 4.1%; n = 5) when compared to that in the corpora cavernosa of the WKY-CTL group (Emax = 100.8 ± 5.0%; n = 7; p < 0.05) (Figure 3b). In the SHR-C50 (Emax = 89.6 ± 3.9%; n = 7), SHR-C100 (Emax = 90.9 ± 2.0; n = 6) and SHR-S1.5 (92.9 ± 8.1, n = 7) groups, the SNP-induced relaxation response in the corpora cavernosa was restored (p < 0.05) (Figure 3b).

2.4 Carvacrol reduces hypercontractility of the corpora cavernosa in SHR

Phe (10 nM - 300 μM) induced significantly greater contraction in the corpora cavernosa of the SHR-CTL group (pD2 = 5.4 ± 0.1; Emax = 146.7 ± 6.0%; n = 5) when compared to that in the corpora cavernosa of the WKY-CTL group (pD2 = 5.0 ± 0.05; Emax = 104.7 ± 8.5%; n = 5) (Figure 4a).

In the SHR-C50 (Emax = 102.8 ± 12.0%; n = 6; p < 0.05) (Figure 4a) and SHR-C100 (Emax = 78.0 ± 10.6; n = 5; p < 0.05) (Figure 4a) groups, the hypercontractility response to Phe that was observed in the SHR-CTL group was reduced (p < 0.05). On the other hand, the SHR-S1.5 group (pD2 = 5.2 ± 0.2; Emax = 155.0 ± 16.1%; n = 6) did not exhibit the hypercontractility response to Phe observed in the SHR-CTL group (Figure 4a).
Figure 4 – Concentration-response curves to Phe (10 nM – 300 μM) (a) and frequency-response curves to EFS (1 – 16 Hz) (b) in rat corpora cavernosa. (c) Original representative tracings showing the contractile frequency-response curves in rat corpora cavernosa. Groups: WKY-CTL (n = 5; ●); SHR-CTL (n = 5; ■); SHR-C50 (n = 6; △); SHR-C100 (n = 5; \(\nabla\)); SHR-S1.5 (n = 6; ◊). Results are expressed as mean ± SEM. Data were analyzed using two-way ANOVA, followed by Bonferroni post-test. *p<0.05 vs WKY-CTL; †p<0.05 vs SHR-CTL.

Similarly, the SHR-C50 (Emax = 118.3 ± 16.9%; n = 5) (Figure 4b, c) and SHR-C100 (Emax = 74.3 ± 13.8%; n = 6) (Figure 4b, c) groups were able to attenuate the hypercontractility response to EFS in the corpora cavernosa that was observed in the SHR-CTL group (Emax = 181.2 ± 25.1%; n = 6) when compared to that in the corpora cavernosa of the WKY-CTL group (Emax = 100.3 ± 19.8%; n = 7).
Similarly to the observed response to Phe, the SHR-S1.5 group ($E_{\text{max}} = 185.7 \pm 25.7; n = 6$) did not exhibit the hypercontractility response to EFS observed in the SHR-CTL group (Figure 4b, c).

2.5 Carvacrol reduces hypercontractility in a similar way to tempol of the corpora cavernosa in SHR

Pre-incubation of the corpora cavernosa with tempol (10 mM) (SHR-CTL-TEMPOL: $E_{\text{max}} = 98.0 \pm 6.4\%$; $n = 5$) reduced the hypercontractility response to Phe observed in the SHR-CTL group ($E_{\text{max}} = 146.7 \pm 6.0\%$; $n = 5$) in the absence of tempol (Figure 5a).

Interestingly, tempol did not modify the contractile response to Phe in the SHR-50-TEMPOL ($E_{\text{max}} = 93.3 \pm 10.2\%$; $n = 5$) (Figure 5b) and SHR-C100-TEMPOL ($E_{\text{max}} = 66.7 \pm 13.6\%$; $n = 5$) (Figure 5c) groups when compared to that in the groups without tempol, SHR-C50 and SHR-100.

On the other hand, with the addition of tempol, the contractile response induced by Phe was reduced in the corpora cavernosa of the SHR-S1.5-TEMPOL ($E_{\text{max}} = 98.0 \pm 6.5\%$; $n = 5$) mice when compared to their corresponding control group (SHR-S1.5, $E_{\text{max}} = 155.0 \pm 16.1\%$; $n = 5$) (Figure 5d) in the absence of tempol.

**Figure 5** – Effect of carvacrol on concentration-response curves to Phe (10 nM – 300 μM), in presence of tempol, in rat corpora. Groups: WKY-CTL (n = 5; ●); SHR-CTL (n = 5; ■); SHR-CTL-TEMPOL (n = 5; □); SHR-C50 (n = 6; △); SHR-C50-TEMPOL (n = 4; ▲); SHR-C100 (n = 5; ▼); SHR-C100-TEMPOL (n = 6; ▽); SHR-S1.5 (n = 6; ◊); SHR-S1.5-TEMPOL (n = 5; ◆). Results are expressed as mean ± SEM.
Data were analyzed using the two-way ANOVA, followed by Bonferroni post-test. *p < 0.05 vs WKY-CTL; ′p < 0.05 vs SHR-CTL.

2.6 Carvacrol reduces reactive oxygen species in the corpus cavernosum of SHR

The DHE probe emitted baseline fluorescence in the rat corpora cavernosa sections of all experimental groups. The SHR-CTL showed an increase in DHE fluorescence intensity (226.0 ± 13.1%; n = 5), when compared to the DHE fluorescence intensity of the WKY-CTL group (103.3 ± 12.0%; n = 5) (Figure 6a, b).

The corpora cavernosa sections of the SHR-C50 (101.3 ± 10.1%; n = 5), SHR-C100 (103.4 ± 5.0%; n = 6) and SHR-S1.5 (142.5 ± 6.1%; n = 6) groups showed a significant reduction in the fluorescence intensity emitted by the DHE probe when compared to that emitted by the DHE probe in the SHR-CTL group.

**Figure 6** – Effect of carvacrol on ROS in rat corpora cavernosa. Basal fluorescence intensity of DHE in transverse sections of rat corpora cavernosa. Groups: WKY-CTL (n = 5); SHR-CTL (n = 5); SHR-C50 (n = 5); SHR-C100 (n = 6); SHR-S1.5 (n = 6). a) Basal fluorescence intensity emitted by the DHE in transverse sections of rat corpora cavernosa (objective 10X). b) Measurement of basal fluorescence intensity of DHE (%) in transverse sections of corpora cavernosa. The data are expressed as mean of percentage of fluorescence in relations to the control ± SEM. *p < 0.05 vs WKY-CTL, ′p < 0.05 vs SHR-CTL.

3. Discussion

This study revealed that carvacrol improves the erectile function of SHR likely through a mechanism involving restoration of the endothelial dysfunction and reduction of rat corpus cavernosum hypercontractility.

First-line therapy for the treatment of ED consists of the administration of PDE-5 inhibitors (iPDE-5). However, their efficacy is reduced when NO bioavailability is decreased. Thus, endothelial dysfunction may limit the efficacy of this therapy [24]. Therefore, it is necessary to develop new therapeutic options that may attenuate the progression of the problem in patients with this condition [1].
The use of plant products has been an important alternative and has been the subject of several studies demonstrating their potential in the treatment of ED [11, 13, 25]. In this context, monoterpenes, particularly the isomers carvacrol and thymol, stand out in the literature due to their important activity on the cardiovascular system [22, 23, 26]. In general, these compounds have antioxidant activity [16] and may, thus, be indicated for the protection of cellular constituents susceptible to chronic oxidative damage [15].

Studies conducted by our research group have demonstrated beneficial effects of thymol on ED in streptozotocin-induced diabetic animals (unpublished data). When taking into account these data, together with hypotensive activity [22] and the fact that both compounds are isomers, the present study sought to show the possible beneficial effects of carvacrol on ED in spontaneously hypertensive rats.

During treatment, the SHR-CTL group exhibited higher blood pressure levels than did the WKY-CTL group, which confirms the development of hypertension in this group, similarly to other studies in the literature [27]. In the present study, the reduction in SBP levels in animals treated with carvacrol was also observed, and there was no significant difference between doses (50 or 100 mg/kg) at the end of treatment, demonstrating the potential antihypertensive activity of this substance. The SHR-S1.5 group also exhibited reduced blood pressure levels, which is in agreement with data in the literature showing reduction of SBP after two months of treatment [28]. Interestingly, carvacrol or sildenafil treatments were able to reduce SBP to, virtually, the same level.

Erectile function was assessed by the ICP/MAP ratio. The ICP/MAP ratio in the SHR-CTL group was significantly reduced when compared to that in the WKY-CTL group, confirming the development of ED in SHRs, as previously observed in the literature [7]. Strikingly, the SHR-C50 group exhibited partially reversed erectile function, only at the maximum frequency tested. In contrast, the SHR-C100 group exhibited a restored erectile function in most of the stimulation frequencies tested, when compared to the SHR-CTL group. These results demonstrate that carvacrol, in addition to having an antihypertensive effect, which may contribute to improve or delay the development of ED, also has an influence on the erectile function, capable of reversing hypertension-associated ED. This statement is in agreement with the results showing that the reduction in pressure levels alone is not effective in improving ED, as demonstrated by the effects induced by some antihypertensive drugs, such as beta-blockers and thiazide diuretics [29, 30]. The SHR-S1.5 group presented an improved erectile function, corroborating with reports in the literature [31-33]. Interestingly, no differences were observed in terms of the erectile function of the SHR-C100 and SHR-S1.5 animals, demonstrating that these treatments have beneficial effects on the treatment of hypertension-associated ED.

Since NO is considered the main peripheral pro-erectile physiological regulator and changes in its synthesis or bioavailability may lead to the development of ED [34], the activity of ACh, which is an endothelial muscarinic agonist, was assessed to determine whether there is any impairment of the endothelium-dependent relaxation mediated by this molecule. Thus, in the present study, ACh-mediated endothelium-dependent relaxation was significantly impaired in the corpus cavernosum strips from the SHR-CTL, when compared to the WKY-CTL group. Similar results are described in the literature, which prove that ED in hypertensive animals involves endothelial dysfunction [7, 27].

The SHR-C50 group did not demonstrate improvement of endothelial dysfunction. In contrast, carvacrol 100 mg/kg completely restored the endothelial function in SHR corpus cavernosum, which may suggest the involvement of this mechanism in reversing erectile dysfunction. Sildenafil significantly improved endothelial dysfunction in SHR. This effect corroborates data from the literature, which demonstrate that different antioxidant substances also improve the endothelial function in rat corpora cavernosa [11, 35].

In addition to endothelial dysfunction, hypertension may cause impairment of pathways directly involved in the relaxation of smooth muscle cells of the penile tissue [7]. Therefore, SNP, a NO donor, was used to assess endothelium-independent relaxation. In response to SNP, a reduction of the maximum effect on the SHR-CTL group was observed when compared to the WKY-CTL...
group. This effect suggests that hypertension decreases the response to the components of the NO-mediated signaling cascade in the smooth muscle cells of rat corpora cavernosa. In addition, these findings corroborate the results presented in the literature, which state that the reduction of this effect may be associated with a decrease in the formation of cGMP [36]. Carvacrol and sildenafil treatments improved the SNP-mediated response similar to the results observed for the WKY-CTL group. These data suggest that the abovementioned treatments induced an improvement in the NO-mediated signaling cascade. This result is quite significant compared to data in the literature that showed that treatment with other antioxidants, such as α-tocopherol, were not able to change this parameter [11].

Penile erection requires adequate blood inflow into the cavernous tissue, which depends on the degree of relaxation of the smooth muscle of the cavernous tissue. In hypertension, some of the pathophysiological mechanisms involved in the development of ED are related to changes in peripheral sympathetic activity and structural modifications, such as erectile tissue remodeling and increased cavernous smooth muscle hypertrophy. These mechanisms are involved in the increase of erectile tissue tonus and, therefore, reduced blood flow and intracavernosal pressure [27, 37]. Thus, we investigated the effect of different contracting and relaxing agents on strips of corpora cavernosa in the different experimental groups.

In our study, at the end of the four weeks of treatment, the SHR-CTL group demonstrated increased contractile response of corpora cavernosa to Phe and to EFS when compared to that of corpora cavernosa to Phe and to EFS in the WKY-CTL group. Divergent results were observed by Toblli (2007), who showed a reduction of contractility to Phe in corpora cavernosa strips of 24-week-old SHR. Interestingly, treatment with carvacrol at both doses (50 or 100 mg/kg) reduced both hypercontractility to Phe and to EFS in SHR. These results suggest that the improvement in the contractile response induced by carvacrol seems to involve a common pathway that is mediated by a reduction in the sensitivity of smooth muscle machinery to contracting substances, which contributed to an improvement in the erectile function.

However, treatment with sildenafil was not able to reduce the contractility of the corpora cavernosa in hypertensive rats, suggesting that the improvement in erectile function does not involve the modulation of the muscle contractile response. Similar results were observed by Toblli (2007), who showed increased contractility in strips of rat corpora cavernosa after treatment with sildenafil.

Oxidative stress-mediated neurovascular alteration appears to play a crucial role in the development of ED. O₂⁻, which is mainly formed by NADPH oxidase, has an important vasoconstrictor effect [38]. The oxidative stress state can be reversed by both increasing antioxidant agents and by limiting the activity of ROS-generating enzymes [9].

Several studies have shown that free radical scavengers can restore erectile function [39, 40]. Studies in animal models of diabetic erectile dysfunction have revealed that conventional antioxidants, such as vitamin E, tempol and selenate, could improve erectile capacity by modulating ROS scavenging activities [41-43].

Therefore, the modulation by O₂⁻ of Phe-induced contraction in corpus cavernosum strips of rats pretreated with tempol, which is a SOD mimetic, was evaluated. This enzyme is responsible for transforming O₂⁻ into H₂O₂ [44]. The present study demonstrated that pre-incubation with tempol significantly reduced the maximal effect on Phe-induced contraction in SHR-CTL rats. These data suggest that the reduced levels of O₂⁻, evoked by pre-incubation with tempol, negatively modulate the Phe-induced contraction in the corpora cavernosa of SHR-CTL rats.

The pre-incubation with tempol in SHR-C50 and SHR-C100 rats had no additive effect in reducing Phe-induced contraction when compared to the effect in the absence of this scavenger. We suggest that treatment with carvacrol at both doses probably reduces contractility through a mechanism similar to that induced by tempol. Conversely, pre-incubation with tempol in the SHR-S1.5 group reduced the Phe-mediated contractile response when compared to the SHR-CTL group, suggesting that treatment with sildenafil seems to improve erectile function mainly by other mechanisms.
To confirm the modulating effect of oxidative stress, we evaluated whether treatment with carvacrol was able to modify baseline ROS levels in corpus cavernosum sections of SHR by measuring the DHE fluorescence intensity.

In these experiments, the SHR-CTL group showed a significant increase in the DHE fluorescence intensity in the penile tissue when compared to that in the penile tissue of the WKY-CTL group, demonstrating the increase in the levels of superoxide anion and oxidative stress in the corpora cavernosa of SHR. This result agrees with data in the literature showing an increase in oxidative stress in the corpora cavernosa of SHR [36].

Treatment with carvacrol in the two doses studied (50 and 100 mg/kg) significantly reduced DHE fluorescence levels when compared to those of the SHR-CTL group, presenting superoxide anion levels similar to those of the WKY-SHR group. Animals from the SHR-S1.5 group also showed a reduction in the DHE fluorescence intensity; however, this response was not reversed to the baseline levels of the WKY-CTL group. This result suggests that treatment with sildenafil reduces oxidative stress; however, this does not appear to be the main mechanism by which erectile function is improved.

As previously mentioned, the increase in superoxide anion production in hypertension-associated ED is related to increased NADPH activity [38]. Studies have shown that carvacrol reduces ROS formation through the modulation of NADPH expression, suggesting that carvacrol can prevent the formation of pathological lesions of atherosclerosis [45].

Finally, TRPC3 and TRPC4 channels are involved in the positive modulation of oxidative stress. The TRPC3 channel amplifies ROS formation through an interaction with NADPH oxidase (Nox2), which causes the stabilization of this protein complex and consequently increases ROS production, leading to functional alteration of the cells that culminates in tissue damage. The initial increase in oxidative stress leads to the formation of a heterometric complex between the TRPC3 and TRPC4 channels, leading to an increase in ion influx to the cell and in oxidative stress [46, 47]. Considering the wide action of carvacrol on the blockade of TRP receptors, we can hypothesize a possible involvement of the monoterpene studied in the inhibition of these receptors for a more pronounced reduction of oxidative stress, which needs future investigations.

4. Materials and Methods

4.1 Animals

All protocols were carried out using 12-week-old Wistar Kyoto (WKY) and SHR from the IPeFarM Animal Production Unit of the Federal University of Paraíba, where the rats were held under a controlled temperature (21 ± 1 °C) in a 12-hour light/12-hour dark cycle with water and food ad libitum. All experimental protocols were approved by Animal Research Ethics Committee (CEUA) of the Federal University of Paraíba under certificate nº 132/2017.

4.2 Study design

The rats were randomly divided into five experimental groups: normotensive control (WKY-CTL; n = 8); hypertensive control (SHR-CTL; n = 8); hypertensive rats treated with 50 mg/kg/day carvacrol (SHR-C50; n = 8) or 100 mg/kg/day carvacrol (SHR-C100; n = 8); and hypertensive rats treated with 1.5 mg/kg/day sildenafil (SHR-S1.5, n = 8). All animals received their treatments intragastrically for four weeks.

4.3 Reagents

To carry out the experiments, the following substances were used: sodium heparin, L(-)-phenylephrine (Phe) hydrochloride, acetylcholine (ACh) hydrochloride, sodium nitroprusside (SNP), dihydroethidium (DHE), 4-hydroxy-TEMPO (tempol) and Cremophor®; all of which were obtained from SigmaAldrich Brazil Ltda. (São Paulo-SP, Brazil); xylazine hydrochloride and ketamine hydrochloride, which were both obtained from Syntec (Santana de Parnaíba - SP, Brazil);
and sildenafil citrate, which was obtained from the Sigma-Aldrich Brazil Ltda. (São Paulo-SP, Brazil). Carvacrol (5-isopropyl-2-methylphenol) was obtained from Sigma-Aldrich Brazil Ltda. at 98% purity, with reference 28219710G, batch #STBH1940, molecular formula C10H14O, molecular mass 150.217 g/mol and density 0.98 g/mL. The stock solutions of carvacrol were prepared from the solubilization of this substance with Cremophor®. The desired concentrations were obtained by diluting it in saline solution. The concentrations of Cremophor® in solution did not exceed 0.03%.

4.4 Systolic blood pressure measurement

The systolic blood pressure (SBP) of the rats was measured weekly using the tail-cuff method (Panlab, Harvard Apparatus, Spain). To measure the blood pressure, the rats were kept in a heated acrylic container (28-30°C) for 10 minutes prior to the measurement to make the caudal artery pulsation more readily detectable. At least three successive measurements were recorded in the data acquisition system (LabChart® software, version 7.1; ADInstruments, Colorado Springs, CO) to obtain the mean SBP.

4.5 Intracavernosal pressure (ICP) measurement

Under anesthesia, a small incision was made in the cervical region for dissection and exposure of the right common carotid artery, where a polyethylene (PE) catheter (PE-10 tubing welded to a PE-50 catheter) was implanted to gain access to the thoracic aorta and to perform continuous measurement of the mean arterial pressure (MAP). Then, the major pelvic ganglion was exposed and stimulated using a bipolar bronze electrode (Animal Nerve Stimulating Electrode, MLA0320, ADInstruments) connected to a stimulus generator (Stimulus generators contained in PowerLab®, ADInstruments). Consecutive electrical stimulation with pulses for 1 ms and at 6 V at different frequencies (0.2, 0.4, 0.6, 1, 2, 4, 8 and 12 Hz) and with durations of 45 s were induced in the ganglion to measure ICP and to construct a frequency-response curve. ICP was measured by the introduction of a 30 G needle connected to a catheter in the crural region of the left corpus cavernosum. The variations in MAP and ICP were measured using pressure transducers (Disposable BP Transducer, MLT0699, ADInstruments) coupled to the PowerLab® data acquisition system (LabChart® software, version 8.1; ADInstruments, Colorado Springs, CO).

4.6 Functional studies in strips of rat corpora cavernosa

For dissection and removal of the rat corpora cavernosa, the penis was removed at the level of the attachment to the ischium and immersed in Krebs solution with the following composition (mM): 118.0 NaCl; 4.7 KCl; 1.56 CaCl2; 2H2O; 1.2 KH2PO4; 1.17 MgSO4; 25.0 NaHCO3 and 5.5 glucose. Strips of corpus cavernosum were suspended by metal rods connected to a force transducer and inserted into tanks for isolated organ baths (Panlab Multi Chamber Organ Baths, ADInstruments) containing 10 mL of solution at 37°C that was continuously bubbled with a mixture of 95% O2 and 5% CO2 (pH 7.4). The strips were maintained under a basal tension of 0.5 g for a stabilization period of 60 minutes. The tension changes were measured using isometric transducers (MLT020, ADInstruments, Australia) and recorded in the PowerLab® system (ML870/P, LabChart version 7.0, ADInstruments, Australia).

The contractility of the rat corpora cavernosa was evaluated by increasing the cumulative concentration of Phe (10 nM - 300 μM) and via electrical field stimulation (EFS). The EFS was performed using 50 V electric pulses having a duration of 1 ms at different frequencies (1, 2, 4, 8 and 16 Hz) with a duration of 10 seconds for each frequency. The contractile response of the corpora cavernosa to Phe was also evaluated by pre-incubation for 30 minutes with tempol (1 mM), which is a superoxide dismutase (SOD) mimetic. The relaxation response of the corpora cavernosa in the different treatment groups was assessed by increasing the cumulative concentration to ACh (0.1 nM - 100 μM), which is an endothelial muscarinic agonist, or to SNP (10 nM - 300 μM), which is an spontaneous NO donor.
4.7 Evaluation of superoxide anion production

ROS generation in the rat corpus cavernosum was detected with the fluorescent dye dihydroethidium. The rat corpora cavernosa were isolated, embedded into the Tissue Tek Compound (OCT) embedding medium and frozen in liquid nitrogen. Subsequently, sections of corpora cavernosa (10 μM) were incubated with 5 μM DHE at 37 °C for 30 minutes in a humid chamber and protected from light. The intensity of the fluorescence emitted by DHE was used to measure the superoxide anion production in the different groups. The digital images were captured using a fluorescence microscope (NIKON Eclipse Ti-E, NIKON, Japan) for further analysis.

4.8 Statistical analysis

The data are expressed as the mean ± SEM. For statistical analysis, one-way or two-way ANOVA was used followed by the Bonferroni post-hoc test. The differences between the means were considered significant when p < 0.05. The data were analyzed and plotted in the statistical software GraphPad Prism 7.0®. The maximum relaxation corresponded to the maximum effect (Emax) for the highest concentration used. Pharmacological potency was determined using the EC50 (concentration that induces a response halfway between the baseline and maximum).

5. Conclusions

In conclusion, our results demonstrate that treatment with carvacrol improves hypertension-associated erectile dysfunction in SHR, at least, by a mechanism involving endothelial function restoration and reduction of rat corpora cavernosa hypercontractility.

Author Contributions: Tays A. F. Felisberto Gonçalves, designed the study, conducted the research, analyzed the results and drafted the manuscript. Priscilla M. P. Maciel, Larissa I. M. Villanueva, Pablo F. dos Santos, Ismael de L. O. Júnior conducted the research and analyzed the results. Robson C. Veras, analyzed the results and helped to review the manuscript. Islania G. A. Araújo, design of the study and helped to draft the manuscript. Isac A. de Meideiros takes primary responsibility for the paper, conceived and coordinated the study and helped to draft the manuscript.

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**Sample Availability:** Samples of the compounds ...... are available from the authors.