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Treatment with a combination of ginger, L-citrulline, muira puama and Paullinia cupana can reverse the progression of corporal smooth muscle loss, fibrosis and veno-occlusive dysfunction in the aging rat

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Abstract

Aims—Aging associated erectile dysfunction is characterized within the corpora by a progressive apoptosis of the smooth muscle cells and their replacement by collagen. Nitric oxide from iNOS has been shown to inhibit these histological changes in the corpora while PDE5 inhibitors as well as certain nutraceuticals such as ginger, paullinia cupana, muira puama and L-citrulline are known to enhance the effects of NO. We evaluated whether the daily oral administration for 2 months with a combination of ginger, paullinia cupana, muira puama and L-citrulline (COMP-4) can effectively delay the ongoing corporal fibrosis, smooth muscle cell apoptosis and cavernosal veno-occlusive dysfunction (CVOD) seen in middle aged rats similar to that seen with tadalafil.

Methods—10 Month old Fisher 344 rats were treated or not for two months with COMP-4, tadalafil or a combination of tadalafil plus COMP-4. CVOD was determined by dynamic infusion cavernosometry. Penile sections of the corpora cavernosa were subjected to Masson trichrome staining to evaluate fibrosis and immunohistochemistry for desmin as a marker of smooth muscle content and inducible nitric oxide synthase (iNOS) followed by image analysis. Oxidative stress levels were determined by GSH/GSSG ratio in whole blood.

Results—a decline in the non-treated rat's erectile function is evident by 10-12 months of age and is accompanied by a decrease in the corporal smooth muscle content determined by desmin expression and an increase in corporal fibrosis. The daily treatment for two months with COMP-4 reverses this process by reducing systemic oxidative stress and increasing desmin and iNOS expression, similar to that seen with tadalafil or the combination of COMP-4 plus tadalafil.

Conclusion—An oral combination of ginger, muira puama, Paullinia cupana and L-citrulline seems to be as effective as daily PDE5 inhibitor therapy in either delaying or reversing the onset of the histological and functional characteristics of aging related erectile dysfunction.

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Keywords

Ginger; Paullinia cupana; Muira Puama; L-Citrulline corporal smooth muscle; corporal fibrosis; corporal veno-occlusive dysfunction

Introduction

Erectile dysfunction (ED) associated with the aging process is characterized histologically within the cavernosa by a progressive apoptosis or loss of the corporal smooth muscle cells (SMC) and replacement of these cells with collagen (1-3). It has been estimated that once approximately 15 -20% of the corporal SMCs have been lost, venous leakage or corporal veno-occlusive dysfunction (CVOD) becomes clinically apparent (4). We have previously demonstrated that as these aging-related histological changes begin to occur in the cavernosa, the SMCs themselves attempt to combat these apoptotic and fibrotic changes by upregulating inducible nitric oxide synthase (iNOS) (3). It is believed that the high output of nitric oxide (NO) produced intracellularly by iNOS can act in this setting and in other conditions as an anti-apoptotic and anti-fibrotic factor (5-7). This anti-fibrotic effect of iNOS is evident not only in aging related ED (ARED) (3,8) but also in the bilateral cavernosal resection rat model of ED where the histological changes that occur in the cavernosa resemble a more accelerated version of ARED (9-12).

Nutraceuticals are substances that may be considered as food or be a part of it that offers health or medicinal benefit, including prevention and/or treatment of diseases (13). To date, the only nutraceutical that has been shown to enhance iNOS activity is ginger (*Zingiber officinale* Roscoe) albeit in an in vitro setting (14,15). In fact, ginger has been used successfully in the treatment of liver fibrosis in vivo (16). Besides ginger, L-arginine, the amino acid that provides the nitrogen moiety for NO during its synthesis, has by itself or in combination with PDE5 inhibitors also shown to have some beneficial effects in minimizing fibrosis both in vivo and in vitro (17-20). Two other nutraceuticals, muira puama and Paullinia cupana which are both indigenous to the Amazon rainforests, have been reported to enhance erectile function (21). Paullinia cupana also exhibits *in vitro* protective effects against cytotoxicity and oxidative stress in NIH-3T3 embryonic fibroblasts cells induced by SNP exposure, thereby suggesting that Paullinia cupana has an *in vitro* bioactive action on NO modulation (22). In addition, we have shown that both P. cupana and muira puama taken orally can each individually and in combination further increase the expression of iNOS in the corpora (personal observation).

The favorable ethnopharmacological properties of these four natural products make them potential suitable candidates for inhibiting or delaying the onset or progression of the naturally occurring apoptosis and fibrosis that is seen in ARED and in turn the resultant CVOD or venous leakage. The aim of the present study is to determine whether in middle aged rats of approximately 10 months of age, the daily oral administration for 2 months of a combination of these four products: ginger, Paullinia cupana, muira puama and L-citrulline (as a substitute for L-arginine) can be effective in retarding and possibly reversing the ongoing corporal SMC loss and fibrosis, and the subsequent CVOD associated with aging.

The efficacy of this combination of these four natural products will be compared to the known anti-oxidant and anti-fibrotic effects with chronic PDE5 inhibitor treatment (8-12).

Materials and Methods

Animal treatments

Middle aged 10 month-old Fisher 344 rats (Harlan Sprague–Dawley, San Diego, CA) were divided in 4 groups and each group (n = 6) was treated daily for 2 months as follows: a) control: 12 month-old at the time of death receiving by retrolingual administration vehicle only composed of 10% Dimethyl sulfoxide, peanut butter and water **(12 mo)**; b) Composition 4 **(COMP 4)** (Naturex, Hackensack, NJ) : a combination of muira puama (45 mg/Kg B.W), Paullinia cupana (45 mg/Kg B.W), ginger (45 mg/Kg B.W) and L-Citrulline (133 mg/Kg B.W) dissolved in vehicle c) tadalafil **(TAD)** (Lilly, Indianapolis, IN) : 2.5 mg/Kg B.W tadalafil dissolved in vehicle d) Composition 4 + Tadalafil (**COMP4 + TAD**): a combination of b and c at the same doses. Doses were selected based on what is recommended for humans and corrected for rat body surface. The dose selected are equivalent to a human dose of 500 mg/day ginger rhizome, 1500 mg/day of L-Citrulline, 500 mg/day Paullinia cupana, and 500 mg/day muira puama in a 70 Kg man. These doses are below the toxicity level for each nutraceutical. Tadalafil dose was also corrected for body surface and is equivalent to 22 mg/day in a 70 Kg men. This dose is slightly higher than the maximum recommended dose in humans and is one-half of what we have used in previous studies (12).

Dynamic Infusion Cavernosometry

Cavernosometry was performed as previously described (8-12). Briefly, under deep anesthesia the penis was exposed and a cannula was inserted into the corpora cavernosa and the basal intracavernosal pressure **(ICP)** was recorded. Papaverine in a dose of 5 mg/Kg as previously reported (11) was administered through the cannula. The ICP during tumescence was recorded as ICP after papaverine **(ICPAP)**. Saline was then infused through another cannula, increasing infusion rate by 0.05 ml/min every 10 seconds, until the ICP reached the maintenance rate at 80 mmHg. The “drop rate” was determined by recording the fall in ICP within the next 1minute after the infusion was stopped (7,8,10-12).

Histochemistry and immunohistochemistry

After cavernosometry, animals were euthanized and the skin-denuded middle part of penile shafts was fixed overnight in 4% buffered p-formaldehyde, and stored for 24 hs in alcohol 70 % at 4°C until processed for paraffin embedded tissue sections. Five micrometer anatomical matched sections were used for (7,8,10-12): a) Masson trichrome staining for collagen (blue) and smooth muscle (red); b) immunodetection with: polyclonal antibody against desmin, a marker of smooth muscle cell content. c) polyclonal antibody against iNOS (Calbiochem, La Jolla, CA). The specificity of the antibodies was validated by western blot.

Sections were then incubated with biotinylated anti-Rabbit IgG followed by ABC complex (Vector labs, Temecula, CA) and 3,3 'diaminobenzidine (Sigma) Sections were counter-

stained with haematoxylin. Negative controls in the immunohistochemical experiements were done by replacing the first antibody with IgG isotype.

Quantitative image analysis

Quantitative image analysis was performed by computerized densitometry using the ImagePro 7.1 software (Media Cybernetics, Silver Spring, MD), coupled to an Olympus BHS microscope equipped with a Retiga digital camera (7,8,10-12). For Masson staining, 40× magnification pictures of the penis comprising half of the corpora cavernosa were analyzed for smooth muscle (stained in red) and collagen areas (stained in blue) excluding the sinusoidal spaces, and expressed as the smooth muscle/collagen ratio. For desmin and iNOS staining, 200× magnification pictures of the corpora cavernosa were analyzed in a computerized grid and expressed as % of positive area vs. total area of the corpora cavernosa. In all cases, four fields at 40×, or eight fields at 200×, were analyzed per tissue section, with at least 4 matched sections per animal and 6 animals per group.

Estimation of Gluthatione reduced (GSH) and Gluthatione oxidized (GSSG)

For the measurement of GSH/GSSG ratio (23), blood was collected with or without 1-methyl-2 vinylpyridinium trifluoromethane sulphonate (M2VP) scavenger of reduced glutathione, described in the commercial kit protocol (‘Bioxytech GSH/GSSG-412 kit’ from Oxis Health Products, Portland, OR). The omission or addition of M2VP allows the measurement of GSH and GSSG, respectively. The spectrophotometric detection was recorded at 412 nm for 3 min after the addition of 3.8 μmol NADPH. GSH/GSSG ratio inversely proportional to oxidative stress levels and monitors the effectiveness of antioxidant intervention strategies.

Statistical analysis

Values were expressed as mean ± SEM. The normality distribution of the data was established using the Wilk-Shapiro test. Multiple comparisons of the 12 month old groups were analyzed by a single factor analysis of variance (ANOVA), followed by post-hoc comparisons with the Student-Neuman-Keuls test, according to the GraphPad Prism V 5.1. Differences were considered significant at $P<0.05$. The use of six animals per group was selected to obtain a 80% power at alpha 0.05. For additional comparison, we compared the results of the 12 month old animals to the cavernosometry values obtained from earlier studies in 5 mo old non treated animals (8). In the immunohistochemistry and histochemistry studies, penile sections of untreated 5 month old animals were run in parallel for comparison and included in the statistical analysis.

Results

Figure 1 shows that the control 12 mo rats receiving only vehicle had a considerable decrease (28%) in the ICP after intracavernosal papaverine administration **(top)**, and a concomitant 1.7-fold increase in the drop rate **(bottom)** when compared to our previously published historical data in young 5 mo old (young) control animals. This indicates the presence of ED (as measured by the ICPAP) and a moderate venous leak or CVOD (as measured by the drop rate) in the 12 mo rats. The treatment of these 10 mo old animals for 2

months with daily PDE5 inhibitor, tadalafil, increased the papaverine response **(top)** and decreased the drop rate when compared to the 12 mo control animals and is in agreement with previous results in aged animals who were treated daily via the drinking water with another PDE5 inhibitor, sildenafil (8).

Daily administration of COMP-4 for two months increased the papaverine-induced erection **(top)** and reduced the drop rate **(bottom)** to values not significantly different from the ones treated with daily tadalafil or the young 5 mo of age non-treated animals. The combination of COMP-4 plus TAD was similar to the ICPAP response for either COMP-4 alone or TAD alone, without any synergistic effect between TAD and COMP4.

As seen in Figure 2, the presence of venous leakage as determined by the drop rate observed in the 12 mo control animals was also accompanied by a 70% decrease in the smooth muscle (SMC)/collagen ratio as determined by Masson trichrome staining. In agreement with previous published results with daily and continuous sildenafil in the drinking water (8), daily oral treatment with the PDE5 inhibitor TAD improved significantly the SMC/collagen ratio by 60% when compared to the 12 mo control animals, although the ratio still remained lower than that seen in the 5 mo control. However, daily treatment with COMP-4 alone restored the SMC/collagen ratio to levels similar to those of the young 5 mo controls while the combination of COMP4 with TAD further improved the levels above the 5 mo controls.

To determine what effect treatment for two months with COMP-4 with or without TAD had solely on the smooth muscle content of the corpora of these 10 mo old animals, a marker of smooth muscle, desmin, was evaluated by immunohistochemistry in adjacent sections to those employed to determine the aforementioned SMC/collagen ratio. Figure 3 shows as expected a significant 52% reduction of desmin expression at 12 mo when compared to the 5 mo animals. Treatment for 2 months with either COMP-4 or COMP-4 +TAD resulted in a marked increase in desmin expression approaching levels seen in the young 5 mo old animals. As would be expected, there is a 38% increase in the desmin expression in the TAD treated group when compared to the 12 mo old non-treated controls but this level does not quite reach those levels seen in young 5 mo old animals. Furthermore, COMP-4 and COMP-4 +TAD groups produced a more pronounced effect with an increase in desmin expression by 54% with respect to the 12 mo group reaching the same level of expression as the historical 5 mo control group.

In order to elucidate whether the improvement in penile dynamics and the reduction of fibrosis seen in these treated animals are mediated by an increase in iNOS expression, iNOS was assessed by immunohistochemistry. Fig 4 shows, as expected with aging, that there was a significant increase in iNOS expression in the 12 mo control animals with respect to the historic 5-mo controls. With daily tadalafil for 2 months, there was a non-significant but slight increase in iNOS expression compared to the control 12 mo animals. However, treatment with COMP-4 produced a significant increase of 36% when compared to the 12 mo controls. The combination of COMP-4 +TAD showed a similar significant increase in iNOS expression as the COMP-4 group alone when compared to the 12 mo controls.

To determine whether the improvement of erectile function and reduction in corporal fibrosis observed by the treatment with COMP-4 for two months can be attributed to a reduction in oxidative stress which is one the presumptive mechanisms by which NO from iNOS exerts its anti-fibrotic effect, the GSH/GSSG ratio, which is a method of measuring oxidative stress, was determined in the whole blood of these animals. Figure 5 shows that the 12 mo non-treated control animals exhibited levels indicative of considerable oxidative stress levels (a very low ratio) when compared to young 5 months old animals (a high ratio). We found that two months of daily treatment with COMP-4 effectively increased the GSH/GSSG ratio to levels (less oxidative stress) similar to those found in 5 mo old animals. The TAD and COMP-4 +TAD animals also showed increases in the ratio but did not achieve the levels seen in the young 5 mo or the 12 mo COMP4 treated animals.

Discussion

Our results indicate that in the laboratory rat a decline in the rat's erectile function as measured by both by the ICPAP and the drop rate is evident by 12 months of age. This decline is accompanied histologically by a decrease in the corporal smooth muscle content, its replacement by collagen and a resultant increase in corporal fibrosis. The daily treatment of these animals for two months with COMP-4 which contains a mixture of muira puama, Paullinia cupana, ginger and L-citrulline, seems to reverse this process in that CVOD as measured by the drop rate is improved, smooth muscle content is increased, and corporal fibrosis is reduced. In addition, COMP-4 reduced oxidative stress as measured by the GSH/GSSG levels in the blood.

Our theory of aging related ED is that it occurs in an environment of high oxidative stress and is most likely due to a genetically predetermined apoptosis of the corporal smooth muscle with replacement of the apoptotic cells by collagen resulting in an increase in corporal fibrosis. One of the ways the SMC tries to combat this high oxidative stress and apoptotic process is by inducing iNOS which theoretically produces high levels of intracellular NO that can act as an anti-oxidative and an anti-fibrotic molecule (24). In a previously published study, we have shown in a markedly aged rat of about 21 months of age that the daily and continuous use of the PDE5 inhibitor, sildenafil, which acts on the NO-cGMP pathway to enhance the effect of NO presumably derived from iNOS, not only halted the aging related corporal SMC apoptosis but seemed to reduce the formation of fibrosis within the corpora (8). In addition, in the cavernosal nerve resection rat model in which the animals were about 5 mo of age, it has been shown experimentally that continuous daily treatment with PDE5 inhibitors can retard the apoptosis of the corporal SMC and minimize the development of corporal fibrosis normally seen when the cavernosal nerve is iatrogenically damaged (9-11, 24-26). In addition, in Peyronie's disease which is another penile fibrotic condition albeit in the tunica albuginea rather than in the corporal tissue, gene therapy with an iNOS cDNA has been shown to reverse the fibrosis that occurs in this experimentally induced Peyronie's disease rat model (6).

COMP-4 was initially conceived as a compound to delay the onset of erectile dysfunction centered on the observation that one of its constituents, ginger, was found in vitro at a certain concentration to increase both the expression of iNOS and its production of NO (14,15), and

in vivo to reduce oxidative stress in diabetic rats (21). Since the nitrogen molecule of NO emanates solely from the conversion of L-arginine to L-citrulline and because oral L-citrulline by itself resulted in higher levels of blood L-arginine levels than if L-arginine itself was ingested (28), L-citrulline was substituted for L-arginine as one of the constituents of COMP-4. Finally, two other compounds, muira puama and Paullinia cupana, have been reported individually to have aphrodisiac properties as well as enhancing effects on erectile function and orgasm (18) and to increase cAMP levels in rabbit corporal tissue as well as induce corporal smooth relaxation and reducing oxidative stress (29, 30). Furthermore, both of these compounds have been shown to induce iNOS expression in the corpora (unpublished observations). Together, these four compounds comprise COMP-4.

Although COMP-4 seemed to at least halt and possibly reverse the histological changes seen in the aging penis, we essentially saw similar improvement with tadalafil alone. Daily tadalafil for 2 months not only improved the erectile response to intracavernosal papaverine but also decreased the drop rate. Although its effects on the SMC/collagen ratio and desmin expression were less evident, daily tadalafil nevertheless did show an improvement in this ratio. When COMP-4 was given together with tadalafil, this combination had a similar effect as COMP-4 alone on the ICPAP, drop rate and desmin staining although the SMC/collagen ratio of the COMP-4 + TAD animals was slightly better than TAD alone suggesting that the combination of COMP-4 + TAD seemed to produce better outcomes in the amelioration of corporal fibrosis. These results are in agreement with our previous study where 21 month old animals that were treated with daily and continuous sildenafil demonstrated an improvement in the histological changes known to be associated with ARED (7).

The treatment with either COMP-4, TAD by itself or the combination of COMP4 + TAD increased iNOS expression. It is thought that iNOS and hence a high NO output acts as an antifibrotic defense mechanism by inhibiting collagen synthesis and myofibroblast differentiation (31,32). This is clearly seen in Peyronie's disease where treatment with an iNOS cDNA gene ameliorated the fibrosis (6) whereas the administration of an iNOS inhibitor (L-N-[1-iminoethyl]-lysine acetate) enhanced the fibrosis (32). Our results demonstrate that certain nutraceutical products may act as anti-fibrotic agents by increasing intracellular NO. As such, COMP-4 as a nutraceutical may be a promising candidate when the goal is to either reverse or delay the onset or progression of ARED.

Another rationale for using this combination of 4 nutraceuticals is that each nutraceutical in COMP-4 has been shown to either decrease oxidative stress (ginger and muira puama), increase iNOS and NO bioavailability (Paullinia cupana and ginger), or simply possess anti-fibrotic activity (L-Citrulline), These are all the elements required to improve erectile function in aging by combating those aging related physiological processes that impact the normal functioning of the corporal tissue.

The novelty of this present study in which we compared the nutraceutical combination of COMP-4 to the PDE5 inhibitor, tadalafil, is that the use of natural interventions can have similar effects and outcomes as PDE5 inhibitors at a potentially much reduced cost. One limitation of this study is that we did not determine whether COMP-4 modulates cGMP levels since this would explain whether the observed effects of COMP-4, which were similar

to those seen with the PDE 5 inhibitor tadalafil, is due to an increase in NO and cGMP modulating oxidative stress, fibrosis and erectile function.

Conclusion

Oral combination of L-citrulline, ginger, muira puama and Paullinia cupana seems to be effective in either retarding and/or reversing those histological and functional characteristics of ARED in a manner similar to that we have seen experimentally with PDE5 inhibitors. Further studies are necessary to determine whether one or a combination of these four natural occurring products holds potential as a treatment to either delay the onset and/or possibly reverse those histological changes associated with ARED.

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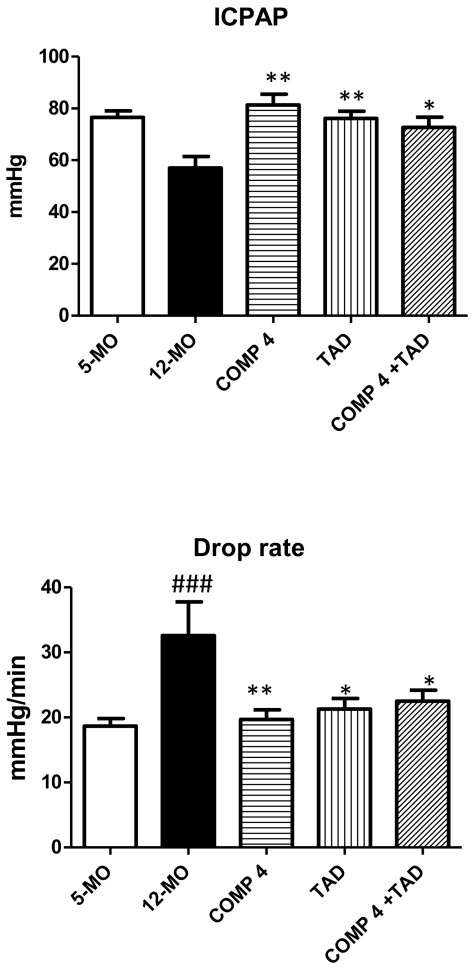

Figure 1. Prevention of CVOD in middle aged Fischer 344 rat by oral treatment with tadalafil (TAD), COMP4 and combination of COMP4 + TAD, as determined by dynamic infusion cavernosometry

5MO: Five month-old animals at the time cavernosometry and euthanasia; **12 MO** : Middle aged control, 12 month-old at the time of cavernosometry and euthanasia. **COMP4**: 10 mo old animals treated with composition 4 for two months and sacrificed at 12 months; **-TAD**: 10 month old animals treated with tadalafil for 2 months and sacrificed at 12 mo; **COMP4+TAD**: 10 mo old animals animals treated with a combination of composition 4 and tadalafil for 2 months and sacrificed at 12 months of age. **p<0.01; * p<0.05 respect to 12 MO. ### p<0.005 respect to 5-MO. (n=6)

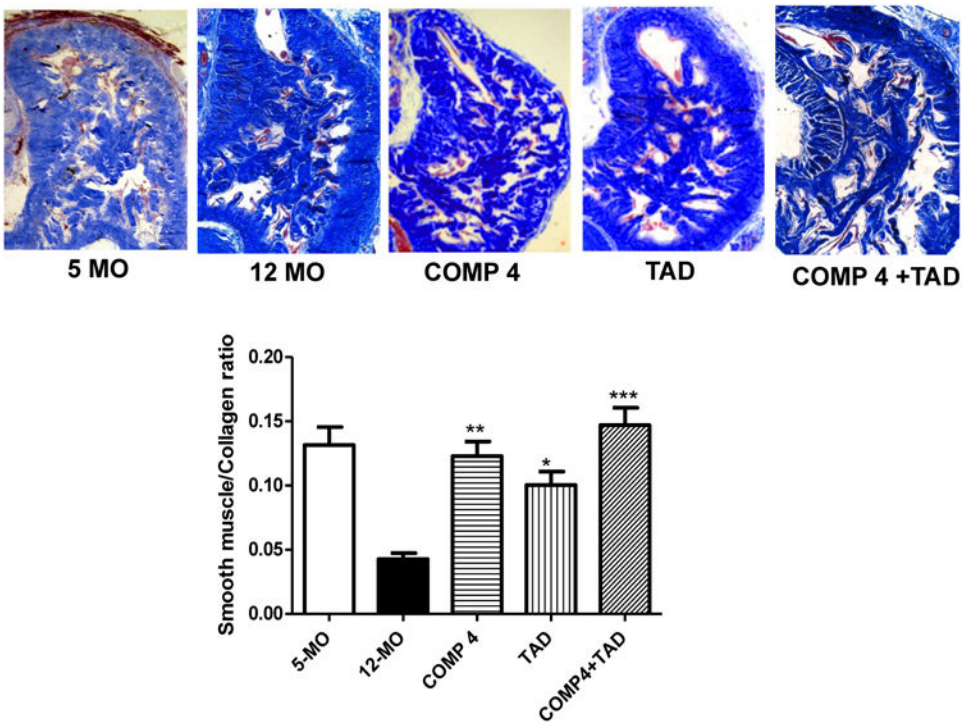

Figure 2. Partial normalization by TAD and complete normalization by COMP4 and COMP4+TAD of the reduced smooth muscle/collagen ratio and increased collagen content in the middle aged rat corpora cavernosa

Top: representative pictures (40×, Bar=50 μm) of penile corpora cavernosa tissue sections from the rat groups presented on Figure 1, stained with Masson trichrome. Bottom: quantitative image analysis of the SMC/collagen ratio. *p<0.05, **p<0.01, ***p<0.005 respect to 12 MO. (n=6)

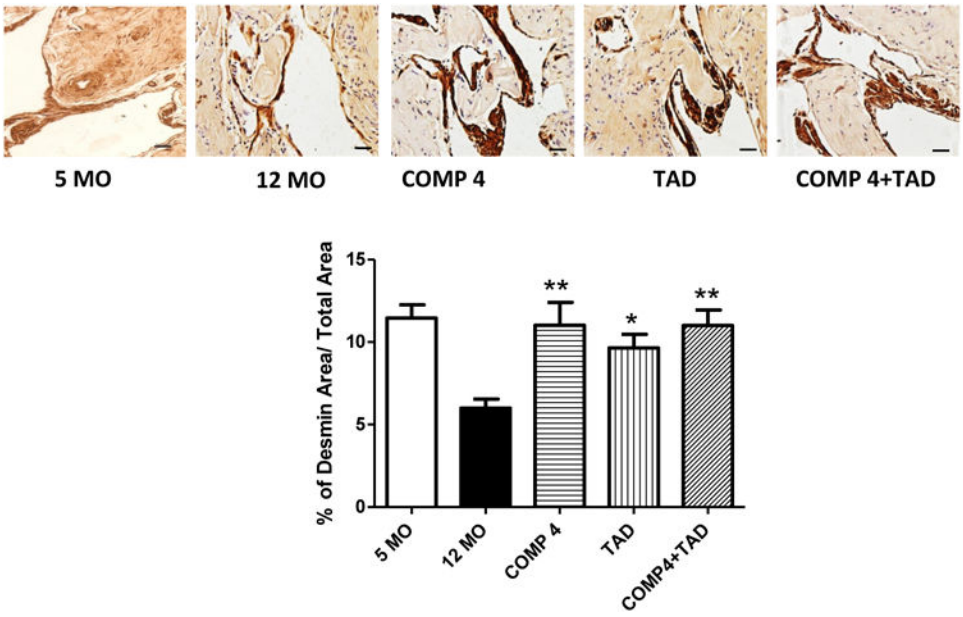

Figure 3. Effect of TAD, COMP4, and COMP4+TAD on the preservation of smooth muscle content in the middle aged rat corpora cavernosa

Top: representative pictures (200×, Bar=50 μm) of penile corpora cavernosa sections adjacent to those presented on Fig. 2, immunostained for desmin as a smooth muscle cell marker. Bottom: quantitative image analysis. *p<0.05, **p<0.01, respect to 12 MO (n=6)

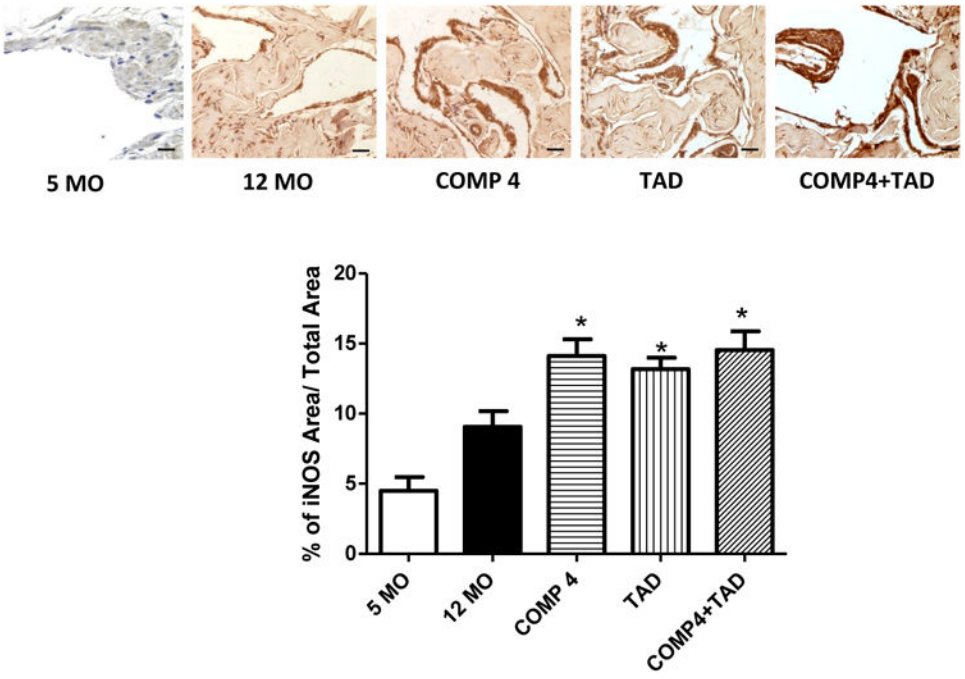

Figure 4. iNOS expression in the middle aged rat corpora cavernosa is up regulated by COMP4 and by COMP 4 + TAD

Top: representative pictures (200×, Bar= 50μm) of penile corpora cavernosa sections adjacent to those presented in previous figures that were subjected to immunodetection for iNOS. Bottom right: quantitative image analysis * $p<0.05$. (n = 6).

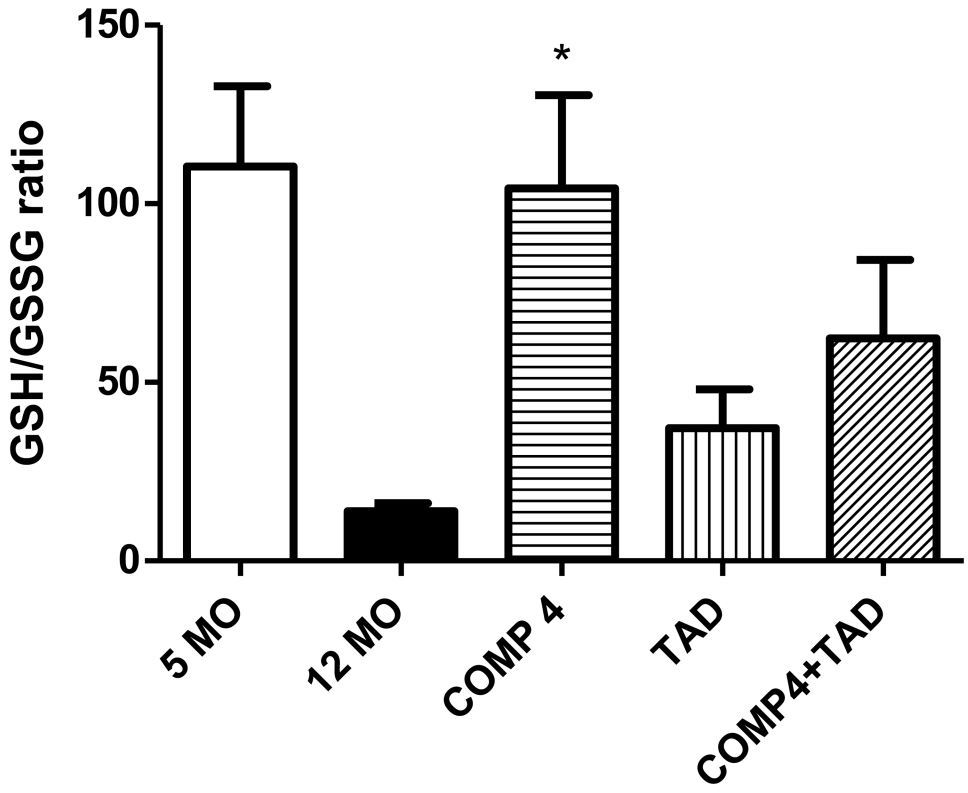

Figure 5. COMP4 reduces oxidative stress in whole blood of middle aged rat

Oxidative stress was determined by measuring GSSG and GSH in whole blood after the addition or not of M2VP respectively. The GSH/GSSG ratio was calculated as (GSH-2*GSSG)/GSSG. *$p<0.05$ respect to 12 MO. (n=6)