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# Spice up the hypertension diet - curcumin and piperine prevent remodeling of aorta in experimental L-NAME induced hypertension

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## Abstract

**Background:** Increase of blood pressure is accompanied by functional and morphological changes in the vascular wall. The presented study explored the effects of curcuma and black pepper compounds on increased blood pressure and remodeling of aorta in the rat model of experimental NO-deficient hypertension.

**Methods:** Wistar rats were administered for 6 weeks clear water or L-NAME (40 mg/kg/day) dissolved in water, piperine (20 mg/kg/day), curcumin (100 mg/kg/day) or their combination in corn oil by oral gavage. The systolic blood pressure was measured weekly. Histological slices of thoracic aorta were stained with hematoxylin and eosin, Mallory's phosphotungstic acid hematoxylin (PTAH), orcein, picosirius red and van Gieson staining and with antibodies against smooth muscle cells actin. Microscopic pictures were digitally processed and morphometrically evaluated.

**Results:** The increase of blood pressure caused by L-NAME was partially prevented by piperine and curcumin, but the effect of their combination was less significant. Animals with hypertension had increased wall thickness and cross-sectional area of the aorta, accompanied by relative increase of PTAH positive myofibrils and decrease of elastin, collagen and actin content. Piperine was able to decrease the content of myofibrils and slightly increase actin, while curcumin also prevented elastin decrease. The combination of spices had similar effects on aortic morphology as curcumin itself.

**Conclusions:** Administration of piperine or curcumin, less their combination, is able to partially prevent the increase of blood pressure caused by chronic L-NAME administration. The spices modify the remodeling of the wall of the aorta induced by hypertension. Our results show that independent administration of curcumin is more effective in preventing negative changes in blood vessel morphology accompanying hypertensive disease.

**Keywords:** piperine, curcumin, L-NAME, hypertension, aorta, remodeling

## Background

N-nitro-L-arginine-methylester (L-NAME) is a nonspecific inhibitor of all three NO synthase (NOS) isoforms (neuronal - nNOS; inducible - iNOS; endothelial - eNOS) and causes an increase of blood pressure in a dose-dependent manner when administered to the experimental animals [1,2]. The adaptation process to the increased blood pressure may include several morphological changes in the blood vessel wall. Hypertension causes

thickening of the vascular wall [3,4], however, the role of blood pressure in the inner diameter enlargement is currently unclear since some claim its importance [5,6] while others present its insignificant role in this process [7,8].

Most of the morphological changes appear in the *tunica media* formed by smooth muscle cells (SMC) and the extracellular matrix (ECM). Under normal conditions mature SMC exhibit low synthetic activity [9] but unlike skeletal and heart muscle, the vascular SMCs are able to change their phenotype and start producing large amounts of ECM components and increase their

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proliferation and migration [10]. Actin polymerization is one of the important SMC phenotype changes [11].

Collagens in the aortic wall are represented mainly by collagens of type I and type III [12]. Collagen type I (Col I) is mechanically firm and hypertension induced by long term administration of L-NAME leads to an increase of its synthesis in blood vessels [13]. Collagen type III (Col III) forms a net of fine fibers which enables to maintain blood vessel elasticity and their damage results in increased vessel wall rigidity [14,15]. The protein responsible for the blood vessel elasticity is elastin [16] which is in comparison to collagen an extremely stable polymer with low level biological turn-over [17]. When mechanically damaged by hypertension it can be newly synthesized in higher amounts [18].

Nutrition and lifestyle play a vital role in prevention of hypertension and therefore there is a continuous search for dietary components having positive effect on blood pressure. Curcumin is the main polyphenol of *Curcuma longa*, a spice of yellow color. It is currently known due to its chemopreventive [19], antioxidant and anti-inflammatory [20] properties. Moreover, curcumin is able to inhibit proliferative activity of vascular SMC *in vitro* and induce their apoptosis and thus to participate in prevention of pathological changes in blood vessels [21].

Piperine is responsible for the pungency of spices gained from *Piper nigrum* and *Piper longum* (sources of black or long pepper) [22]. Its main biological significance lies in its ability to influence metabolism of other substances (including drugs) and if consumed together it can significantly increase the bioavailability of curcumin [23]. The hypotensive effect of piperine on blood pressure has been verified after acute intravenous administration [24] or per orally in high doses (50 mg/kg) [25]. However, piperine in higher concentrations leads to increased hydroxyl radical production while in low concentrations it acts as an antioxidant [26] and it is important to establish whether piperine is able to influence the blood pressure after peroral administration of lower doses.

The aim of the presented study is to evaluate the preventive effects of piperine and curcumin on blood pressure increase and the pathological changes in blood vessel morphology in the experimental hypertension induced by L-NAME administration.

## Methods

### Experimental protocol

All procedures and experimental protocols were approved by an ethical committee and conform to the European Convention on Animal Protection and Guidelines on Research Animal Use. The animals were housed in an air-conditioned room at a steady temperature (22-24°C) and humidity (45-60%) on a 12:12 h light/dark

cycle and maintained on a standard pellet diet and tap water *ad libitum*. During the experiment, three animals (one from L-NAME group, two from L-NAME + curcumin + piperine group) died from technical problems.

Adult 12-week-old male Wistar rats were divided into 8 groups with 6 animals in each group: control group (Con, receiving pure water and corn oil), group treated with 40 mg/kg/day of L-NAME (L) dissolved in the drinking water and administered orally; groups receiving piperine (20 mg/kg/day) in corn oil by oral gavage with L-NAME (LP) or without it (P); groups receiving curcumin (100 mg/kg/day) in corn oil (max 0.4 ml per animal) by oral gavage with L-NAME (LC) or without it (C); groups receiving curcumin and piperine in the same manner with L-NAME (LCP) or without it (CP). The dose of curcumin was chosen according to previous positive results on abdominal aorta [27]. The selected dose of piperine was reported to be able to enhance the bioavailability of curcumin [28].

All compounds were administered daily for 6 weeks and the experiment was ended by an overdose of thiopental. Daily water consumption and the body weight were estimated one week before the experiment and controlled during the treatment. There were no statistically significant changes of the body weights of animals in different experimental groups at the beginning or at the end of the experiment.

### Blood Pressure Measurement

The systolic blood pressure (BP) was measured non-invasively in awaked animals by the tail-cuff plethysmography by using blood pressure module (NIBP Controller, ADInstruments, Spechbach, Germany) connected through the manometer and Powerlab 8/30 module (ADInstruments, Spechbach, Germany) to the computer. The last measurement was taken at the end of the 5th week of administration. The final value was calculated from five successive measurements.

### Histology Staining

Specimens of the middle part of thoracic aorta were fixed 24 h in 10% formalin, routinely processed in paraffin and 5 µm thick slices were stained with hematoxylin and eosin, Mallory's phosphotungstic acid hematoxylin (PTAH), van Gieson collagen staining and orcein elastin staining using standard procedures.

Smooth muscle actin was detected by mouse monoclonal antibodies (DAKO) diluted 1:100 in Antibody Diluent (DAKO) applied on specimens with previously heat-retrieved epitopes in 10 mmol/L Tris buffer, 1 mmol/L EDTA, pH 9.0. The slides were incubated with the primary antibody for one hour at laboratory temperature, then washed in TBS (pH 7.4) and incubated with anti-mouse peroxidase complex (EnVision<sup>®</sup> kit,

DAKO), washed in TBS and the color reaction was developed with diaminobenzidine (DAKO). The immunohistochemical staining was performed in DAKO Autostainer staining automat.

Sirius red F3BA dissolved in a saturated picric acid stains collagens type I and III, which can be distinguished with the use of polarized light, and their content evaluated by computer assisted morphometry is in concordance with the results obtained by immunohistochemistry and evaluation of mRNA levels [29,30]. Modified technique with picosirius red [31] was used as follows: the slides were submerged in 0.2% phosphomolybdic acid, then stained with 0.1% sirius red F3BA in a saturated water solution of picric acid for 90 min, washed in 0.01 N HCl, dehydrated and mounted. Slides were then viewed under polarized light, in which Col I has red to yellow color because of thickness of its bundles, while thin collagen type III. fibers are green [30].

If not stated otherwise, all chemicals were purchased in Sigma Chemie (Germany).

#### Digital Morphometry

All slides were evaluated in a Leica light microscope (Leica Systeme, Wetzlar, Germany). The slides stained by hematoxylin and eosin were photographed by digital photographic camera (S 50, Canon, Japan) using the same conditions (microscope magnification 45× and camera settings) for each slide and for Burkner cell counting chamber. Based on the known size of the Burkner chamber's grid the evaluating programme (ImageJ 1.42q, National Institute of Health, USA) was preset to calculate the results in SI units. Internal circumference along the vessel *lamina elastica interna* and external circumference along *lamina elastica externa* were then measured. Inner diameter, tunica media thickness and its cross-sectional area (CSA) were calculated.

For all other stainings, at least five random places were selected on each slide and documented. The pictures were digitally processed in Active pixels software (Idea systems, USA) to limit the measurement to aortic media (the background and other parts of aorta were deleted) and afterwards evaluated with the ImageJ software. Threshold values were determined for the colors or the intensity of staining representing the analyzed compounds of aortic media. Cross-sectional area of media was estimated for every taken picture and percentage of the specific color covering that particular area was measured. Proportional amounts were then matched together with results obtained by the morphometric evaluation (CSA size in square millimeters) used in order to calculate the "absolute" amount of the stained components of aortic media, as well as the amount of unstained components.

#### Statistics

The results were statistically analyzed by GraphPad Prism (GraphPad Software, USA) using two-way ANOVA with Bonferroni test for blood pressure (time and given substances being the two variables) or one-way ANOVA with Keuls-Neumann test, and expressed as mean  $\pm$  standard deviation. Values with  $p < 0.05$  were considered significant.

#### Results

##### Blood pressure

At the beginning of the experiment (week 0) the systolic blood pressure was without any differences among the groups. Administration of curcumin, piperine and their combination caused no significant changes in blood pressure. Administration of L-NAME induced a significant increase in blood pressure already after the first week (to  $157.4 \pm 14.0$  mm Hg) and continuing administration caused even further increase ( $168.8 \pm 11.1$  mm Hg the 5th week) (Figure 1).

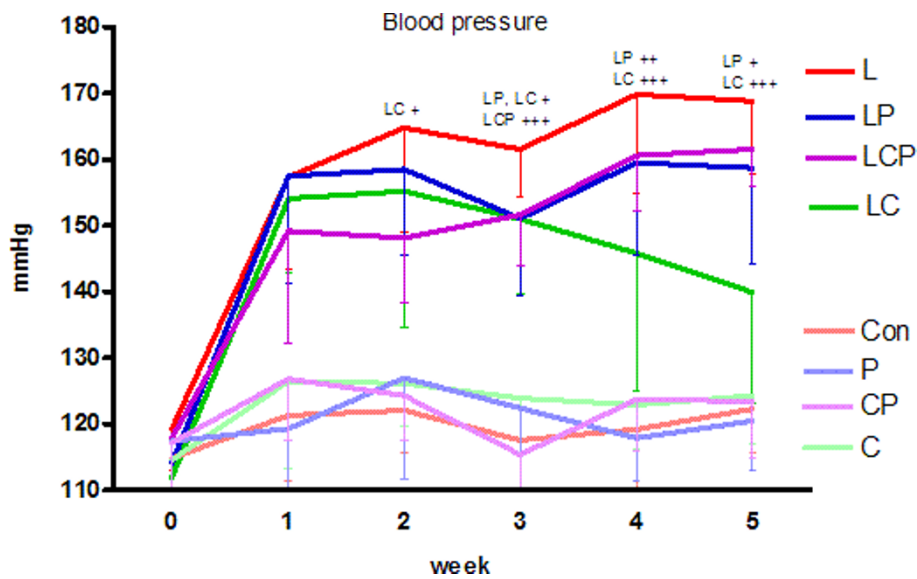
Treatment with piperine caused a significant decrease in blood pressure in comparison to the L-NAME group following the third week of administration, however, the blood pressure remained above the control until the end of the experiment. Curcumin treatment caused a significant drop of the increased blood pressure starting the second week and this reduction continued in the following weeks reaching a decrease of 28.9 mm Hg at the end of the experiment. The combination of spices showed a tendency to decrease the blood pressure but these changes were statistically significant only in the third week (Figure 1).

##### Morphometry

Administration of the spices alone did not cause any significant change in the inner aortic diameter, aortic media thickness or the cross section area (CSA) when compared to the control. Six weeks of L-NAME-induced hypertension caused a significant enlargement of the aorta and co-administration of either curcumin or piperine was not able to prevent it. Combination of the spices was able to significantly reduce the aortic wall thickness in animals with hypertension to average values of the CSA that did not significantly differ from the control (Figure 2).

##### Fibrillar proteins stained by Mallory's phosphotungstic acid hematoxylin (PTAH)

Neither of the spices alone caused any significant changes in the proportional amount of myofibrils stained by PTAH in the aortic media. L-NAME-induced hypertension inflicted a significant gain in their relative content which was reduced by simultaneous administration of curcumin and/or piperine, with curcumin being significantly more effective than piperine (Table 1). The absolute amount of



**Figure 1** Effect of piperine and curcumin on systolic blood pressure in L-NAME-induced hypertension. Con - control, L - L-NAME induced hypertension, P - piperine, C - curcumin. +  $p < 0.05$ , ++  $p < 0.01$ , +++  $p < 0.001$  versus L.

PTAH-positive myofibrils in the CSA was not affected by the spices alone but was increased in hypertensive animals. Piperine had no effect on this increase, curcumin and combination of the spices brought about a significant decrease of the absolute amount of PTAH positive myofibrils to the control level (Figure 3).

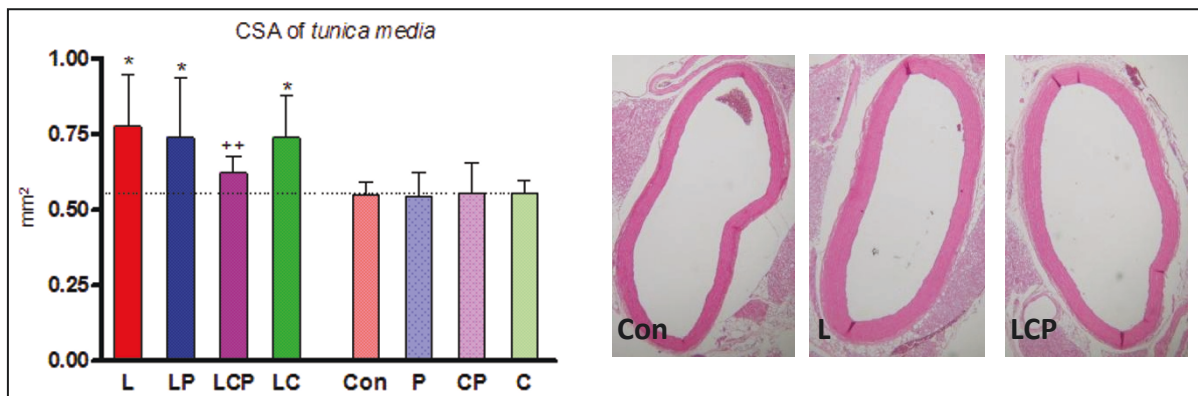
#### Smooth muscle cell actin

The proportional content of smooth muscle cell actin in the aortic media was not changed by curcumin and/or piperine administration. Six weeks of L-NAME-induced hypertension lead to a significant decrease in the actin relative amount. Piperine administration had no significant effect but the relative content of actin was

normalized by curcumin (with or without piperine) to the level of controls (Table 1). The absolute content of actin counted in square millimeters of aortic media cross section area was not influenced by the spices alone and was decreased in hypertensive animals. Piperine with or without curcumin returned the absolute amount of actin in these animals to the control level while curcumin led to an increase of the actin amount significantly above the level of controls (Figure 4).

#### Collagens stained by van Gieson and picrosirius red staining

In specimens stained with picrosirius red staining the spices alone did not change the proportion of collagen in



**Figure 2** Changes in cross sectional area (CSA) of the media of aorta induced by hypertension and the effect of piperine and curcumin in the graphic form (left) and examples of the aorta stained with hematoxylin and eosin (right). Con - control, L - L-NAME induced hypertension, P - piperine, C - curcumin. \*  $p < 0.05$  versus Con, ++  $p < 0.01$  versus L.



**Table 1 Content of various proteins detected in the aortic media**

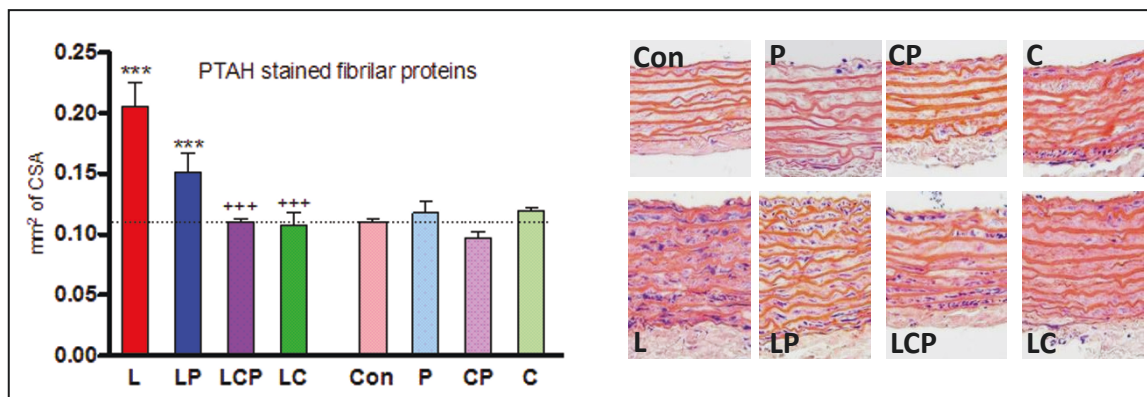
	L	LP	LCP	LC	Con	P	CP	C
<b>PTAH</b>	26.38 ± 11.65* +	21.06 ± 10.52 +	17.69 ± 5.02 + +	15.01 ± 8.52 + ++	20.07 ± 7.12	21.58 ± 13.41 +	17.45 ± 4.88 ++	21.50 ± 6.92 +
<b>actin</b>	18.87 ± 13.19*** +	24.81 ± 10.39* +	26.81 ± 8.41 + +	27.76 ± 8.50 + ++	31.03 ± 9.00	32.69 ± 7.34 + ++	30.48 ± 8.11 ++ +	27.96 ± 6.25 ++
<b>elastin</b>	25.75 ± 3.93***	28.06 ± 3.92***	33.73 ± 3.09 + ++	30.95 ± 8.36 +	34.12 ± 7.88	33.26 ± 9.02 + ++	33.78 ± 9.40 ++ +	34.74 ± 5.57 ++ +
<b>collagen I</b>	12.48 ± 6.77**	10.60 ± 8.23***	10.85 ± 3.45***	7.22 ± 4.76***	20.20 ± 12.10	22.67 ± 7.18 + +	18.67 ± 11.09 +	22.11 ± 10.12 + +
<b>collagen III</b>	0.23 ± 0.16*	0.20 ± 0.17**	0.30 ± 0.18*	0.22 ± 0.24**	0.50 ± 0.39	0.61 ± 0.44 +	0.60 ± 0.51 ++	0.58 ± 0.43 ++
<b>total collagen</b>	12.75 ± 6.89**	10.81 ± 8.36***	11.15 ± 3.56***	7.44 ± 4.95***	20.71 ± 12.27	23.29 ± 7.41 + ++	19.27 ± 11.46 + ++	22.69 ± 10.38 + ++
<b>van Gieson</b>	15.14 ± 6.78***	17.45 ± 4.22***	17.35 ± 2.08***	13.98 ± 4.15***	25.57 ± 6.27	27.01 ± 5.42 + ++	25.20 ± 3.34 ++ +	26.08 ± 3.82 ++ +
<b>CSA</b>	0.77 ± 0.17*	0.73 ± 0.19*	0.62 ± 0.05 ++	0.73 ± 0.13*	0.55 ± 0.04	0.54 ± 0.07 +	0.55 ± 0.09 +	0.55 ± 0.03 +
<b>thickness</b>	0.14 ± 0.03*	0.14 ± 0.02**	0.12 ± 0.01	0.14 ± 0.02***	0.11 ± 0.01	0.10 ± 0.01 ++	0.11 ± 0.01 +	0.11 ± 0.01 +
<b>inner diameter</b>	1.42 ± 0.09	1.51 ± 0.15	1.43 ± 0.03	1.47 ± 0.12	1.54 ± 0.09	1.53 ± 0.12	1.58 ± 0.11	1.49 ± 0.13

The table summarizes the values of the relative content of various proteins in the media of the aorta expressed in% of the total cross sectional area (CSA), which is given in mm<sup>2</sup>, thickness and inner diameter in mm. Con - control, L - L-NAME induced hypertension, P - piperine, C - curcumin. + p < 0.05, ++ p < 0.01, +++ p < 0.001 versus L; \* p < 0.05 versus Con, \*\* p < 0.01 versus Con, \*\*\* p < 0.001 versus Con.

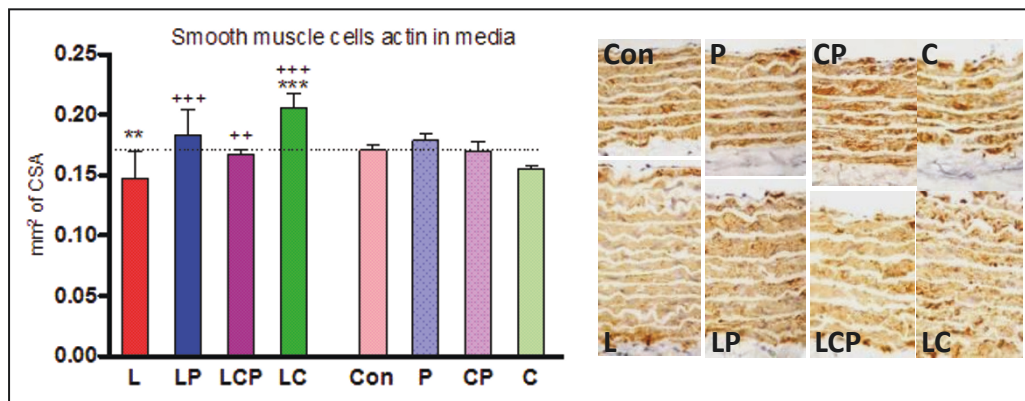
comparison to the control. L-NAME-induced hypertension led to a significant decrease in the relative content of total collagen and also of Col I and Col III, in the aortic media. These changes were not prevented by the spices co-administration (Table 1). The staining by van Gieson provided data similar to those obtained with picosirius red. The absolute amount of collagen was not changed by the spices and was decreased in hypertensive animals. Piperine administration induced a significant decrease of the absolute amount of collagen in these animals. Curcumin administration (with or without piperine) caused a further decreased of the absolute amount of collagen in the aortas of hypertensive animals (Figure 5).

**Elastin stained by orcein**

Administration of the spices did not change the proportional amount of elastin in the aortic media. This was significantly decreased in hypertensive animals. The effect of L-NAME-induced hypertension was not prevented by piperine alone, but curcumin with or without piperine caused an increase of elastin proportional content reaching the level of controls when combined with piperine (Table 1). The calculated absolute amount of elastin disclosed that spices alone caused no change in its amount. Surprisingly, the absolute content of elastin was increased by L-NAME-induced hypertension and administration of curcumin and/or piperine lead even to its further increase (Figure 6).



**Figure 3** The absolute amount of myofibrils in the aortic media identified with phosphotungstic acid hematoxylin was significantly increased in hypertensive animals (L), curcumin caused their return to the level of controls. Graphic form of the results (left) obtained from aorta specimens stained with PTAH (right). CSA - cross sectional area, Con - control, L - L-NAME induced hypertension, P - piperine, C - curcumin. +++ p < 0.001 versus L, . \*\*\* p < 0.001 versus Con.



**Figure 4** Smooth muscle cell actin in the media of aorta was decreased in hypertensive animals, both spices substances reversed this decrease. Immunohistochemical detection of smooth muscle actin (right) and evaluation in graphic form (left). CSA - cross sectional area, Con - control, L - L-NAME induced hypertension, P - piperine, C - curcumin. ++ p < 0.01 versus L, +++ p < 0.001 versus L, \*\* p < 0.01 versus Con, \*\*\* p < 0.001 versus Con.

#### Ground substance

Mathematically calculated changes of the ground substance were expressed in percentage and in absolute values (squared millimeters) of CSA of the aortic media. Since only average numbers were used for the calculation no statistical significance was stated and the results are considered as illustrational.

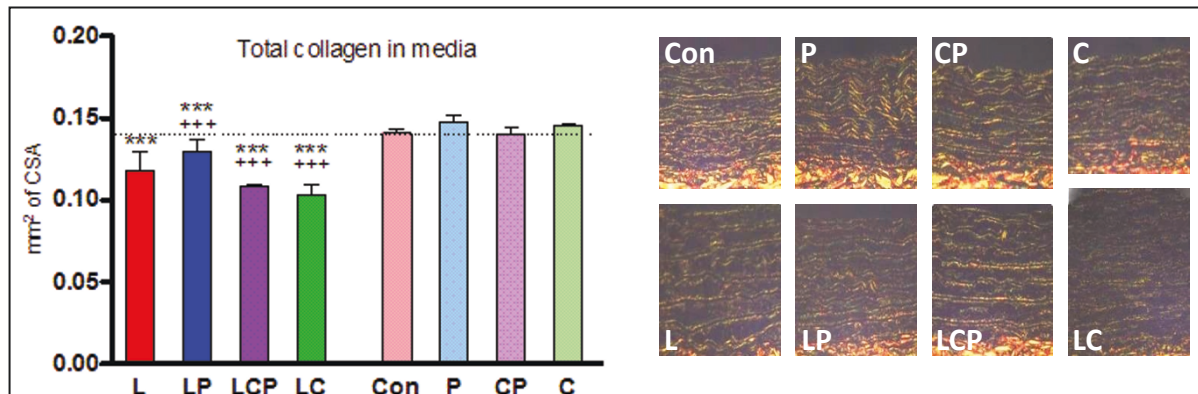
After administration of the spices the relative contents of the components of the aortic media as well as of the ground substance were not changed when compared to the control. Long term L-NAME administration with hypertension development caused an increase of the proportional amount of the ground substance (from 9.28% to 40.24%) as well as its absolute content (from 0.05 mm<sup>2</sup> to 0.31 mm<sup>2</sup>). The absolute amount of the ground substance in the aortic media of animals with induced hypertension changed after six weeks of spices

administration as follows: piperine 29.68% (0.21 mm<sup>2</sup>), curcumin 27.31% (0.20 mm<sup>2</sup>) and combination of the spices 22.11% (0.13 mm<sup>2</sup>) (Figure 7).

#### Discussion

Six weeks of L-NAME administration causes a chronic increase in blood pressure in rats previously described by other authors even after shorter duration of the experiment [32]. The increase in blood pressure after L-NAME administration is connected with inhibition of NO synthases and decrease in NO production [33,34]. Down regulation of eNOS may also participate at lower NO availability, however, the changes in iNOS expression may vary depending on the length of L-NAME administration [35,24].

Administration of piperine or curcumin to animals with L-NAME-induced hypertension lead to a decrease



**Figure 5** The total content of collagen in the aorta was significantly decreased in hypertensive animals. The spices did not substantially correct this drop. Graph (left) with results from van Gieson staining, specimens stained with picosirius red evaluated in polarized light (right). Con - control, L - L-NAME induced hypertension, P - piperine, C - curcumin. \*\* p < 0.01 versus Con, \*\*\* p < 0.001 versus Con.



pressure induced by L-NAME administration [34,41]. NO-deficient hypertension in our experiment is accompanied by morphological changes of the blood vessel wall. According to our results and the results of others [7,8,41,42], L-NAME administration does not affect the inner diameter of the thoracic aorta but it increases the cross-sectional area and the thickness of the aortic media. This change appears mostly due to the increase of ground substance and water [43,44] that can affect vessel wall elasticity and the changes of the proportional amount of other components in the media of the aorta. Described remodeling effects of L-NAME-induced hypertension could be effectively prevented only by the combination of piperine and curcumin while the spices given independently did not have a significant effect. Reduction of oxidative stress in the aortic media by the spices [34,23] might contribute to reduction of water content in the ground substance and leading to reduction of the cross-sectional area of the aorta in hypertensive animals. Inhibitory effect of piperine on vascular remodeling has been described through activation of superoxide dismutase [45] or differential expression of calcium regulating channels [46]. Both spices influence remodeling by different mechanisms, thus, their combination resulted in an additive reducing effect on vascular remodeling.

The relative content of elastin and collagen was decreased in the aortas of hypertensive animals. The decrease of relative elastin content might be the result of the effect of increased blood pressure and has been also detected in spontaneously hypertensive rats [47]. The absolute amount of elastin in the aortic media, however, was increased when compared to control, which could be ascribed to the increased synthetic activity of vascular wall cells as a response to the increased fatigue of elastin [48] depicted also in other models of experimental hypertension [18]. The spices, and especially curcumin alone or in combination with piperine, caused a further increase of elastin content in the blood vessel wall probably also due to the inhibitory effect on elastases [49,50] or on the matrix metalloproteinase 2 [51]. In case of curcumin the just mentioned effects probably lead also to the significant increase of the relative amount of elastin in our experimental setting.

The changes of collagen content in the aorta will relate to the used experimental model, as some authors described its increase [52] while others did not observe any changes [48,53]. Decrease of collagen content seen in our experimental setting was probably caused by increased activity of matrix metalloproteinases, the higher activity of which has been described in spontaneously hypertensive rats [54]. The effect of the spices on the degrading enzymes as well as on the synthetic activity reflected the absolute amount of collagen in the

aorta. Piperine in this process presumably inhibits the matrix metalloproteinase 13 and thus may support the collagen amount increase [55]. However, this effect is not sufficient to compensate the probable inhibition of collagen synthesis by curcumin [56].

The spices elicited a significant effect on smooth muscle cells. L-NAME-induced hypertension caused a decrease in the amount of actin. This might be the result of increased proliferation of SMCs synthesizing less of this protein [57]. At the same time the proportional amount of myofibrils increased in muscle cells as we already have described in the NO-deficient hypertension [32], probably because of the changes in globular and fibrillar actin [58]. This conclusion was drawn from the differences resulting from the used detection methods. The antibodies bind to actin regardless its quaternary structure [59], which on the other hand is crucial for the phosphotungstic acid hematoxylin staining [60]. Curcumin has been shown to suppress the vascular smooth muscle cells migration and proliferation [61] and to differentially interfere with proteins translation [62], which may correlate with correction of the fibrillar proteins content in the aortic media.

The effect of hypertension on myofibrils was partially preventable by curcumin and/or piperine administration, piperine being less effective than curcumin. The effect of both spices can be explained by influencing the actin polymerization [21]. Piperine is able to block the influx of calcium which is needed for the change of G-actin to F-actin [63]. The administration of curcumin leads to an increase of actin content above the level of controls and it can be assumed that this effect is caused by an increased reaction of the injured muscle cells to stimulation of its synthesis. When the spices are combined, the protective effect of piperine on muscle cells [57] probably prevents the overreaction of SMSs to curcumin and leads to the normalization of the actin amount in the vessel wall.

In conclusion, the increase of blood pressure caused by L-NAME can be partially prevented by piperine or curcumin, the result of their combination being less significant. L-NAME-induced hypertension leads to an increase of the aortic media thickness and its cross-sectional area, which are accompanied by the increased proportional amount of PTAH-positive myofibrils, decrease of elastin, collagen and actin. Piperine is able to decrease the content of myofibrils and slightly raise the proportion of actin, while curcumin also prevents the decrease of elastin. The combination of the spices has a similar effect on the vessel morphology as curcumin alone. The effects on the morphology of the aorta wall may be mediated by different mechanisms of action of the spices. Our results show that independent administration of curcumin is more effective in preventing



negative changes in blood vessel morphology accompanying hypertensive disease.

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#### Authors' contributions

LH conceived of the study, and participated in its design and coordination and drafted the manuscript, AJ carried out the immunohistochemical staining and evaluation of the slides, UO was involved animal handling and obtaining of specimens, PJ carried out the experiment preparation, was involved in statistical analysis and slides evaluation, AC was involved in animal handling, obtaining of specimens and slides evaluation, PB overviewed the experiment, was involved in slides evaluation, manuscript drafting and has given final approval of the manuscript to be published. All authors read and approved the final manuscript.

#### Competing interests

The authors declare that they have no competing interests.

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