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EXPERIMENTAL STUDY

Piperine, active substance of black pepper, alleviates hypertension induced by NO synthase inhibition

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Abstract: *Objectives:* The presented study is aimed on exploring the effects of black pepper on blood pressure in the rat model of experimental hypertension induced by chronic NO synthesis inhibition. Background: Piperine, the compound of black pepper, can cause a significant decrease of blood pressure in normotensive rats possibly via calcium channel blockade, a pathway that is known to be effective in prevention of L-NAME (N(G)-nitro-L-arginine methyl ester) induced hypertension.
Methods: Wistar rats were administered clear water (C), L-NAME (40 mg/kg/day, L), piperine (20 mg/kg/day) in corn oil by oral gavage with L-NAME (LP) or without it (P) for 6 weeks. The systolic blood pressure was measured weekly. Specimens of thoracic aorta were processed in paraffin and histological slices were stained with hematoxylin and eosin, Mallory's phosphotungstic acid hematoxylin (PTAH), orcein, antibodies against inducible NO synthase (iNOS) and smooth muscle cells actin (SMCA). Microscopic pictures were digitally processed and morphometrically evaluated.
Results: L-NAME increased the blood pressure, cross-sectional area of aorta, media thickness, elastin and SMCA synthesis and PTAH positive myofibrils relative and absolute content in the aortic media, wheras it decreased percentual content of iNOS, elastin and SMCA. Piperine decreased the blood pressure rise from the third week of treatment, synthesis of elastin and the percentual and absolute content of PTAH positive myofibrils, however, it did not affect other parameters.
Conclusion: Oral administration of piperine is able to partially prevent the increase of blood pressure caused by chronic L-NAME administration. This effect is probably caused by the blockage of voltage-dependent calcium channels and supported by filamentous actin disassembly *(Tab. 1, Fig. 2, Ref. 35)*. Full Text in free PDF *www.bmj.sk.*
Key words: piperine, L-NAME, hypertension, black pepper, actin, aorta.

Piperine, the compound responsible for the pungency of peppers derived from *Piper nigrum* (black pepper) and *Piper longum*, is not only a food additive but it also has various biological effects. Its main significance lies in the ability to affect metabolism and bio-availability of other substances, mostly through inhibition of cytochromes P450 (Atal et al, 1985).

Our knowledge about the effect of piperine on blood pressure is limited. The few published works suggest that dosage of piperine is crucial for its effects. It is known that intravascular (i.v.) administration of high dose of piperine (650 nmol/kg, approximately 180 mg/kg) is able to stimulate adrenal glands to secrete catecholamines (Kawada et al, 1988). However, lower doses (10 mg/kg and lower, i.v.) of piperin cause significant decrease of blood pressure in normotensive rats which is possibly mediated through Ca^{2+} channel blockade (Taqvi et al, 2008). Large amounts of piperine may increase the production of reactive oxygen species while at low concentrations piperine acts as an antioxidant (Mittal and Gupta, 2000).

Increased levels of free radicals can also be observed in hypertension caused by chronic administration of L-NAME (Nůnitro-L-arginine methyl ester) and they are involved in the process of vascular remodeling (Tsukahara et al, 2000, Husain a Hazelrigg, 2002). Remodeling of the vascular wall is an important compensatory mechanism that in pathological circumstances may contribute to the chronic increase of blood pressure by decreasing blood vessel elasticity. Aortic stiffness is an important risk factor for cardiovascular mortality, coronary morbidity and mortality, fatal strokes, renal failure or diabetes mellitus (Laurent et al, 2005) and worsens the known malicious effect of chronic hypertension. For these reasons the presented study is aimed on exploring the possible preventive effects of black pepper consumption on blood pressure and aortic wall remodel-

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ing in the model of experimental hypertension induced by chronic L-NAME treatment.

Methods

All procedures and experimental protocols were approved by the Ethical Committee of the Institute of Normal and Pathological Physiology SAS, 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 stable temperature (22-24 °C) and humidity (45-60 %) on a 12:12 hour light/dark cycle and maintained on a standard pellet diet and tap water *ad libitum*. During the experiment, one animal (from L-NAME group) died because of trauma caused by gavaging.

Adult 12-week-old male Wistar rats were divided into 4 groups with 6 animals in each group: control group (C), group treated with 40 mg/kg/day of L-NAME (L) dissolved in the drinking water and administered orally; group receiving piperine (20 mg/kg/day) in corn oil by oral gavage with L-NAME (LP) or without it (P). All compounds were administered daily for 6 weeks and the experiment was ended by thiopental overdose. 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 realized 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 hours in 10% formalin, routinely processed in paraffin and 5 ěm thick slices were stained with hematoxylin and eosin, Mallory's phosphotungstic acid hematoxylin (PTAH) and orcein.

Inducible NO synthase (iNOS) in aortic media was visualized by rabbit anti-iNOS antibodies (Santa-Cruz Biotechnology, California, USA) diluted 1:200 in Antibody Dilutent (DAKO, Glostrup, Denmark). After overnight incubation at 4 °C the slides were washed in PBS (phosphate buffered saline, pH 7,4) for 10 minutes, incubated for 30 min with anti-rabbit peroxidase complex (EnVision® kit, DAKO), washed in PBS and the color reaction was developed with diaminobenzidine (DAKO).

Smooth muscle actin was detected by mouse monoclonal antibodies (DAKO, Glostrup, Denmark) diluted 1:100 in Antibody Dilutent (DAKO, Glostrup, Denmark) used 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® kit, DAKO), washed in TBS and the color reaction was developed with diaminobenzidine (DAKO).

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

Digital Morphometry

The slides were studied in a Leica light microscope (Leica Systeme, Wetzlar, Germany). At least five random places were selected on each slide and documented with a digital photographic camera S 50 (Canon, Japan). 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 ImageJ software (National Institute of Health, Bethesda, USA).

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 the percentage of the specific color covering that particular area was measured, in order to avoid the results to be influenced by any changes in the size of aortic media.

ImageJ software was also used to measure the inner and the outer circumference of the aortas (excluding adventitia) and the obtained numbers were used for calculation of the cross-sectional area of the blood vessels. This value was then used to count the "absolute" content of the compounds expressed in squared millimeters.

Statistics

The results were statistically analyzed by two-way (for blood pressure) or one-way ANOVA with Keuls-Neumann test, and expressed as mean±standard deviation. Values with $p<0.05$ were considered significant.

Results

Blood pressure

Blood pressure of the rats was not significantly different between the groups at the beginning of the experiment. Piperine slightly, but statistically significantly decreased the blood pressure rise from the third week of treatment when compared to the group receiving L-NAME only, and the blood pressure remained lower (by 10.1 mmHg in average) to the end of the experiment. Administration of piperine alone did not cause any change in the blood pressure (Fig. 1).

Cross-sectional area, inner diameter, wall thickness

The inner diameter was not significantly changed in any study group. The wall thickness as well as the cross-sectional area (CSA) were significantly increased (thickness by 27.3 %, CSA by 41.8 %) after L-NAME administration in comparison to controls. Piperine did not cause a significant change in the wall thickness or the CSA in animals without L-NAME and did not prevent the effect of the NOS inhibitor when co-administered.

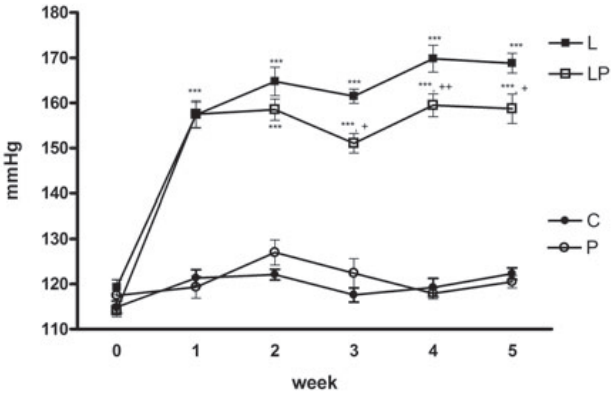

Fig. 1. Blood pressure during administration of L-NAME (L), piperine (P), L-NAME + piperine (LP) for 6 weeks, last measurement was made on 5th week of experiment. Control is labeled as C. Graph represents average values and standard errors. Compared to control (C) *** p<0.001, compared to L-NAME group (L) + p<0.05, ++ p<0.01.

Inducible NO Synthase

There was a low level reaction of antibodies against iNOS in the aortic media of the control group and the group receiving only piperine. This reaction was significantly decreased (by 91.8 % L vs C) in groups receiving L-NAME, administration of piperine did not prevent this effect.

Elastin

Cross-sectional area of the evaluated part of the aorta contained 33.73±8.3 % of elastin in control animals and it was not changed by the piperine administration. L-NAME caused a significant decrease in the elastin relative content in the aortic wall (by 19.8 %) and piperine did not prevent this effect. In absolute numbers, the elastin content after L-NAME administration was slightly (by 12.9 %) but significantly increased when compared to controls. Simultaneous administration with piperine caused a return of this parameter to control levels. Piperine itself did not cause changes in the absolute amount of elastin in the aortic media.

Smooth muscle actin

Administration of L-NAME caused a significant decrease (by 21.0 %) in immunohistochemical positivity of smooth muscle actin in the aortic media when compared to control. Piperine given together with L-NAME or by itself did not significantly change the smooth muscle actin. Interestingly, in absolute values, the smooth muscle actin was significantly increased (by 11.5 % L vs C) in both L-NAME groups with and without piperine compared to control.

PTAH positive myofibrils

In the PTAH stained slices (Fig. 2), the relative content of myofibrils in the aortic media in the control group was not influenced by piperine, but L-NAME significantly increased this parameter (by 40 %). Co-administration of L-NAME and piperine resulted in a significant decrease of myofibrils proportion in the aortic media when compared to the L-NAME group (by 21.2 %). In absolute numbers, the differences were even more prominent, since L-NAME increased the content of myofibrils by 97.1 % when compared to controls. Piperine administration caused a significant decrease when administered together with L-NAME (21.2 % L vs LP) and even without it (13.7 % C vs P).

Results are summarized in Table 1.

Tab. 1. Percentual and absolute content of compounds in aortic media after L-NAME and piperine treatment.

	Elastin	
	%	mm2
C	33.73±8.33	0.186±0.004
L	27.04±6.87 ***	0.210±0.012 ***
P	33.26±9.02 +++	0.187±0.007 +++
LP	27.21±4.69 ***	0.191±0.007 +++
	Diameter mm	Thickness mm
C	1.55±0.10	0.11±0.01 *
L	1.42±0.10	0.14±0.03
P	1.54±0.12 ++	0.11±0.01 ++
LP	1.52±0.16 *	0.14±0.02 **
	CSA mm^2	iNOS %
C	0.55±0.04	4.33±1.94
L	0.78±0.17 **	0.47±0.39 ***
P	0.56±0.08	3.86±2.28 +++
LP	0.70±0.16	0.62±0.30 ***
	Smooth muscle cell actin	
	%	mm^2
C	45.76±8.14	0.252±0.003
L	36.16±16.31 **	0.281±0.028 **
P	47.08±6.71 +++	0.264±0.005
LP	40.17±11.36 *	0.297±0.022 ***
	Myofibrils (PTAH)	
	%	mm^2
C	18.89±7.27	0.104±0.003
L	26.44±10.99 **	0.205±0.019 ***
P	21.48±12.79 +	0.121±0.010 *** +++
LP	20.81±9.82 +	0.146±0.015 ** +++

C – control, L – L-NAME, P – piperine, LP – L-NAME + piperine, * p<0.05 vs C, ** p<0.01 vs C, *** p<0.001 vs C, + p<0.05 vs L, ++ p<0.01 vs L, +++ p<0.001 vs L, CSA – cross-sectional area of aortic media, iNOS – inducible NO synthasis, PTAH – Mallory's phosphotungstic acid hematoxylin. Numbers represent the average value ± standard deviation.

Discussion

Oral administration of piperine was able yo partially prevent the hypertensive effect of L-NAME on blood pressure, even though the blood pressure drop was mild. This result suggests

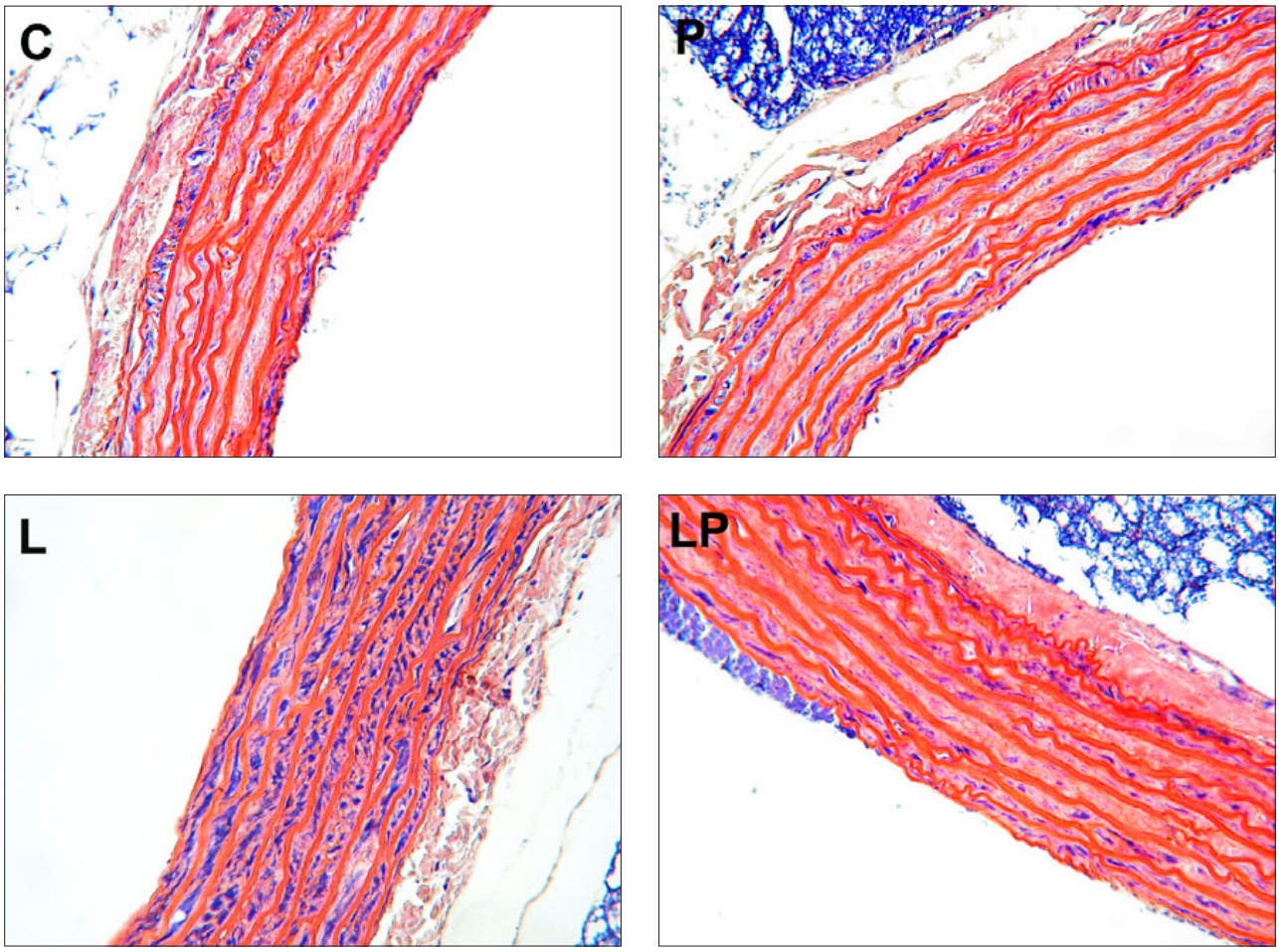

Fig. 2. Mallory's phosphotungstic acid hematoxylin (PTAH) staining of aortic media after 6 weeks of administration of L-NAME (L), piperine (P), L-NAME + piperine (LP). Control is labeled as C. Dark blue color represents PTAH positive myofibrils, connective tissue is stained red. Magnification 400x.

that piperine can be included in prevention of hypertension as a dietary component. The blood pressure lowering effect of piperine has previously been described after the intravenous administration to anesthetized rats. This effect of piperine was dose dependent with the mean arterial pressure declined by 9.34±5.45 % after 1 mg/kg of piperine intravenously (Taqvi et al, 2008). Comparable results were obtained also in the present study due to availability of piperine in blood after oral intake. The pharmacokinetics of piperine seems to depend on the vehicle used for its administration and in rat it may reach 2.83±0.46 μmg/ml after oral administration of 20 mg/kg (Bajad et al, 2002).

Mechanisms of the piperine effects were tested on isolated rat aorta preparations, in which piperine induced endothelium-independent relaxation and inhibited high K^+-induced contractions. Contraction of aortic preparations induced by high K^+ depends on the influx of Ca2+ into the cells through voltage-dependent calcium channels, therefore a substance that can inhibit high K+-induced contractions is considered to be a Ca^{2+} channel blocker. This is supported by similar results obtained by verapamil administration in the same experiment (Taqvi et al, 2008).

Verapamil has already been proven to be able to prevent L-NAME induced hypertension, although it does not affect the contraction response to potassium in this type of hypertension (Küng et al, 1995). However, from other studies it is know that verapamil can cause changes in aortic morphology (Zanchetti et al, 1998) namely through inhibition of vascular smooth muscle cells proliferation (Leszczynski et al, 1994). If the effects of piperine are similar to those of verapamil, its influence might be demonstrated on the aortic media compounds.

The role of hypertension in the aortic diameter enlargement is currently unclear. Some authors confirm the main role of hypertension in this process (Wolinsky, 1970, Vardulaki et al, 2000, MacSweeney, 1994). Others claim its role to be unimportant (Vasan et al, 1995, Mitchell et al, 2003, Agmon et al, 2003) and our results support this assumption. On the other hand, the wall thickness and the cross-sectional area are usually increased in hypertension (Xu et al, 2001, Wolinsky, 1970) and remain so even after blood pressure normalization (Amann et al, 1997). This can be observed also in L-NAME induced hypertension (Nadaud et al, 2009) and it was demonstrated by our results as well.

This mass increase may be caused by various substances. Already in the early fifties (Tobian and Binion, 1952) it has been known that aortas of hypertensive rats had significantly higher water content and that hypertension caused gradual increase of ground substance in spontaneously hypertensive rats (Olivetti et al, 1982). Expanded ground substance was found to be one of the factors leading to increased arterial stiffness and was sometimes accompanied by calcium depositing (Laurent and Boutouyrie, 2007). Increase of water or ground substance might result in a decrease of the relative content of various compounds. It was therefore important to assess both relative and absolute content.

We have observed a significant decrease in relative elastin content caused by L-NAME that was not prevented by piperine. Elastin is an extremely stable molecule with a chemical half life measurable in decades and with very low turn-over (Debelle and Tamburro, 1999). The decrease of its relative content in the aortic media could also be seen in renal clip and Dahl salt-sensitive rat models of hypertension (Keeley and Alatawi, 1991). Content of elastin in absolute numbers, however, showed an increased synthesis of elastin in the aortic media after L-NAME treatment. Similar synthetic response had been also proven in other types of hypertension (Keeley and Alatawi, 1991). Administration of piperine prevented the increase of elastin synthesis, but its effect was not reflected in elastin relative content. It could be assumed that the slight increase in elastin synthesis was a reaction to a disturbance of its proper function, because of the fatigue of elastic fibres (Arribas et al, 2006) and its fragmentation and calcifications (Safar et al, 2003) both reported during hypertension. It cannot be excluded, that the lower elastin synthesis after piperine intake was caused by some currently unknown effect of piperine on elastin fibers preservation.

The increase of ground substance and the difference of relative and absolute content can be also seen on smooth muscle actin in the aortic media. The relative content of actin is decreased in L-NAME-induced hypertension compared to controls according to our measurements. On the other hand, the absolute amount of actin expressed in squared millimeters of aortic media CSA is significantly increased indicating an elevated actin synthesis and hypertrophy of vascular smooth muscle cells, well known in hypertension (Owens et al, 1981). Interestingly, L-NAME hypertension increases proportional and absolute content of the PTAH positive myofibrils in aortic media (Babal et al, 1997) and piperine is able to prevent this effect. It was shown by Clancy et al (1995) that increased nitric oxid level is able to decrease filamentous actin content in neutrophils and later Maneen et al (2006) reported that peroxinitrite induces filamentous actin depolymerization in vascular smooth muscle cells in cerebral arteries. Therefore NO synthesis inhibition by L-NAME is likely to cause the increase of filamentous actin that can be manifested by increased PTAH positive myofibrils.

The histochemical analysis showes a decreased expression of inducible NOS (usually associated with peroxinitrite production) in the aortic media after L-NAME administration when compared to control. This is consistent with the results obtained from hearts after 4-weeks of L-NAME intake (Spániková et al, 2008), but different from the results from aortas after 8 weeks of administration (Luvarŕ et al, 1998). However, piperine has no significant effect on iNOS protein synthesis and its effect on filamentous actin is likely to be mediated through a different pathway.

It was already suggested that the effect of piperine on blood vessels is similar to calcium channel blocker, verapamil. Verapamil is known to induce reduction of intracellular F-actin in T--lymphocytes (Blaheta et al, 2000) and accelerated actin degradation in neonatal rat heart myocytes (Sharp et al, 1993), suggesting that this may be the pathway of piperine preventive effect on the increase of PTAH positive myofibrils caused by L--NAME administration.

In conclusion, oral administration of piperine is able to partially prevent the increase of blood pressure caused by chronic L-NAME administration. This effect is probably caused by the blockage of voltage-dependent calcium channels and supported by filamentous actin disassembly.

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