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Hypercholesterolemia does not alter endothelial function in spontaneously hypertensive rats

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Hypercholesterolemia and endothelial function.

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Abbreviations: **Ach –** acetylcholine; **SNAP** - S-nitroso-N-acetyl-penicillamine; **L-NAME** - L-N^G-nitroarginine methyl ester; **PGI_2** - prostacyclin**, SHR** - spontaneously hypertensive rats; **WKY** - Wistar-Kyoto rats; **MCP-1** - monocyte chemoattractant protein 1; **COX** - cyclooxygenase**.**

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Abstract

In humans hypercholesterolemia and hypertension are associated with endothelial dysfunction. Here we assess whether hypercholesterolemia induces endothelial dysfunction in rats with pre-existing hypertension. Spontaneously hypertensive rats (SHR) and normotensive controls (WKY) were fed with a high-cholesterol diet for 12 weeks, and endothelial function was assessed in isolated thoracic aortic rings. In SHR and WKY rats, the hypercholestrolemic diet resulted in the elevation of total cholesterol and LDL levels by approximately 2.5 and 4.5 fold, respectively. However, in aorta the basal nitric oxide (NO) production – as assessed by the magnitude of L-NAME-induced vasoconstriction as well as the NO-dependent relaxation induced by acetylcholine or histamine – were not diminished either in SHR or in WKY rats fed with the hypercholestrolemic diet. Interestingly, prostacyclin (PGI_2) production in aortic rings from SHR rats was higher than in the aorta from WKY rats. However, the hypercholestrolemic diet had no further effects on PGI_2 production in the aorta either of SHR or WKY rats. MCP-1 level in plasma was slightly elevated in SHR and WKY rats fed with the hypercholestrolemic diet as compared to their normocholesterolemic counterparts. In summary, even in the presence of pre-existing hypertension, hypercholesterolemia fails to modify NO-dependent and PGI_2-dependent endothelial function in SHR rats; it also does not induce a robust inflammatory response. Both are prerequisites for the development of atherosclerosis.

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Introduction

Endothelial dysfunction with its proinflammatory and prothrombotic phenotype is a key element in the pathogenesis of atherosclerosis in humans. All known risk factors, such as hypertension, hypercholesterolemia, diabetes, and obesity, are associated with endothelial dysfunction, and the diagnosis of endothelial dysfunction has recently gained in prognostic significance in respect of atherosclerosis (Bonetti et al., 2003;Chlopicki and Gryglewski, 2004). In particular, hypertension and hypercholesterolemia alone impaired endothelium-dependent vasodilation, and their combination led to additive impairment of the NO-dependent endothelial function (Bonetti et al., 2003).

The endothelial function was also impaired in hypercholestrolemic rabbits (Verbeuren et al., 1990), mice (d'Uscio et al., 2001), as well as in other species (Shaul, 2003). It has repeatedly been shown that a high-cholesterol diet in non-human primates, swine, rabbits, mice and pigeons led to intimal lesions and to the development of atherosclerotic plaques (Faggiotto et al., 1984;Jerome and Lewis, 1985;Rosenfeld et al., 1987). Although the precise mechanisms of plaque development in response to hypercholesterolemia are still not clear, the involvement of platelets, monocytes, and limfocytes, endothelial dysfunction and systemic inflammatory response are all well documented (Hansson et al., 2002).

Interestingly, in numerous studies rats were resistant to the development of hypercholesterolemia-induced atherosclerosis (Moghadasian, 2002;Nakamura et al., 1989;Pisulewski et al., 2005). For example, electron microscopic studies revealed subtle endothelial changes in rat aorta only after they had been fed a high-fat diet for 12 months (Still and O'Neal, 1962). In fact, normotensive rats fed with a high-cholesterol diet did not develop endothelial dysfunction (Pisulewski et al., 2005).

In contrast to the apparent resistance of rats to hypercholesterolemia-induced endothelial dysfunction and atherosclerosis, hypertension in rats is linked to endothelial dysfunction, as is the

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case in many other species. Indeed, the impairment of NO-dependent endothelial function as well as endothelial inflammation was repeatedly detected in Salt-Dahl rats, DOCA-salt hypertensive rats, spontaneously hypertensive rats (SHR), as well as in 1-kidney, 1-clip (1K1C) model of hypertension (Ortiz and Garvin, 2001). Importantly, lowering blood pressure in hypertensive rats by various pharmacological interventions normalized endothelial function (Hoshino et al., 1994). Furthermore, non-hypotensive drugs endowed with endothelial action such as antioxidants, a xanthine oxidase inhibitor (allopurinol) or statins, improved endothelial function in rats with hypertension (Carneado et al., 2002;Ulker et al., 2003).

In humans (Bonetti et al., 2003) and in some species of laboratory animals, coexistence of hypertension and hypercholesterolemia leads to additive effects on endothelium. For example, in hypertensive Watanabe hypercholestrolemic rabbits vascular lesions were greater than in normotensive rabbits with hypercholesterolemia (Chobanian et al., 1989).

That is why in the present study we tested whether pre-existing hypertension would facilitate the development of endothelial dysfunction in hypercholesterolemic rats. Accordingly, spontaneously hypertensive rats (SHR) and normotensive controls (WKY) were fed with a hypercholestrolemic diet for 12 weeks, after which the functional activity of endothelial NO and PGI_2 was assessed in isolated aortic rings. In addition, the intensity of hypercholesterolemia-induced inflammation was assessed by measurement of monocyte chemoattractant protein 1 (MCP-1) in plasma. Aortic root tissue was also examined for the presence of early atherosclerotic plaques.

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Methods

The experiments were conducted according to the Guidelines for Animal Care and Treatment of the European Community and were approved by the Local Animal Ethics Committee.

Animals

12 months-old SHR (n=20) and WKY (n=20) male rats, weighing 350 –550 g were obtained from the Animal Laboratory of Polish Mother's Research Institute Hospital in Lodz, Poland. The rats were housed individually in stainless-steel, wire-bottomed cages in an isolated room at controlled temperature and ambient humidity. Lights were maintained on a 12h light-dark cycle. The animals were acclimatised to these conditions for 1 week and given free access to water and the basal semi-purified AIN-93M diet. After the adaptation period, the SHR and WKY rats were randomly divided into four groups to receive one of the following type of diet for the 12 week period: the control diet (basal dry rat AIN-93M diet) or the hypercholestrolemic diet (basal AIN-93M diet + 1g/100g cholesterol + 0.5g/100g cholic acid + 20g/100g butter) (Reeves, 1997). The final composition of both types of diet is given in Table 1.

Indirect blood pressure (BP) was monitored by the tail-cuff method at starting point of the experiment and at the end of the experiment after 12 weeks of the respective diet. Mean BP at the beginning of the experiment was significantly higher in the SHR than in the control WKY (189±6.7 mmHg *vs* 119±10.4 mmHg, respectively). The type of diet did not influence BP significantly in SHR or WKY rats.

Determination of lipid profile in the SHR and WKY rat

After decapitation blood samples for lipid analyses were collected in test tubes and centrifuged (4000 g, 10 min.) to obtain serum. Samples of serum were immediately frozen (-20^{o}C)

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and stored until assayed. Using commercially available kits the serum samples were analysed for total cholesterol and triglycerides (CORMAY, Lublin, Poland) and for HDL-cholesterol, LDL-cholesterol (Olympus Diagnostica GmbH, Hamburg, Germany), and expressed in mmol/l.

Determination of MCP-1 level in SHR and WKY rat serum

For determination of levels of MCP-1, blood samples were collected and centrifuged (4000g, 10 min.) to obtain serum. Samples were stored at -70°C until assayed using the commercially available enzyme immunoassay kits (BioSource, Camarillo, California, USA). The level of MCP-1 in serum was expressed in pg/ml.

Protocol of experiments in isolated aortic rings

Rats were anesthetised with thiopental-sodium (120-150 mg kg^{-1} body weight) (Biochemie GmbH, Kundl-Rakusko, Austria) and the thoracic aorta was quickly removed by central sternotomy, after which it was gently washed through the lumen with ice-cold saline and placed in cold Krebs-Heinseleit buffer of the following composition (mmol/l): NaCl 118, $CaCl_2$ 2.52, $MgSO_4$ 1.64, $NaHCO_3$ 24.88, $K2PO_4$ 1.18, KCl 4.7, glucose 10.0. Loose connective tissue was removed and each aorta was cut into 6 rings 3-4 mm in lenght. Vascular rings were then transferred to organ chambers filled with 5 ml of Krebs- Heinseleit solution maintained at 37°C, pH 7.4 and gassed with carbogen. Rings were mounted between 2 hooks attached to an isometric force transducer (Biegastab K30 type 351; Hugo Sachs March-Fr, Germany) with continuous recording of tension (Graphtec WR3320, UK). After mounting the rings, the resting tension was stepwise increased to reach a final 4g, following which the rings were incubated to equilibrate for 30 minutes. The viability of the tissue was documented by a contractile response to potassium chloride (KCl) at a final concentration of 3×10^{-2} to 9×10^{-2} mol/l). Next the aortic rings were precontracted with phenylephrine (final concentration of 10^{-8} – 3×10^{-7} mol/l) to obtain submaximal contraction (60-80% of KCl-induced maximum response), and after obtaining a stable plateau phase of contraction

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the integrity of the endothelium was assessed with acetylcholine (Ach) 10^{-6} mol/l. Then relaxation to cumulative concentrations of Ach (10^{-9}-10^{-5} mol/l) or histamine (10^{-8} – 3×10^{-4} mol/l) was induced. The endothelium independent vasorelaxation was evoked by S-nitroso-N-acetyl-penicillamine (SNAP, 10^{-9}-10^{-5} mol/l). To test the involvement of NO in acetylcholine or histamine-induced responses these responses were repeated in the presence of an inhibitor of nitric oxide synthesis – L-N^G-nitroarginine methyl ester (L-NAME, $3x10^{-4}$ mol/l). Rings were incubated with L-NAME for at least 15 minutes before eliciting vasodilator responses. The plateau of L-NAME-induced contraction (after phenylephrine precontraction) was seen approximately 10 minutes after addition of L-NAME. Thus, 15-minutes of incubation period with L-NAME was chosen for experiments.

The relaxation or contraction response was expressed as a percentage of the contraction induced by phenylephrine (10^{-8} -3×10^{-7} mol/l) or KCl (9×10^{-3} mol/l).

Basal production of NO by aortic rings was determined on the basis of the magnitude of contraction induced by L-NAME. In this experiment rings were pre-contracted with phenylephrine (10^{-8} -5×10^{-8} mol/l to obtain 10-30% pre-contraction of maximal KCl-induced contraction) and then L-NAME (3×10^{-4} mol/l) was added. In order to see a vasoconstrictor response to L-NAME, vessels needed to be precontracted with phenylephrine (by 10-30%). The plateau phase of L-NAME-induced contraction was determined and expressed as a percentage of the maximum KCl-induced contraction.

Determination of basal prostacyclin production in aortic rings

Prostacyclin (PGI_2) release by aortic rings was analyzed by measuring its stable hydrolysis product 6-keto-$PGF_{1\alpha}$ in an organ bath medium. Commercially available EIA kits (Cayman Chemical Company, MI, USA) were used. Aortic rings were washed with fresh Krebs-Heinseleit solution and then incubated for 30 minutes under 4 g stretch as for the functional experiments. Samples of supernatant were collected after 20 minutes of incubation and then processed for level

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of 6-keto-$PGF_{1\alpha}$ according to the manufacturer's instructions. Aorta rings were collected after the experiments, dried for 48 hours in 56°C and weighted. PGI_2 production was expressed as pg/ml/mg of dry weight of aortic rings. The enzymatic source of PGI_2 was assessed using non-selective COX or selective COX-2 inhibitors such as indomethacin (5×10^{-6} mol/l) or rofecoxib (10^{-6} mol/l), respectively. The rings were pre-incubated with inhibitors for at least 20 minutes.

Oil Red O (ORO) staining of aortic roots and liver

The heart and ascending aorta were snap-frozen in OCT (Optimal Cutting Temparature) gel (Tissue-Tek, USA) and stored in –80°C. Cryosections were cut in a Leica 3050 cryostat using a standardized protocol. Briefly, serial cryostat sections were cut from the aortic root upwards from the point, where all three cusps were visible. Then 10 sections were collected at every 100 μm over a segment of approximately 5-10 mm of the aortic root before being fixed with formaldehyde. Livers from the rats were also taken to OCT and cryosections were prepared by standard procedure.

Sections of aorta and liver were stained with oil red-O (ORO) and mounted in water-soluble mounting media (Kaiser's glycerol gelatin). For each section, images were captured by digital camera and analyzed.

Statistical analysis

Results are expressed as means ± SEM. Comparison of means was assessed by ANOVA and post hoc Sheffe's test. $P<0.05$ was considered significant.

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Results

Effect of hypercholesterolemic diet on the serum lipid profile in SHR and WKY rats

After 12 weeks of the hypercholesterolemic diet, the total cholesterol concentration in plasma increased from 2.5±0.66 to 6,6±2,98 mmol/l and from 2.6±0.61 to 6.5±0.94 mmol/l, in SHR and WKY rats respectively. LDL-cholesterol levels increased from 1.08 to 7.12 mmol/l and from 1.1 to 5.4 mmol/l in SHR and WKY rats respectively . In contrast, levels of HDL and TG were not modified by the hypercholesterolemic diet (*fig. 1*).

Effects of the hypercholesterolemic diet on endothelium-dependent and endothelium-independent vasodilation in aorta in SHR and WKY rats

In all four experimental groups (SHR control, SHR hypercholesterolemia, WKY control, WKY hypercholesterolemia) acetylcholine (Ach) induced a concentration-dependent vasodilation in aortic rings with an intact endothelium (*fig. 2A).* The maximum vasodilation induced by Ach (10^{-5} mol/l) did not differ between the 4 experimental groups. Lack of effect of the hypercholesterolemic diet in either SHR or WKY rats on endothelium-mediated response was also noted for another endothelium-dependent vasodilator, histamine *(fig. 2B).* Maximum vasodilation induced by histamine was obtained with 3×10^{-4} mol/l histamine and did not differ between the 4 experimental groups (*fig. 2B*).

The endothelium-independent vasorelaxation induced by SNAP was not influenced by the type of diet either in SHR or WKY rats (*fig. 2C*). In all cases SNAP (10^{-5} mol/l) induced vasodilation that amounted to approximately 100%.

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Effect of L-NAME on endothelium-dependent responses in SHR and WKY rats fed with control and hypercholesterolemic diets

Addition of L-NAME to aortic rings slightly precontracted with phenylephrine induced further vasoconstriction. The magnitude of L-NAME –induced response was not diminished but slightly increased in SHR and WKY rats fed with the hypercholesterolemic diet as compared to their counterparts fed with the control diet. However, only in SHR rats did this difference reached statistical significance ($p<0.05$) (*fig. 3*).

In the presence of L-NAME Ach-induced vasodilation was abrogated in all four experimental groups (SHR hypercholesterolemia, SHR control, WKY hypercholesterolemia, WKY control). Moreover, in the presence of L-NAME acetylcholine induced concentration-dependent vasoconstriction that amounted to approximately 30-40% of maximal contraction induced with KCl (for 10^{-5} mol/l Ach). Again, Ach-induced vasoconstriction was not significantly influenced by the hypercholesterolemic diet either in SHR or in WKY rats (*fig. 4)*. Only in the WKY rats with hypercholesterolemia did the vasoconstriction produced by Ach in the presence of L-NAME tend to be modified as compared to the WKY control rats, though the difference did not reach statistical significance.

Effects of the hypercholesterolemic diet on basal prostacyclin production by aortic rings

The basal release of prostacyclin in aortic rings was approximately four- fold higher in SHR then in WKY rats ($p<0,001$) (*fig. 5*). However, neither in SHR nor in WKY rats did the hypercholesterolemic diet modify the basal level of prostacyclin production as compared to their counterparts fed with the control diet (*fig. 5*). In the presence of indomethacin (5×10^{-6} mol/l) or rofecoxib (10^{-6} mol/l) basal prostacyclin production by aortic rings was markedly decreased in SHR and in WKY rats irrespective of the type of diet (*fig. 5*).

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Effects of the hypercholesterolemic diet on MCP-1 concentration in serum of SHR and WKY rats

Serum concentration of MCP-1 in SHR and WKY rats fed with the control diet was similar. The hypercholesterolemic diet induced a slight increase in MCP-1 levels in the SHR and WKY rats as compared to their control counterparts, however only in the case of WKY rats did this increase reach statistical significance *(fig. 6)*.

Oil Red O (ORO) staining of aortic roots and liver

The oil red-O staining of aortic sections did not reveal lipid accumulation in aortic roots from SHR or WKY rats fed with the hypercholesterolemic diet (*fig. 7*). In contrast, lipid deposits were abundant in the liver of both the SHR and the WKY rats fed with the hypercholesterolemic diet (*fig. 8*).

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Discussion

Previously, we showed that hypercholesterolemia did not induce endothelial dysfunction in Wistar rats (Pisulewski et al., 2005). These findings stay in line with the known resistance of normotensive rats to developing hypercholesterolemia-induced atherosclerosis (Moghadasian, 2002;Nakamura et al., 1989). Here, using SHR rats, we comprehensively demonstrated that even in the presence of pre-existing hypertension, the implementation of hypercholesterolemia still remained without a significant effect on the endothelial function. Indeed, 12-week long hypercholesterolemia did not affect the vasodilator activity of NO that was assessed on the basis of the magnitude of L-NAME-induced vasoconstriction and the NO-dependent vasodilation induced by acetylcholine and histamine. 12-week long hypercholesterolemia in SHR rats was also without an effect on the basal activity of PGI_2 as well as on the functional activity of the vasoconstrictor response to acetylcholine that could be attributed to the endothelium-derived contracting factor (EDCF). Furthermore, hypercholesterolemia was not associated with a robust inflammatory response, but only with mildly elevated MCP-1 levels, and was not associated with atherosclerotic plaque formation as evidenced by a lack of fatty-streak formation in the aortic roots. Accordingly, together with our previous report (Pisulewski et al., 2005) we claim that, irrespective of the presence or absence of hypertension, hypercholesterolemia in rats does not impair the activity of endothelial NO and PGI_2 and does not induce a significant inflammatory response of the endothelium that could lead to the development of atherosclerosis.

The NO-dependent function was slightly impaired in the SHR and the WKY rats, and the 12-week long hypercholesterolemia did not modify it further. The lack of effect of hypercholesterolemia on the NO-dependent function in the SHR rats was also found by Lindberg et al. (Lindberg et al., 1995). In contrast, Cappelli-Bigazzi et al. (Cappelli-Bigazzi et al., 1997) showed mild impairment of acetylcholine-induced vasorelaxation in SHR rats fed with the hypercholestrolemic diet, and more profound impairment of achetylcholine-induced and nitroglycerine-induced vasorelaxation in SHR rats fed with an atherogenic diet (1% cholesterol and

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vitamin D_2). The reason for this discrepancy is not clear. However, there are differences in experimental design between the study of Cappelli-Bigazzi et al., and our own. For example, Cappelli-Bigazzi et al. used 6 week-old rats, while in our study 12-month-old rats were used. Moreover, Cappelli-Bigazzi et al. analyzed endothelium-dependent responses in the presence of indomethacin (Cappelli-Bigazzi et al., 1997). Whether the elimination of COX-derived products by indomethacin could uncover the impairment of the NO-dependent function in hypercholesterolemia remains to be elucidated.

In contrast to Cappelli-Bigazzi et al. (Cappelli-Bigazzi et al., 1997), we also assessed effects of hypercholesterolemia on basal PGI_2 production and on the magnitude of vasoconstrictor response to acetylcholine that could be attributed to EDCF. Neither of them was changed by hypercholesterolemia.

Indeed, basal PGI_2 production was not affected by hypercholesterolemia either in the SHR or in the WKY rats. Interestingly, PGI_2 production in aorta was higher in the SHR rats as compared with the WKY rats, though in both strains of rats it was markedly inhibited by rofecoxib. Thus, the major source of vascular PGI_2 in the aorta from the SHR and WKY rats is COX-2 and not COX-1 (Hocherl et al., 2002). Previously, it was found that COX-1 and PGI_2 synthase in SHR thoracic aorta are significantly higher than in WKY rats (Numaguchi et al., 1999). Whether COX-2 is also overexpressed in SHR rats remains to be tested. In rabbits, a hypercholesterolemic diet as short as 4 weeks led to the substantial inhibition of PGI_2 production (Dembinska-Kiec et al., 1977). In SHR or in WKY rats a hypercholesterolemic diet lasting 12 weeks did not influence vascular PGI_2 production. These results further support the notion that hypercholesterolemia in SHR rats does not lead to the development of endothelial dysfunction. Indeed, the impairment of the NO-dependent function goes along with the impairment of COX-1 derived PGI_2 production (Frein et al., 2005) or compensatory overproduction of COX-2 derived PGI_2 (Belton et al., 2000). Neither of these responses was noted in our study.

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It was repeatedly shown that the impairment of endothelial activity of PGI_2 and NO is associated with a concomitant inflammatory activation of endothelium, reflected e.g. in an increased release of soluble adhesion molecules (e.g. sICAM-1, sVCAM-1) or chemokines (e.g. MCP-1). Indeed, PGI_2 as well as NO inhibits the inflammatory response of endothelium so their down-regulation may importantly accelerate the inflammation of endothelium (Chlopicki and Gryglewski, 2004). Notably, MCP-1 was shown to be down-regulated by exogenous NO (Zeiher et al., 1995). Although we found a moderate increase in MCP-1 plasma levels by hypercholesterolemia, it reached significance in the WKY rats but not in the SHR rats. Furthermore, the increase in MCP-1 levels was much less pronounced than in other species of animals fed with a hypercholesterolemic diet (Kowala et al., 2000).

In various experimental settings the impairment of NO-dependent function was demonstrated to be associated with enhanced activity of the endothelium-derived contracting factor (EDCF) (Vanhoutte et al., 2005). It was also shown in the SHR rats that, in the presence of L-NAME, acetylcholine caused vasoconstriction that was mediated by TP-receptors (Gluais et al., 2005). This response was attributed to EDCF (Vanhoutte et al., 2005). In our experiments, a vasoconstrictor response to acetylcholine in the presence of L-NAME could also be blocked by a TP receptor antagonist (SQ 29548) both in SHR and WKY rats (data not shown). We showed that the magnitude of this response – which could be attributed to EDCF - was not influenced by the hypercholestrolemic diet either in the SHR or in the WKY rats.

Altogether we demonstrated that a 12-week long period of hypercholesterolemia did not influence the activity of NO, PGI_2 , EDCF and MCP-1 either in the SHR or in the WKY rats significantly. It was therefore not surprising that we did not find signs of atherosclerotic plaque development in aortic roots that is believed to be a primary place for plaque development in hypercholesterolemia-induced atherosclerosis in rodents (Bonthu et al., 1997).

We did not investigate mechanisms that could be involved in keeping the endothelial function insensitive to hypercholesterolemia in normotensive or hypertensive rats. However, the

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fact that in rats a diet enriched with 1% cholesterol and 20 % butter led only to a modest increase of total and LDL-cholesterol should be taken into account. Such an increase could be just insufficient to induce alterations in vascular function. Indeed, in our experiments as well as in studies of other authors (Cappelli-Bigazzi et al., 1997;Ren et al., 2001) the total cholesterol and LDL cholesterol in rats fed with a high-cholesterol and high-fat diet rose to approximately 6 and 7 mmol/l, respectively, while in rabbits a similar type of diet resulted in a total cholesterol level of above 25 mmol/l (Chiba et al., 1997;Zulli et al., 2003). Also in apoE-/- or ApoE/LDLR-/- mice that develop spontaneous hypercholesterolemia, total cholesterol levels reached 15 mmol/l or 25 mmol/l, respectively. When apoE-/- mice were fed with a Western-type diet, then total cholesterol levels amounted to approximately 40 mmol/l (Breslow, 1996;Plump et al., 1992). Thus, it could well be that endothelial dysfunction and the progression of atherosclerosis develop in response to a much higher levels of cholesterol than that achieved in rats. Accordingly, it could well be that cholesterol transport through the vascular wall and reverse cholesterol transport is so efficient in rats that it prevents the development of severe hypercholesterolemia and lipid accumulation in the vascular wall. In our study, oil red staining was negative and did not reveal lipid deposits in the subendothelial space of the aortic roots of the hypercholesterolemic SHR or WKY rats. On the other hand, the liver in the SHR or WKY rats fed with the hypercholesterolemic diet was heavily overloaded with lipids. The adventitia of the descending aorta was also overloaded with lipids (data not shown). These results could support the hypothesis of the highly efficient cholesterol transport through the vascular wall in rats. Interestingly, a hypercholesterolemic diet in normotensive Wistar rats led to the increased density of adventitial vasa vasorum (Kai et al., 2002) that could also facilitate outflow of cholesterol from the vessel wall.

HDL plays a role in the reverse cholesterol-transport as well as it possesses direct anti-atherosclerotic effects, including the stimulation of NO and PGI_2 release from the endothelium (Nofer et al., 2002). In our experiments, the level of HDL was not modified by a hypercholestrolemic diet. Thus, it still remains to be determined what is the contribution of the

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reverse cholesterol transport, protective properties of HDL *per se*, and other mechanisms to the resistance of the rat aorta to lipid accumulation in the subendothelial space.

In summary, in spontaneously hypertensive rats hypercholesterolemia failed to modify the NO-dependent and PGI_2-dependent endothelial function and did not induce a robust inflammatory response. Accordingly, we postulate that irrespective of the presence or absence of hypertension, rats are resistant to hypercholesterolemia-induced atherosclerosis because hypercholesterolemia in this species does not lead to the impairment of activity of endothelial NO and $PGI_{2,}$ and the substantial accumulation of lipids in subendothelial space. Further studies are needed to elucidate the mechanisms involved.

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Footnotes

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Legends for figures

Fig. 1. Influence of the hypercholestrolemic diet on the lipid profile in serum from the SHR and WKY rats.

* indicates $p<0.05$ *vs* respective control, # indicates $p<0.001$ *vs* respective control

Fig. 2. Effects of hypercholesterolemia on NO-dependent vasodilation. Concentration-dependent curves for vasodilation induced by acetylcholine (A), histamine (B) and SNAP (C) in isolated aorta from the SHR and WKY rats fed the control or the hypercholestrolemic diet. Values are expressed as means ±SEM.

Fig. 3. Effects of hypercholesterolemia on basal NO production as assessed by the magnitude of the L-NAME ($3x10^{-4}$ mol/l)- induced vasocontraction in phenylephrine pre-contracted aortic rings from the SHR or WKY rats. The difference for L-NAME response between SHR control vs SHR hypercholesterolemia groups and WKY control vs WKY hypercholesterolemia groups could not be related to the different levels of phenylephrine-induced precontraction. For the WKY control, WKY hypercholesterolaemia, SHR control, and SHR hypercholesterolemia rats the average magnitude of phenylephrine-induced precontraction were as follows: 12,5%, 8,9%, 27,2% and 27,03% of the maximum KCl-induced contraction respectively. Data are shown as means ±SEM. * indicates $p<0.05$ between the SHR hypercholesterolemia and the SHR control group.

Fig. 4. Effect of hypercholesterolemia on acetylcholine-induced vasocontraction in the presence of L-NAME ($3x10^{-4}$ mol/l) in phenylephrine pre-contracted aortic rings from the SHR and WKY rats. Data shown as means ±SEM.

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Fig. 5. The role of COX-2 in basal prostacyclin (6-keto-$PGF_{1\alpha}$) production by aortic rings in the SHR and WKY rats fed with the control or hypercholesterolemic diets. Results are expressed in pg/ml/mg of the dry weight of the aorta. The value represent means ±SEM. * indicates $p<0.05$ between the SHR control and the WKY control rats, # indicates $p<0,001$ *vs* respective control.

Fig. 6. Effect of hypercholesterolemia on the serum level of MCP-1 in the SHR and WKY rats. Data are means ±SEM. * indicates $p<0.05$ between the WKY control and the WKY hypercholesterolemia groups.

Fig. 7. Lack of lipid deposition in wall of aorta from the WKY (A, B) or SHR (C, D) rats fed with the hyperholesterolemic diet (B, D) as evidenced by Oil red-O staining of a cross-section of the aortic root tissue. One representative microphotograph from each of the four experimental groups is shown. Original magnification: ×40

Fig. 8. Intracellular accumulation of lipids in the liver in the WKY (A, B) and in the SHR (C, D) rats fed with the hypercholesterolemic diet (B, D) (Oil red-O staining). One representative microphotograph from each of the four experimental group is shown. Original magnification: ×40

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Tables

Tab. 1. Composition of the control (AIN-93M) diet and the hypercholesterolemic diet.

Composition (%)	Control	Hypercholesterolemic
Cornstarch	62,249	44,749
Casein	14	14
Sucrose	10	10
Soybean oil	4	0
Butter	0	20
Fiber	5	5
Mineral Mix	3,5	3,5
Vitamin Mix	1	1
Choline bitartrate	0,25	0,25
Tert-Butylhydroquinone (TBHQ)	0,0008	0,0008
Cholesterol	0	1
Cholic acid	0	0,5

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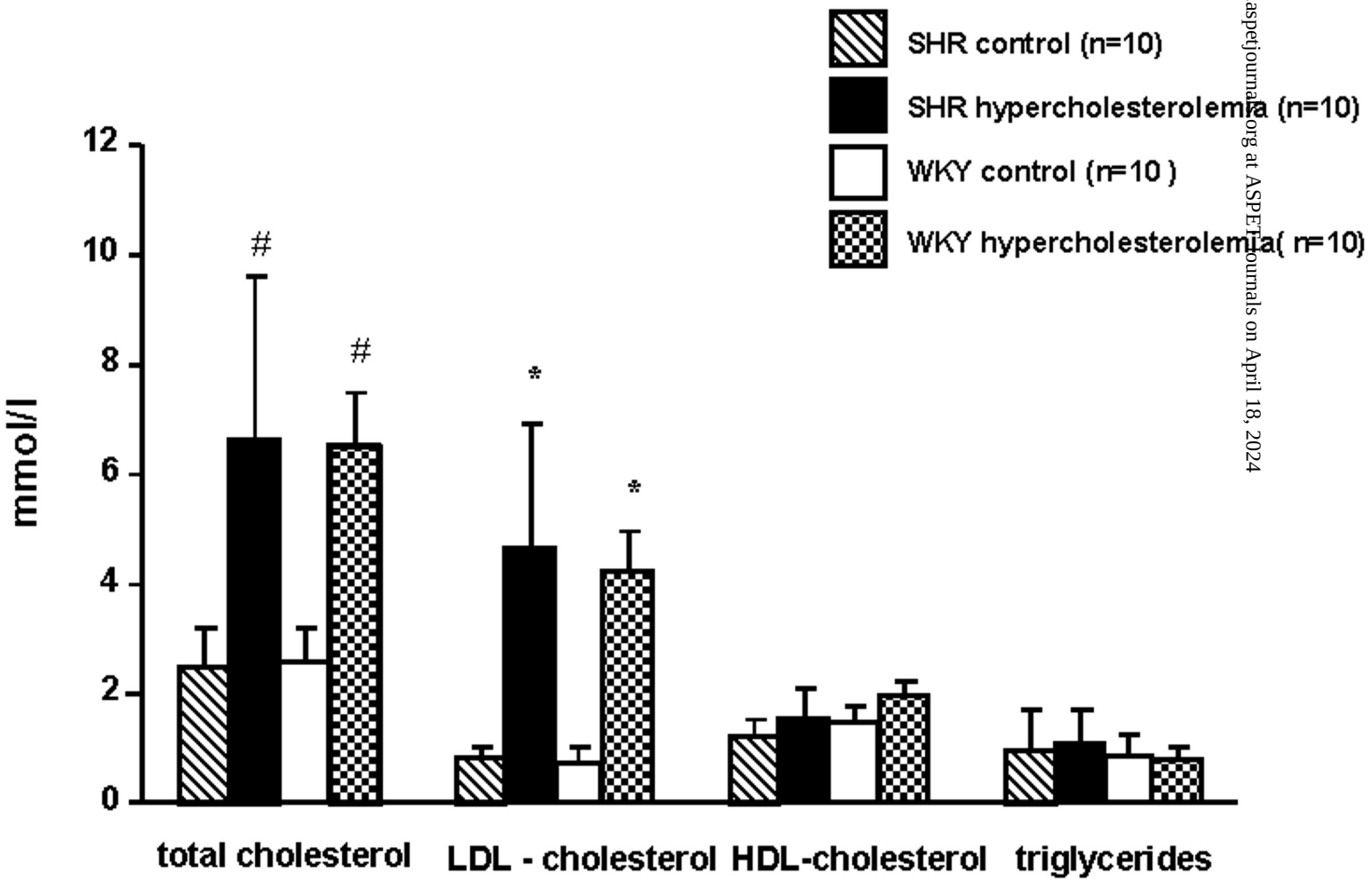
Fig. 1.



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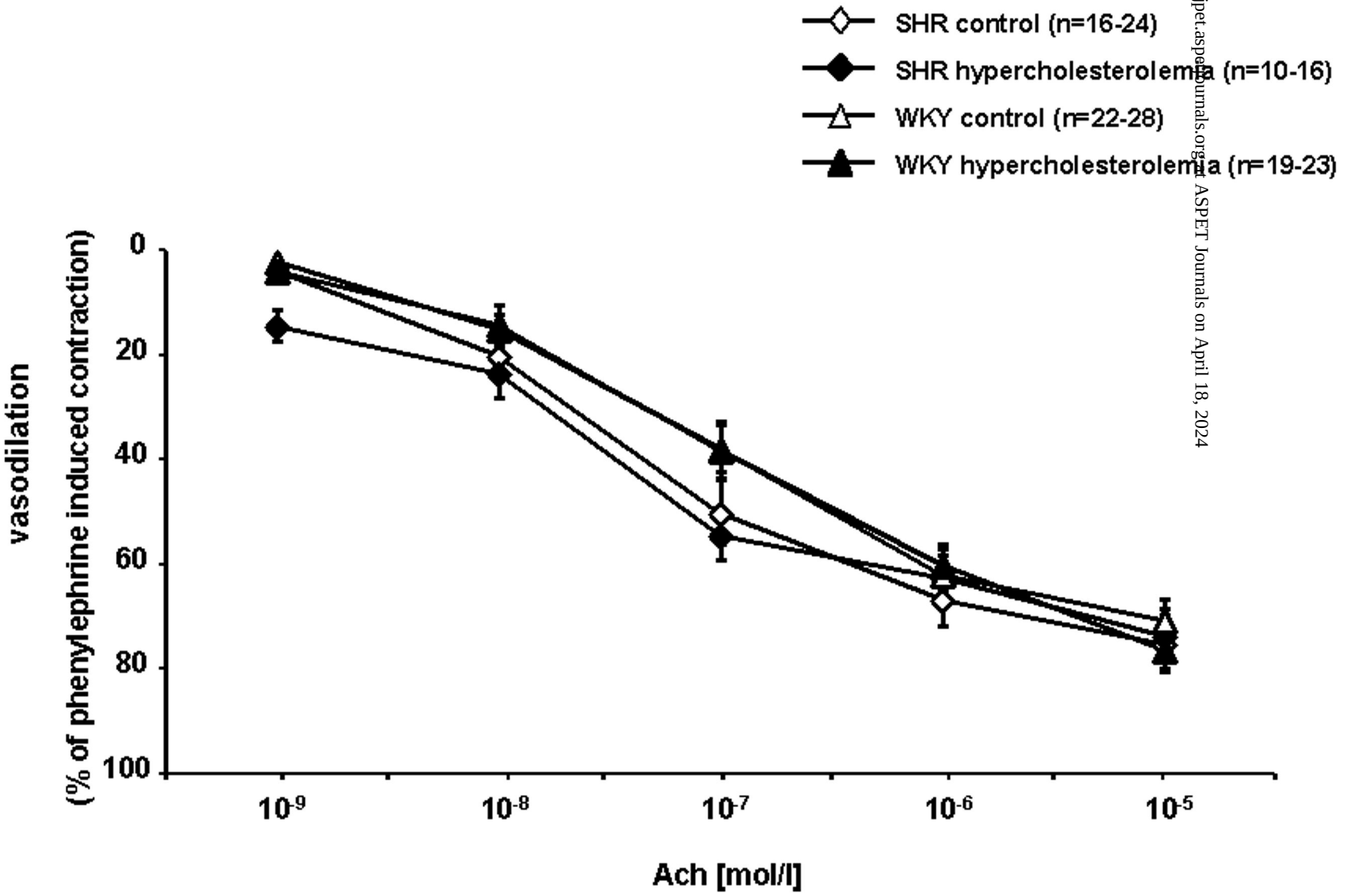

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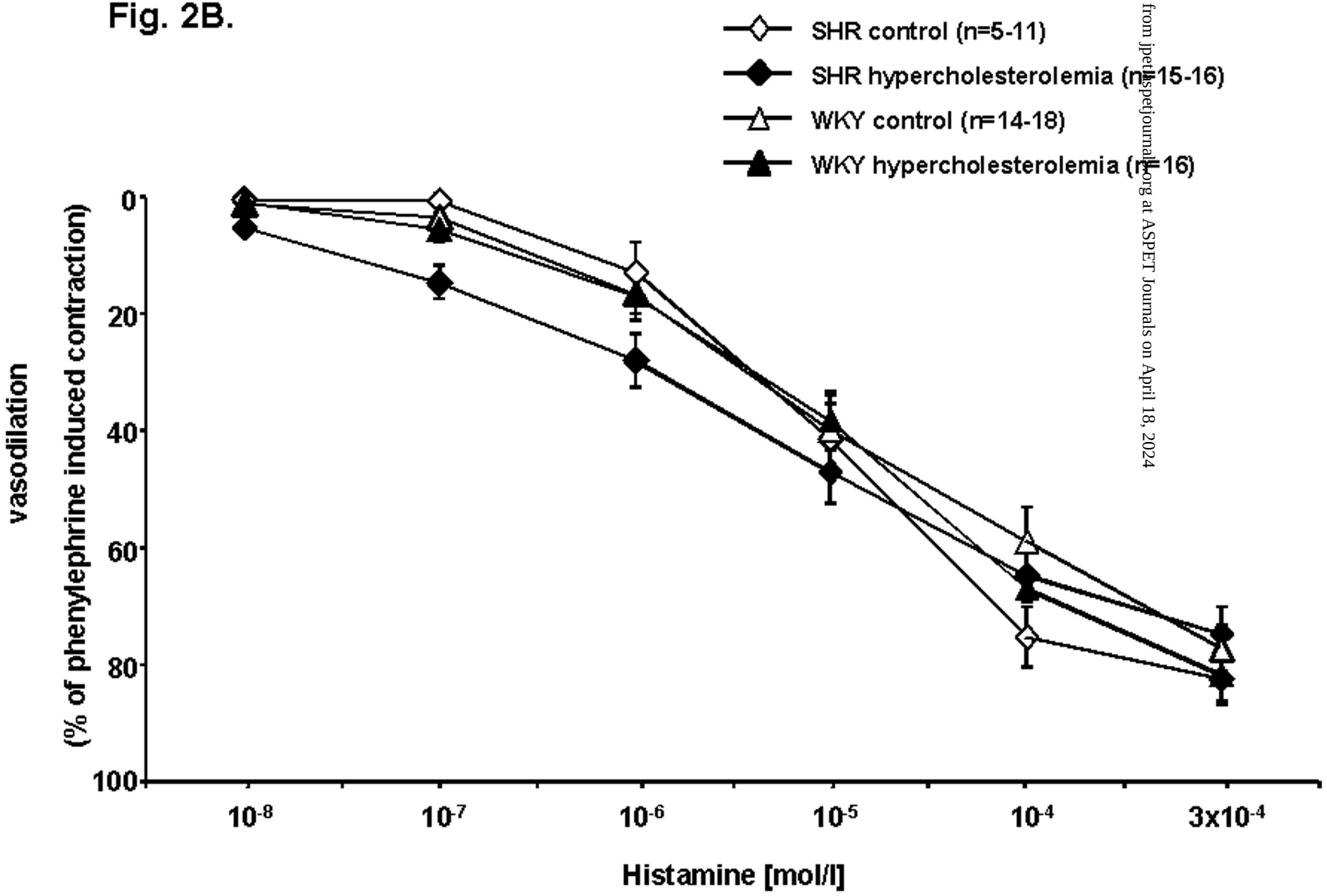

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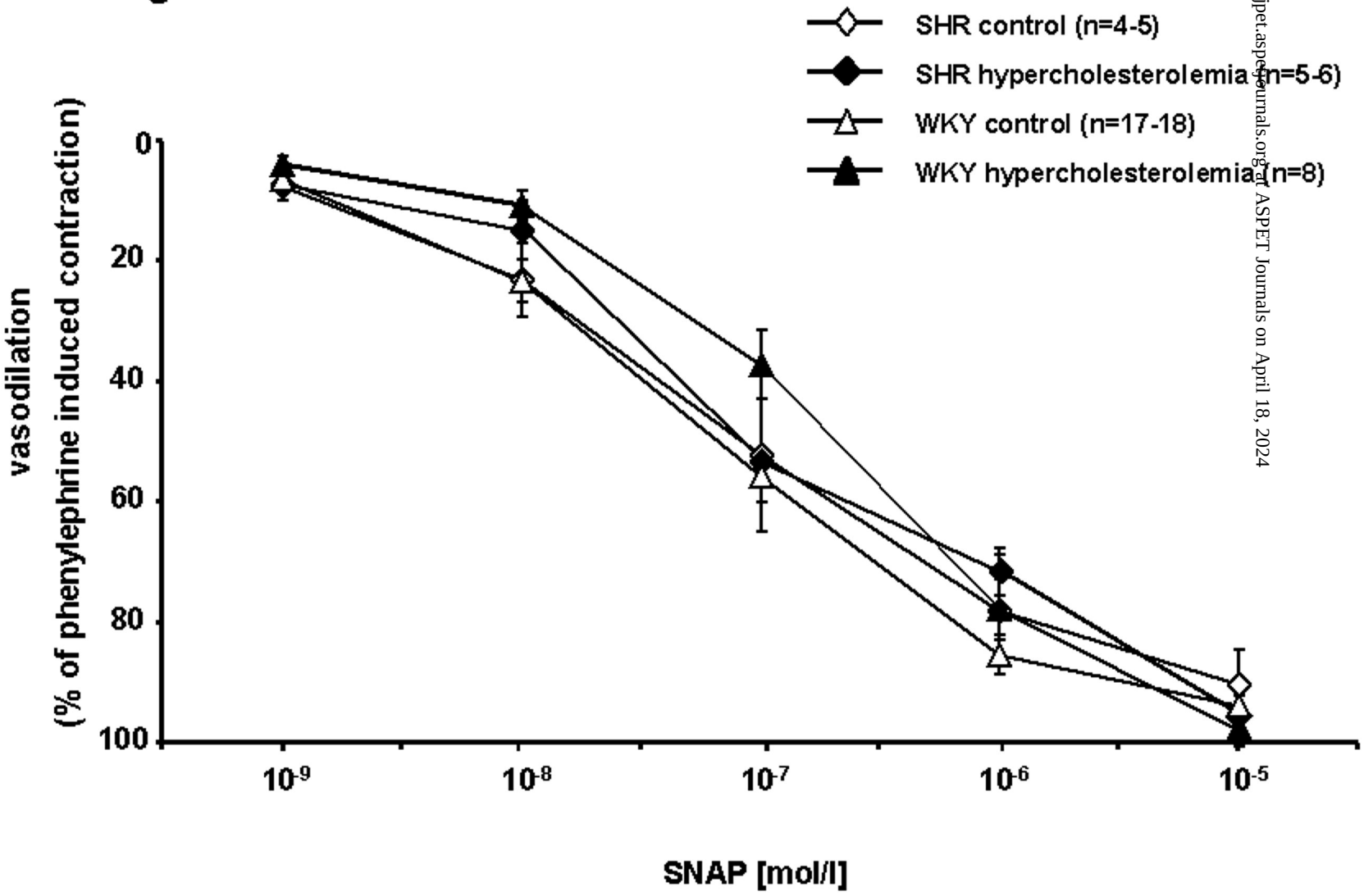

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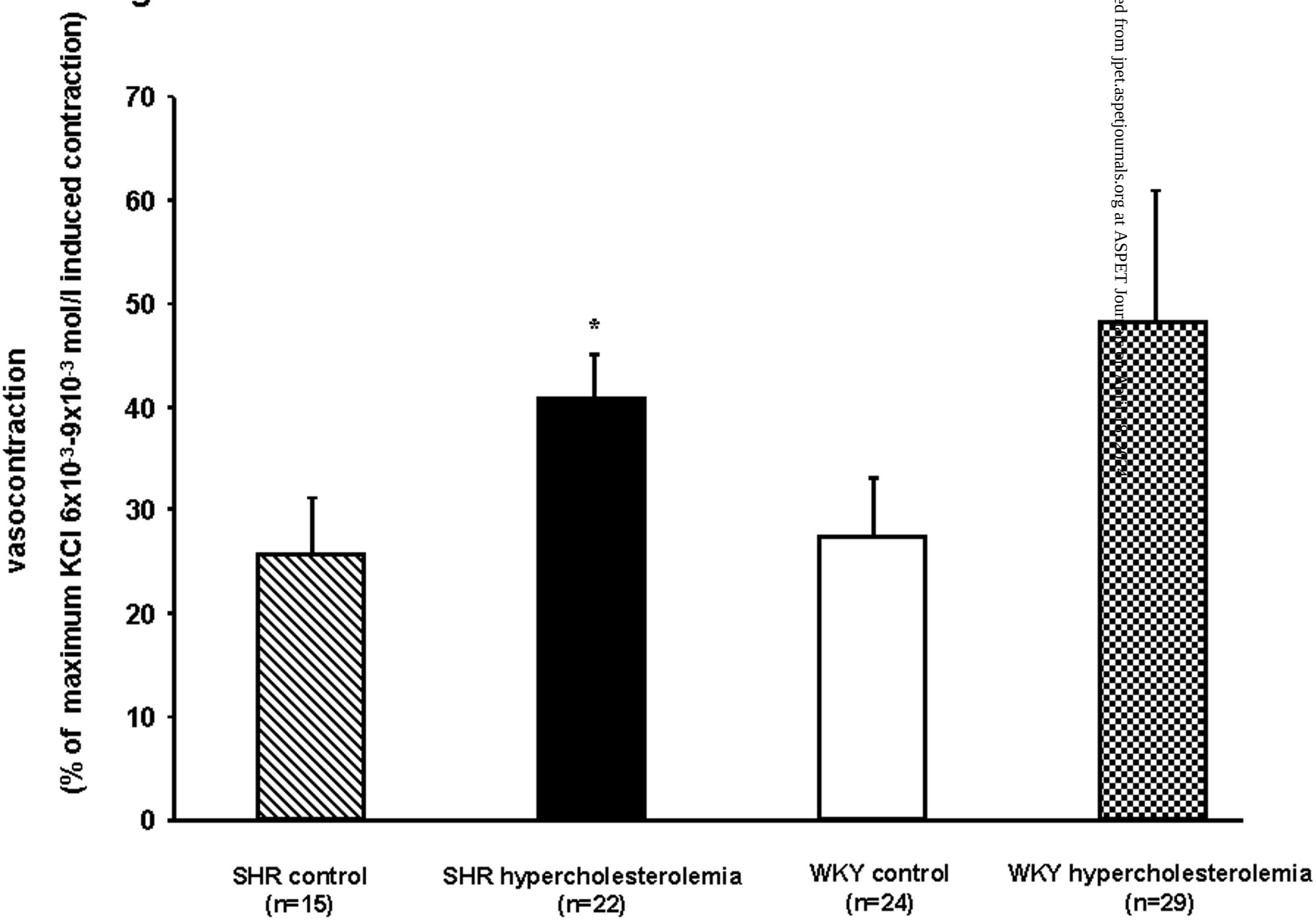

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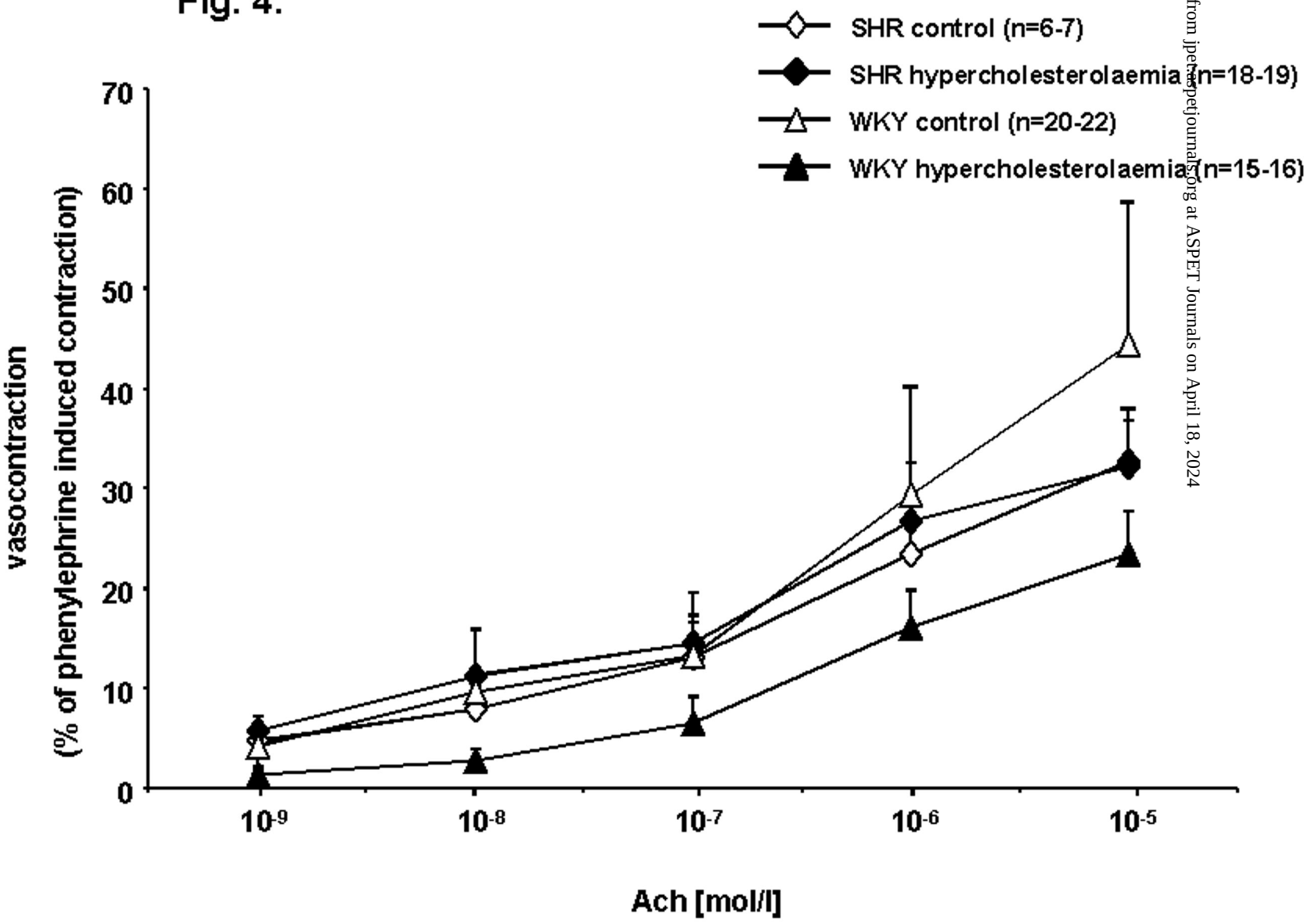

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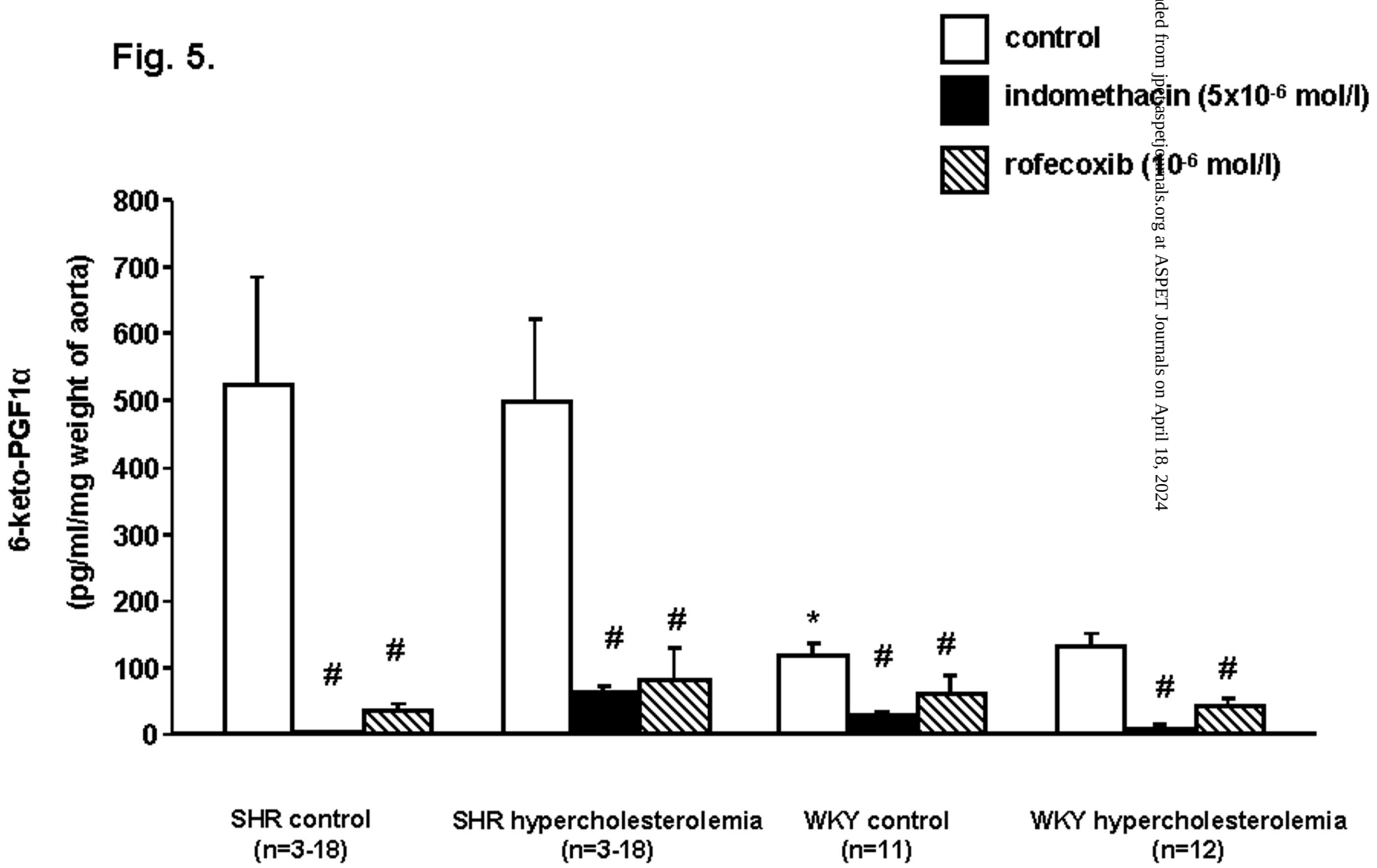

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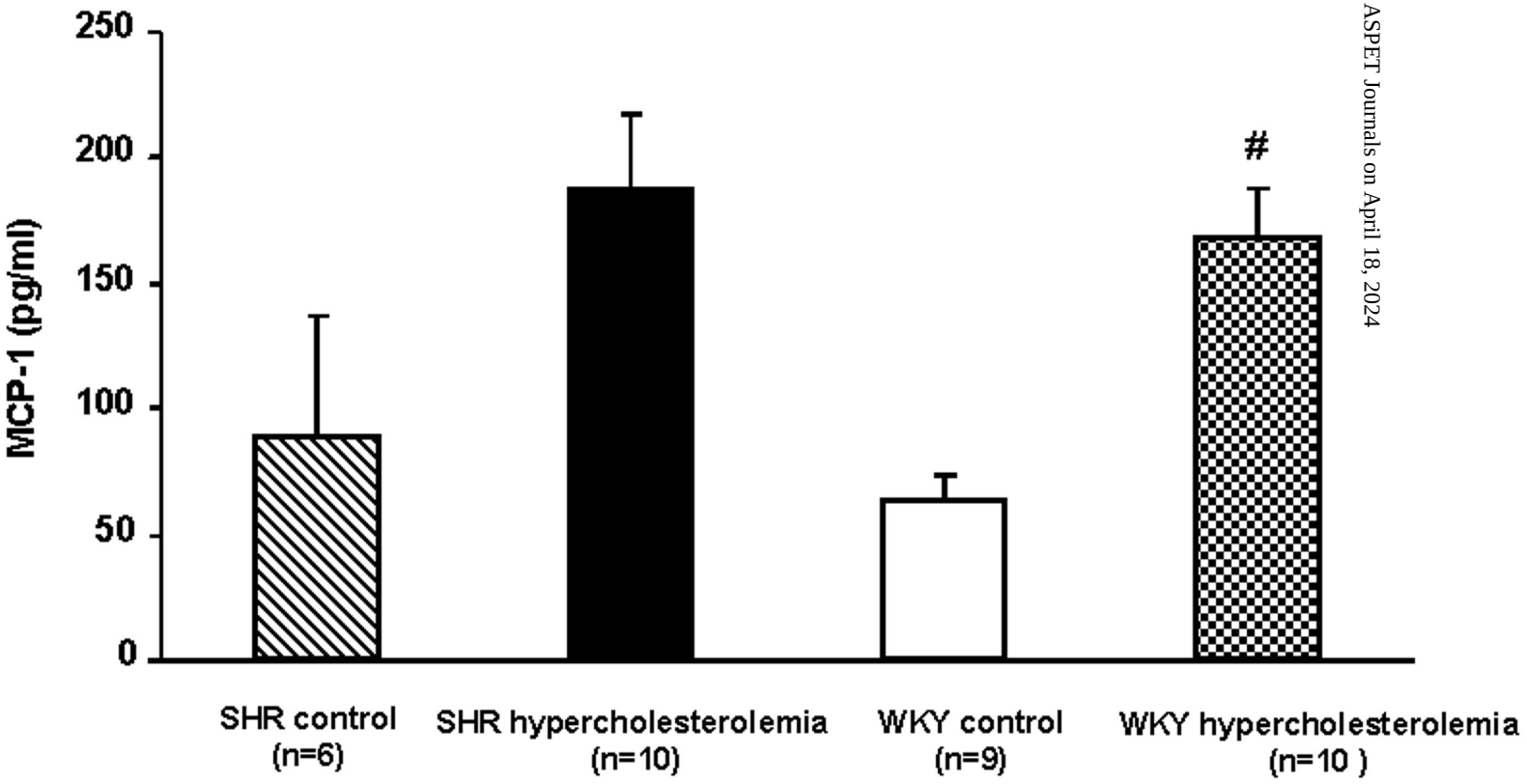

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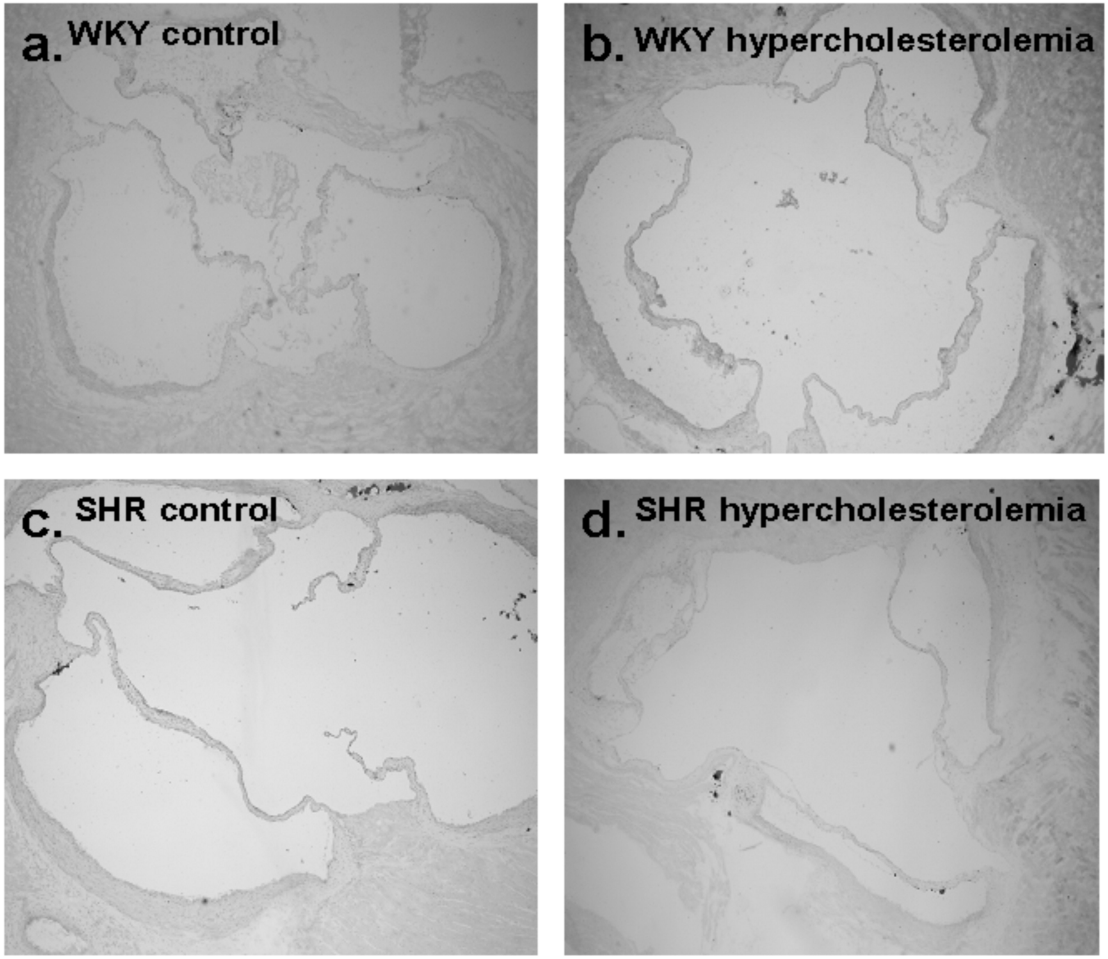

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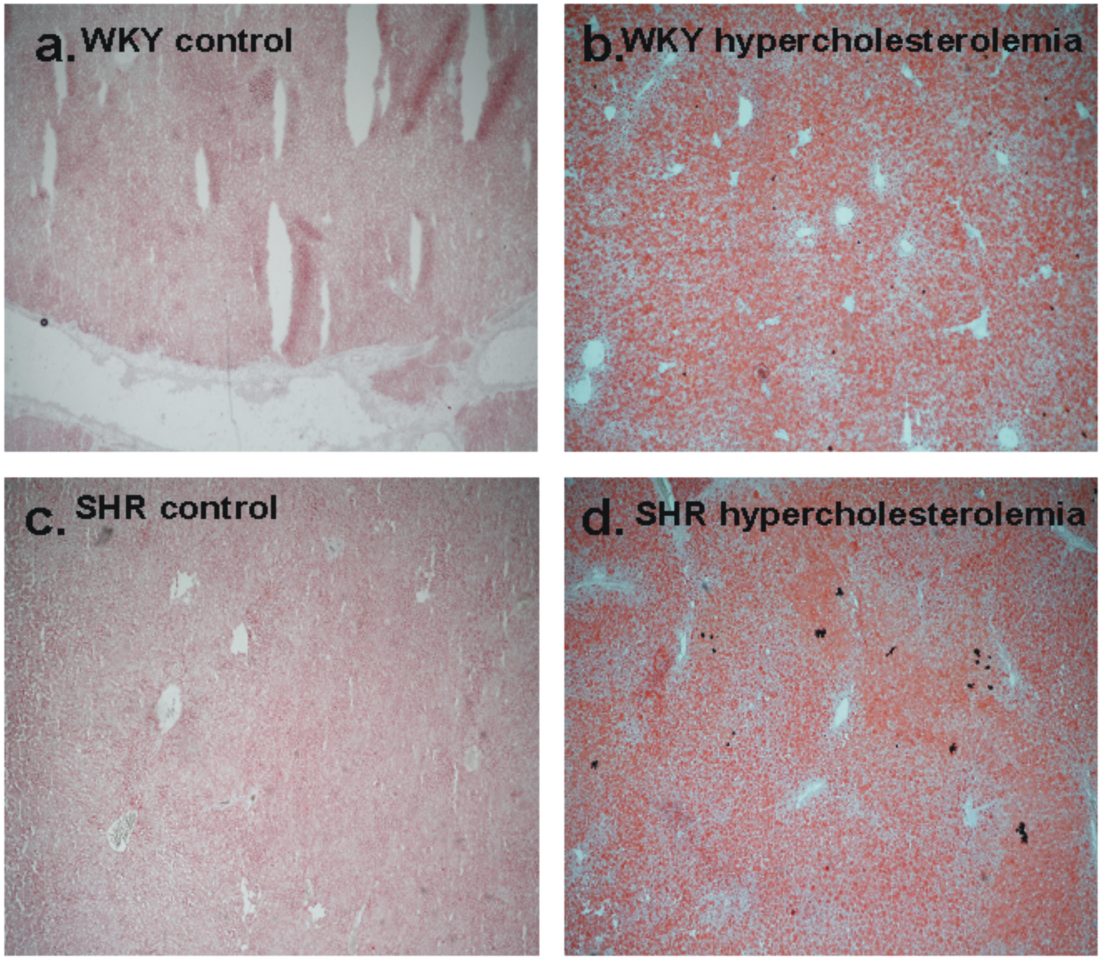

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