Androgens deficiency impairs thoracic aortic vascular function by promoting smooth muscle cell proliferation via an autophagy dependent manner

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Abstract

Background

Androgens play an essential role in maintaining vascular hemostasis via the regulation of autophagy, but the underlying mechanisms remain unknown. Here we investigated the effects of androgen deficiency in a rat castration model and the effects of androgen treatment in human umbilical vein smooth muscle cells (HUVSMCs). Male Sprague–Dawley rats were divided into two groups: (A) control group, (B) castration group. The vasodilation and vasoconstriction effect of dihydrotestosterone (DHT) were detected on rat thoracic aortic rings. Transmission electron microscopy was used to determine the levels of autophagy. Masson's trichrome staining, MTT assay and flow cytometry were performed to detect the levels of HUVSMC cell proliferation.

Results

Androgen deficiency led to significantly enhance in the vasoconstriction and reductions in the vasodilatation of rat thoracic aortic rings. Treatment with rapamycin or 3-MA attenuated and enhanced, respectively, the vasorelaxation effect of androgen deficiency. Autophagy was significantly increased in the endothelial cells and SMCs of the thoracic aortic rings of model rats. Moreover, androgen deficiency induced excessive proliferation of SMCs. Treatment of HUVMSCs with high concentrations of DHT increased the percentage of cells in G0/G1 phase and reduced the percentages of cells in the G2 phase and S phase.

Conclusions

It was concluded that vascular injury resulting from androgen deficiency is mediated, in part, by increased autophagy. By promoting SMCs proliferation, androgen deficiency results in alteration of the aortic structure and aggravates vascular injury. Androgen treatment can inhibit the excessive proliferation of SMCs.

Background

Androgens, a type of steroid hormones, function in both physiological conditions and pathophysiological states via non-DNA binding-dependent actions [1]. They have multiple actions in their target organs, particularly in the process of sex differentiation and sexual maturation. However, recent studies have provided evidence that androgens are also involved in many diseases, including those of the cardiovascular system. In a clinical study, administration of androgen suppression therapy in patients with prostate cancer increased patients' risk of fatal cardiovascular disease and myocardial infarction [2]. Testosterone is the principal member of the androgen family, and low serum testosterone levels were found to be common among patients with coronary disease and to negatively impact their survival [3–5].

Androgens were also shown to have vasodilatory effects in different vascular beds, leading to disruption of vascular homeostasis [6]. One study showed androgen deprivation can reduce the angiogenic capability in mice [7].

The endothelial cell lining of blood vessels is known to be a crucial regulator of vascular homeostasis, angiogenesis, and vascular remodeling. Upon vascular injury, the endothelium mediates the recruitment of pro-inflammatory leukocytes through expression of multiple cytokines and cell adhesion molecules [8, 9]. Activation of the endothelium causes vascular smooth muscle cells (VSMCs) to display an altered phenotype and subsequently to migrate and proliferate within the intimal compartment, with the result being the generation of a fibromuscular plaque [10, 11]. VSMC phenotypic modulation is an adaptive response for vascular homeostasis maintenance, but excessive stimuli-induced uncontrolled VSMC adaptation has been shown to lead to severe vascular injury [12, 13]. The effects of androgens on the phenotype of VSMCs remains unclear at present. Although it has been demonstrated that both low and high concentrations of testosterone can increase cardiovascular risk by promoting excessive proliferation of VSMCs [14], the underlying mechanisms remain largely unknown.

In our previous research, we found in the corpus cavernosum of a castration rat model that the smooth muscle content was significantly decreased and collagen deposition was remarkably increased [15]. Moreover, it was shown that low androgens levels induce corporeal fibrosis by promoting apoptosis and inhibiting autophagy [16]. These results suggest that androgens may exert an essential role in maintaining vascular hemostasis by counter-regulating autophagy. Furthermore, inhibition of dihydrotestosterone (DHT) by long-term treatment with 5a-reductase inhibitors decreased the rate of autophagy and increased the rate of apoptosis via decreased LC3-Ⅱ and Beclin 1 expression [17]. Despite these investigations of the roles of androgens in vascular disease, the effects of androgens on thoracic aortic vascular function and the underlying mechanisms remain largely unclear.

In the present study, we investigated the effects of androgen (testosterone) deficiency on vasomotoricity in the thoracic arteries of the castrated rat model and determined the underlying mechanisms by which androgens counter-regulate autophagy in human umbilical vein smooth muscle cells (HUVSMCs).

Results

Effects of androgen deficiency on thoracic arterial vasomotricity

Rat thoracic aortic rings harvested from the castration group exhibited enhanced endothelium-mediated contraction (Fig. 1A) and reduced endothelium-mediated relaxation (Fig. 1B, C) upon exposure to PE and acetylcholine Ach or to SNP, respectively, compared with those from the control group. These results suggest that the vascular endothelial cells and smooth muscle cells in the thoracic aortic rings were injured following castration of the rats.

To investigate whether this DHT-induced vascular injury was associated with autophagy, the rat thoracic aortic rings were pre-treated with rapamycin (autophagy promoter) or 3-MA (autophagy inhibitor) before treatment with PE and then increasing concentrations of DHT. The results showed that the androgen-induced vascular relaxation was significantly attenuated following treatment of the thoracic aortic rings with rapamycin (Fig. 2). By contrast, the thoracic aortic rings treated with 3-MA showed stronger vasodilation than those treated with rapamycin. These findings suggest that autophagy is involved in the vascular injury induced by androgen deficiency.

Effect of androgen deficiency on autophagy in vascular endothelial cells and VSMCs

The morphology of the vascular endothelial cells and VSMCs within the aortic rings from each group of rats was observed by TEM. Compared with samples from the control group, those from the castration group showed increases in the number and size of vacuoles and lipid droplets in the cytosol (Fig. 3B) as well as an increase in autophagosomes (yellow arrows, Fig. 3A). Quantitative analysis indicated that the numbers of vacuoles (Fig. 3C, $P < 0.001$) and autophagosomes (Fig. 3D, $P < 0.001$) were significantly increased in the vascular endothelial cells and VSMCs of thoracic aortic samples from the castration group compared with the control group. Correspondingly, the relative area of autophagosomes within the cytoplasm also was remarkably greater for the castration group compared with the control group (Fig. 3E, $P < 0.001$). These findings suggest that androgen deficiency promotes autophagy among vascular endothelial cells and VSMCs in the rat castration model.

Regulatory role of androgens in VSMCs

The staining of hematoxylin-eosin (HE) was employed to evaluate the histological changes of thoracic aortic, and the histological changes between the castration group (Fig. 4B) and control (Fig. 4A) was not so obvious. Masson's trichrome staining showed that the rat thoracic aortic rings of the castration group (Fig. 4D) had an increased area of smooth muscle (red stain) compared with those of the control group (Fig. 4C). This observation suggests that androgen deficiency can lead to excessive proliferation of VSMCs.

Effect of androgen treatment on HUVSMC proliferation

To investigate the proliferation of HUVSMCs exposed to androgen, HUVSMCs in culture were treated with different concentrations of DHT (0, 100, or 200 nM). The results showed that the HUVSMC proliferation rate was significantly inhibited by DHT treatment, and this inhibitory effect increased with increasing DHT concentration (Fig. 5A). To confirm this observation, flow cytometric analysis was performed to evaluate the cell cycle distribution among HUVSMCs treated with DHT. After exposure to DHT in culture, the percentage of HUVSMCs in the G0/G1 phase was significantly increased, whereas those in the G2 phase and S phase were markedly reduced (Fig. 5B). These results indicated that a high concentration of

androgen altered the cell cycle progression of HUVSMCs by increasing the percentage of cells in G0/G1 phase and reducing the percentages of cells in G2 phase and S phase (Fig. 5B, C).

Discussion

In the present study, we found that androgen deficiency in the rat castration model led to enhanced endothelium-mediated contraction (Fig. 1A) and reduced endothelium-mediated relaxation (Fig. 1B, C) of rat thoracic arteries. Moreover, androgen-induced vasorelaxation could be attenuated by pre-treatment of the rat thoracic aortic rings with rapamycin, a promoter of autophagy, whereas pre-treatment with 3-MA, an autophagy inhibitor significantly increased vasodilation in the thoracic aortic rings. Furthermore, increases in the number and size of vacuoles and lipid droplets were observed in the cytosol of cells with the thoracic aortic rings of the model rats, along with the increased appearance of autophagosomes and apoptotic bodies in the endothelium and VSMCs. Masson's trichrome staining showed an increase in the area of smooth muscle in thoracic aortic rings of the model rats. Additionally, high concentrations of DHT inhibited HUVSMC proliferation *in vitro*.

The incidence rates of hypertension and coronary artery disease have long been known to be higher in men than in women [18–20], but only recently has evidence begun to accumulate indicating that low concentrations of androgens are associated with vascular injury. Notably, male patients receiving androgen deprivation therapy have an increased risk of cardiovascular disease [21], while exogenous testosterone supplementation protects cardiac myocytes [22]. Experiments in male mice showed that testosterone protects against atherosclerosis by targeting thymic epithelial cells [21]. In addition, androgens were shown to play an antihypertensive role in spontaneously hypertensive rats, while androgen deficiency could trigger the development of hypertension [23]. The vasorelaxing effect of androgens has been previously identified in various types of isolated vascular beds, but few studies have examined their effects on thoracic arteries. Research has shown though that different types of blood vessels have differing sensitivity to androgens [6]. For example, the coronary artery is more sensitive to androgen-mediated vasorelaxation [6]. In the present study, we investigated the effects of an androgen, DHT, on isolated thoracic aortic rings from male castrated rats. Adding to the previous observations that androgens exert a relaxing effect on both phenylephrine- and KCl-induced contraction in the mesenteric artery and thoracic aorta of male hypertensive rats [24], we further confirmed in the thoracic aortic rings that androgen deficiency led to exhibited enhanced PE-induced contraction and reduced Ach- and SNP-induced vasorelaxation. These results indicate that androgen deficiency can cause vascular injury and negatively affect vascular homeostasis, with additional research needed to elucidate the underlying mechanisms.

We observed in thoracic aortic rings that DHT-induced vascular relaxation could be attenuated by treatment with rapamycin and DHT-induced vasodilation could be increased by 3-MA. In the cytosol of cells with in the thoracic aortic rings, the number and size of lipid droplets were increased in the rat castration model. We previously found in this model that the number of autophagosomes is decreased in

cavernous smooth muscle cells [16]. In contrast, the present study revealed increased numbers of vacuoles, autophagosomes, and apoptotic bodies in specimens from the castration group. These findings demonstrate that androgen deficiency led to increased autophagy in the thoracic arteries. The discrepancy between the findings of the previous study and our present study are likely due to apoptosis among endothelial cells as a result of vascular injury.

In response to endothelial cell dysfunction, VSMCs, the main components of the arterial vessel wall, undergo a phenotypic switch from a contractile phenotype to a synthetic phenotype that is essential for vascular remodeling [25]. This VSMC phenotypic modulation is regulated by autophagy, and a previous study reported that the expression of the autophagy marker Beclin 1 is increased in shear stress–induced VSMC phenotypic modulation [26]. Autophagy plays an essential role in maintaining cellular homeostasis in physiological conditions [27, 28] and participates in various physiological cellular processes as well as some pathological processes. A relationship between autophagy and cell proliferation has been demonstrated in many conditions [29, 30]. Cell proliferation is controlled by progression through the cell cycle. The percentage of cells in the S phase were increased and the percentage of those in the G0/G1 phases were decreased when cell proliferation was enhanced. Previous research found that transitions from the G1 into the S phase of pulmonary arterial smooth muscle cell were enhanced under hypoxic conditions. And the enhancement of pulmonary arterial smooth muscle cell proliferation is related to the activation of autophagy [31].

Androgens were shown to be crucial for the survival and proliferation of cells within the prostate gland [32], and adrenal androgen dehydroepiandrosterone sulfate can inhibit VSMC proliferation [33]. Our results demonstrated that androgen deficiency lead to increased proliferation of VSMCs, and treatment of HUVSMCs with high concentrations of DHT increased the percentage of cells in G0/G1 phase and reduced the percentage of cells in G2 phase and S phase. These results revealed that androgens deficiency could markedly enhance the proliferation of HUVSMCs, and the enhancement of HUVSMCs proliferation may related to the activation of autophagy.

A major limitation of this current study is that we did not evaluate HUVSMC phenotypes. Moreover, how HUVSMC autophagy affects vascular homeostasis remains largely unknown. Additional studies are necessary to explore the direct effects of androgen-induced autophagy on HUVSMC phenotypes and vascular function both *in vitro* and *in vivo*.

Conclusions

In conclusion, androgen deficiency in rats resulting from castration can led to significant enhanced vasoconstriction and reduced vasodilatation through the induction of autophagic vascular endothelial cell death. Autophagy is essential for androgen deficiency–induced vascular injury in rat thoracic arteries. In addition, androgen deficiency altered structural features of the thoracic aorta and aggravated vascular injury by promoting proliferation of VSMCs. The results of the present study provide insight toward a better understanding of the effects of androgens in vascular diseases.

Abbreviations

VSMCs	Vascular smooth muscle cells
HUVSMCs	human umbilical vein smooth muscle cells
DHT	dihydrotestosterone
PE	Phenylephrine
Ach	acetylcholine
TEM	Transmission electron microscopy

Methods

Animal model and sample collection

Male Sprague–Dawley rats (weighing 200–300 g) were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and randomly divided into two groups: a sham group (control group, n = 7) and a castration group (n = 7). The rats were maintained in specific pathogen free conditions in the Department of Laboratory Animal Science of Fudan University, and bred by professional breeders. At 14 days after castration of the rats in the castration group, the rats in both groups were euthanized by intraperitoneal injection of sodium pentobarbital for isolation of thoracic aortic rings. The Animal Experiment Committee of Huashan Hospital, Fudan University approved the present study, which was conducted in accordance with the ethical standards of the responsible Institutional Committee on Animal Experimentation.

Assessment of vasomotricity

A PowerLab data acquisition system was used to record the tension responses of the isolated thoracic aortic rings. Phenylephrine (PE), over a concentration range of 1×10^{-8} to 5×10^{-5} M, was added in the organ bath, and the PE-induced contraction curve was recorded. Then cumulatively increasing concentrations of acetylcholine (Ach, 1×10^{-8} to 5×10^{-5} M) were added when a steady tonic response was reached, and then relaxation curves were recorded. The rings were rinsed several times until the tension was restored to the baseline level. Next sodium nitroprusside (SNP, 1×10^{-8} to 5×10^{-5} M) was added into the organ baths for recording of the relaxation curves and effective medium concentration (EC_{50}) after re-contraction induced by PE (5×10^{-5} M). The contractility of smooth muscle induced by PE and Ach or SNP was denoted as F1 and F2, respectively. The diastolic rate was calculated according to the formula (F1-F2)/F1.

Thoracic aortic rings also were pretreated with 100 nM rapamycin (a promoter of autophagy) or 10 nM 3-methyladenine (3-MA; an inhibitor of autophagy) for 30 min. Then increasing concentrations of dihydrotestosterone (DHT, 10^{-8} to 10^{-5} M) were added sequentially to the organ baths after induction of

pre-contraction by PE (5×10^{-5} M). The relaxation curves were recorded, and the rate of relaxation was calculated according to the formula (F1-F2)/F1.

Transmission electron microscopy (TEM)

The harvested rat aortic rings were fixed in glutaraldehyde for 2 h followed by post-fixation in a 1% osmic acid solution at 4 °C for 2 h. The samples were then embedded in epoxy resin 618 embedding solution (TAAB Laboratories Equipment, Berks, UK). Thin sections were cut using an LKB V ultra-thin microtome (LKB, Sweden) and stained with lead citrate. Cytoplasmic vacuoles, lipid droplets, and autophagosomes were visualized by TEM (Philips CM-120, Eindhoven, The Netherlands). The degree of autophagy was quantified according to the number of autophagosomes as well as the ratio of autophagic vesicles to the total cytoplasmic area.

Masson's trichrome staining

According to our previously described protocol [17], Masson's trichrome staining was carried out using a Masson's trichrome kit (Sigma-Aldrich, St. Louis, MO, USA). Briefly, paraffin-embedded sections of thoracic aorta samples were stained with acid ponceau, differentiated in phosphomolybdic acid aqueous solution, and stained with aniline blue. With this staining protocol, the muscle fibers were stained red, and the collagen fibers were stained blue.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

HUVSMCs were seeded in 96-well culture plates at a cell density of 2×10^3 cells per well in 100 μl culture medium (Dulbecco's Modified Eagle Medium [DMEM] containing 10% fetal bovine serum). After culture in an incubator at 37 °C for 1 day, the culture medium was replaced with serum-free DMEM. After 24 h in culture, DHT, was added at a concentration of 0, 100, or 200 nM to the cells for incubation for different periods (1, 2, or 3 days). At the end of the culture period, the cells were incubated with MTT solution (10 μl) for 3 h at 37 °C followed by 100 μl dimethyl sulfoxide (DMSO) for 10 min at 37 °C. A microplate reader (Tecan, Nanoquant, Switzerland) was used to measure the optical density (OD) value at 490 nm of the solution in each well.

Flow cytometric analysis

HUVSMCs were seeded in 6-well culture plates and dispersed into a single cell suspension after treatment with DHT (0, 50, or 200 nM). Aliquots of 1 million cells in 10 μl were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) for 10–15 min at room temperature protected from light. Then 400 μl buffer solution was added to each sample, and all liquid in each sample was transferred to a flow analysis tube. Flow cytometry was used to analyze the cell cycle distribution within each sample.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Data were compared among the groups by one-way analysis of variance (ANOVA) using SPSS software (SPSS21.0, Inc., Chicago, IL, USA). Differences were considered significant if $P < 0.05$.

Declarations

Ethics approval and consent to participate

The Animal Experiment Committee of Huashan Hospital, Fudan University approved the present study, which was conducted in accordance with the ethical standards of the responsible Institutional Committee on Animal Experimentation. Ethical approval number: 2020 Huashan Hospital, Fudan University JS-240

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZMG: designed and supervised this investigation. ZNN, LM, GFB and WXJ: performed this investigation, and they contributed equally to this work. DF and SYF contributed to the data collection. ZS, CSW and DQ: provided technical or material support. All authors read and approved the final manuscript.

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Figures

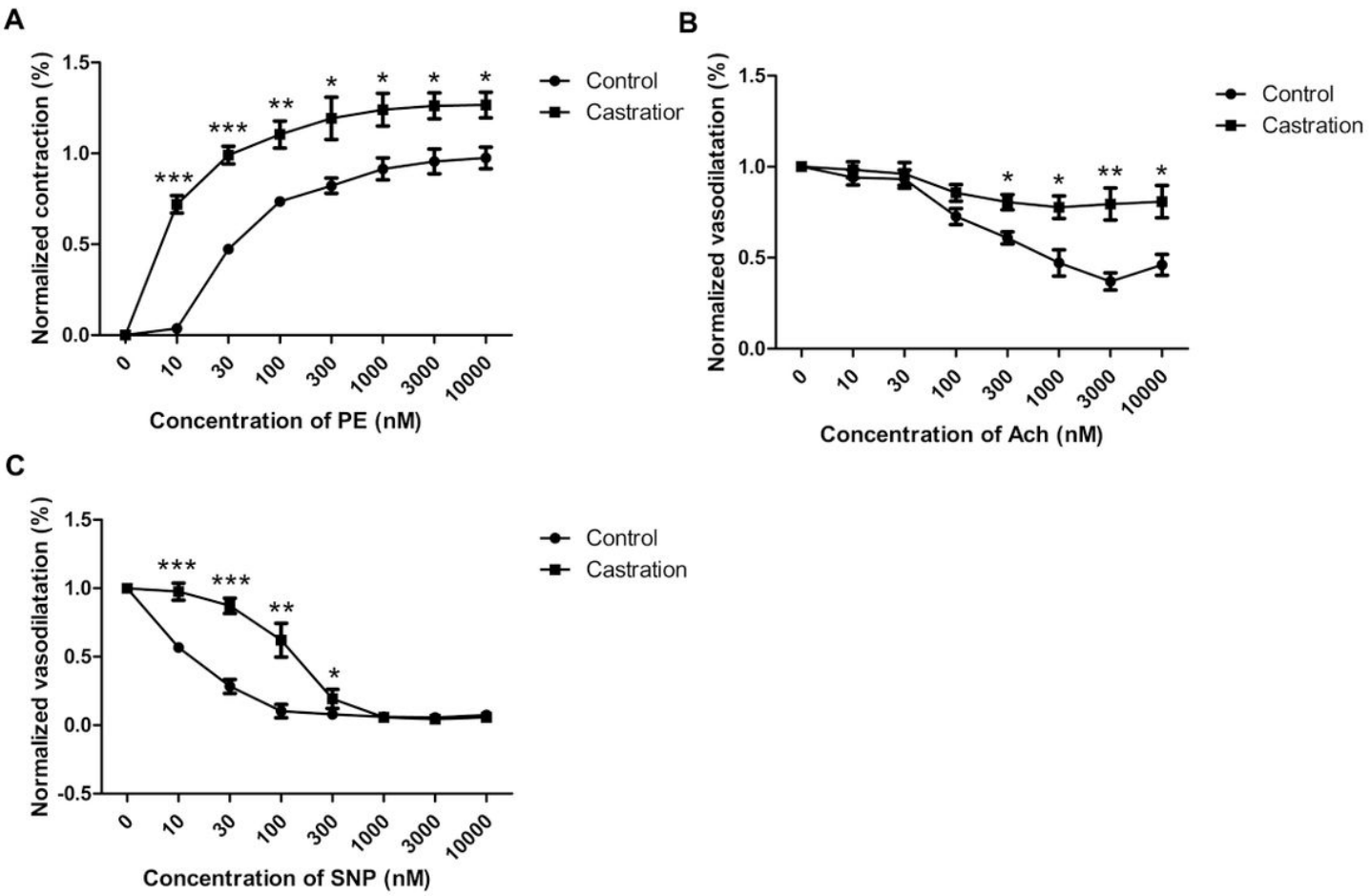

Figure 1

Endothelium-mediated vasocontraction and relaxation of rat thoracic aortic specimens from the control and castration groups. (A) Contraction response to treatment with phenylephrine (PE). (B) Relaxation response to treatment with acetylcholine (Ach). Relaxation response to treatment with sodium nitroprusside (SNP). n=4 in each group. *P<0.05, **P<0.01, ***P<0.001.

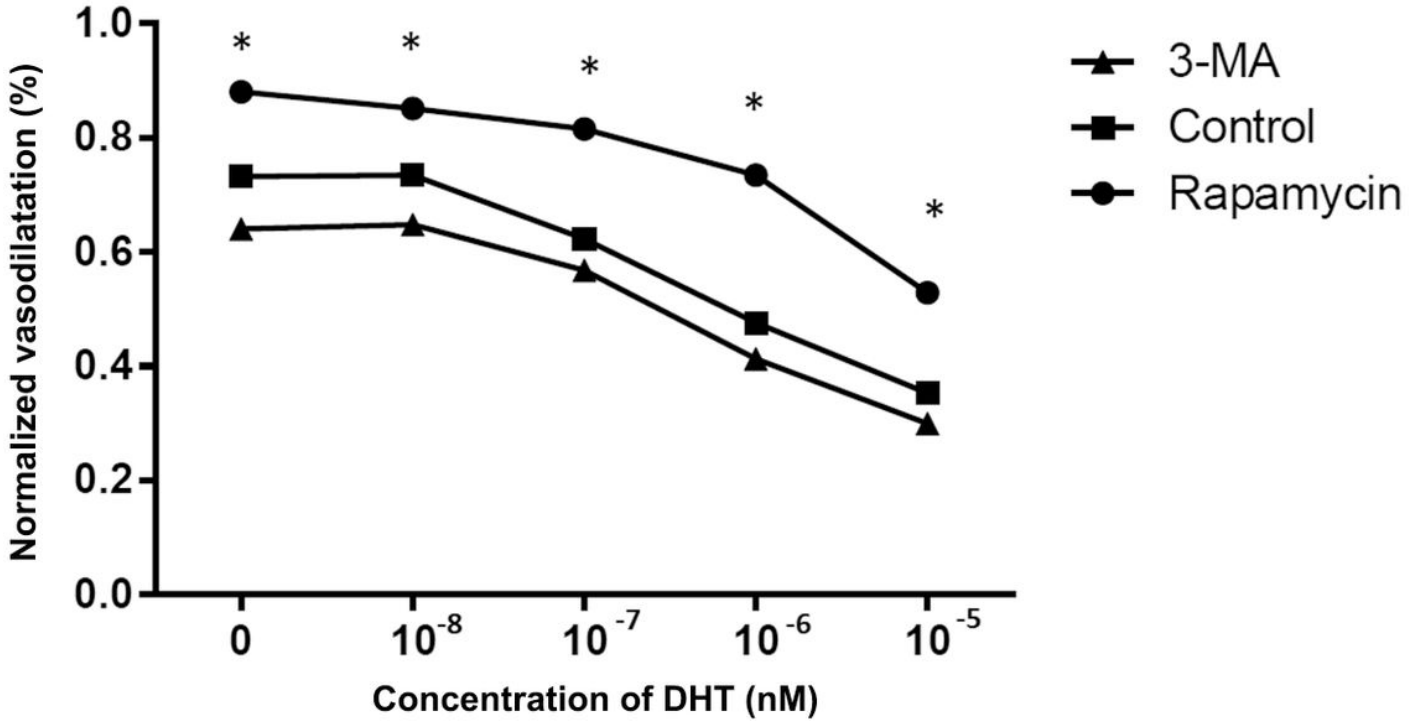

Figure 2

Involvement of autophagy in dihydrotestosterone (DHT)-induced vascular relaxation. Vasorelaxation, as assessed by the diastolic rate, of rat thoracic aortic rings after pre-treatment with rapamycin or 3-MA before sequential treatment with PE and DHT. n=4 in each group. *P<0.05 for control group versus rapamycin group.

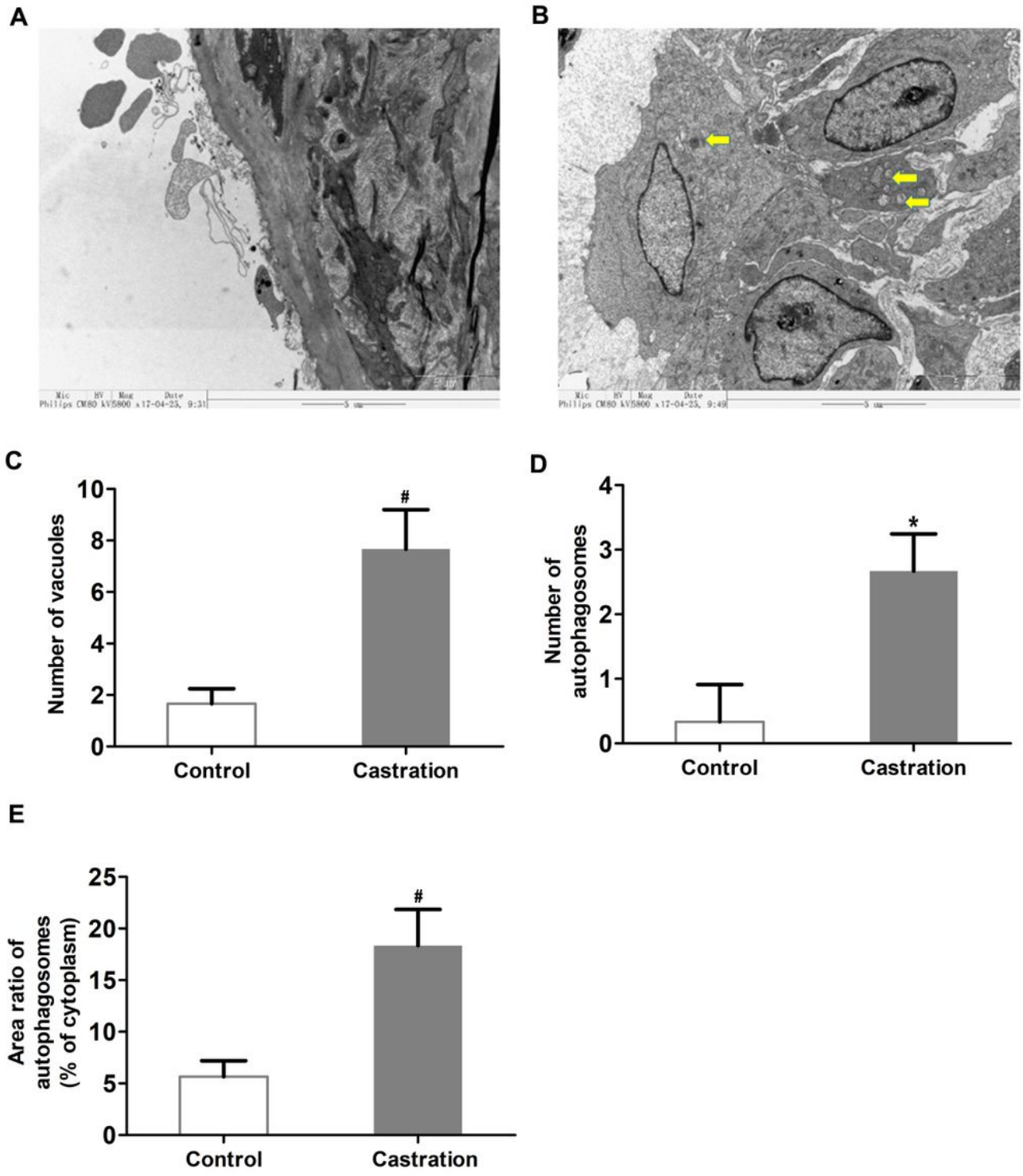

Figure 3

Autophagy of vascular endothelial cells and vascular smooth muscle cells (VSMCs) within rat thoracic arteries. Transmission electron microscopy (TEM) showed that the numbers of vacuoles, lipid droplets (LDs), and autophagosomes (yellow arrows) were greater in the vascular endothelial cells and VSMCs of the thoracic arteries of specimens harvested from the castrated rats (B) than in those from control rats (A). Numbers of vacuoles (C) and autophagosomes (D) in vascular endothelial cells and VSMCs of

specimens from the control and castration groups. (E) Ratio of the area of autophagosomes to the total cytoplasmic area in cells within specimens from each group. n=3 in each group. *P<0.01, #P<0.001.

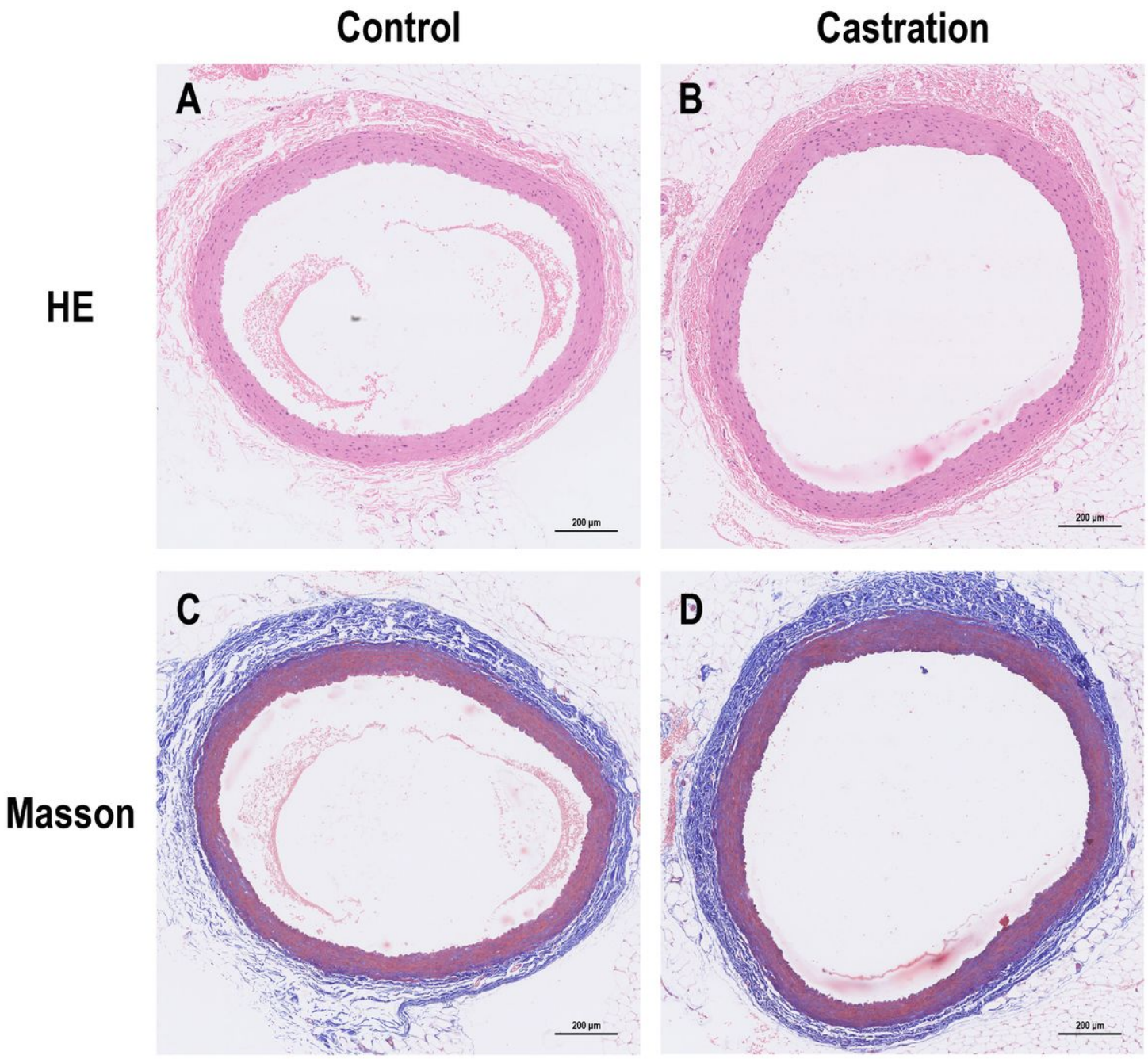

Figure 4

Representative images of rat thoracic aortic rings from the control and castration groups stained with hematoxylin-eosin (HE, A, B) and Masson's trichrome stain (C, D) to reveal structural features.

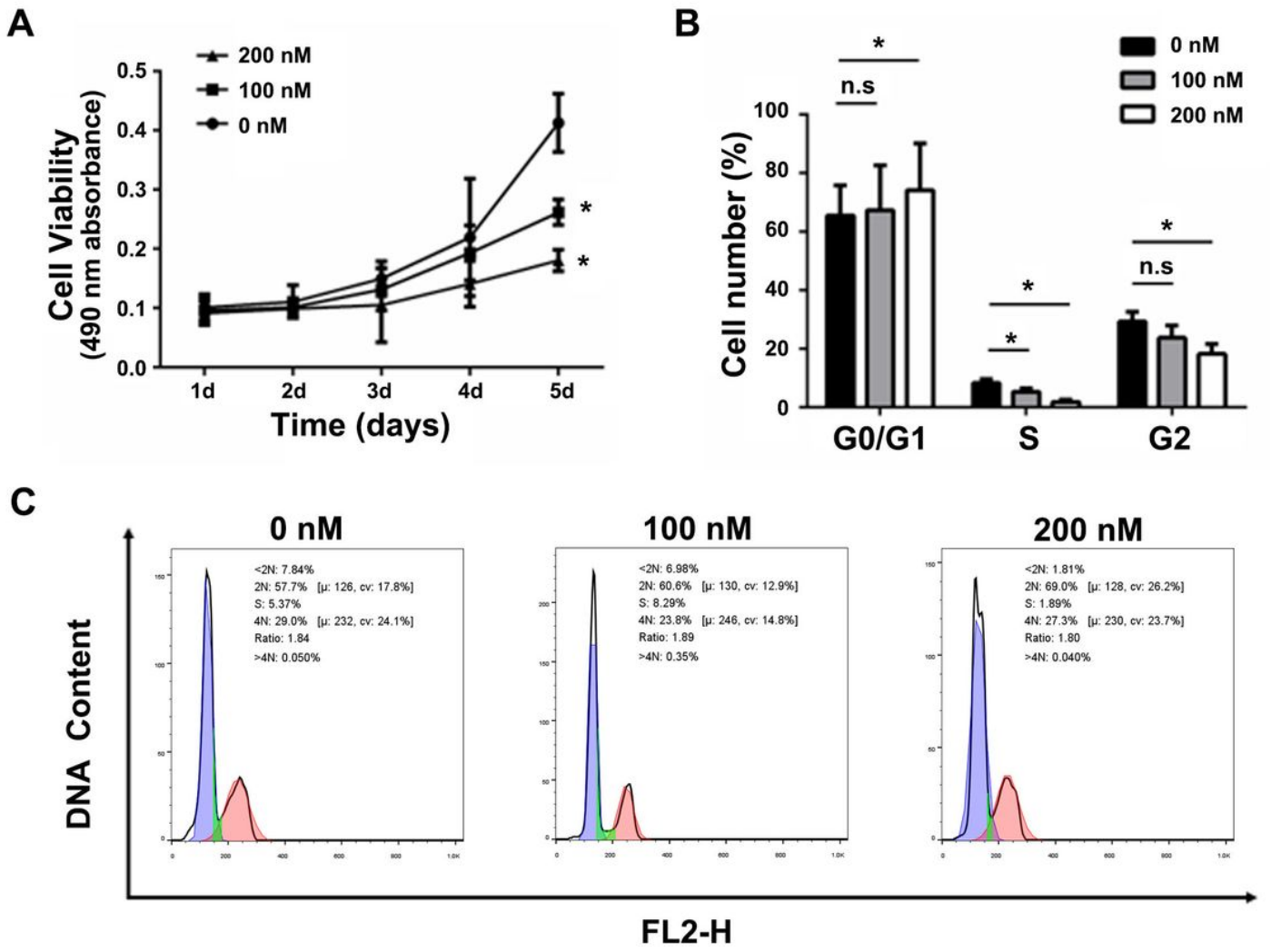

Figure 5

Inhibitory effects of DHT on HUVSMC proliferation. (A) MTT assay results showing proliferation of HUVSMCs exposed to different concentrations of DHT. (B, C) Cell cycle distribution of HUVSMCs exposed to different concentrations of DHT. n.s: not significant, *P<0.05.

Supplementary Files

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