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Chronic administration of minoxidil protects elastic fibers and stimulates their neosynthesis with improvement of the aorta mechanics in mice

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ABSTRACT

Arterial wall elastic fibers, made of 90% elastin, are arranged into elastic lamellae which are responsible for the resilience and elastic properties of the large arteries (aorta and its proximal branches). Elastin is synthesized only in early life and adolescence mainly by the vascular smooth muscles cells (VSMC) through the cross-linking of its soluble precursor, tropoelastin. In normal aging, the elastic fibers become fragmented and the mechanical load is transferred to collagen fibers, which are 100–1000 times stiffer than elastic fibers. Minoxidil, an ATP-dependent K^+ channel opener, has been shown to stimulate elastin expression *in vitro,* and *in vivo* in the aorta of male aged mice and young adult hypertensive rats. Here, we have studied the effect of a 3-month chronic oral treatment with minoxidil (120 mg/L in drinking water) on the abdominal aorta structure and function in adult (6-month-old) and aged (24-month-old) male and female mice. Our results show that minoxidil treatment preserves elastic lamellae integrity at both ages, which is accompanied by the formation of newly synthesized elastic fibers in aged mice. This leads to a generally decreased pulse pressure and a significant improvement of the arterial biomechanical properties in female mice, which present an increased distensibility and a decreased rigidity of the aorta. Our studies show that minoxidil treatment reversed some of the major adverse effects of arterial aging in mice and could be an interesting anti-arterial aging agent, also potentially usable for female-targeted therapies.

1. Introduction

In order to conduct blood to small arteries, capillaries and tissues, the aorta and its proximal branches act as an elastic buffering chamber downstream from the heart [1]. During the systole, these large elastic arteries are able to accommodate the ejected blood volume by distending and storing energy in the elastic fibers present in theirs walls. During the diastole, elastic fibers release the accumulated energy by compressing the stored blood, which maintains diastolic high pressure and flow in the arteries. This phenomenon, known as the Windkessel effect, helps to decrease the heart afterload, improves coronary blood flow and progressively smoothens the originally discontinuous blood ejection from the heart into a more regular/continuous blood flow and pressure in the vasculature [2–5].

The normal functioning of the large elastic arteries is related to the key elements of the arterial extracellular matrix (ECM): elastin and collagen. ECM, produced by all vascular cells, confers a structural support to vessels and defines the mechanical properties of the arteries, especially elasticity provided by elastic fibers and rigidity provided by collagen. The core of elastic fibers consists of elastin (90%), which is a polymer of the monomeric precursor tropoelastin and the dominant contributor to the fiber elastic properties. The peripheral components of elastic fibers (10%) are made of fibrillin-rich microfibrils [6,7]. Elastin is synthesized in the aorta only in early life and adolescence, essentially by vascular smooth muscles cells (VSCMs), then mainly arranged into concentric circumferentially-oriented lamellae of elastic fibers *-i.e.* elastic lamellae- around the arterial lumen, alternating with layers of smooth muscle cells [3,8,9].

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During aging, the structure of the arterial wall changes as a consequence of collagen accumulation [6] and fractures of the elastic fibers [10]. Elastin and elastic fibers are broken down both by mechanical extension cycles (elastin fatigue) and enzymatic processes involving an increasing imbalance between anti-proteases and proteases [11]. In particular, elastin degrading enzymes, *i.e.* elastases, include several matrix metalloproteases (MMP), such as MMP-2 and MMP-9, and cathepsins, such as cathepsins S, K and L [6,12]. Due to these processes, elastic arteries undergo two major physical changes with age: dilation and stiffening, the latter also resulting from collagen cross-linking by advanced glycation endproducts (AGEs) [11,13]. Vascular stiffening induces limited aortic extension and arterial pulse wave reflexions during systole, leading to an increase in systolic and a decrease in diastolic blood pressure, thus increasing pulse pressure. The augmented arterial systolic blood pressure increases the systolic workload of the LV (elevated postcharge), leading to increased oxygen consumption, left ventricular hypertrophy and interstitial fibrosis, all of these events predisposing to development of aging-heart failure [14,15].

In order to counteract the age-related elasticity loss, many strategies either pharmacological (angiotensin converting enzyme (ACE) inhibitors, angiotensin-receptor blockers (ARB), and calcium-channel antagonists) or non-pharmacological (exercise training, low-salt diet) are aimed at moderating the vascular stiffening and the resulting cardiovascular dysfunction [16]. Minoxidil, an anti-hypertensive K_{ATP} channel opener [17], has previously been shown to increase elastin expression *in vitro* in cultured skin fibroblast or VSMCs [18,19], and *in vivo* in arteries of young mice genetically deficient for elastin or rats [20–22]. Also, nicorandil, an antihypertensive agent that belongs to the ATP-dependent potassium (K_{ATP}) channel opener family [23], has been shown to increase the proportion of elastin and the number of elastic lamellae in the aortic wall of adult rats [24]. Several other potassium channel opener derivatives have been shown to stimulate elastin production by cultured rat VSMCs [20,25,26]. Also, we have recently demonstrated that minoxidil is able to stimulate the resynthesis of elastin and elastic fibers in the aorta of aged male mice, when this process is normally stopped at this age [27].

Based on the aforementioned evidences, and because the aging processes often present gender-dependent differences in various organs, such as the skin [28] or arteries [29] in relation with elastic fiber alterations, we compared the potential of minoxidil to counteract the deleterious effects of age on arteries in male and female mice. Our results show that minoxidil is able to protect pre-existing aortic elastic fibers, re-activate elastic fiber synthesis and improve arterial function, more efficiently in females than in males.

2. Material and methods

2.1. Animals and chronic treatment by minoxidil

A total of forty five adult (6-month-old) and aged (24-month-old) C57Bl/6 J mice, females and males, were treated with minoxidil for 3 months, at a concentration of 120 mg/L in drinking water [22]. Minoxidil was purchased from Molecula (London, UK). In addition, three untreated adult female mice (6-month-old) were used for cell culture and subsequent ELISA experiments. All housing and surgical procedures were in accordance with institutional guidelines and country regulations.

2.2. Blood pressure

Blood pressure was measured at the tail artery in awake animals by using a CODA tail-cuff recorder (Kent Scientific, Torrington, USA). Measurements were repeated for 3 days, two times a day. The values obtained at days 2 and 3 were averaged for each animal, as described [27].

2.3. Body and heart weights

Mice were weighed (body weight: BW) and anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg). Hearts were collected, washed and weighed (wet weight). Left ventricle (LV), right ventricle (RV) and septum (S) were then dissected, washed, and weighed (wet weight). Total heart weight (HW) to body weight (HW/BW), as well as left ventricle + septum weight to body weight ratio (LV + S/BW) and right ventricle weight to body weight ratio (RV/BW) were calculated.

2.4. Surgical procedure, abdominal aorta mechanics and reactivity

Mice were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg) and the abdominal aorta was excised and placed in a physiological buffer composed of: 135 mM NaCl 5 mM KCl, 1.6 mM $CaCl_2$, 1.17 mM $MgSO_4$, 0.44 mM KH_2PO_4, 2.6 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 5.5 mM D-glucose, 0.025 mM EDTA, 10 mM HEPES (pH 7.4). The vessels were then cannulated and mounted onto a pressure myograph -placed under a videomicroscope- allowing for aorta bathing and filling with physiological solution at 37 °C. By using the homemade videoanalysis software (WinDiam) coupled to a video-microscope, the inner and outer diameters were measured when changing the intraluminal pressure of the physiological buffer, as described [27]. Below 125 mmHg, the inner diameter was calculated, as described [30,31].

Distensibility, *i.e.* the change in relative luminal volume (percentage) per mmHg [32], was then calculated. Here, we have used the distensibility per 25 mmHg increments (D_{25}).

Circumferential midwall strain (ε, relative increase in diameter, at a given pressure, as compared to the diameter at no pressure), circumferential wall stress (σ, forces that are circumferentially applied to each small portion (surface) of the vessel wall) and incremental elastic modulus (Einc, indice of wall stiffness) were calculated according to classical formulas [33]. Details are presented in the online data suplement.

Variations of the abdominal aorta diameter in response to 10^{-5} M phenylephrine (PE), a VSMC-dependent vasoconstrictor mainly acting through the alpha1-adrenoceptors, then 10^{-5} M acetylcholine (ACh), an endothelial cell–dependent vasodilator, were measured at 75 mmHg [31]. The effect of phenylephrine was expressed as the decrease in inner diameter (in %) induced by phenylephrine at an intraluminal pressure of 75 mmHg. The effect of acetylcholine was expressed as the restoration percentage of the diameter decrease induced by PE at an intraluminal pressure of 75 mmHg.

2.5. Arterial total protein, desmosine and hydroxyproline contents

3 mm long carotid artery segments were used to determine arterial wall protein content. Carotid arteries were used here in order to reduce the number of animal used and because the elastin, collagen and total protein contents are close between the abdominal aorta and the carotid artery [3,31]. In the tissue hydrolysates, hydroxyproline levels, considered as representative of the collagen content, and total protein were determined by amino-acid analysis using high-pressure ion-exchange chromatography on a Biochrom 30 amino acid analyzer (Cambridge, UK). Results were expressed as amino-acid mass per artery segment length unit (μg/mm), as described [27]. Desmosine levels, considered as representative of the elastin content, were determined by radioimmunoassay, as described [34].

2.6. Histological examination

After mouse anesthesia by intraperitoneal injection of pentobarbital (60 mg/kg), an intracardiac perfusion of 4% paraformaldehyde (PFA) was performed in order to maintain vessels morphology under pressure.

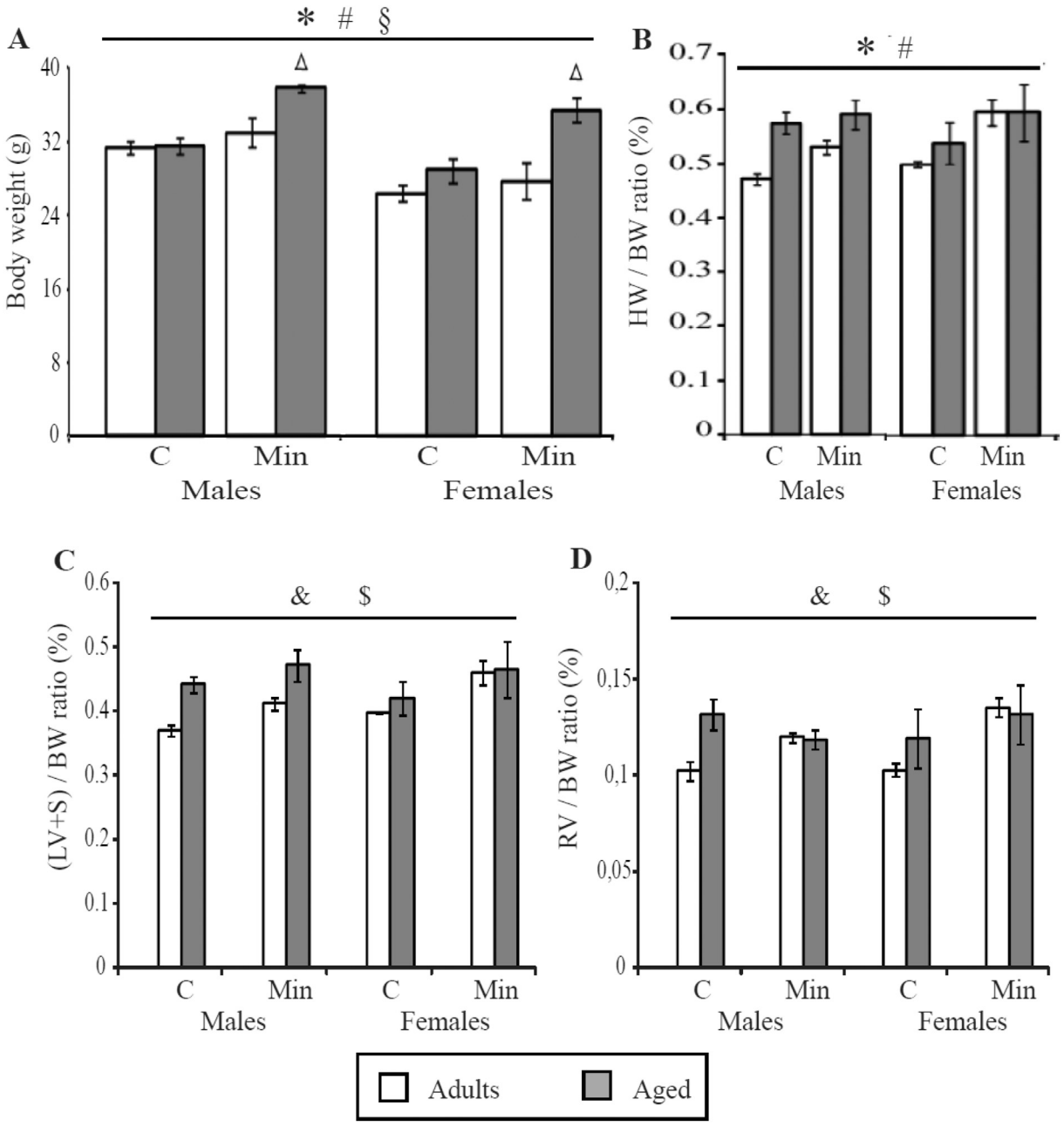

Fig. 1. Comparison of body weight (BW) (A), total heart weight (HW)/BW ratio (B), left ventricular + septum weight (LV + S)/BW (C) and right ventricular (RV)/BW (D) ratios, as a function of treatment (minoxidil or control), gender and age of mice. Values are mean ± SEM. Min: Minoxidil, C: Control (= untreated animals). Δ: significant difference between minoxidil-treated mice and age-matched controls (3-way ANOVA followed by LSD test, $P \leq 0.05$). *,& General significant difference between control and minoxidil-treated mice, independent of age and gender (3-way ANOVA or Mann-Whitney *U* test, respectively, $P \leq 0.05$). #,$ General significant difference between adult and aged mice, independent of treatment and gender (3-way ANOVA or Mann-Whitney U test, respectively, $P \leq 0.05$). § General significant difference between male and female mice, independent of treatment and age (3-way ANOVA, $P \leq 0.05$). n = 4–8 in each group.

The abdominal aorta was excised and conserved in 4% PFA for one night at 4 °C and embedded in paraffin. Five-micrometer sections were cut, deparaffinized with xylene, hydrated in ethanol, and then rinsed with water. Two typical stains were used: Haematoxylin-Eosin-Safran (HES) staining for cytoplasm, nuclei and collagen, and Weigert (resorcin-fuschin) staining for elastic fibers. After staining, the tissue sections were examined under a light microscope (Nikon, France) and elastic lamellae disruptions were counted under microscope (40× objective). In order to obtain the number of disruptions per elastic lamella in a given vessel, we ratioed the total number of lamella disruptions to the number of elastic lamella in the same vessel.

2.7. RNA analyses

Total RNA was extracted from thoracic descending aorta with the E.Z.N.A.® total RNA kit I (Omega Biotek, Inc., Norcross, GA, USA) and genomic DNA was digested with DNase I. Gene expression levels were evaluated by real-time PCR using a CFX96® real-time system with the IQ™ SYBR® Green supermix (Bio-Rad, Marnes-la-Coquette, France) after oligodT- primed reverse transcription of 200 ng of total RNA. Expression of mRNAs were normalized against total mRNA, quantified using the Quant-iT™ Ribogreen® RNA assay kit (InVitrogen, Cergy-Pontoise, France). Amplification primers for the tested gene (tropoelastin) were: 5'-AAGCTGCTGCTAAGGCTGC-3′ (antisense) and 5'-TGC

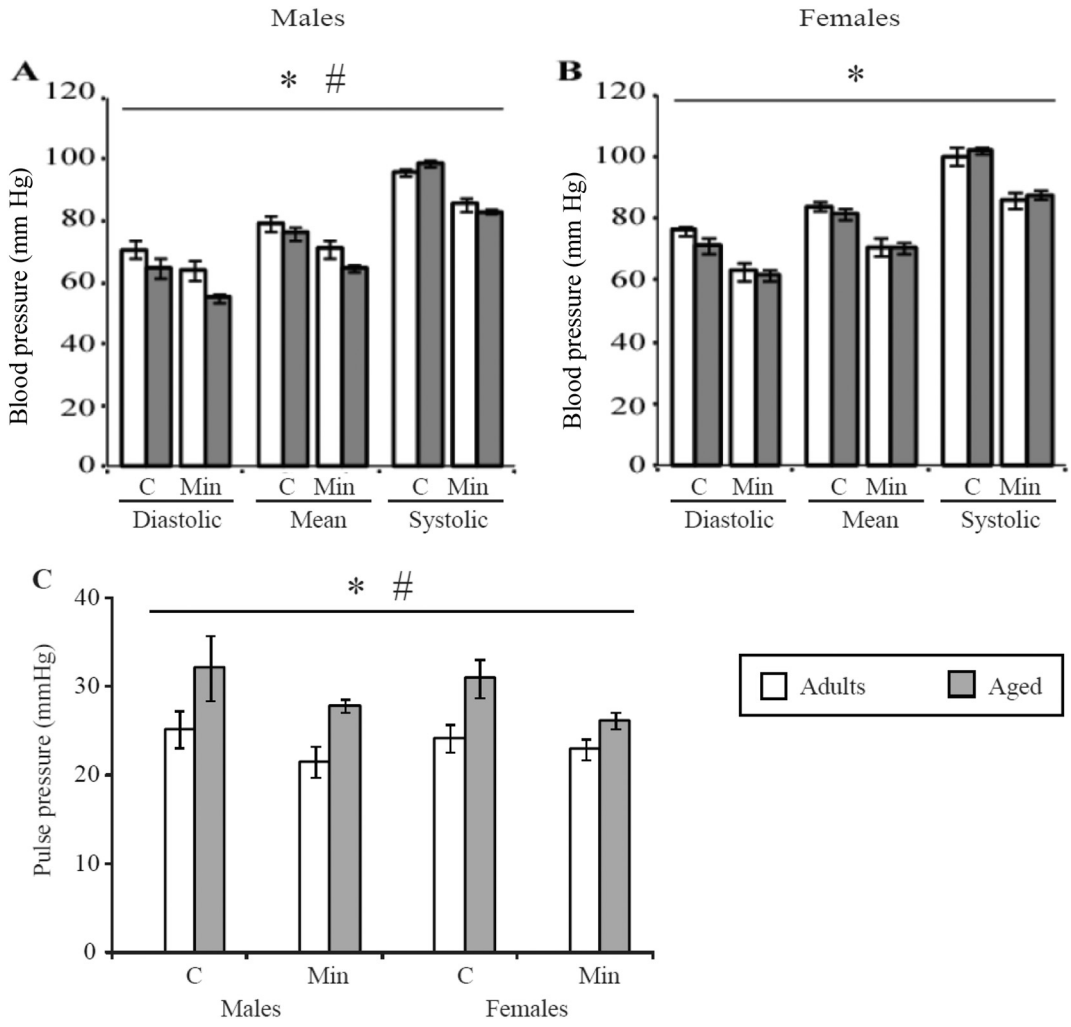

Fig. 2. Effect of minoxidil treatment and age on blood pressure (A and B) and pulse pressure (C) in male and female mice. Pulse pressure = Systolic Pressure - Diastolic Pressure. Min: Minoxidil, C: Control (= untreated animals). Values are mean ± SEM. *General significant difference between control and minoxidil-treated mice, independent of age (3-way ANOVA, $P \leq 0.05$). # General significant difference between adult and aged mice, independent of treatment (3-way ANOVA, $P \leq 0.05$). n = 4–6 in each group.

AACTCCTCCACCTGGGAA-3′ (sense) [3].

2.8. Elastin produced by cultured VSMCs stimulated with minoxidil

2.8.1. Cell culture

Vascular smooth muscles cells (VSMC) were isolated from the mouse thoracic aorta, after removal of the adventitia, by enzymatic digestion with collagenase type 2 (1 mg/mL) and elastase (0.5 mg/mL) for 40 min at 37 °C. The suspension was centrifuged at 600 × *g* for 10 min, and the cells were collected and placed in Dulbecco's modified Eagle's medium (DMEM), containing 20% bovine fetal serum (FBS), 1% (*v*/v) penicillin/streptomycin solution and 1% Non-Essential Amino Acids Solution (NEAA), and maintained in 5% CO2 humidified air, at 37 °C. After confluence, cells were isolated by trypsinization and used between passage numbers 4 to 6.

2.8.2. Extracellular elastin quantification

The protocol established for this study is based on the ELISA technique, as described by Vallet and Wiel (2001). Confluent cultures of VSMC were placed in fresh 1% FBS-DMEM with 1 or 2 mM -final concentration- minoxidil. Minoxidil-sulfate was also used in other *in vitro* experiments, rather than minoxidil, since minoxidil-sulfate is a more active metabolite of minoxidil on vasomotricity (see online data supplement) [35]. Minoxidil-sulfate is normally produced from minoxidil metabolism by the liver in *in vivo* experiments, but minoxidil conversion into minoxidil-sulfate does not spontaneously occur in VSMC cultures. Dexamethasone (0.1 μM) was used as a positive control of elastin expression stimulation [36]. After 48 h, extracellular elastin was quantified by measuring the absorbance of the wells at 450 nm.

Complete protocol for ELISA assays as well as reagent references are described in the online data supplement.

2.9. Statistics

Prior to further statistical analysis, the normality and homogeneity of variance were verified by the use of the Kolmogorov-Smirnov's test and the Levene's test, respectively. The results of these tests are provided in the online data supplement. In the cases where the assumptions of normality and homogeneity of variance were verified, the comparisons of treated and control mice body weights, heart weight/body weight ratios, blood pressure, vessel diameter (OD and ID), wall thickness, distensibility, elastic modulus in response to transmural pressure or vasoactive agonists, morphometric measurements, protein dosages, ELISA assays and mRNA levels were assessed using 1-, 2- or 3-way ANOVA followed when necessary by Fisher's Least Significant Difference test (LSD) for paired value comparisons. When the criteria of normality and/or homogeneity of variance were not met, non-parametric Mann-Whitney *U* tests were used for comparisons. Unless

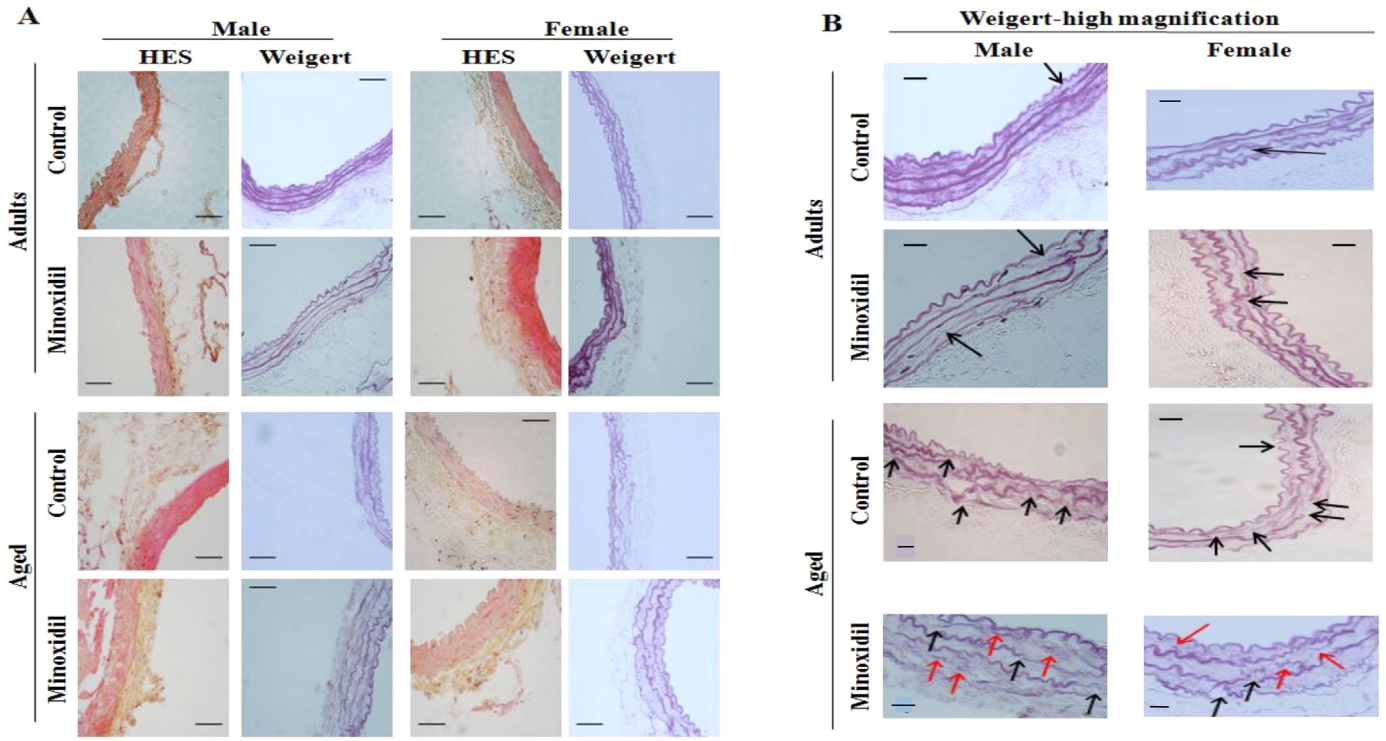

Fig. 3. (A) Histological examination of cross-sections of the abdominal aorta from adult and aged mice (untreated and minoxidil-treated) of both genders, stained with haematoxylin–eosin–safran (HES) or Weigert colorations. Bar size: 50 µm. (B) Cross-sections of the abdominal aorta stained with Weigert coloration at high-magnification. Bar size: 20 µm. Control: untreated animals. Black arrows: elastic lamella disruptions. Red arrows: neosynthesized elastic fibers. HES staining is specific for cytoplasm, nuclei and collagen. Weigert (resorcin-fuschin) staining is specific for elastic fibers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

otherwise indicated, the results are presented as mean values ± SEM, and P values ≤ 0.05 were considered as statistically significant.

3. Results

3.1. Body weight, heart weight and blood pressure

Chronic minoxidil treatment resulted in significant body weight (BW) gain in male and female groups of both ages, when compared to control animals. The impact of minoxidil on BW was of higher amplitude in aged animals (male groups: 31.5 ± 0.9 g *vs.* 37.8 ± 0.3 g; female groups: 28.4 ± 1.3 g *vs.* 34.7 ± 1.3 g) (Fig. 1A). In order to determine whether minoxidil affects cardiac morphology, total heart weight-, left ventricle plus septum weight- and right ventricle to body weight ratios (HW/BW, LV + S/BW and RV/BW, respectively) were measured in all animal groups. Treatment with minoxidil as well as age caused a general cardiac hypertrophy independent of gender (Fig. 1B), by increasing the LV + S/BW and RV/BW, although the effect of age on the RV/BW ratio was minimal in minoxidil treated-mice (Fig. 1C,D). As expected, minoxidil also decreased blood pressure (diastolic, mean and systolic) in all treated groups, independently of age and gender, when compared to untreated groups (Fig. 2A,B). In 24-month-old mice, mean arterial blood pressures (MAP), in control and after minoxidil treatments, were 76 ± 2 mmHg *vs.* 64 ± 1 mmHg in male groups and 82 ± 2 mmHg *vs.* 70.5 ± 1.8 mmHg in female groups, respectively. Pulse pressure (PP = Systolic Pressure - Diastolic Pressure), an index of arterial stiffness [37] underwent an -expected- important age-dependent increase in untreated groups (+27% in males and females). PP was generally decreased by minoxidil treatment and, interestingly, the age-dependent increase in PP was lowered by minoxidil-treated mice in both sexes (Fig. 2C).

3.2. Abdominal aorta morphology

After haematoxylin-eosin-safran (HES) or Weigert (specific for elastic fibers) staining, no clear overall difference could be observed between the abdominal aorta walls of minoxidil-treated and untreated mice, except for an increase in wall thickness induced by minoxidil in aged mice (Fig. 3A). When examining the details of the aorta walls, Weigert staining (specific of elastic fibers), however, revealed the presence of numerous disruptions of the elastic lamellae in aged mice, less in adult animals (Fig. 3A,B). The number of elastic lamellae (≃4) in the media of the abdominal aorta was unchanged by aging (Mann-Whitney *U* test: males P = 0.23, females P = 0.81) and treatment (Mann-Whitney U test: males P = 0.26, females P = 0.08) (Fig. 4A, B). Of particular interest, many additional neosynthesized elastic fibers were present in the aortic interlamellar spaces of aged female and male mice treated with minoxidil, when compared to their controls (Fig. 3B). These neosynthesized elastic fibers were rare or absent in the aorta wall from adult mice of both genders. Surprisingly, in both genders, treatment with minoxidil also significantly reduced the elastic lamella disruptions observed in aortic elastic lamellae of mice (Figs. 3B and 4C,D). In aged mice, the numbers of disruption per elastic lamella of minoxidil-treated mice (*vs.* age-matched controls) were 3.87 ± 0.06 *vs.* 5.16 ± 0.44 (males) and 3.24 ± 0.33 *vs.* 4.61 ± 0.33 (females), respectively (Fig. 4C,D). These results indicate that chronic treatment with minoxidil both protects pre-existing elastic fibers and induces elastic fiber neosynthesis.

3.3. Arterial desmosine, hydroxyproline and total protein contents

The degree of aortic cushioning (the conversion of pulsatile flow into a continuous flow) is dependent on the amounts and proportions of elastin and collagen in the aorta. Desmosine and hydroxyproline contents were therefore measured in the left carotid artery, as indicators of

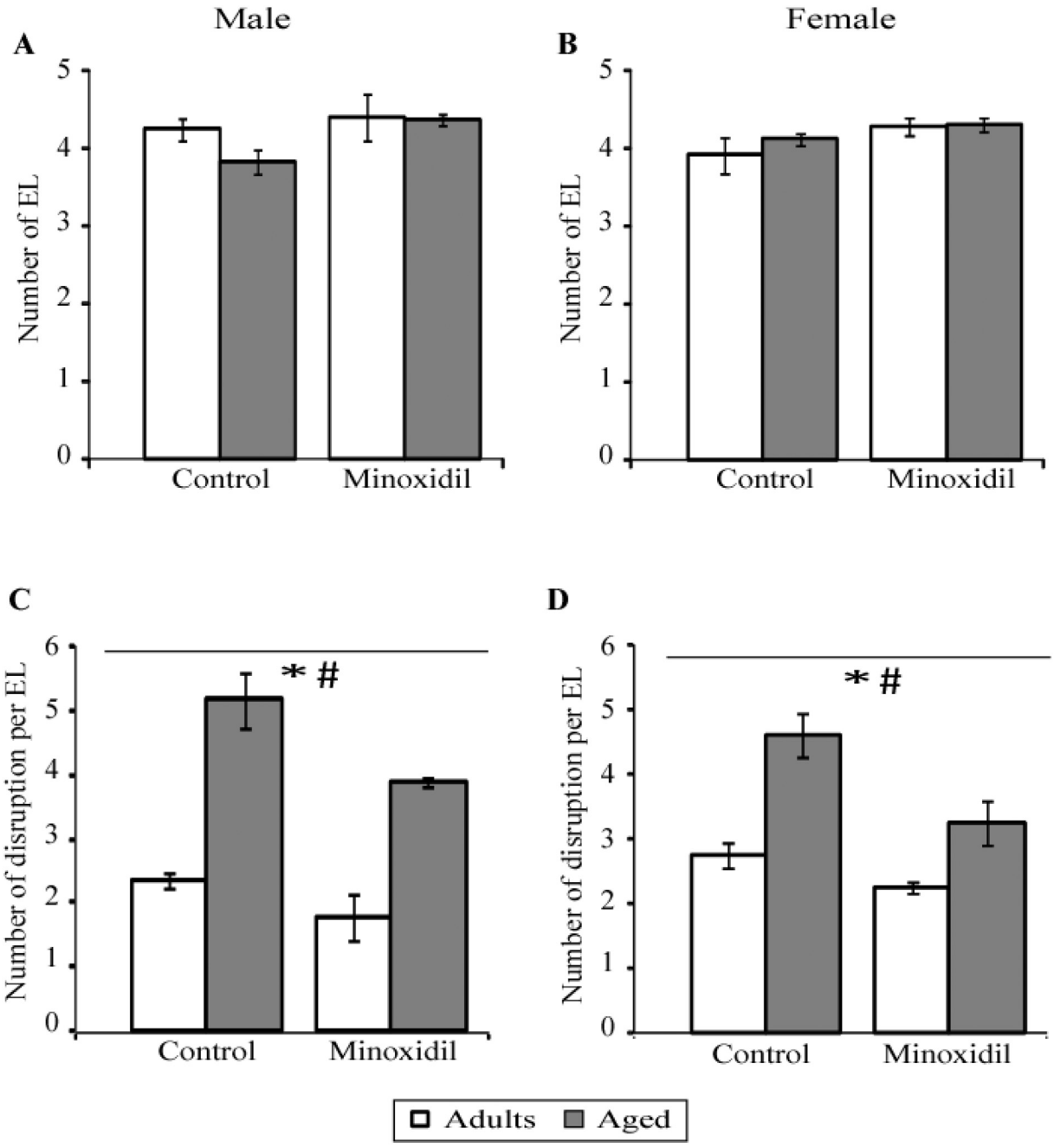

Fig. 4. Morphometric parameters derived from the histological sections of the abdominal aorta wall. Number of elastic lamellae (A and B) and total number of ruptures per elastic lamella (C and D) in the abdominal aorta of adult and aged mice of both genders, untreated (controls) or chronically treated with minoxidil. EL: Elastic lamella. Values are mean ± SEM. Male mice: A, C, female mice: B, D. *General significant difference between control and minoxidil-treated mice, independently of age (males: Mann-Whitney *U* test, $P \leq 0.05$; females: 2-way ANOVA, $P \leq 0.05$). # General significant difference between adult and aged mice, independently of treatment (males: Mann-Whitney U test, $P \leq 0.05$; females: 2-way ANOVA, $P \leq 0.05$). Measurements were made in aortae from 3 different animals in each group.

elastin and collagen contents, respectively. In male mice, surprisingly, desmosine content increased with age independently of treatment, but no significant change in desmosine, hydroxyproline or total protein levels was observed in response to chronic minoxidil (Fig. 5A-C). In female mice, minoxidil treatment generally increased desmosine and hydroxyproline levels -not total protein- in the arterial wall, independently of age (Fig. 5G, H, I), despite a trend (LSD test, P = .06) towards an age-related increase in total protein content in untreated females only (Fig. 5I). To better understand the functional significance of the differences in collagen and elastin levels, both measurements were normalized to the total protein content of the vessel wall, in order to provide an indication of the local matrix composition. Minoxidil increased the desmosine to total protein ratio independently of age (possibly because of both increased desmosine and slightly decreased total protein levels in aged animals) in females, not in males (Fig. 5D,J). Also, aging increased the desmosine to total protein ratio independently of the treatment in female mice, not in males (Fig. 5D, J). No clear change in hydroxyproline to protein ratio could be detected in all groups (Fig. 5E,K). Desmosine to hydroxyproline ratio, an index of elasticity, showed a strong trend towards elevation in females after a 3-month treatment by minoxidil (LSD test, P = 0.07), although not reaching the significance threshold, while it was clearly unchanged in

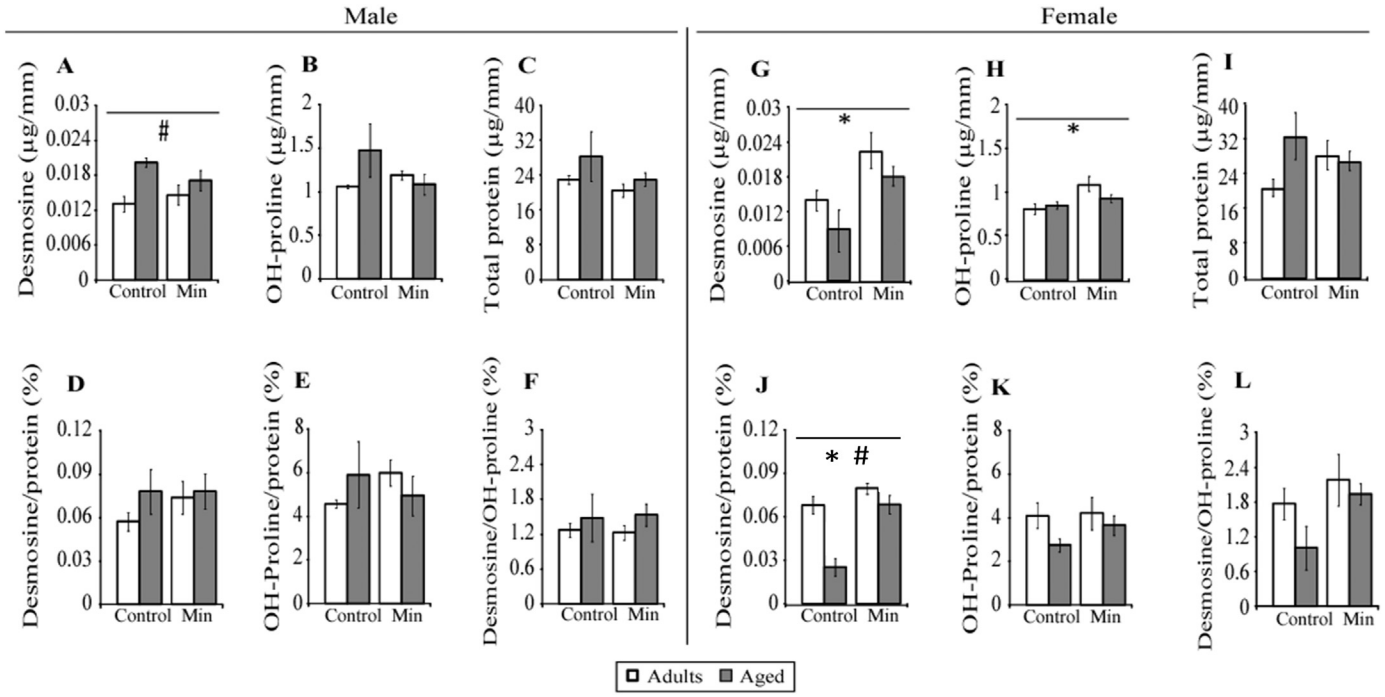

Fig. 5. Desmosine, hydroxyproline and total protein levels in the carotid artery of adult and aged mice untreated or chronically treated with minoxidil, males (A-C) or females (G-I). Desmosine, hydroxyproline and total protein levels are expressed as mass per artery segment length unit (µg/mm). The desmosine and hydroxyproline ratios relative to total protein content were presented for male (D, E) and female (J, K) animals. The desmosine to hydroxyproline ratios, indicative of tissue elasticity, were presented for male (F) and female (L) animals. OH-proline: Hydroxyproline, Min: Minoxidil. Males: A-F, females: G-L. Values are mean ± SEM. *General significant difference between controls (= untreated) and minoxidil-treated mice, independently of age (2-way ANOVA, $P \leq 0.05$). # General significant difference between adult and aged mice, independently of treatment (2-way ANOVA, $P \leq 0.05$). n = 4–6 per group.

male mice (Fig. 5F,L).

3.4. Abdominal aorta mechanics

The impacts of minoxidil treatment on the abdominal aorta mechanics of adults and aged mice of both genders was investigated by correlating the arterial inner (ID) and outer (OD) diameter values to the transmural pressure (0 to 175 mmHg). Virtually all minoxidil-treated mice were found to undergo structural modifications in the arterial wall, with OD, ID and wall thickness generally higher than those of corresponding control mice in animals of both genders and ages (Fig. 6A-F). The only exception was the absence of significant change in ID following minoxidil treatment in female mice, despite a trend towards ID elevation ($P = 0.17$) (Fig. 6D). A general age-dependent enlargement of the aortic outer diameter and increase in wall thickness was also observed in female mice (Fig. 6B,F), not in males (Fig. 6A,E).

The mechanical parameters derived from the diameter- or wall thickness-pressure relations were then calculated. Distensibility, an index of aortic elasticity [38], was significantly reduced by aging in male mice, not in females, independently of treatment, especially at physiological pressures (Fig. 7A,B). Chronic treatment with minoxidil generally increased the distensibility of the abdominal aorta in females, not in males, independently of age (Fig. 7A,B). Consistently, in the 50–150 mmHg pressure range (more physiological, excluding the extreme non-physiological 0–25 mmHg and 150–175 mmHg intervals), the abdominal aorta presented a significantly reduced elastic modulus (Einc, indicative of vessel wall stiffness) after minoxidil treatment in female mice, not in males, independently of age (Fig. 7C,D). The impact of minoxidil was below the statistical significance threshold when the whole 0–175 mmHg range was taken into account. This is because of the very low and virtually identical Einc values in all groups in the 0–25 mmHg interval, and because of the variability of Einc in minoxidil-treated female groups (adult and aged) in the extreme 150–175 mmHg interval. In the abdominal aorta of minoxidil-treated aged mice of both genders, as well as minoxidil-treated adult males (not females), the circumferential stress values were decreased at any given strain, probably due to the increased wall thickness in these animals compared to age-matched controls. In particular, in minoxidil-treated aged groups, this led to a right-shift of the strain-stress curve, closer to that found in younger adult mice (Fig. 7E,F).

3.5. Ex vivo response of the abdominal aorta to vasoactive agents

The abdominal aorta of mice treated with minoxidil presented a decreased vasoconstriction in response to phenylephrine when compared to that of untreated mice, independently of age and gender. In aged mice, the vasoconstriction percentages decreased from 36% (males) and 40% (females) in control animals to 23% (males) and 22% (females) in minoxidil-treated mice (Fig. 8A, B). However, treatment with minoxidil had no significant effect on the endothelium-dependent relaxation of the aorta in response to acetylcholine, independently of age and gender (Fig. 8C, D). Besides, it could be observed that aging significantly decreased the Ach-induced vasodilation in males ($P \leq 0.05$) and induced a strong trend towards a reduced response to the acetylcholine-induced vasodilation in females ($P = 0.08$), independently of treatment (Fig. 8C, D).

3.6. Impact of treatment with minoxidil on elastin production by cultured VSMCs and tropoelastin gene expression in vivo

Elastin is synthesized and secreted by VSMCs, conferring elasticity to blood vessel wall. After addition of minoxidil to cultured VSMCs for 48 h, a significant increase in elastin production was observed at both tested doses, 1 mM minoxidil inducing an elevation close to that triggered by the known elastin expression inducer dexamethasone [36] (Fig. 9A).

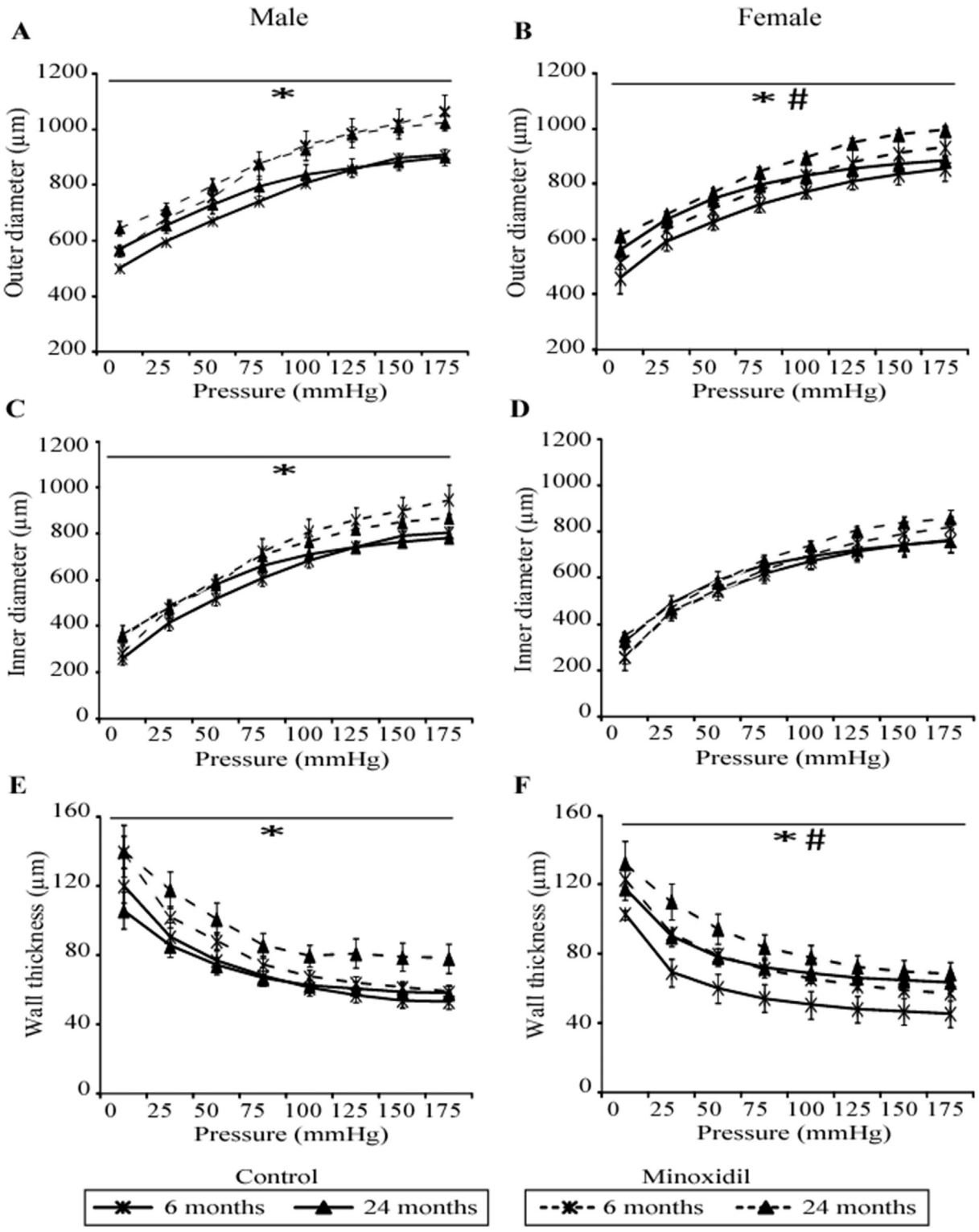

Fig. 6. Diameter-pressure and wall thickness-pressure curves in the abdominal aorta. The outer diameter (A and B), inner diameter (C and D) and wall thickness (E and F) of the abdominal aorta are presented as a function of intraluminal pressure in adult and aged mice, untreated and minoxidil-treated, of both genders. Male mice: A, C, E, female mice: B, D, F. *General significant difference between control (= untreated mice) and minoxidil-treated mice, independently of pressure and age (Mann-Whitney *U* test, $P \leq 0.05$). #General significant difference between adult and aged mice, independently of pressure and minoxidil treatment (Mann-Whitney U test, $P \leq 0.05$). n = 4–6 per group.

In addition, gene expression of tropoelastin was quantified. Chronic treatment with minoxidil induced a strong trend towards increase in tropoelastin mRNA level in male mice, not females, independently of age (2-way ANOVA, P = 0.08) (Fig. 9B). Nevertheless, a trend towards elevation of tropoelastin mRNA level after minoxidil treatment was observed in young females only, not in aged females (1-way ANOVA, P = 0.14) (Fig. 9C).

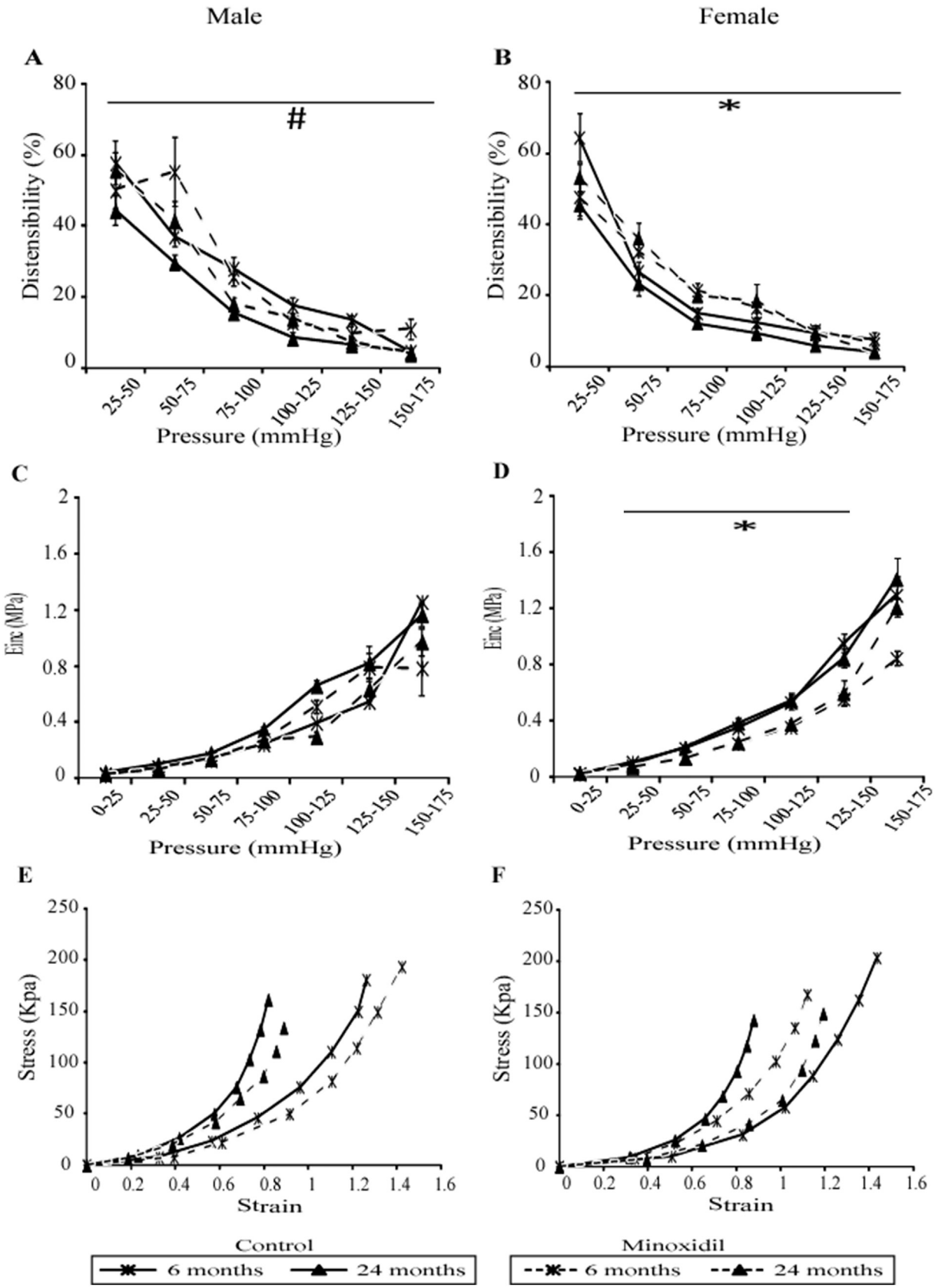

Fig. 7. Effect of minoxidil treatment and age on the distensibility (A and B), incremental elastic modulus (Einc) (C and D) and stress-strain relation (E and F) in the abdominal aorta from mice of both genders. Male mice: A, C, E, female mice: B, D, F. *general difference between control and minoxidil-treated mice, independently of pressure and age (Mann-Whitney U test, $P \leq 0.05$). #General significant difference between adult and aged mice, independently of pressure and minoxidil treatment (Mann-Whitney U test, $P \leq 0.05$). n = 4–6 per group.

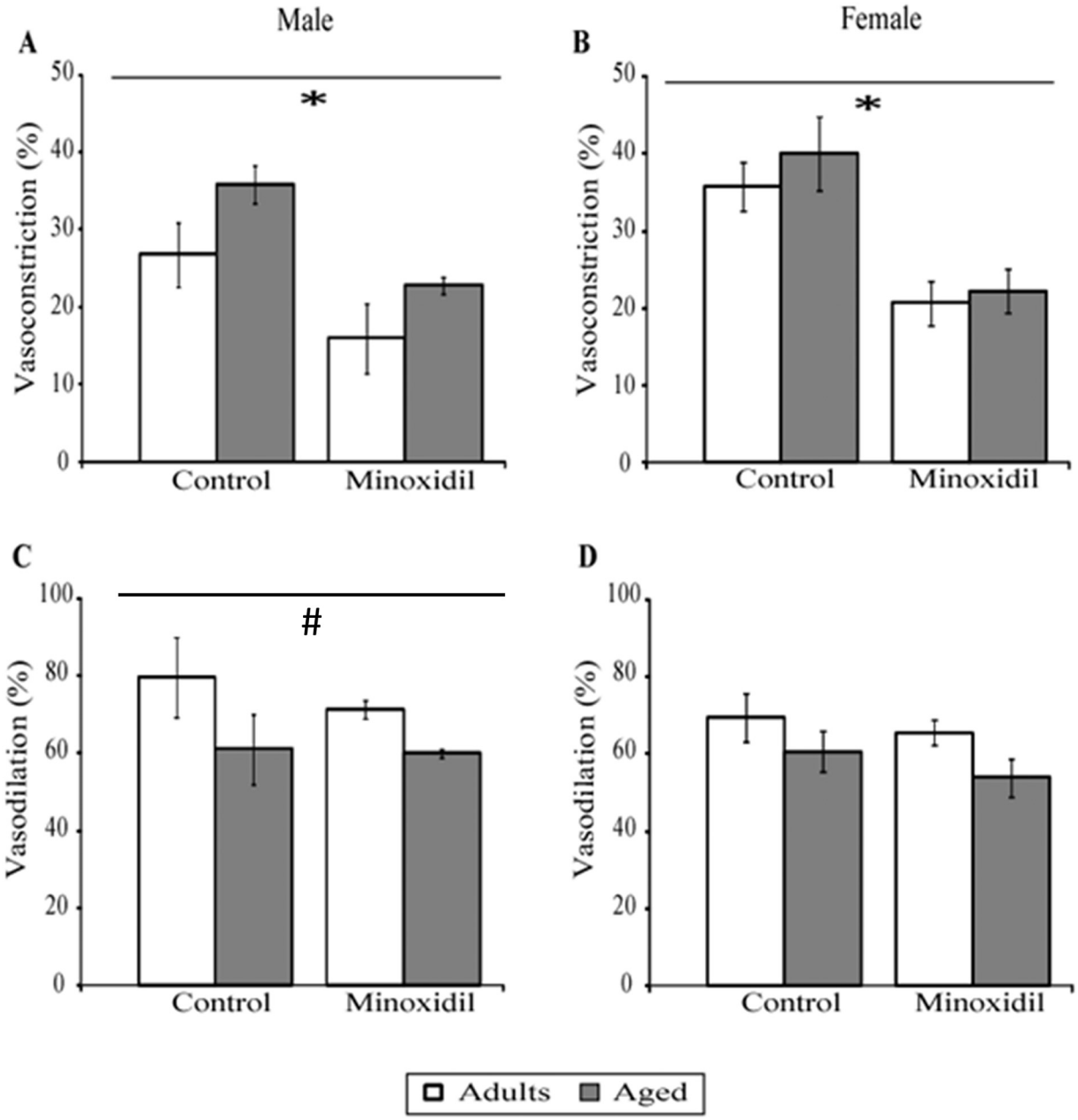

Fig. 8. Effect of age and treatment with minoxidil on the abdominal aorta reactivity in response to vasoactive agonists in adult and aged male (A,C) and female (B,D) mice. A and B: relative decrease in arterial inner diameter due to the action of the vasoconstrictor phenylephrine (10^{-5} M). C and D: relative restoration of the diameter from the level induced by the action of phenylephrine, due to the action of the vasodilator acetylcholine (10^{-5} M). *General significant difference between control and minoxidil-treated mice, independently of age (2-way ANOVA $P \leq 0.05$). #General significant difference between adult and aged mice, independently of pressure and minoxidil treatment (2-way ANOVA $P \leq 0.05$). n = 4–6 per group.

4. Discussion

Elastin synthesis and elastic fiber formation in blood vessels occurs during development and child growth, and is scarce in adults. In human beings, age-related dysfunction of elastic arteries is associated with aneurysms, hypertension and vascular stiffness [3,39], leading to altered hemodynamics and increased risk of organ dysfunction. Hence, a strategy for stimulation of elastic fiber production in adult and aged persons could lead to a decrease in cardiovascular dysfunction and the age-dependent pathologies linked to improper organ perfusion. Minoxidil, an anti-hypertensive ATP-dependent K^+ channel opener, is known to stimulate elastin expression in vascular smooth muscle cells *in vitro* [18], as well as *in vivo* in young male rats [20,22] and young mice genetically deficient for elastin [21]. Minoxidil has also recently been shown to stimulate elastin production and elastic fiber neosynthesis in the thoracic aorta of aged male mice [27]. Because some aging processes are different in males and females [28,29], we have compared the effects of chronic treatment with minoxidil, in young and aged male and female mice, on the functional and structural properties of the abdominal aorta. We have paid particular attention to the efficiency of minoxidil to stimulate the elastic fiber synthesis *in vivo*, which does not normally occur after the end of growth, *i.e.* during adulthood or aging. This study shows that minoxidil was able to improve the aortic structure and function of the treated mice, with some gender differences.

First, our results indicate that a mild cardiac hypertrophy is induced by minoxidil (2–17%, depending on the group), as expected, in a similar range to that observed in the rat [20]. Treatment with minoxidil also reduces the number of elastic lamella disruptions in the abdominal

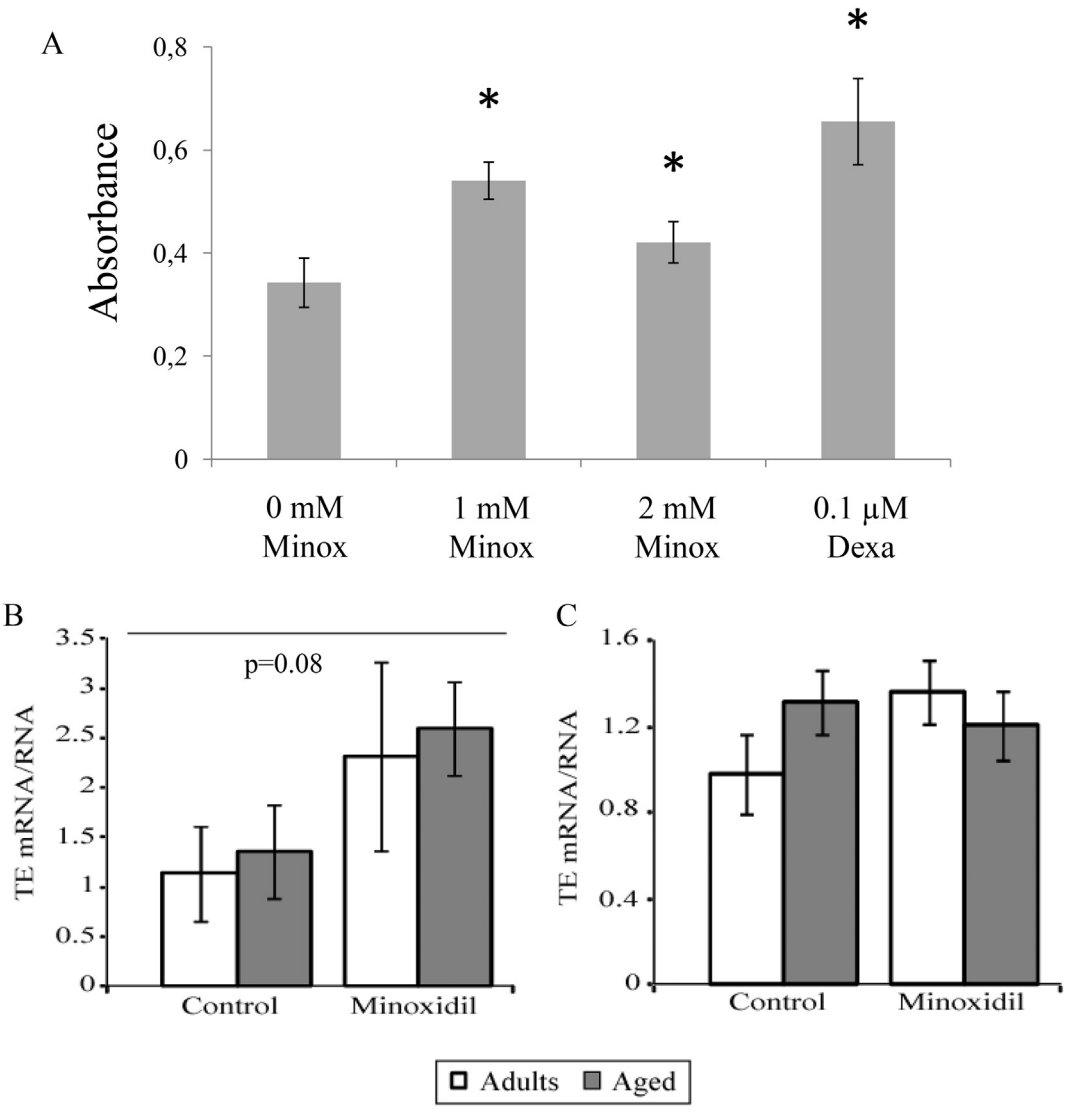

Fig. 9. Impact of treatment with minoxidil on elastin production by cultured VSMCs and tropoelastin gene expression *in vivo*. Effect of minoxidil on elastin production by cultured vascular smooth muscle cells (A). Dexamethasone (Dexa) was used as positive control. Minox: minoxidil. The absorbance is representative of extracellular elastin quantity. n = 8–11 in each group. Tropoelastin (TE) gene expression was measured in the descending aorta from adult and aged male (B) and female (C) mice, untreated or treated with minoxidil. n = 4–6 per group. *Significantly different from 0 mM minoxidil (2-way ANOVA followed by LSD tests, $P \leq 0.05$). p = 0.08 in panel B indicates a general trend towards a difference between untreated and minoxidil-treated mice, independently of age (2-way-ANOVA).

aorta by 16–27% in male and female mice. Since the main causes of elastic fiber fragmentation are mechanical (fatigue failure) and enzymatic (driven by MMP and other enzyme activities) [11], minoxidil treatment could make the elastic fiber more resistant to mechanical fatigue and/or enzymatic digestion. This hypothesis is supported by previous results showing that chronic minoxidil decreases tissue elastase activity in the abdominal aorta [22]. Moreover, our previous *in vitro* data show that minoxidil substantially decreases (−20%) the capacity of elastin to form advanced glycation end products (AGEs) [27], which are known to trigger rapid generation of reactive oxygen species (ROS) and up-regulation of inflammatory pathways resulting in MMP transcription and perturbations of elastic fiber homeostasis [40]. This is consistent with the previous finding that minoxidil application reduces the production of ROS in the aorta [41], which may limit inflammation. Thus, minoxidil might play a multiple protective role for elastic fibers by reducing both deleterious elastin crosslinking with AGEs and elastase activities, which decrease arterial elasticity, and reducing ROS production, therefeore potentially contributing to the limitation of inflammation-related aging (inflamm'aging). Furthermore, numerous neosynthesized elastic fibers were observed after chronic minoxidil treatment in the aorta wall of aged mice from both genders, in accordance with the observations that minoxidil treatment tends to enhance tropoelastin mRNA levels *in vivo* (except in aged females, but desmosine levels were increased by minoxidil in females) and elastin synthesis in cultured VSMCs. This is consistent with previous studies which have showed that treatment with minoxidil increases mRNA levels of tropoelastin and lysyl-oxidase (LOX, cross-linking enzyme which is a major contributor to elastic fiber assembly), and elevates elastin production or arterial content [18–20,22,27]. This is also consistent with the fact that chronic treatment with another potassium channel opener, nicorandil, increases the number of elastic lamellae and elastin proportion in the aorta wall of adult rats [24]. Taken together, these data show that minoxidil not only induces an increase in elastin production but also triggers the complex multi-molecular processes which are necessary for the assembly of mature elastic fibers, even in aged animals in which elastic fiber synthesis is normally stopped. The mechanism by which minoxidil induces neosynthesized elastic fiber production is probably related to its action on K^+ channels and the Ca^{2+}-ERK (extracellular signal-regulated kinases)–dependent pathway [42]. Minoxidil, through induction of K^+ efflux and subsequent

hyperpolarisation of VSMCs, closes the voltage-dependant membrane calcium channels and thereby decreases intracellular calcium [43], which stimulates elastin expression. This has been demonstrated by treatment of VSMC with A23187, a calcium ionophore triggering intracellular calcium level elevation, which induces ERK1/2 phosphorylation and, consequently, a decrease in elastin pre-mRNA and mRNA levels [42].

Regarding the blood vessel biomechanics, it is known that collagen fibers mediate arterial stiffness at high pressure, while elastin provides support and elasticity at a lower pressure [39,44]. Because of the aging process, elastin itself becomes stiffer, due to disruptions, calcification and formation of AGE-related cross-links, a process that affects collagen even more strongly [4,11]. Hence, age-related alterations of elastic lamellae likely lower the mechanical role of elastic fibers (elasticity/distensibility) and increase the role of the collagen fibers (stiffness) in arteries in the lower pressure range. Interestingly, our data indicate that chronic treatment with minoxidil in aged mice induces an arterial enlargement in both genders, consistent with the literature [21,27]. Our results also show that minoxidil treatment increases aortic distensibility and reduces aortic stiffness (Einc) at physiological pressures in female mice, independently of age, not in males (except for a trend in aged males). Two separate actions of minoxidil may be involved in these changes. First, the improvement of the aortic biomechanics might be due to the minoxidil-induced enhancement of neosynthesized elastic fiber production and/or the protection of elastic lamellae. A second explanatory mechanism might be related in part to the anti-hypertensive effect of minoxidil treatment due to its vasorelaxant properties, since pulse pressure, an index of arterial stiffness [45], is markedly decreased after minoxidil. The minoxidil-triggered decrease in aortic stiffness in female mice of both ages, absent in males (except for a trend in aged males), could possibly be explained by the increase in elastin/total protein ratio and the strong trend towards an increase in the elastin/collagen ratio, representative of elasticity or distensibility, observed only in female mice.

Finally, minoxidil generally decrease the contractile response to phenylephrine in all groups of mice, likely due to the desensitization of the adrenergic receptors after a long treatment with minoxidil (3 months). Actually, it has previously been shown that minoxidil activates the adrenergic nervous system [46], which leads to chronic stimulation of the adrenergic receptors causing their desensitization [47]. This long term effect of minoxidil could be beneficial by limiting the adrenergic/sympathetic nervous system-induced reduction of arterial compliance during aging [48].

The present study demonstrated that chronic treatment with minoxidil improved elastic lamellae integrity in all animal groups, re-induced elastic fiber synthesis in aged animals of both genders and improved abdominal aorta biomechanics in young and aged female mice. In addition to its anti-hypertensive and anti-hair loss effects, minoxidil could be considered as a potential new anti-arterial aging molecule. Minoxidil could also be an interesting agent for future female-targeted therapies of arterial dysfunctions.

Declaration of competing interests

The authors declare that they have no conflict of interest in the work presented in this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.05.018.

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