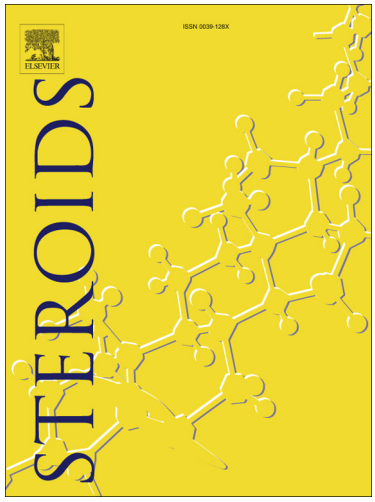

Nandrolone combined with strenuous resistance training reduces vascular nitric oxide bioavailability and impairs endothelium-dependent vasodilation.

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ABSTRACT

Anabolic Androgenic Steroids (AASs) misuse has increased among adolescents and recreational athletes due to their potential effects on muscle hypertrophy. On the other hand, AAS might induce alterations on cardiovascular system, although some controversies regarding AAS on vascular properties remain unknown. To address this question, we aimed to investigate the effects of high doses of nandrolone combined with strenuous resistance training (RT) on function and structure of thoracic aorta. Rats were randomized into four groups: non-trained vehicle (NTV), trained vehicle (TV), non-trained nandrolone (NTN), and trained nandrolone (TN), and submitted to 6 weeks of treatment with nandrolone (5mg/kg, twice a week) and/or resistance training. *In vitro* response of thoracic aorta to acetylcholine (ACh) was analyzed. Vascular nitric oxide (NO) and reactive oxygen species (ROS) synthesis were evaluated using 4,5-diaminofluorescein diacetate (DAF-2) and hydroethidine fluorescent techniques, respectively. Thoracic aorta was processed for microscopy analyses and tunica media thickness was measured. ACh-mediated relaxation response was impaired in endothelium intact aortic rings isolated from trained rats (TV and TN) as compared with their matched non-trained groups. TN rats showed reduced ACh-mediated vasodilatation than NTN rats. NO production and bioavailability decreased in thoracic aorta of nandrolone-treated rats in relation to their matched non-trained group (NTN *vs.* NTV; TN *vs.* TV). ROS production and tunica media thickness were increased in TN rats when compared with TV rats. These findings indicate that high doses of nandrolone combined with strenuous RT affect NO bioavailability and might induce endothelial dysfunction and arterial morphological alterations.

Key words: nandrolone, resistance training, vasodilatation, nitric oxide, reactive oxygen species.

1. Introduction

The anabolic-androgenic steroids (AAS) are synthetic substances derived from testosterone hormone, which are designed for clinical applications [1,2]. Although numerous epidemiological and case-series studies of AAS misuse have been conducted, either in professional and amateur athletes, the effects of high doses of AAS in recreational athletes are poorly understood. Also, ASS misuse has grown among adolescents boys [3] mainly because they combine AAS intake with resistance training (RT). Inconsistently, AASs have been associated with increased performance due to their substantial effects on muscle hypertrophy and strength, which might evoke interest of adolescents, recreational athletes and casual fitness enthusiasts [4]. Because of its potent anabolic power in comparison with other steroids, nandrolone decanoate (DECA-Deca-Durabolin®) is one of the most used AAS among them [5–7]. Likewise, ASSs are commonly used with no medical support, and can promote serious side effects [8–10]. For example, AAS have been shown to induce alterations on cardiovascular system [11], such as increased blood pressure [12], left ventricular hypertrophy [13,14], cardiac fibrosis [15], myocardial infarction, arrhythmias, stroke [16–18] and death [19]. On the other hand, experimental animals have been shown to be an appropriate subject of study, given that most of studies in humans exhibits methodological limitations [20,21], such as case studies with no placebo-controlled conditions.

We have previously reported that high doses of nandrolone, combined or not with RT affected glycogen content in skeletal and cardiac muscles [22], increased blood pressure, induced cardiac hypertrophy, prolonged the duration of the QTc interval [23], increased ventricular collagen content, reduced cardiac index and alpha-myosin heavy chain gene expression as well as impaired both diastolic and systolic function [24]. Moreover, strenuous RT changed the

proteomic profile of cardiac tissue of rats, inducing the expression of proteins related to heart failure [25]. Even though, controversies regarding to strenuous RT and AAS effects on vascular properties remains unclear [2,26], we demonstrated that high doses of nandrolone blunted aorta subsensitivity to phenylephrine (PE) in rats submitted to RT, suggesting that nandrolone might impair vascular function by nitric oxide (NO) [27]. Whether reactive oxygen species (ROS) generation might be involved in that response is also worth of investigation. Therefore, the aim of this study was to investigate the effects of high doses of nandrolone combined with strenuous RT (high intensity, elevated frequency of training and short rest period) on thoracic aorta relaxation and NO bioavailability (NO/ROS levels) in rats. We hypothesized that nandrolone plus RT might induce endothelial dysfunction in thoracic aorta of rats by inhibiting NO and elevating ROS production.

2. Experimental

Twenty-four male Wistar rats (90 days old) were housed in a temperature-controlled room (22±2°C) with a 12:12-hour light-dark cycle. Water and rodent chow were provided *ad libitum*. After approval by the institutional Committee for Ethics in Animal Research (protocol number 944-1), rats were randomized into 4 groups: non-trained vehicle (NTV), trained vehicle (TV), non-trained nandrolone (NTN), and trained nandrolone (TN). Nandrolone decanoate (5mg/Kg; Organon) or vehicle propilenglycol (0.2 mL/Kg) were injected twice a week, intramuscularly for 6 weeks [22,24,27,28]. Doses of 10mg/Kg have been suggested as high doses of nandrolone for rats, usually 10-100 fold higher than therapeutic doses for humans [2,29–31].

2.1 RESISTANCE TRAINING PROTOCOL

The exercise protocol has been previously described in detail [22,27]. Animals were adapted to the exercise training protocol (jumping in a cylinder containing the water) during 5 days, carrying a load correspondent to 50% body weight attached to the chest. After the adaptation period, trained rats (TV and TN groups) performed jump sessions protocol during 5 days/week for 5 weeks. The load (fishing sinkers) was attached to the rat's chest not only to avoid flotation but also to make them exercise against an overload [27]. The load was also adjusted for each week of training (50% body weight over the 2nd week of training; 60% body weight over the 3rd and 4th weeks of training; 70% body weight over the 5th week of training). Trained rats performed 4 sets of 10 jumps with 30 seconds of rest between each set of jumps. Rats performed jump sessions in a poly (vinyl chloride) PVC cylinder tank measuring 50cm in height, 25cm in diameter, containing water (30±2°C) to a depth of 38cm. One complete jump was determined when rats reached the top of PVC cylinder and breath.

2.2 AORTA REACTIVITY TO ACh IN VITRO

After 48h the last training session, rats were euthanized and vasodilatation function was assessed in isolated intact thoracic aortic rings as previously described [32]. After 60 min for stabilization, the intact endothelium was assessed by determining the vasodilatation to 10 μmol/L acetylcholine (ACh) in pre-contracted rings with phenylephrine (PE) (0.1 μmol/L). Only aortic rings which presented >70–80% relaxation were used. Aortic rings were rinsed three times with Krebs buffer and allowed to re-equilibrate for 45–60 min. Concentration-response curves to ACh were obtained by cumulative addition of molar concentration of the drug (0.1 nM to 0.1 mM), increasing by one-half log intervals (10^{-10} to 10^{-4} mol/L). Agonist concentration-response curves were fitted using nonlinear interactive fitting software (Graph Pad Prism 5.0, Graph Pad

Software Inc., USA). Maximal responses (Emax) or potencies ($-\log EC_{50}$) to each agonist were expressed as mean ±SEM, and confidence interval respectively.

2.3 4,5 - DIAMINEFLUORESCEIN DIACETATE (DAF-2) - NITRIC OXIDE SYNTHESIS

NO synthesis was evaluated using 4,5-diamino fluorescein diacetate (DAF-2), an NO-sensitive fluorescent dye [33]. Thoracic aorta was quickly dissected, frozen, and embedded in a freezing medium (TBS – Triangle Biomedical Sciences). Transverse sections (7 µm) were obtained on a cryostat, collected on glass slides, and incubated at 37 °C with 12.5 µmol/L of DAF-2 in phosphate buffer (0.1 M – pH 7.4) containing 0.45 mM $CaCl_2$. After 1.5 h, the sections were stimulated with 100 µM of acetylcholine [34]. Control sections received the same volume of phosphate buffer. After additional 1 h, digital images were collected on a microscope (Carl Zeiss, Germany) equipped for epifluorescence and a standard fluorescein filter. The images were analyzed using Image J software (NIH, USA) by measuring the mean optical density of the fluorescence observed in throughout the aorta in relation to the background staining [35].

2.4 HYDROETHIDINE ASSAY - REACTIVE OXYGEN SPECIES (ROS) SYNTHESIS

Different segments of thoracic aorta were used to measure ROS levels. The oxidation sensitive fluorescent dye dihydroethidium (DHE) was used to evaluate the *in situ* concentration of ROS, as previously described [36]. Thoracic aortic were dissected, embedded in a freezing medium and frozen in -80°C until the determination of ROS. Transverse sections (7 µm) were obtained in a cryostat from previously frozen thoracic aortas, collected on glass slides and incubated for 30 minutes at 37°C in phosphate-buffer saline (PBS, 0,1M, pH 7.4). Fresh PBS containing DHE (2,5 µM) was applied to each tissue and incubated for 30 minutes at 37°C. Images were obtained using Nikon E1000 microscope equipped for epifluorescence within a rhodamine filter

(excitation at 488 178 nm; emission at 610 nm). The images were analyzed using Image J software (NIH, USA) by measuring the mean optical density of the fluorescence observed throughout the aorta in relation to the background staining [37]..

2.5 AORTIC THORACIC WALL THICKNESS

The bottom portion of thoracic aorta was isolated to microscopy analyses. Tissues were fixed in Karnovsky solution for 6 h at 4° C and post-fixed in 1% osmium tetroxide in phosphate buffer, pH 7,2, for 2 h in room temperature [38]. After, aortas were dehydrated in solutions of acetone (50%, 70%, 90% and 100%) for 10 min and included in resin for 48 h at 60° C. The tissues were cut at 1 µm of thickness in an ultra micrometer (MT2B Sorvall Porter Blum) with glass blades. The cuts were dye with 0,5% blue toluidine and 0,5% sodium borate for 50 seconds and 0,5% fuchsin for 30 seconds. The images were digitalized in a digital camera (Leica, DFC 280) attached to an upright light microscopy (Leica, DM LP) using Leica Application Suite software (LAS Version 2.8.1; 2003-2007) at 20x magnification. Sections of aortas were obtained from five rats per group (n=5) for light microscopy analysis. Tunica media thickness was considered the distance between the internal and external elastic lamina [39].

2.6 STATISTICAL ANALYSIS

Statistical differences were determined by two-way analysis of variance (ANOVA) followed by the post-hoc Tukey test. Differences were considered significant at $p<0.05$. The results are presented as means ± standard error of the mean (SEM).

3. Results

The initial and final body weights (BW) of experimental groups are presented in Table 1. In trained groups, the final BW decreased when compared with their matched non-trained rats (TV *vs.* NTV and TN *vs.* NTN; $p<0.0001$). NTN rats showed lower final BW than that observed in NTV rats (NTN *vs.* NTV; $p=0.01$).

Percentages of maximal relaxation ($E_{máx}$) of thoracic aorta from experimental groups are shown in Table 2. RT reduced significantly ACh-induced vasodilatation of thoracic aortas in vehicle-treated rats (TV *vs.* NTV; $p<0.0001$). Furthermore, TN rats showed impaired ACh-mediated vasodilator responses of thoracic aorta when compared with NTN and TV groups (Figure 1; $p<0.0001$ and $p=0.02$, respectively).

No changes in NO levels of thoracic aorta were observed in basal condition (Figure 2A). After ACh stimulation, vascular NO production significantly decreased in TV rats when compared with non-trained animals (*vs.* NTV; $p=0.003$). NTN and TN groups showed lower NO production than their matched vehicle-treated groups (NTN *vs.* NTV; TN *vs.* TV; $p<0.0001$) (Figure 2B).

ROS levels in thoracic aorta are demonstrated in Figure 3A. While ROS production decreased in TV rats in relation to NTV animals, elevated production of ROS was observed in aortas from TN group when compared with TV rats (Figure 3B; $p=0.008$). Nandrolone induced marked reduction in NO/ROS levels (NTN *vs.* NTV; TN *vs.* TV; $p<0.0001$) (Figure 3C).

Thickness of tunica media of thoracic aorta increased in TV when compared with NTV rats (Figure 4A and 4B). Whereas thinner tunica media was observed in NTN than NTV rats, TN group showed thicker tunica media in relation to NTN rats (TN vs. NTN; p=0.0001).

4. Discussion

This study showed that high-intensity RT, combined or not with high doses of nandrolone, may induce endothelial dysfunction in the thoracic aorta from rats, supported by lower vasodilation response to ACh and reduced NO bioavailability.

The RT reduced the final body weight of rats in relation to non-trained rats indicating that the high-intensity exercise training may alter body composition. The lower final BW of trained animals may be a consequence of increased lipid oxidation following post-exercise recovery period [40], increasing caloric expenditure and reducing body fat [41]. It has been reported either inhibition [42,43] or increase [44] in BW after administration of excessive AASs doses as well as in glucose metabolism [45]. In fact, we observed that nandrolone reduced BW, corroborating with results from a previous study [46].

Along with AAS-related changes in body composition and lipid metabolism [22,27], cardiovascular changes have been documented in consequence of AAS abuse [47]. In this sense, we have previously demonstrated that high doses of nandrolone canceled the RT-induced subsensitivity to PE in thoracic aorta. However the mechanisms involved in this response was not investigated.

Aerobic exercise training has been shown to attenuate endothelium-mediated vasoconstrictor response to PE [48–50] while enhancement of endothelium-mediated vasodilation was observed in animals [51]. Whereas improvement of ACh-induced vasodilation has been reported [52–54], we observed impairment of ACh-induced vasodilation of aorta in trained rats, treated or not with nandrolone (TV *vs.* NTV and TN *vs.* NTN). TV and TN rats exhibited 79% and 51% of maximum relaxation respectively, when compared with their matched non-trained-rats . Differences in exercise training protocols could be contributing to the disparate results. We speculate that RT used in this study might induce different metabolic adaptations than other exercise training protocols for rats, once they performed a strenuous RT (high-intensity with short rest periods during five consecutive days of training) [25]. Our findings are in agreement with studies using high-intensity exercise training protocols [55,56]. Moreover, short rest periods, as commonly used by bodybuilders and recreational athletes, lead to increases in inflammatory process [57], circulating epinephrine, norepinephrine and cortisol [58], which might contribute to our findings. To our best knowledge, this is the first time documented that RT at high intensity and frequency of training along with short periods of rest evoked impairment of ACh-induced vasodilatation in thoracic aortas.

Furthermore, the combination of high doses of nandrolone and RT impaired the ACh-induced vasodilation (Figure 1), which is in agreement with others [50]. In fact, nandrolone has been shown to affect aorta relaxation in rabbits [59] and reduce aortic sensitivity to PE in rats [27]. Accordingly, NO has been suggested as the main modulator of those responses. Therefore, the impairment of ACh-induced vasodilation could be associated with the decreased NO production observed in trained nandrolone-treated rats (Figure 2B and 2C). Indeed, our findings are in accordance with others that showed impaired reactivity to ACh in mesenteric artery, concomitant

to decreases in NO levels [60]. Of note, our previous study reported increased low density lipoprotein (LDL) in rats treated with nandrolone [27], which might support the impaired ACh-mediated vasodilatation described in this study. Increased LDL levels inhibit vasodilator response to ACh in aorta [61] and nandrolone administration has also been shown to abrogate the exercise-induced vascular adaptive response in rats [62]. Thus, we suggest that the impaired vasodilatation in trained rats treated with high doses of nandrolone was, at least in part, due to a decrease of NO bioavailability.

In order to investigate whether the impairment of ACh-induced vasodilation could be related with endothelium-derived relaxing factors, we measured endothelial NO levels in thoracic aorta. In fact, we observed that high doses of nandrolone reduced vascular NO levels in thoracic aorta (Figure 2C). In addition to changes in NO, ROS levels were elevated in response to nandrolone administration (Figure 3A and 3B). Taken together, the concomitant decreases in NO levels and increased generation of ROS could be related to endothelial dysfunction [63,64]. Therefore, the reduction in vascular levels of NO induced by high doses of nandrolone supports our initial hypothesis. Along with decreased NO levels, marked reduction of NO bioavailability could be related with impaired ACh-mediated vasodilatation observed in TN group (Figure 3C). Changes in endothelial growth, apoptosis, and modification of intracellular calcium concentration have been reported in human vascular endothelial cells treated with nandrolone [65]. Truly, Ca^{+} ions are the most important signaling involved on NO synthesis through eNOS activation [66], which could be determinant for nandrolone-induced reduction of NO bioavailability. Alterations in acetylcholine-muscarinic receptor, reduced eNOS activation (reduced nicotinamide adenine dinucleotide phosphate - NADPH and tetrahydrobiopterin - BH4), altered expression and functional activity of eNOS and increased

production of endothelium-derived vasoconstrictors also emerge as potential candidates involved in nandrolone-induced decrease of NO bioavailability [67].

We also hypothesized that aortic muscle structure could be altered in both nandrolone-treated groups, since NO has been demonstrated to be associated with arteriogenesis and angiogenesis [68]. While low concentration of NO seems to favor cell proliferation, high levels of NO induces cell apoptosis [69]. Indeed, we observed increased tunica media thickness of thoracic aorta in TN rats, where NO production was markedly reduced (Figure 4A, 4B and 2C, respectively). In this context, our results are in agreement with those from Sader et al. (2001) [70] who observed increased carotid intima-media thickness in AAS users. For our understanding, no previous studies have reported the effects of high doses of nandrolone in combination with high intensity RT on morphological adaptations of arterial wall.

Moreover, our findings corroborate with others, showing that exercise training triggers smooth muscle hypertrophy [71,72]. Nevertheless, prolonged exercise training has been shown to cause NO-dependent arterial remodeling to normalize the exercise-induced increase in blood flow and the shear stress [73–75]. However, as we also observed reduction in NO production after RT, the increased tunica media thickness after RT could also be explained by decreased vascular NO levels in TV group.

In conclusion, we demonstrated that strenuous RT combined or not with high doses of nandrolone, reduced NO bioavailability, impaired ACh-induced vasodilatation and increases tunica media of thoracic aorta. Our findings suggest that such combination commonly used by recreational athletes might increase the risk of vascular maladaptive responses, which might lead to endothelial dysfunction and morphological alterations in arterial wall.

Declaration of interest

The authors declare that there are no conflicts of interest.

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Tables

Table 1

Initial and final body (BW) weight of experimental groups.

	NTV	TV	NTN	TN
Initial BW (g)	260 ± 18	268 ± 14	256 ± 15	266 ± 13
Final BW (g)	377 ± 28	324* ± 16^{+}	347* ± 25	314$^{\&}$ ± 17

The values are expressed as means ± SEM. Initial BW: body weight of experimental groups before the first RT session. Final BW: body weight of experimental groups 48h after the last session of RT. Groups: non-trained vehicle (NTV); trained vehicle (TV); non-trained nandrolone (NTN), and trained nandrolone (TN). Two-way ANOVA showed significant main effect of nandrolone factor ($p=0.01$) and training factor ($p<0.0001$). Tukey's post-test ($p<0.05$): * vs. NTV; $^{\&}$ vs. NTN, n = 8/group.

Table 2.

$E_{máx}$ of experimental groups.

	NTV	TV	NTN	TN
$E_{máx}$	100 ± 0	78.9 ± 16.6*	97.8 ± 0.8	50.8 ± 8.4$^{\&}$

$E_{máx}$: percentage of maximal relaxation in thoracic aorta of non-trained vehicle (NTV, n=7); trained vehicle (TV, n=5); non-trained nandrolone (NTN, n=8) and trained nandrolone (TN, n=6) rats.Two-way ANOVA showed significant main effect of nandrolone factor ($p=0.02$) and training factor ($p<0.0001$). Tukey's post-test ($p<0.05$): * vs. NTV; $^{\&}$ vs. NTN.

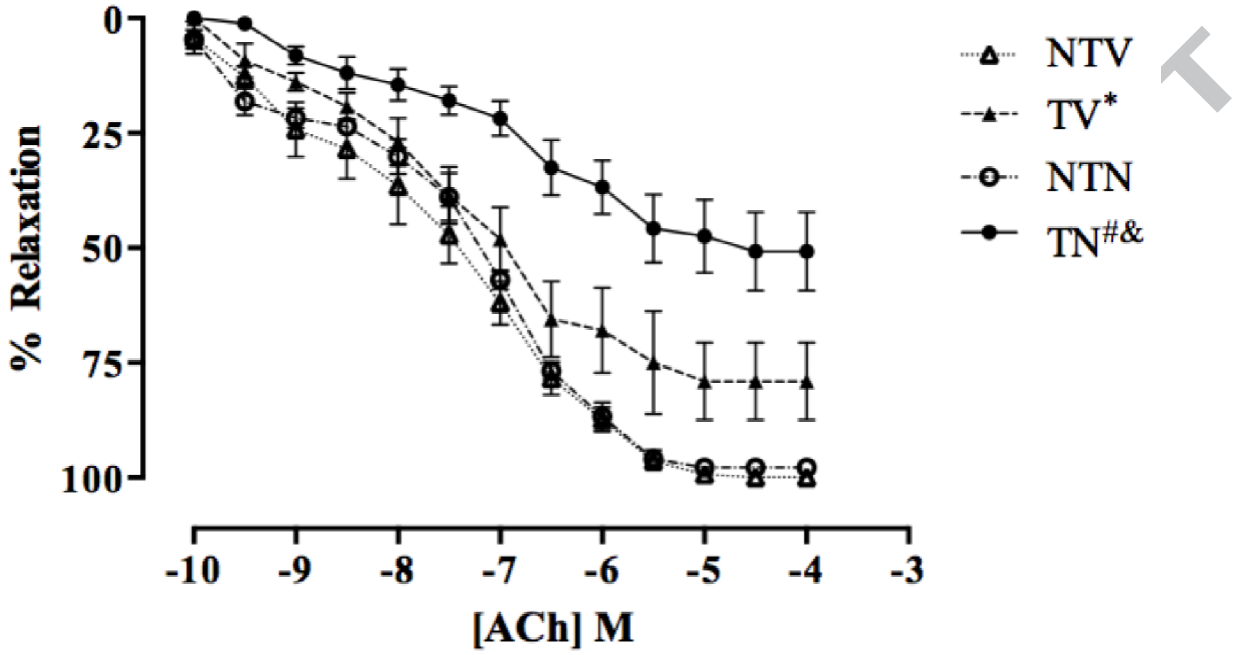
Figures Legends

Fig. 1. Dose–response curves to acetylcholine (ACh) in aortic rings, pre-contract with phenylephrine (PE). Groups: non-trained vehicle (NTV); trained vehicle (TV); non-trained nandrolone (NTN), and trained nandrolone (TN). Two-way ANOVA showed significant main effect of training factor ($p<0.0001$) and nandrolone factor ($p=0.02$). Tukey's post-test ($p<0.05$):.* vs. NTV; $^{\#}$ vs. TV and $^{\&}$ vs. NTN, n = 5/group.

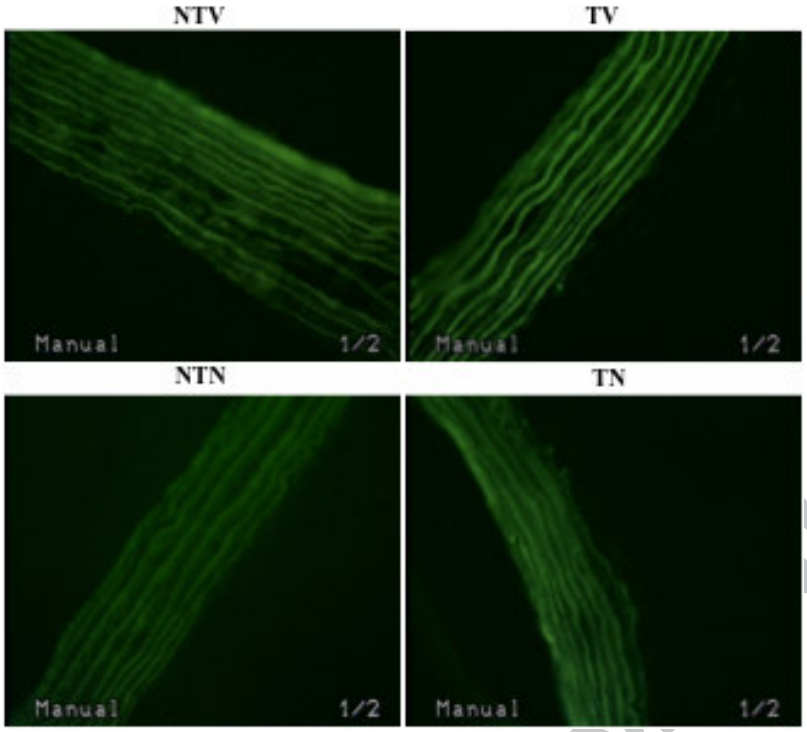
Fig. 2. Longitudinal sections of thoracic aorta treated with dyaminefluorescein diacetate (DAF-2) in basal condition (A) and stimulated with ACh (B). NO levels are expressed as fluorescence arbitrary units (C). The values are presented by means ± SEM. Groups: non-trained vehicle (NTV); trained vehicle (TV); non-trained nandrolone (NTN), and trained nandrolone (TN). Two-way ANOVA showed significant main effect of training factor ($p=0.003$) and nandrolone factor ($p<0.0001$) in B. Tukey's post-test ($p<0.05$):.* vs. NTV; $^{\#}$ vs. TV, n = 5/group.

Fig. 3. Longitudinal sections of thoracic aorta treated with hydroethidine (A). ROS levels (B) and NO/ROS levels are expressed as fluorescence arbitrary units (C). The values are presented by means ± SEM. Groups: non-trained vehicle (NTV); trained vehicle (TV); non-trained nandrolone (NTN), and trained nandrolone (TN). Two-way ANOVA showed significant interaction ($p=0.008$) in B and main effect of nandrolone factor ($p<0.0001$) in C. Tukey's post-test ($p<0.05$):.* vs. NTV; $^{\#}$ vs. TV, n = 5/group.

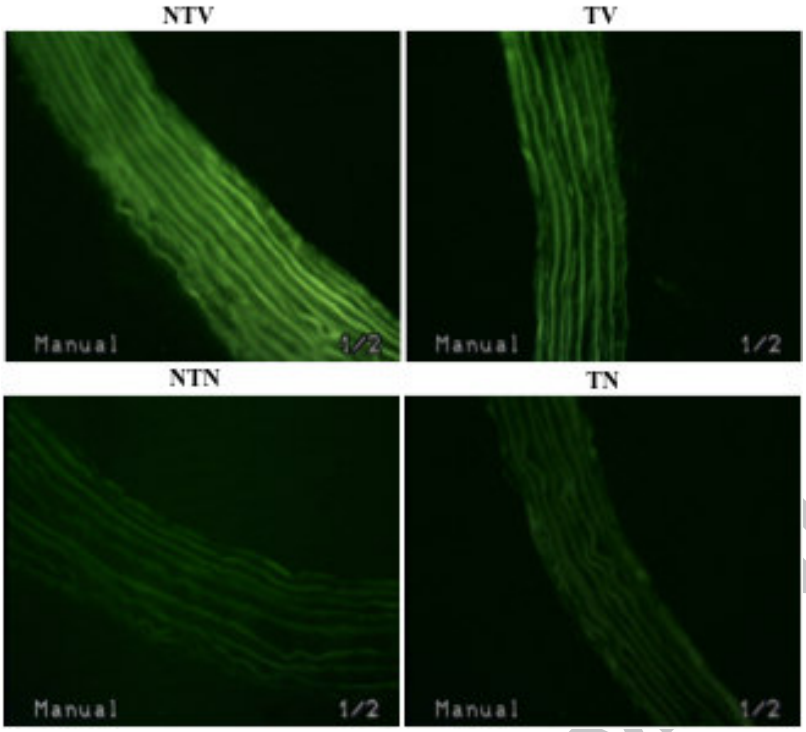
Fig. 4. Images of thoracic aortas at 20x magnification (A). Tunica media thickness (μm) of thoracic aorta (B). The values are presented by means ± SEM. Groups: non-trained vehicle (NTV); trained vehicle (TV); non-trained nandrolone (NTN), and trained nandrolone (TN). Two-way ANOVA showed significant interaction ($p=0.001$) in B. Tukey's post-test ($p<0.05$): * vs. NTV; $^{\&}$ vs. NTN, n = 5/group.



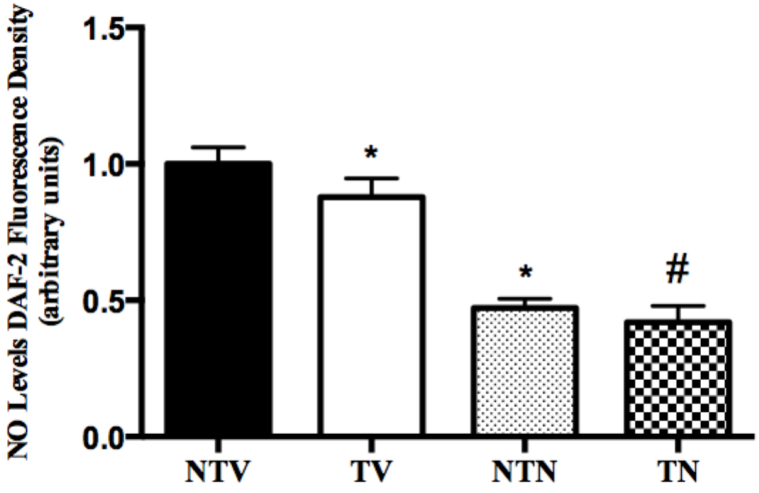
A - basal



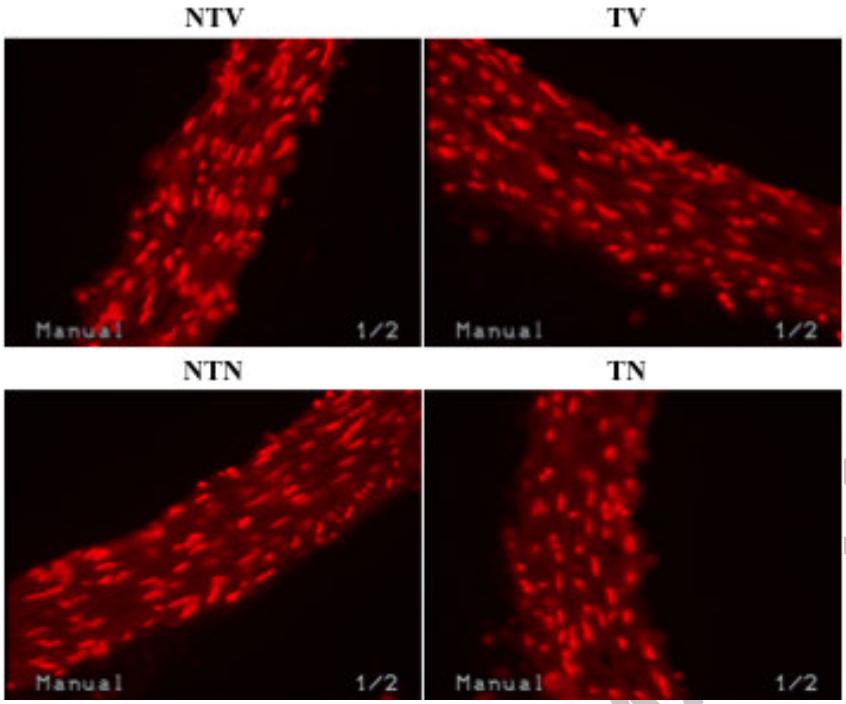
B – stimulated with ACh



C



A



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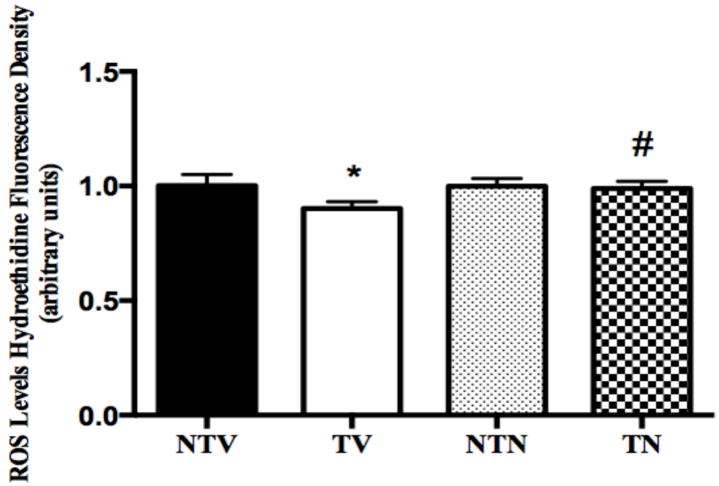

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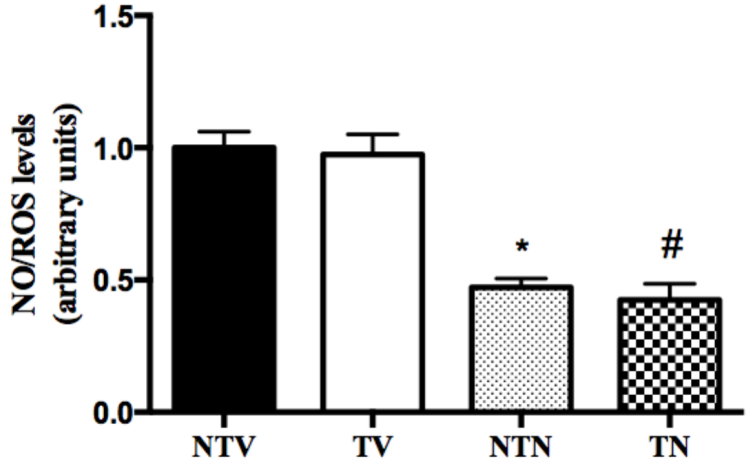
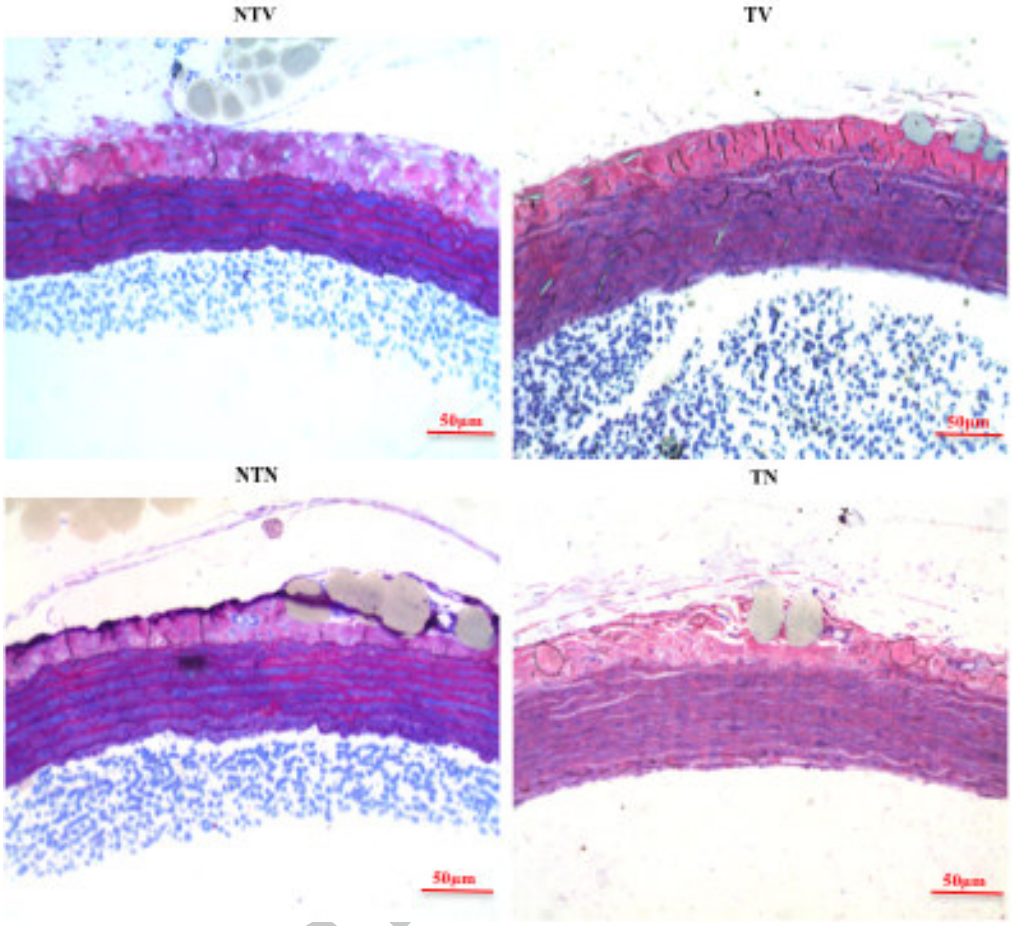
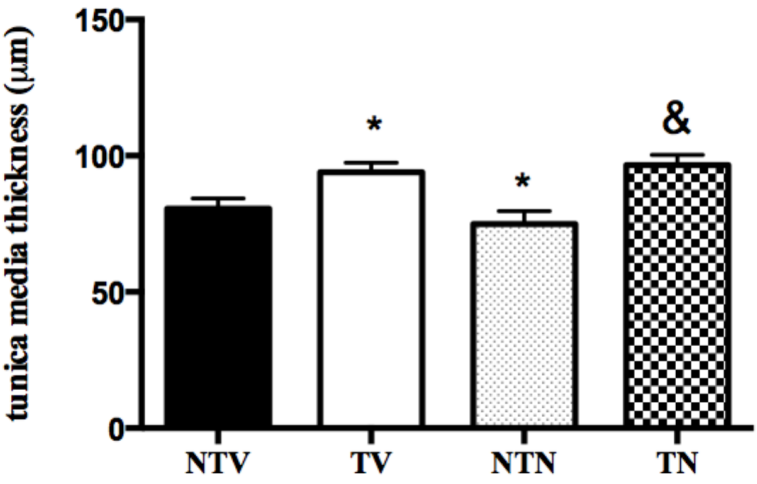


A



B



Highlights

- Nandrolone plus strenuous RT impairs acetylcholine-mediated aorta vasodilation.
- Nandrolone plus strenuous RT increased reactive species of oxygen levels.
- Nandrolone plus strenuous RT dramatically reduced vascular NO bioavailability.
- Nandrolone plus strenuous RT increased arterial wall thickness.
- Combination of nandrolone and strenuous RT might lead endothelial dysfunction.