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Original Research

Androgenic Anabolic Steroids Cause Thiol Imbalance in the Vascular Endothelial Cells

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Abstract

Background: Androgenic anabolic steroids (AASs) are synthetic drugs structurally related to testosterone, with the ability to bind to androgen receptors. Their uncontrolled use by professional and recreational sportspeople is a widespread problem. AAS abuse is correlated with severe damage to the cardiovascular system, including changes in homeostasis and coagulation disorders. AASs alter vascular function by blocking nitric oxide (NO)-mediated dilation, impairing endothelial growth and by potentiating vasoconstrictor signals. **Methods**: This paper demonstrated that long-term use of AASs (nandrolone and boldenone), negatively affects the basic cell functions of vascular endothelial cells. The susceptibility of endothelial cells to AASs depends on the expression of androgen receptors, although cells without androgen receptors can also be affected by high doses of AASs to a limited extent. Seven-day incubation with AASs diminishes endothelial cell proliferation and migration (determined by transwell and scratch migration assay) and monolayer formation (using transendothelial electrical resistance assay). **Results**: Disturbances in cell function were accompanied by downregulation of peroxiredoxins (PRDX1 and PRDX2), involved in maintaining the thiol-disulphide balance. In addition, AASs increased oxidation of the non-enzymatic thiol buffer, glutathione (GSH), reduced secretion of thiol oxidoreductase protein disulphide isomerase (PDI) from endothelial cells and affected the thiol pattern of PDI. **Conclusions**: These changes may be related to a thiol-disulfide imbalance and vascular endothelium dysfunction, that are often correlated with abnormal platelet aggregation, inflammation, increased vascular permeability, and vascular smooth muscle cell proliferation—all of which are observed in athletes who abuse AASs.

Keywords: androgenic anabolic steroids; disulphide bonds; oxidation-reduction reactions; protein disulphide isomerase; thiol groups; vascular endothelium

1. Introduction

The androgenic anabolic steroids (AAS) include the natural testosterone, along with a range of synthetic testosterone variants used predominantly for illegal doping in sport. The long-term side effects of steroid supplementation are related to the chemical structure of AAS, their dosage and frequency of use, concurrent use of other performance-enhancing drugs and sex of the user. The side effects of AAS abuse can be life threating, with the best documented including damage to the reproductive tract, liver and cardiovascular system [1].

Natural androgens such as testosterone or dihydrotestosterone are either regarded a risk factors or protective agents against the development and progression of cardiovascular diseases [2–4]. Testosterone reduces myocardial ischemia, increases vasodilation in coronary arteries [5], improves symptom scores of angina, insulin sensitivity and body composition of patients with cardiovascular diseases [6,7], although administration of testosterone to hypogonadal men could result in impaired vascular reactivity [8]. Natural androgens affect the coagulation cascade by modulating the vascular endothelium and blood platelet function [9,10]. In older men, low testosterone level is usually associated with a greater risk of adverse thrombotic events including stroke, myocardial infarction, and deep vein thrombosis [11–13]. Klinefelter syndrome, characterised by low testosterone level, is related to increased mortality from cerebrovascular disease and vascular insufficiency [14].

Androgens stimulate fibrinolysis by initiating the release of Tissue-type Plasminogen Activator (t-PA) from the vascular endothelium, a fibrinolytic agent that restores blood flow in occluded arteries by lowering Plasminogen Activator Inhibitor-1 (PAI-1) level [15,16]. Under basal conditions, dihydrotestosterone reduces platelet reactivity in response to arachidonate, collagen and ADP. Both testosterone and dihydrotestosterone are associated with the downregulation of the activated integrin αIIbβ3: the receptor for fibrinogen, which is crucial for the interaction between platelets and the vascular endothelium [13]. However, incubation of platelets with testosterone leads to decrease in platelet nitric oxide (NO) level, which results in the synthesis of pro-aggregatory prostaglandins [17]. In men, testosterone replacement therapy has been associated with abnormal platelet aggregation initiated by the overexpression of pro-thrombotic thromboxane A2 receptors [18,19]. It has been suggested that only high levels of circulatory testosterone display negative effects on vascular endothelium and are able to stimulate platelet aggregation [10].

AAS are considered to be more dangerous to the cardiovascular system than natural androgens. AAS abuse leads to life-threatening coagulation abnormalities [20,21], that may result in cerebrovascular accidents, pulmonary emboli and acute coronary thrombi [22,23]. In pharmacological doses, some AAS have been shown to increase fibrinolysis by lowering PAI-1 levels, similar to natural androgens. However, in general, AAS are considered pro-aggregatory agents [21,24]. Especially in male abusers, the impact of AAS may change from anti- to pro-thrombotic very rapidly, without other symptoms, leading to serious harm or even death [25].

Androgen receptors have been identified on some types of vascular endothelial cells, which suggests that vascular endothelium constitutes a direct target for testosterone and AAS [26]. Nevertheless, scientific reports describing the role of androgens in vascular endothelial cells are inconsistent and sometimes contradictory. Suppression of dihydrotestosterone was shown to stimulate vascular endothelial cell growth [27], as low doses of androgens promote their proliferation through mechanisms regulated by cyclins and vascular endothelial growth factor [28,29]. In cultured human vascular endothelial cells, administration of low doses of testosterone or dihydrotestosterone was shown to stimulate mitogen-activated protein kinases (MAPKs) and creatine kinase. Dihydrotestosterone induces endothelial nitric oxide (NO) production by up-regulating the expression of endothelial NO-synthase (eNOS) and extracellular signal-regulated kinases (ERK1/2) signalling. Dihydrotestosterone induces the production of endothelial nitric oxide (NO) by upregulating endothelial NO synthase (eNOS) expression. Additionally, testosterone and dihydrotestosterone stimulate NO release in a dose-dependent manner in human umbilical vein endothelial cells (HUVECs) through the activation of phosphoinositide 3-kinases/protein kinase B (PI3K/Akt) and ERK1/2 pathways [30].

In human aortic endothelial cells and HUVECs, testosterone suppresses tumor necrosis factor-α (TNF-α)-induced expression of vascular cell adhesion molecule 1 (VCAM-1), a protein that regulates the adhesion of lymphocytes to the vascular endothelium [31], while dihydrotestosterone treatment results in elevated expression of VCAM-1. Therefore, high doses of androgens increase the adhesion of monocytes to endothelial cells, which may be considered an early stage of vascular endothelium inflammation [32]. Adhesion of monocytes to endothelial cells may be associated with androgen-dependent endothelial dysfunction caused by reduced NO availability [33] or inflammation [34], although exposure of vascular endothelial cells to dihydrotestosterone or testosterone is correlated with the attenuation of TNF-α/lipopolysaccharide-dependent inflammation [35,36].

Endothelial dysfunction is characterized by various changes in biochemical and physiological parameters, including a pro-fibrinolytic or pro-thrombotic state, increased production of pro-inflammatory cytokines, heightened monolayer permeability, and elevated production of reactive oxygen species (ROS) [37]. One of the most important markers of vascular endothelial dysfunction is homocysteine, a thiol-containing homologue of cysteine with additional methylene bridge. Homocysteine impairs the vascular endothelium function through dysregulation of NO production, increasing level of oxidative stress and atherogenic development [38]. Elevated levels of homocysteine were observed in the blood of long-term AAS users both 'on-cycle' (during AAS administration) and 'off-cycle' (after a three-month abstention), suggesting that AAS-dependent vascular endothelial dysfunction persists for months following withdrawal [39]. Bodybuilders have been found to demonstrate acute hyperhomocysteinaemia immediately after injection of supraphysiological doses of AAS [40].

Among various AAS, boldenone, stanozolol, and nandrolone (nortestosterone) have been described as rapidly inducing oxidative stress in the liver and kidneys [41,42]. High doses of nandrolone were found to cause endothelial dysfunction in the thoracic aorta of bodybuilders by reducing vasodilation and NO production. Abuse of nandrolone by weightlifters resulted in downregulation of eNOS expression, and elevated production of endothelium-derived vasoconstrictors [43–45].

This paper examines the negative effects of prolonged exposure of vascular endothelial cells to AAS, with a particular focus on the possibility that endothelial dysfunction in AAS abusers may be related not only to decreased production of NO, but also to oxidative stress and thiol-disulphide imbalance. Thiols contain a functional carbon-bonded sulfhydryl group (SH) that can be oxidised to form a disulphide bond (S–S) [46]. As essential antioxidant buffers, thiols help preserve a balanced redox state both inside and outside the cell. Rearrangements in the thiol-disulphide balance are regarded as the earliest indicators of protein oxidation, observed in ROS-dependent pathological conditions [47]. They may indicate serious changes in oxidation status: either an increase of ROS production or a reduced detoxification capacity by antioxidants like glutathione or total thiols [48].

Our research was focused on proteins involved in maintaining the thiol-disulphide balance, including peroxiredoxins and thiol oxidoreductases like protein disulphide isomerase (PDI) — an enzyme that plays a crucial role during coagulation cascade via regulation of vascular endothelial cells and platelet cross-talk. PDI exhibits a regulatory role on platelet adhesion to the endothelium, on endothelial cell migration, adhesion to extracellular matrix and on signal transduction [49].

2. Materials and Methods

2.1 Materials

EA.hy926 (CRL-2922) and HMEC-1 (CRL-3243) cells were purchased from ATCC (Manassas, VA, USA);

all culture regents including DMEM, DMEM w/o phenol red, MCDB 131, Opti-MEM w/o phenol red, glutamine, trypsin/EDTA solution, fetal bovine serum (FBS), charcoal-stripped FBS, penicillin-streptomycin, Dulbecco's phosphate-buffered saline (DPBS), DPBS with magnesium and calcium; Pierce BCA Protein Assay Kit, Pierce IP Lysis Buffer, protease inhibitor cocktail, neutravidin-agarose, PrestoBlue Cell Viability Reagent, Glutathione Colorimetric Detection Kit; antibodies for PDI (RL90), protein disulfide-isomerase A3 (ERp57) (PA3-009), protein disulfide-isomerase A6 (PDIA6) (PA3-008), Thioredoxin 1 (TRX) (MA5-38102), peroxiredoxin 1 (PRDX1) (15816-1-AP), peroxiredoxin 2 (PRDX2) (10545-2-AP), β-actin (AC-15) were purchased form Thermo Fisher Scientific (Waltham, MA, USA); hydrocortisone, epidermal growth factor (EGF), testosterone (46923), nandrolone (46476), boldenone (46431), N-(3-maleimidopropionyl)biocytin (MPB), N-ethylmaleimide (NEM), tris(2-carboxyethyl)phospine (TCEP), reduced glutathione (GSH), iodoacetamide (IAA), 5-Sulfosalicylic acid dihydrate (SSA), 2-vinylpyridine, crystal violet, β-mercaptoethanol, were purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA); tissue culture plate inserts for transwell system, sodium pyruvate and 96% ethanol were purchased from VWR International (Radnor, PA, USA), Mycoplasma Detection Kit was purchased from InvivoGen (San Diego, CA, USA).

2.2 Cell Cultures

All cell lines were validated by STR profiling by the manufacturer (ATCC) and tested negative for mycoplasma. Additionally, mycoplasma contamination during the research was ruled out using a Mycoplasma Detection Kit, applied every three weeks or after thawing a new batch of frozen cells. EA.hy926 cells were cultured DMEM supplemented with 10% FBS in Lab-line cell incubator model 460-1CE (LAB-LINE Inc., Melrose Parl, IL, USA). HMEC-1 cells were cultured using MCDB 131 supplemented with 10% FBS, 10 ng/mL epidermal growth factor and 1 µg/mL hydrocortisone; at 37 °C and 5% CO_2 in a humidified atmosphere in Lab-line 460-1CE cell incubator. Before the administration of AAS, the cells were transferred to growth medium w/o phenol red (DMEM with the addition of sodium pyruvate for EA.hy926 and Opti-MEM for HMEC-1) supplemented with 2% charcoal-stripped FBS. AAS was dissolved in pure 96% ethanol to make a starting concentration of 5 mM; this was later subjected to a series of dilutions to reach the expected concentration of AAS, with final ethanol concentration at 0.05% in growth medium. The cells were cultured with AAS for seven days, with a new dose added every 24 hours in fresh growth medium.

2.3 Cell Viability Assay Using PrestoBlue

Proliferation assay was performed as described previously [50]. Briefly, the cells were seeded at the count of 1 $\times$ 10^3/well in a 96-well plate in culture medium w/o phenol red supplemented with 2% charcoal-stripped FBS. After two hours of adhesion, AAS were added to each well at different concentrations: 25 nM, 50 nM, 100 nM, 500 nM, 1 µM, 5 µM, 10 µM. After 24 hours of incubation with AAS, the growth medium was removed, cells were washed with serum free medium to remove damaged or detached cells. PrestoBlue reagent, dissolved in a fresh medium without FBS, was added directly to each well. After one hour of incubation at 37 °C, excitation (595 nm) and emission (535 nm) were measured using a Perkin Elmer Victor 3 multifunctional plate reader (PerkinElmer Inc., Shelton, CO, USA). Alternative experimental variants were also included, which were incubated with AAS for three, five or seven days, with a fresh dose of AAS added every day in culture medium w/o phenol red, supplemented with 2% charcoal-stripped FBS.

2.4 Scratch Migration Assay

Migration assay was performed as described previously [50]. Cells were seeded on 12-well plates at the count of 1 $\times$ 10^4 cells/well and incubated with AAS for seven days in accordance with the protocol described in the cell viability assay. After seven days, the cells were washed with serum-free medium and starved for four hours to minimize the effect of cell division on reduction of scratch area. After starvation, a scratch was made using a sterile 200 µL pipette tip and the first picture was taken, which correspond to the baseline 0 hours. Images were then taken after 24 and 48-hours in serum-free medium, using an inverted Olympus CKX41 microscope combined with an Olympus C3040 camera (Olympus Corporation, Tokyo, Japan). The decrease in scratch area was measured using the ImageJ v. 1.48 with MiToBo plugin (National Institutes of Health (NIH), Bethesda, MD, USA).

2.5 Transwell Migration Assay

Migration assay was performed as described previously [50]. The cells were incubated with AAS for seven days, starved for 24 hours in serum-free medium and transferred to the upper chamber of a transwell system (diameter 6.5 mm, thickness 10 µm, pore diameter 8 µm) at the count of 1 $\times$ 10^4 cells/well. The cells were allowed to migrate for 24 hours to the lower chamber with culture medium w/o phenol red, supplemented with 10% charcoal-stripped FBS, as an attractant. After 24-hours, any cells that had not migrated from the inner side of the upper chamber were removed with a sterile cotton swab. Cells that had migrated through pores to the lower side of the membrane were washed with DPBS, fixed with ice-cold methanol for 10 minutes at 4 °C and stained with crystal violet for 10 minutes at room temperature. The membrane with the stained cells was viewed under the Olympus CKX41 microscope combined with an Olympus C3040 camera; cells from ten randomly selected pictures were counted.

2.6 Transepithelial/Endothelial Electrical Resistance (TEER) Assay

Formation of endothelial monolayer by TEER was measured as described previously [51]. The cells were incubated with AAS for seven days, with a new dose added every 24 hours, using transwell system with pore diameter 0.4 µm (diameter 6.5 mm, thickness 10 µm, pore diameter 0.4 µm). Each day, the *Evom3* system with the addition of *Endohm* chamber (World Precision Instruments, Sarasota, FL, USA) was used to examine the permeability of the endothelial monolayer. Before measurement, growth medium was removed and serum-free medium was added into the upper and lower chamber of *Evom3* system to minimize the effect of pressure on the well membrane. After measurement, a new dose of AAS was added to each chamber with growth medium and the incubation was continued.

The TEER assay evaluated barrier integrity by measuring the permeability of small inorganic ions, of a cellular monolayer based on its electrical resistance. The assay involved culturing cells as monolayer on a semipermeable filter insert that separates an apical/luminal (upper) compartment from a basolateral/abluminal (lower) compartment. Resistance measurements were conducted using two electrodes positioned on either side of the cellular monolayer: one in the upper compartment and the other in the lower compartment. The resistance was determined using Ohm's law: $R = \frac{U}{I}$.

The evaluation procedure involves measuring the blank resistance of the microporous membrane without cells (RBLANK) and measurement of the resistance across the cell monolayer on the membrane filter (RTOTAL). This total resistance comprises the cell layer resistance (RTEER), the resistance of the culture medium (RM), the membrane insert resistance (RI), and the resistance of the electrode-medium interface (REMI). The cell-specific resistance (RTISSUE) can be calcualted as: $R_{TISSUE} = R_{TOTAL} + R_{BLANK}$.TEER values are reported in units of $\Omega \times cm^2$ ($TEER_{REPORTED}$) and calculated as: $TEER_{REPORTED} = R_{TISSUE} \times M_{AREA}$, where M_{AREA} represents the area of the semipermeable membrane (measured in cm^2). The final value is inversely proportional to the permeability of the cellular monolayer [52].

2.7 Sulfhydryl Group Labelling

Free thiol groups and disulphide bonds reduced to free thiols, were labelled as described previously [53] with modifications. Instead of labelling surface proteins on living cells, cell lysates were used to label free thiols and disulphide bonds of intracellular proteins.

After seven days incubation with AAS, the cells were washed with serum-free medium and DPBS with the addition of magnesium and calcium. Cell lysates were papered according to the manufacturer's protocol using Pierce IP Lysis Buffer. After centrifugation of lysates at 15,000 ×g for 10 minutes. at 4 °C, the total amount of protein was measured using a Pierce BCA Protein Assay Kit and Perkin Elmer Victor 3 multifunctional plate reader. In total, 50 µg of proteins were used to label the free thiols.

Labelling of free thiols was performed in mixture of lysis buffer and DPBS (1:1) to maintain neutral pH, since acidic pH can strongly affect labelling process. Labelling of free thiols involves incubation with 50 µM N-(3-maleimidopropionyl)biocytin (MPB) for 30 minutes. at room temperature with gentle mixing. The labelling reaction was quenched by the addition of 100 µM reduced glutathione (GSH) for 15 minutes followed by addition of 200 µM iodoacetamide (IAA) for 15 minutes to stop the reaction. An alternative version of labelling involves blocking free thiol groups in cell lysates by incubation with 20 mM N-ethylmaleimide (NEM) for 30 minutes. at room temperature with gentle mixing. Next, the disulphide bonds were reduced to free thiols using 20 µM tris(2-carboxyethyl)phospine (TCEP), for 30 minutes. at room temperature with gentle mixing. After reduction of disulphides to free thiols, MPB labelling was performed as described previously.

The proteins labelled with MPB were precipitated using a high capacity Neutravidin-Agarose resin overnight at 4 °C with gentle mixing/shaking. Only MPB-labelled proteins were precipitated due to specific interaction between avidin from the resin and biotin part of MPB; no NEM-labelled proteins were precipitated as NEM lacks the biotin fragment. After incubation, the resin was washed 3× with lysis buffer and centrifuged 800 ×g for 5 minutes. at 4 °C to detach any non-precipitated proteins. After washing, the labelled proteins were detached from the resin by a reducing agent (β-mercaptoethanol) and boiled at 99 °C for 10 minutes. Next, the detached proteins were separated using Western blot. Biotinylated proteins containing free thiols or disulphide bonds were visualised using specific antibodies or avidin–horseradish peroxidase. All Western blot analyses were performed using ChemiDoc MP Imaging System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.8 PDI Secretion

Secretion of PDI from vascular endothelial cells (ECs) was performed as described previously [49]. After seven-day incubation with AAS, the cells were treated with serum-free medium without PDI secretion stimulating factors (e.g., thrombin). After one hour of incubation, the medium was collected, centrifuged at 10,000 ×g for 10 minutes. at 4 °C and subjected to Western blot analysis for PDI.

2.9 GSH/GSSG Level Measurement

The levels of total glutathione, reduced glutathione (GSH) and oxidised glutathione (GSSG) were analysed using a commercially-available GSH/GSSG colorimetric kit according to manufacturer's protocol. Briefly, the cell pellets were lysed using 5% SSA and centrifuged at 14,000 ×g for 10 minutes at 4 °C. The samples were then divided into one portion for examining GSH and another for examining GSSG. For the GSSG measurements, the cells were

incubated for one hour with 2-vinylpyridine to block any free GSH in the sample. Then, glutathione reductase and NADPH were added and the absorbance was measured at 405 nm using a Perkin Elmer Victor 3 multifunctional plate reader. Free GSH concentration in the sample was calculated from the difference between the total GSH determined and the GSH generated from oxidized glutathione for the 2-vinylpyridine-treated samples.

2.10 Statistical Analysis

Data is presented as a mean ± standard deviation (SD) or standard error of the mean (SEM). Heteroscedasticity was verified based on the Brown-Forsythe test. According to data normality and homoscedasticity, either Dunnett or Dunns test was used for *post hoc* analysis following the one-way ANOVA, while the Boneferroni test was used for two-way ANOVA. All statistical analyses were performed using Statistica v. 13.1 (Dell Inc. Round Rock, TX, USA), Excel v.4 (Microsoft Corporation, Redmond, WA, USA), GraphPad Prism v.5 (GraphPad Software, Boston, MA, USA)

3. Results

3.1 AAS Negatively Affected Proliferation of Vascular Endothelial Cells

The cells were incubated with different concentrations of AAS in standard growth medium without phenol red (a weak oestrogen) with the addition of 2% charcoal-stripped FBS (does not contain any nonpolar molecules, including hormones). This is the standard procedure for testing AAS and hormones in various cell lines, including vascular endothelial cells, as charcoal-stripping removes steroids present in FBS, such as dihydrotestosterone, methylprednisolone, and cortisol, that can bind to and activate androgen receptors [54–56].

After seven days, nandrolone at a concentration of 1 µM displayed an inhibitory effect on EA.hy926 proliferation. No significant effects were observed after administration of 1 µM testosterone or 1 µM boldenone to EA.hy926 (Fig. 1A). Proliferation of HMEC-1 was not inhibited by 1 µM testosterone, nandrolone or boldenone. Only doses higher than 5 µM displayed inhibitory effect on HMEC-1 proliferation after seven days (Fig. 1B). EA.hy926 expressed greater sensitivity to AAS, especially for nandrolone, which inhibited proliferation of EA.hy926 even at a single dose of 5 µM per week. Similar effects were observed after administration of a 10 µM dose of testosterone or boldenone (**Supplementary Fig. 1A**). To inhibit proliferation of HMEC-1 with a single dose of AAS, the dose must exceed 100 µM (**Supplementary Fig. 1B**).

The following experiments used a dose of 1 µM AAS, this being the lowest dose that can negatively affect vascular endothelial cells proliferation in at least one tested cell line.

3.2 AAS Negatively Affected Migration of Vascular Endothelial Cells

High doses of AAS inhibited the migration of EA.hy926 cells more effectively than that of HMEC-1 cells in the "scratch migration" assay (Fig. 2). Testosterone inhibited the migration of EA.hy926 at 5 µM, while nandrolone and boldenone express similar effects at 1 µM (Fig. 2A, **Supplementary Fig. 2**). Migration of HMEC-1 was inhibited only by high doses of AAS (10 µM for testosterone and boldenone; 5 µM for nandrolone) (Fig. 2B, **Supplementary Fig. 3**). Low doses of AAS, corresponding to normal androgen levels in men or slightly exceeding them, show a stimulatory effect on EA.hy926 migration (100 nM for testosterone and boldenone; 25 nM for nandrolone). Increased migration of HMEC-1 in scratch migration assay was not observed after AAS administration at any tested concentration.

Migration through a polycarbonate membrane demonstrated that AAS-dependent changes in ECs mobility depends on concentration. Treatment with 100 nM nandrolone and boldenone stimulated EA.hy926 migration with a significant effect observed for boldenone. 1 µM testosterone, nandrolone or boldenone inhibited migration of EA.hy926 through membrane (Fig. 3A). The migration of HMEC-1 through the transwell system was affected only by boldenone, without significant difference after administration of testosterone or nandrolone. No increased migration by HMEC-1 was observed after supplementation of AAS (Fig. 3B).

3.3 AAS Negatively Affected the Formation of an Vendothelial Monolayer

The initial signs of diminished monolayer formation by EA.hy926 can be observed after six days of incubation with testosterone, five days with nandrolone and four days with boldenone (Fig. 4A). HMEC-1 monolayer formation is affected by seven-day nandrolone treatment and five-day boldenone treatment (Fig. 4B).

3.4 AAS Downregulated the Expression of Peroxiredoxins and Caused Oxidation of Protein Free Thiol Bonds

Testosterone, nandrolone and boldenone showed no effect on the cellular expression of oxidoreductases involved in thiol bond modifications: PDI, ERp57, PDIA6 and thioredoxin, neither in EA.hy926 (Fig. 5A) nor in HMEC-1 (Fig. 5B). In EA.hy926 all three tested AAS decreased cellular level of peroxiredoxin 1 (PRDX1), while nandrolone and boldenone negatively affected the expression of peroxiredoxin 2 (PRDX2) (Fig. 5C). In HMEC-1, PRDX1 and PRDX2 were downregulated by nandrolone and boldenone, but not testosterone (Fig. 5D). Of both tested endothelial cell lines, only EA.hy926 was found to express an androgen receptor on its surface (Fig. 5E).

In EA.hy926, nandrolone and boldenone caused a significant decrease in total pool of free thiols containing proteins (Fig. 6A) and an increase in proteins containing

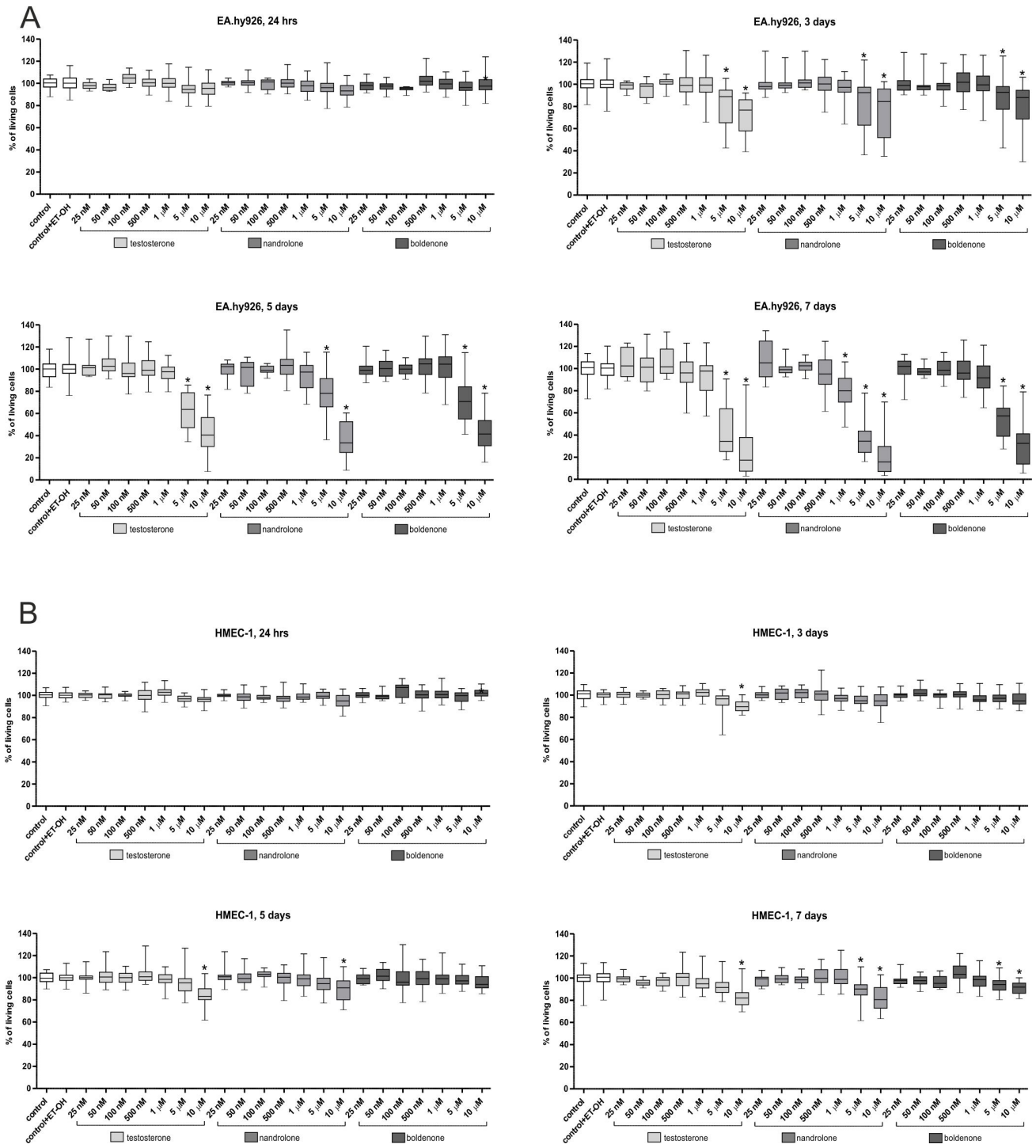

Fig. 1. Cell proliferation of EA.hy926 (A) and HMEC-1 (B) after 24-hour, three-day, five-day and seven-day administration of androgenic anabolic steroids (AAS) at different concentrations. Data presented as mean ± standard deviation (SD); mini-max ranges marked as whiskers; n = 8. Total number of living cells was estimated using PrestoBlue assay with absorbance measurement at 540–570/580–610 nm on a Perkin Elmer Victor 3 Multifunctional Microplate Reader. Significance of differences between control samples and samples with AAS, (* indicating significance at $p < 0.001$), was calculated using one-way ANOVA and the *post hoc* comparisons Dunett test.

disulphide bonds (Fig. 6B). For HMEC-1, only boldenone caused a decrease in free thiols (Fig. 6C) and an increase in disulphide bond-containing proteins (Fig. 6D). While Western blot can be used to determine the prevalence of thiol-containing proteins, signals from different proteins with a similar molecular mass may overlap. Therefore, to give an insight into the oxidation-reduction of the thiol bonds on specific proteins, MPB-labelled proteins precipitated with

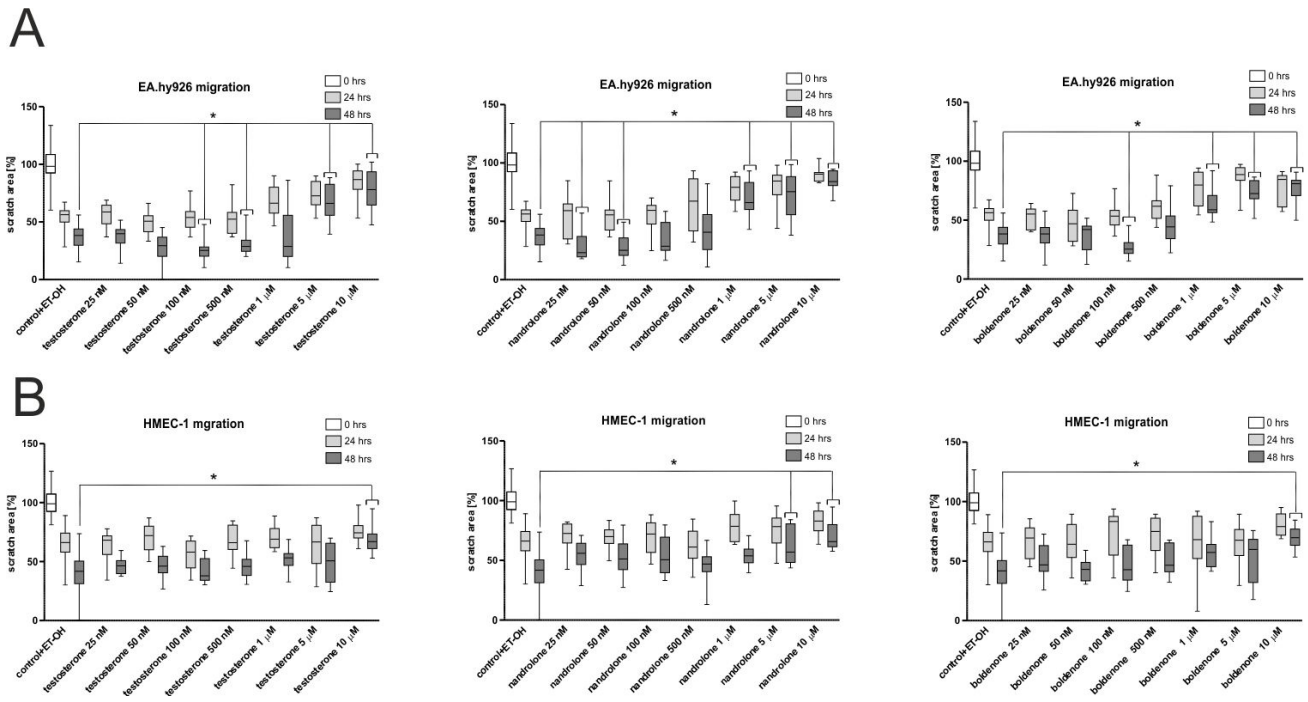

Fig. 2. Migration of EA.hy926 (A) and HMEC-1 (B) after seven-day administration of different concentrations of AAS. Data presented as mean ± SD; mini-max ranges marked as whiskers; n = 6. Significance of differences between control samples and samples with AAS, (* indicating significance at $p < 0.001$), was calculated using one-way ANOVA and the *post hoc* multiple comparisons Dunns test due to the lack of heteroscedasticity verified by the Brown-Forsythe test.

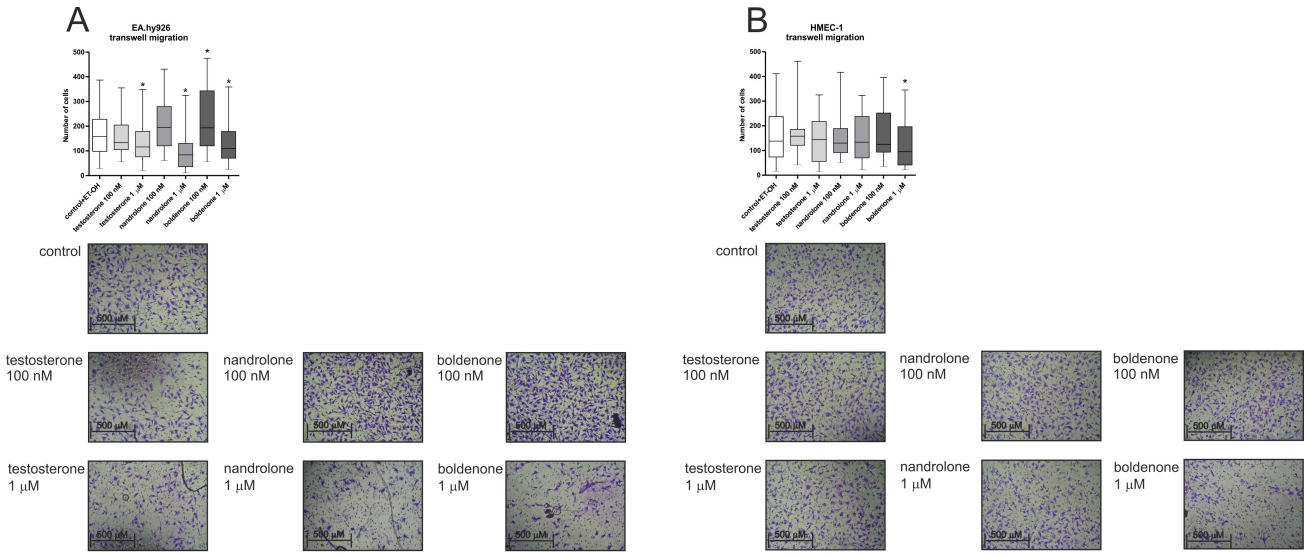

Fig. 3. Migration EA.hy926 (A) and HMEC-1 (B) through transwell system after seven-day administration of different concentrations of AAS. Data presented as mean ± SD; mini-max ranges marked as whiskers; n = 10. Scale bar = 500 μm. Significance of differences between control samples and samples with AAS, (* indicating significance at $p < 0.001$), was calculated using one-way ANOVA and the *post hoc* comparisons Dunett test.

neutravidin-agarose resin were visualised. Despite small changes in thiol-disulphide ratio, PDIA6 and TRX seemed to be not affected by AAS in either tested cell line. Testosterone, nandrolone and boldenone initiated changes in the thiol state of PDI and ERp57 in EA.hy926, decreasing the total pool of proteins with free thiols (Fig. 6E) and increasing the pool of proteins whose thiols were oxidised to form disulphide bonds (Fig. 6F). For HMEC-1 only PDI is affected by AAS: boldenone caused a decrease in free thiols containing PDI (Fig. 6G) while nandrolone and boldenone increased the amount of PDI with disulphide bonds (Fig. 6H).

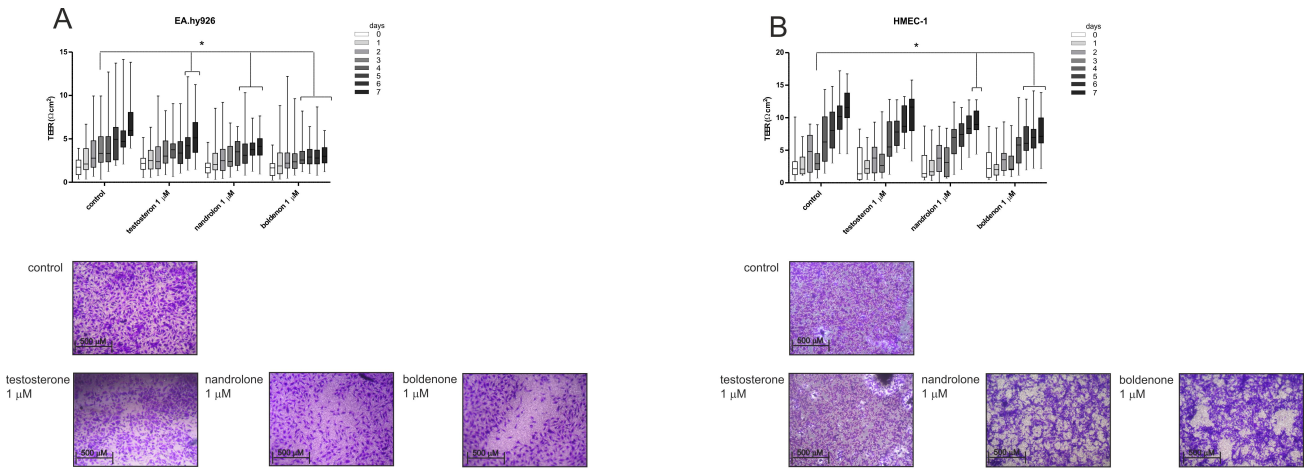

Fig. 4. Monolayer formation of EA.hy926 (A) and HMEC-1 (B) during seven-day incubation with AAS measured by Transepithelial/Endothelial Electrical Resistance (TEER) system. Data presented as mean ± SD; mini-max ranges marked as whiskers; n = 8. Scale bar = 500 µm. Significance of differences between control samples and samples with AAS, (* indicating significance at $p < 0.001$), was calculated using two-way ANOVA and the *post hoc* comparisons Boneferroni test.

3.5 AAS Inhibited Constitutive Secretion of PDI from Endothelial Cells

For EA.hy926, a profound inhibitory effect on PDI secretion was observed after administration of nandrolone (Fig. 7A), but this result could be a cumulative effect of nandrolone-dependent decrease of PDI secretion and nandrolone-dependent decrease in cell proliferation; this was confirmed by cell viability assay using PrestoBlue (Fig. 7B). For HMEC-1, testosterone showed no inhibitory effect on PDI secretion, when nandrolone and boldenone inhibited PDI secretion (Fig. 7C). No changes in cell proliferation were observed after AAS administration to HMEC-1 (Fig. 7D), indicating that lowered PDI secretion is not a result of decreased cell number.

3.6 AAS Caused Oxidation of Reduced Glutathione

Total glutathione level was not affected by the addition of any tested AAS for seven days. The reduction in free reduced glutathione (GSH) level associated with nandrolone and boldenone was accompanied by an increase in oxidised glutathione level (GSSG) in EA.hy926 (Fig. 8A). In HMEC-1, this effect was triggered only by boldenone (Fig. 8B).

4. Discussion

Our findings confirm that basic vascular endothelial function, including proliferation, migration and monolayer formation, could be affected by prolonged AAS administration. The observed results depend on AAS type, dose, time of administration and type of endothelial cell line. In both tested cell lines, testosterone in its natural non-ester form exhibits low or no inhibitory effects on cell function; this form is usually not used by athletes due to its short time of action. More adverse effects can be observed after administration of nandrolone and boldenone. In bovine aortic endothelial cells, testosterone promotes cell proliferation, migration, and vascular tube formation at a dose of 100 nM after a short, i.e., four-hour, incubation followed by a 48-hour proliferation test [57]. This observation, although obtained under different culture conditions, does not contradict our results, since out data indicates that the decrease in proliferation after testosterone treatment is only associated with high doses (>5 µM for EA.hy926 and >10 µM for HMEC-1).

The impact of natural androgens on the cardiovascular system, particularly the vascular endothelium, depends predominantly on the tested dose. Physiological (100 pM–10 nM) and pharmacological (>10 µM) doses of testosterone cause vasodilatation by a dual mechanism. The effect is endothelium dependent at physiological concentrations, but endothelium independent at pharmacological concentrations (100 µM). Typical therapeutic regimens for hypogonadal patients comprise 150–200 mg of the testosterone esters enanthate and cypionate every two weeks or 75–100 mg/week; alternatively undecanoate, a long-acting testosterone analogue, can be given at 750 mg on week 1, followed by 750 mg on week 4, and 750 mg every 10 weeks [58,59]. So called “cycles” of AAS-abuse by athletes involve using more profound doses, and can incorporate one to ten compounds each cycle, such as testosterone enanthate, methandrostenolone, trenbolone, nandrolone, testosterone cypionate, testosterone propionate, boldenone, testosterone blend or oxandrolone. Most AAS abusers chose cycles that alternate between approximately 10.7 ± 4.6 weeks of drug administration and 20.4 ± 28.3 of withdrawal. The doses are chosen based on personal experience and non-medical sources, and ranges from 6.0 mg/week to even 12,000 mg/week and above [60].

According to the World Anti-Doping Agency, while the normal testosterone level should not exceed 10 nM/dL

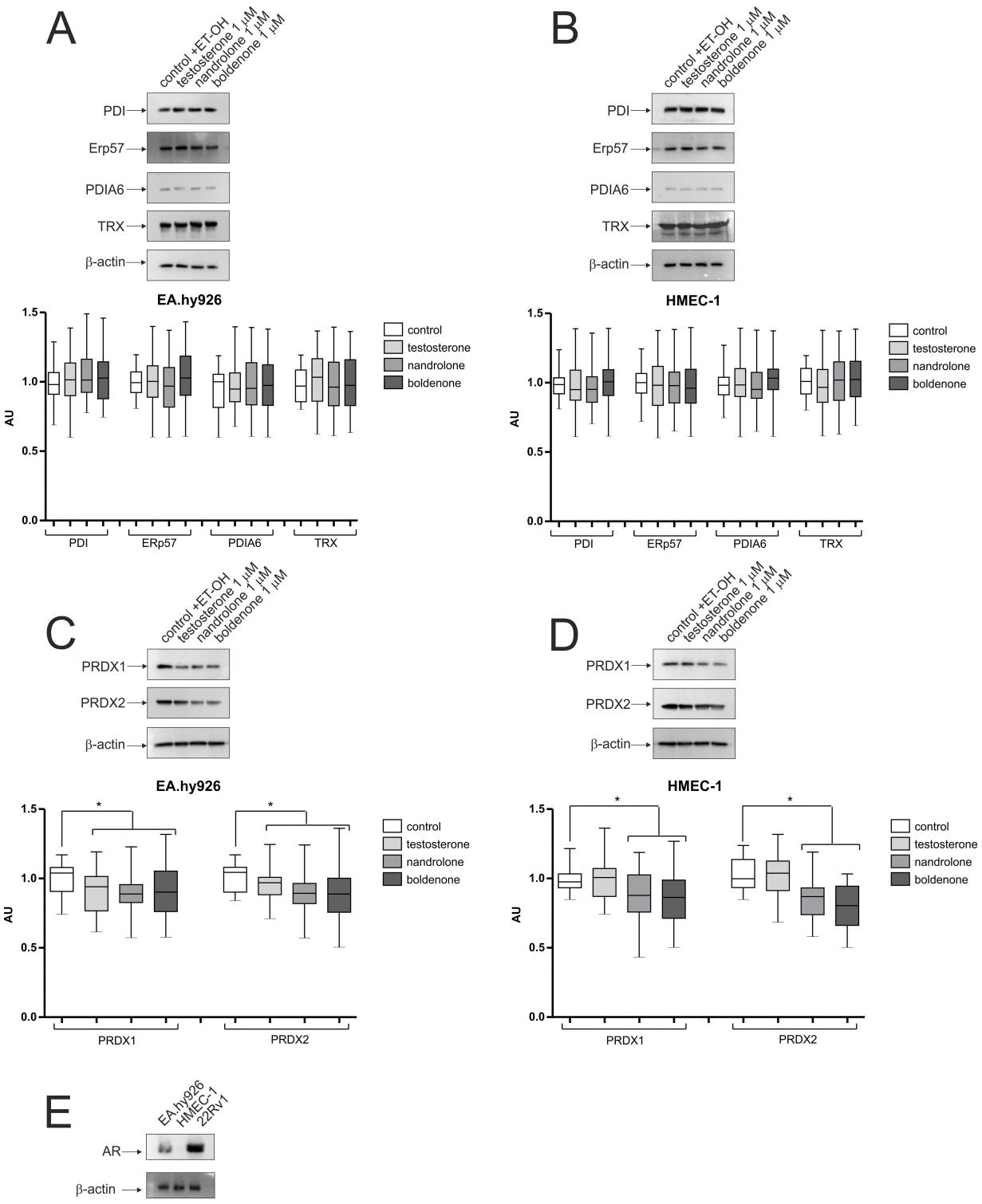

Fig. 5. Overall content of specific oxidoreductases (PDI, ERp57, PDIA6, TRX) in EA.hy926 (A) and in HMEC-1 (B) lysates after seven-day of AAS administration; overall content of peroxiredoxins (PRDX1, PRDX2) in EA.hy926 (C) and in HMEC-1 (D) lysates after 7 days of AAS administration. Expression of androgen receptor (AR) in EA.hy926 and in HMEC-1 (E) with cell lysate from prostate cancer cell line 22Rv1 as a positive control. Analysis of β-actin content in cell lysates serves as a loading control for each experiment. Densitometry analysis performed using National Institutes of Health (NIH) ImageJ program. Data presented as mean ± SD; mini-max ranges marked as whiskers; n = 4. Significance of differences between control samples and samples with AAS (* indicating significance at $p < 0.001$), was calculated using one-way ANOVA and the post-hoc multiple comparisons Dunns test. ERp57, protein disulfide-isomerase A3 (PDIA3); PDI, protein disulphide isomerase; PDIA6, protein disulfide-isomerase A6, PRDX1, peroxiredoxin 1; PRDX2, peroxiredoxin 2; TRX, thioredoxin.

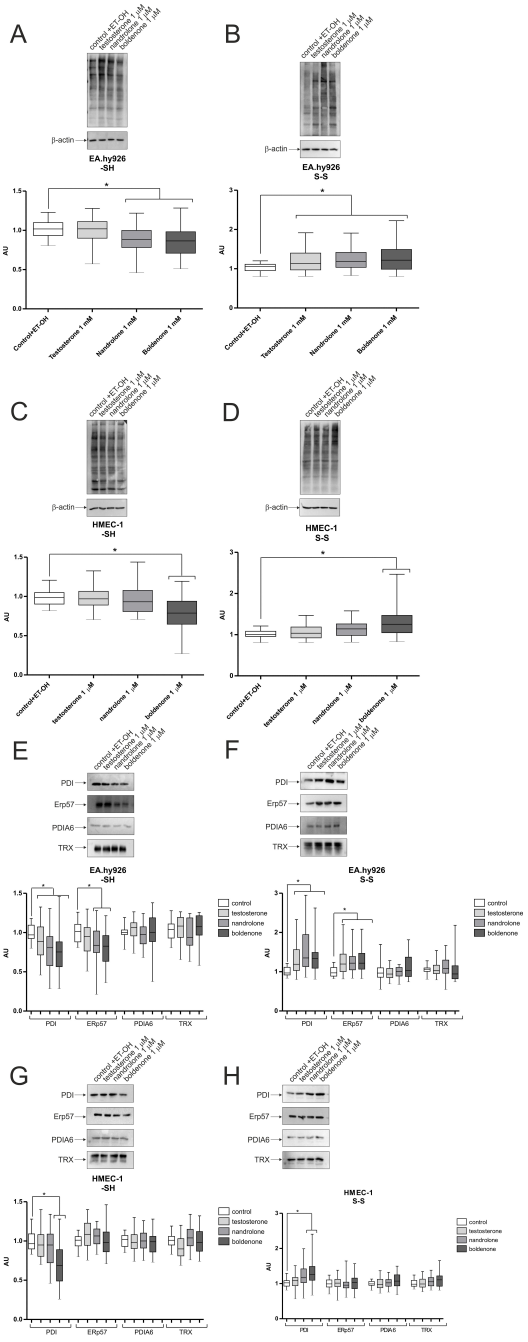

Fig. 6. Overall content of free thiol groups (-SH) and disulphide bonds (S-S) in specific oxidoreductases in EA.hy926 and HMEC-1 lysates after seven days of AAS administration. (A) overall content of thiols in the reduced form of free thiol groups (-SH) in EA.hy926 lysate. (B) overall content of thiols in the oxidised form of disulphide bonds (S-S) in EA.hy926 lysate. (C) overall content of thiols in the reduced form of free thiol groups (-SH) in HMEC-1 lysate. (D) overall content of thiols in the oxidised form of disulphide bonds in (S-S) in HMEC-1 lysate. (E) oxidoreductases (PDI, ERp57, PDIA6, TRX) containing thiols in the reduced form of free thiol groups (-SH) in EA.hy926 lysate. (F) oxidoreductases (PDI, ERp57, PDIA6, TRX) containing thiols in the oxidised form of disulphide bonds (S-S) in EA.hy926 lysate. (G) oxidoreductases (PDI, ERp57, PDIA6, TRX) containing thiols in the reduced form of free thiol groups (-SH) in HMEC-1 lysate. (H) oxidoreductases (PDI, ERp57, PDIA6, TRX) containing thiols in the oxidised form of disulphide bonds (S-S) in HMEC-1 lysate. Western blot analysis of MPB labelled lysates before precipitation with visualising of β-actin level was used as a control for protein loading. Densitometry analysis performed using National Institutes of Health (NIH) ImageJ program. Data presented as mean $\pm$ SD; mini-max ranges marked as whiskers; n = 6. Significance of differences between control samples (white box) and samples with AAS (grey boxes), (* indicating significance at $p < 0.001$), was calculated using one-way ANOVA and the *post hoc* multiple comparisons Dunns test.

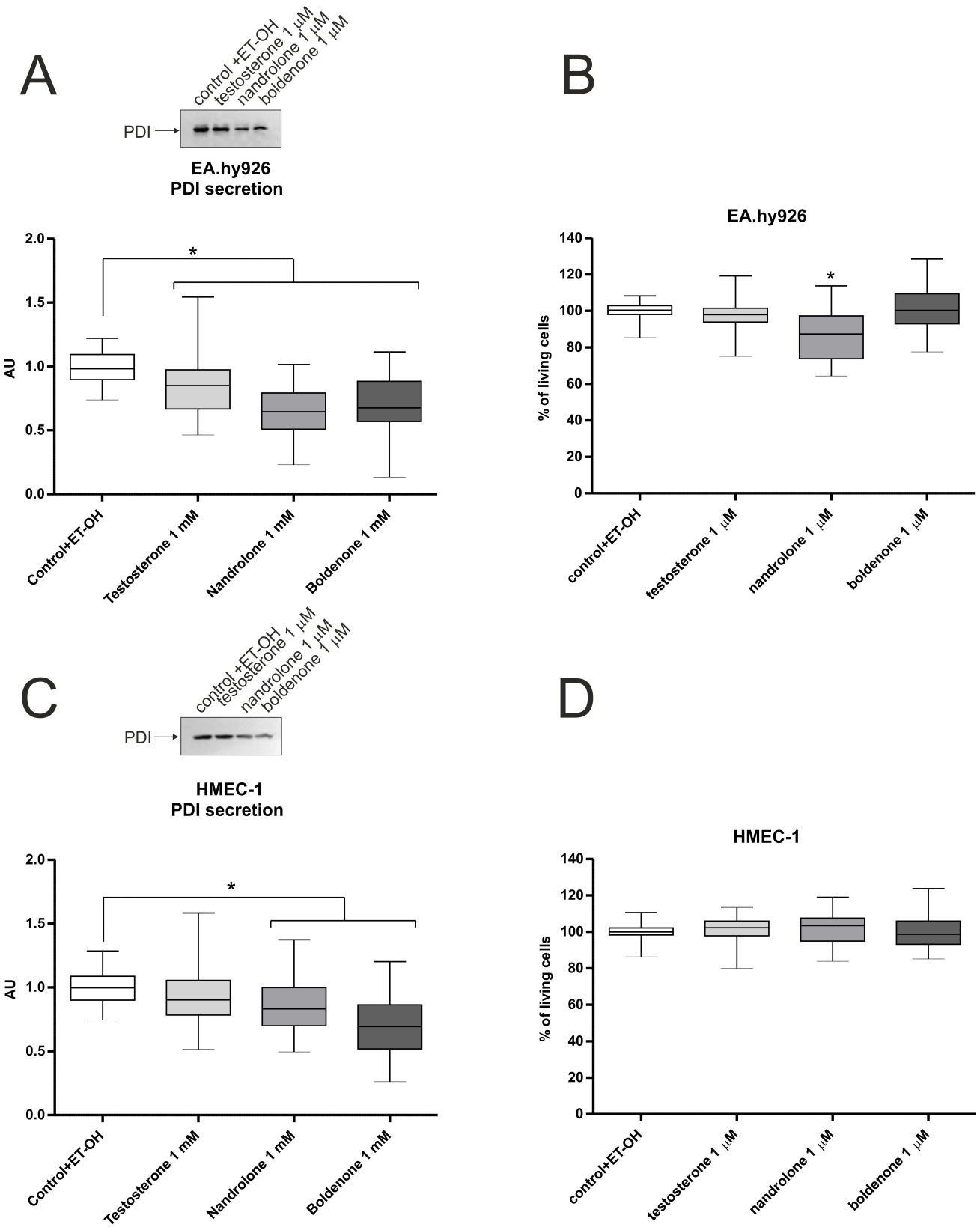

Fig. 7. Constitutive PDI secretion from EA.hy926 (A) and from HMEC-1 (C) after 7 days of AAS administration. Data presented as mean ± SD; mini-max ranges marked as whiskers; n = 6. Significance of differences between control samples and samples with AAS, (* indicating significance at $p < 0.001$), was calculated using one-way ANOVA and the *post hoc* multiple comparisons Dunns test due to the lack of heteroscedasticity verified by the Brown-Forsythe test. Cell proliferation of EA.hy926 (B) and HMEC-1 (D) measured with PrestoBlue assay used as a control of equal number of cells during secretion step.

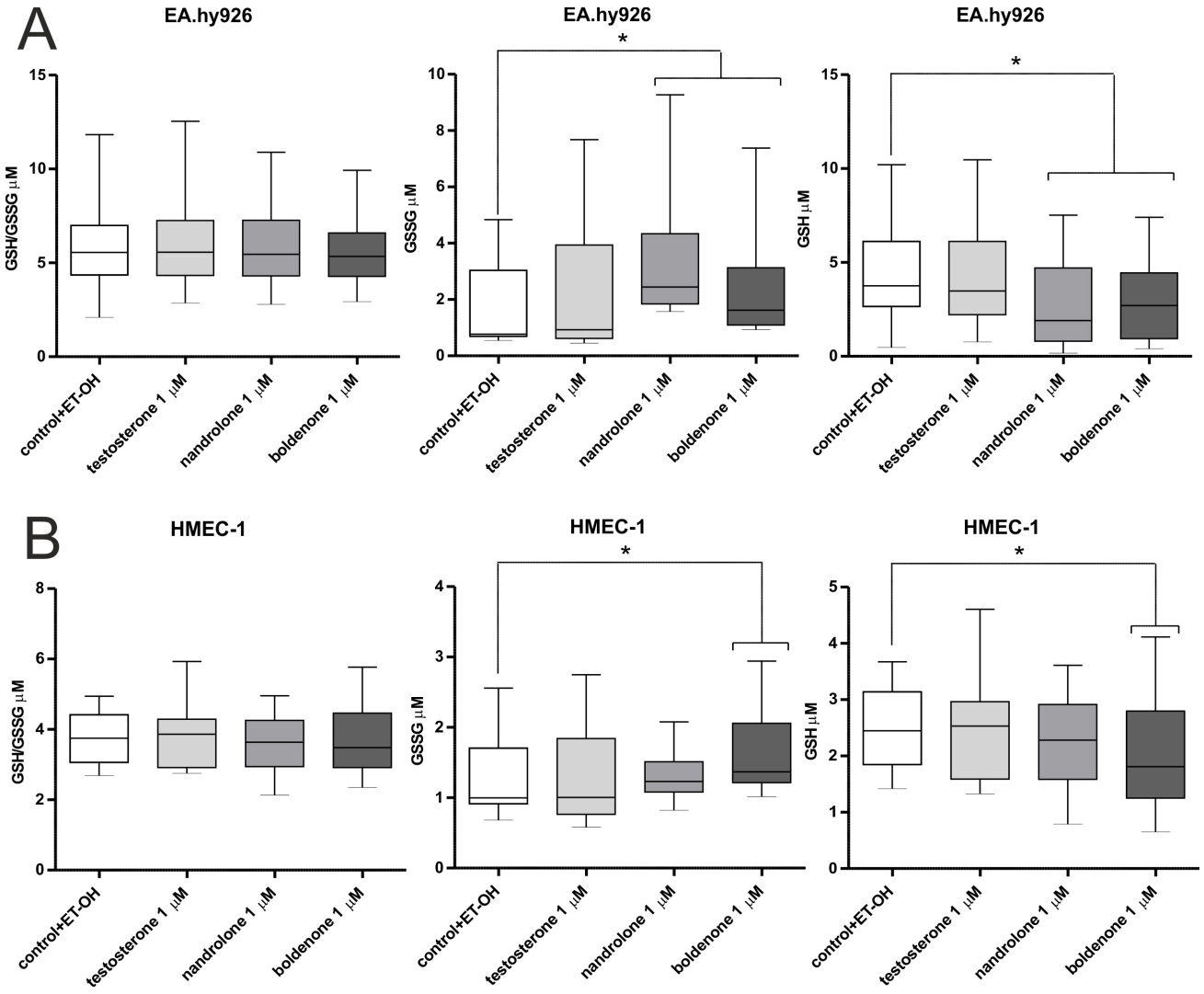

Fig. 8. Contents of total glutathione (GSH/GSSG), oxidised glutathione (GSSG) and reduced glutathione (GSH) in EA.hy926 (A) and HMEC-1 (B) after seven days of AAS administration. Data presented as mean ± SD; mini-max ranges marked as whiskers; n = 8. Significance of differences between control samples and samples with AAS, (* indicating significance at $p < 0.001$), was calculated using one-way ANOVA and the *post hoc* multiple comparisons Dunnett test.

in women, in men, the normal physiological level of testosterone is difficult to estimate, with no clear upper limit, since plasma testosterone levels of sexually-functional men vary considerably, ranging from 10 to 50 nM/dL, with even higher levels often reported [61,62]. The androgen level (testosterone and its derivatives, specific AAS and their derivatives) in the blood of AAS-abusing athletes could exceed normal testosterone level by a few thousand-fold. This is the sole purpose of AAS supplementation, i.e., to promote muscle growth and increase strength and endurance. To achieve this goal, megadoses of steroids are used.

In studies concerning cell cultures, the levels of testosterone and other androgens (e.g., dihydrotestosterone) vary between 1 nM and 10 nM [31,54,56,63,64]. However, doses that exceed normal testosterone level are often employed in research focused on negative effects of AAS. In HUVEC cells exposed to increasing concentrations of testosterone and dihydrotestosterone (100 nM–1 µM), both steroids enhanced eNOS activity and NO synthesis, with maximum effects observed at low concentrations and decreased or inhibited NO synthesis at higher concentrations [30].

It should be noted that the doses used in this study may not reflect exact AAS concentration in AAS-abusing athletes, since levels of AAS and their derivatives vary significantly between patients [54,60]. However, doses that exceed natural testosterone level were selected intentionally to reflect the damage caused by AAS in illegal doping. The study used the lowest concentration of AAS that could inhibit the proliferation of vascular endothelial cells, i.e., 1 µM for nandrolone administered for seven days. IC_{50} values in vascular endothelial cells, as found in other research, were estimated to be 9 µM for nandrolone and 100 µM for testosterone [5]; however, these data were obtained from

experiments in growth conditions including 20% new-born calf serum and 10% FBS. The use of high concentrations of FBS that is not charcoal-stripped and culture medium supplemented with phenol red, may interfere with studies on AAS-induced effects. These components can lead to rapid and abnormal cell division, potentially disrupting the normal oxidative status.

EA.hy926 show greater sensitivity to AAS than HMEC-1. In both lines, cumulative doses of AAS exhibit particularly harmful effects on cell function, with more profound effects observed after administration of nandrolone for EA.hy926 and boldenone for HMEC-1. Unlike EA.hy926, HMEC-1 cells do not express androgen receptors (Fig. 5E), which suggests that the negative effects triggered by boldenone are not directly related to activation of androgen receptor and regulation of gene transcription. One possible explanation for this difference may derive from the boldenone-dependent activation of the oestrogen receptor. Steroid receptors exhibit structural similarity, and it has been suggested that androgen and estrogen receptors may show cross-reactivity following the administration of high doses of steroids [65]. The absence of androgen receptors in HMEC-1, accompanied by high sensitivity to boldenone, indicates that boldenone could cause serious damage in tissues that do not normally express androgen receptors. Boldenone, which is used exclusively as a veterinary AAS and is not approved for human use [66], may be considered one of the most dangerous AAS, with particularly adverse effects on human health.

Nandrolone and boldenone cause significant changes in the thiol-disulphide pattern of intracellular proteins in vascular endothelial cells. This effect is more profound for EA.hy926, where not only the total amount of proteins containing free thiols is decreased and amount of proteins with oxidized thiols increased, but visible changes can be observed. Of the tested oxidoreductases, the thiol pattern of PDIA6 and TRX remains relatively unchanged, which suggest that they are not directly affected by oxidative stress initiated by AAS or they are not involved in preventing AAS-dependent thiol-disulphide imbalance. Increased oxidation of free thiols in PDI after AAS administration could be a direct effect of excess oxidative stress, or it could be a consequence of the PDI-mediated reduction of other protein thiols to prevent the accumulation of oxidized proteins in the endoplasmic reticulum (ER). The reduction in free glutathione level and increased oxidation of glutathione after AAS administration is in agreement with results obtained from cardiomyocytes, where it was proven that high doses of nandrolone cause a cellular redox imbalance, manifested by reduced superoxide dismutase and glutathione reductase activities and reduced total thiol levels [67].

PDI is constitutively secreted from the vascular endothelium and platelets. This process may be increased by thrombin, ADP and other pro-thrombotic factors [68–70]. PDI-mediated isomerisation of disulphide bonds in the extracellular domains of surface receptors called integrins, is considered to be a crucial step in their activation, either in the absence or in the presence of integrin ligands. Integrins are responsible for maintaining cell shape, adhesion to extracellular matrix proteins, ability to move and initiation of signalling pathways. Factors that inhibit PDI enzymatic activity or simply block thiol-disulphide exchange, attenuate interactions between endothelial cells and the extracellular matrix and between endothelial cells and other type of cells, including cancer cells and platelets [50,53]. The release of PDI from platelets and endothelial cells plays a crucial role in the coagulation cascade. Without extracellular PDI, thrombus formation and fibrin generation may be hindered. Platelet activation leads to a 440% increase in surface protein thiol groups and enhanced secretion of PDI molecules. PDI is secreted by activated endothelial cells upon contact with plasma or following vessel wall injury. PDI molecules are present at the injury site even without platelet accumulation. Normal fibrin generation occurs after treatment with platelet aggregation inhibitors, but not after incubation with PDI-blocking antibodies, indicating that PDI is essential for thrombus formation [70–72].

The decreased level of PDI secretion noted after AAS administration could be related to the disturbances in coagulation observed in many AAS-abusing athletes; these are manifested as rapid changes from anti- to prothrombotic states without other symptoms [25]. However, in AAS-abusing athletes, the inhibition of platelet aggregation and thrombus formation due to diminished PDI secretion may be overcome by the pro-aggregatory effects of AAS, or simply by an increased number of blood platelets: this is a very common effect of AAS supplementation [22].

No changes in the expression of oxidoreductases were noted after AAS treatment, but tested AAS were found to alter the expression of two members of the peroxiredoxins family. PRDXs are capable of reducing hydrogen peroxide and are essential for protecting protein free-thiol groups from oxidative damage and enhancing thioredoxin-dependent peroxidase activity. PRDXs are known to regulate various signalling pathways that utilize peroxide as a second messenger [73].

Surprisingly, in response to AAS, the expression of PRDX1 and PRDX2 in vascular endothelial cells does not increase; instead, it is significantly lower than in the control samples. It is possible that this is related to the progression of vascular endothelial dysfunction. The protein levels of PRDX1, PRDX2, PRDX3, and PRDX5 are downregulated in Fuchs' endothelial corneal dystrophy (FECD), a condition arising from severe endothelial dysfunction. In corneal endothelial cells, PRDX1 expression is influenced by excessive oxidative stress [74]. Downregulation of PRDX1 may lead to ferroptotic cell death in an iron-dependent manner; It is characterized by excessive toxic lipid peroxidation resulting from GSH depletion, leading to the inactivation of glutathione peroxidase 4 (GPX4) [75]. In FECD, ferroptosis inhibitors prevent lipid peroxidation and restore cell viability even when PRDX1 is absent [74,76]. Decreased

expression of PRDX1, accompanied by decreased level of GSH in corneal endothelial cells affected by FECD, is in line with our observations that high doses of AAS cause the reduction of GSH levels, increase GSSG levels and trigger PRDX1 downregulation. Similar to FECD, this may be a sign of the early stages of endothelial dysfunction related to excessive redox stress.

PRDX2 is recognized for its role in protecting various vascular endothelial cells from oxidative damage. Downregulation of PRDX2 in immature endothelial cells may be a contributed to the development of malignant vascular tumors, such as angiosarcoma, as oxidative damage has been linked to metastasis of malignant tumors [77]. PRDX2 preserves VEGF signalling in endothelial cells by protecting VEGF receptors from oxidative inactivation caused by oxidation of the essential cysteine residue, Cys1206, in the carboxy-terminal tail. Without PRDX2, cellular H_2O_2 levels rise significantly, making the VEGF receptor inactive and unable to respond to VEGF stimulation to promote endothelial cell growth [78]. During muscle stretching, low doses of testosterone cause an increase in VEGF production and endothelial cell proliferation [79], while nandrolone inhibits production of VEGF mRNA in muscle cells [80]. Further studies are needed to confirm whether AAS can cause H_2O_2-mediated and PRDX2-dependent inactivation of VEGF receptors.

Despite the fact that PRDX1 and PRDX2 use TRX as an electron donor in their redox cycle [81], no significant changes in TRX expression or oxidation status were noted in the present study. However, it cannot be excluded that downregulation of TRX, or its excessive oxidation, occurs at some point during AAS administration.

It is possible that AAS-dependent downregulation of PRDX1 and PRDX2 occurs as a direct consequence of excessive redox stress and depletion of NO caused by AAS abuse. In endothelial cells, stress-related oxygen–glucose deprivation triggers oxidative and nitrosative stress, leading to an initial increase in PRDX1 production and a robust antioxidant response. However, prolonged or intense ischemia-induced nitrosative stress inhibits PRDX1 activity through ubiquitination. PRDX1 undergoes ubiquitination and is targeted for continued degradation, leading to cellular redox imbalance and compromising the integrity of the endothelial blood-brain barrier during ischemia. Under intense oxidative stress, elevated production of NO and superoxide (O_2-) initiate the formation of peroxynitrite, a highly reactive, short-lived oxidant that uncouples endothelial NO synthase, causing it to generate O_2 radicals instead of NO. In oxygen/glucose-deprived endothelial cells, PRDX1 is abnormally ubiquitinated through nitrosative activation of E3 ubiquitin ligase: the E6-associated protein. This ubiquitination results in continuously degradation of the cellular antioxidant defense system. The presence of ubiquitinated PRDX1 indicates an imbalance between the accumulation of toxic unfolded proteins and the proteasomal system's capacity to eliminate them, ultimately causing excessive ER stress [82], that may affect the oxidation status of PDI present in the ER lumen [83]. Since ubiquitination of PRDX1 occurs only after prolonged oxidative stress, we theorise that a similar process could be observed after prolonged administration of AAS.

In conditions of oxidative stress, PRDX2 was found to be inactivated through overoxidation of peroxidatic cysteine residues in balloon-injured rodent carotid vessels and in human atherosclerotic lesions; leading to a selective depletion of PRDX2 in carotid vessels [84]. The exact relationship between AAS-dependent oxidative stress, thiol imbalance and downregulation of peroxiredoxins needs further studies, since PRDX1 serves as a mechanosensitive antioxidant, whose expression is regulated by blood flow [85]. AAS-dependent changes in PRDXs expression and function may be less spectacular when in the context of whole body physiology than in cell culture conditions.

5. Conclusions

In conclusion, we propose that prolonged AAS administration at doses negatively impacting the general cellular function of vascular endothelial cells, downregulates peroxiredoxin levels, alters the oxidation status of thiol-containing proteins, including PDI, and causes oxidation of reduced glutathione. Combined, these observations suggest that AAS may initiate the early stages of thiol-disulfide imbalance, potentially related to excessive oxidative stress. These changes strongly depend on the type of AAS, its dose, and the duration of supplementation.

Abbreviations

AAS, anabolic androgenic steroids; Akt, Protein Kinase B (PKB); AR, androgen receptor; BD, boldenone; ECs, endothelial cells; ERK1/2, Extracellular Signal-regulated Kinases; ERp57, Protein Disulfide-Isomerase A3 (PDIA3); GSH, reduced glutathione; GSSG, oxidised glutathione; MAPKs, Mitogen-Activated Protein Kinases; ND, nandrolone; NO, nitric oxide; PAI-1, Plasminogen Activator Inhibitor-1; PDI, Protein Disulphide Isomerase; PDIA6, Protein Disulfide-Isomerase A6; PI3K, Phosphoinositide 3-kinases; PRDXs, peroxiredoxins; T, testosterone; TNF-α, Tumor Necrosis Factor α; t-PA, Tissue-type Plasminogen Activator; TRX, thioredoxin; VCAM-1, Vascular Cell Adhesion Molecule 1.

Availability of Data and Materials

The datasets generated during the current study will be made publicly available once the data repository of the Medical University of Lodz, adhering to *FAIR* Data principles, is established. In the meantime, all data is available from the corresponding author upon reasonable request.

Author Contributions

MP and MS designed the research study. MP and HP conducted the experiments and analyzed the data. All au-

thors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL26542.

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