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Up regulation of nitric oxide synthase–nitric oxide system in the testis of rats undergoing autoimmune orchitis

S. Jarazo-Dietrich*, P. Jacobo, C.V. Pérez, V.A. Guazzone, L. Lustig, M.S. Theas.

Instituto de Investigaciones Biomédicas, Facultad de Medicina, Universidad de Buenos Aires. Paraguay 2155, Piso 10, C1121 ABG, Buenos Aires, Argentina

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ABSTRACT

Background: Male reproductive tract infection and inflammation are important aetiological factors of infertility. Experimental Autoimmune Orchitis (EAO) is a model of chronic inflammation useful to study mechanisms of inflammatory reactions leading to testicular impairment. EAO is characterised by interstitial cell infiltrate of lymphomonocytes, producers of pro-inflammatory cytokines involved in germ cell apoptosis. Nitric oxide (NO), a free radical promoting immune cell activation and apoptosis, is synthesised by conversion of L-arginine to L-citrulline catalysed by NO synthase (NOS). The NOS isoforms are: constitutively endothelial (e) and neuronal (n) NOS and inducible (i) NOS.
Objectives: Although the NO–NOS system was found to be up-regulated by pro-inflammatory mediators in immune and non immune testicular cells, data on its regulation in chronic inflammatory states is lacking.
Methods and results: EAO was induced in rats by active immunisation with spermatic antigens and adjuvants; control (C) rats were injected with adjuvants. Untreated normal (N) rats were also studied. We demonstrated that iNOS, eNOS and nNOS was mainly expressed by interstitial cells in N and C rats and that in EAO NOS was up-regulated and also expressed by tubular cells. Constitutive and inducible NOS content (Western blot) as well as NO production and activity increased in the testis of rats with EAO. iNOS content and activity were selectively up-regulated in the testis of rats with orchitis. Flow cytometric analysis of NOS isoforms in testicular macrophages (M) showed that the percentage of $ED1^+ED2^-$ and $ED1^+ED2^+$ M subsets, expressing constitutive and iNOS isoforms was significantly higher in EAO, but no change in the percentage of $ED1^-ED2^+$ resident M was observed compared to C rats. M from EAO rats also released more NO than C and N rats.
Conclusions: In testis of rats with EAO, NO–NOS system was up-regulated and both testicular M and cells from seminiferous tubules contributed to NO increase. NO over production in orchitis was generated mainly by increased iNOS content and activity.

Introduction

Infection and inflammation of the male reproductive tract are widely accepted as prevalent causes of infertility; rates up to 15% have been reported for men by andrological examination in infertility clinics. The hallmark of chronic inflammatory reactions in human testis following acute bacterial or viral orchitis as well as those observed amongst infertile men, i.e. infiltration of activated inflammatory T lymphocytes, increased numbers of non-resident macrophages and mast cells, indicate a profound disturbance of local immunoregulation and testicular immune privilege (Schuppe et al. 2008).

Experimental Autoimmune Orchitis (EAO) is a model of chronic inflammation developed in our laboratory in rats by active immunisation with spermatic antigens and adjuvants (Doncel et al. 1989). EAO has proved its usefulness to elucidate mechanisms involved in testicular inflammation and autoimmune based pathology. In line with inflammatory reactions observed in human testis, EAO is characterised by an interstitial cell infiltrate of macrophages and lymphocytes and tubular damage characterised by germ cell apoptosis (Lustig et al. 1993; Rival et al. 2006; Theas et al. 2003).

The free radical nitric oxide (NO) is a reactive molecule that acts as an intra and intercellular messenger in numerous biological systems modulating processes such as immune system activation and inflammation, cell death and proliferation amongst

Abbreviations: EAO, Experimental Autoimmune Orchitis; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; ROS, reactive oxygen species; TNF-α, tumour necrosis factor-alpha; IFN-γ, interferon gamma; LPS, lipopolysaccharide; CFA, complete Freund's adjuvant; Bp, Bordetella pertussis; i.v, intravenous; C, control; N, normal; ST, seminiferous tubules; IC, interstitial cells; BSA, bovine serum albumin; RT, room temperature; CMTM, conditioned media of testicular macrophages.

* Corresponding author. Tel.: +54 11 59509612; fax: +54 11 59509612.
E-mail address: sabrina.jarazo@gmail.com (S. Jarazo-Dietrich).

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others (Pryor et al. 2006). NO is synthesised by enzymatic conversion of L-arginine to L-citrulline catalysed by nitric oxide synthase (NOS). There are three isoforms of NOS: constitutively active and Ca^{++} dependent endothelial (eNOS) and neuronal (nNOS) NOS and an inducible isoform (iNOS) (Thomas et al. 2008). NO and its derivatives (peroxinitrite and nitrogen dioxide) are considered a subclass of reactive oxygen species (ROS) that damage biological molecules. In testis, the NO–NOS system has been found to be up-regulated under different stimuli such as testosterone withdrawal, ageing and heat, known to generate testicular ROS (Turner and Lysiak 2008). Moreover, pro-inflammatory factors such as tumour necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ) and LPS up-regulate NOS expression in testis (Bauché et al. 1998; Gerdprasert et al. 2002).

ROS can be produced by macrophages and neutrophils, spermatozoa and other cell types in pathological conditions. Levels of NO produced by macrophages have been reported to depend on stimuli; for example activation of Toll-like receptors in response to cytokines and LPS generates high amounts of NO (Thomas et al. 2008). The ability of macrophages to secrete NO and other biologically active molecules highlights the capacity of these cells to participate in paracrine interactions with testicular cells. In rats with EAO, *in vivo* administration of liposomes containing clodronate led to a reduction in the number of testicular macrophages, which correlated with decreased incidence and severity of testicular damage, suggesting a pathogenic role of macrophages in orchitis (Rival et al. 2008). Testicular rat macrophage population is heterogeneous and includes a subset of resident macrophages that express the scavenger receptor CD163 (recognised by the ED2 antibody, $ED2^+$ cells) and monocytes recently arrived from circulation that express the lysosomal antigen CD68 (recognised by the ED1 antibody, $ED1^+$ cells). It has been proposed that circulating monocytes could differentiate into resident macrophages in a testicular immunosuppressor micro-environment through an intermediate cell type that expresses both markers ($ED1^+ED2^+$ cells) (Dijkstra et al. 1985; Hedger 1997; Wang et al. 1994; Rival et al. 2008). Since the $ED1^+ED2^+$ subset has been only indirectly defined, we analysed the presence of this subpopulation by flow cytometry and confocal microscopy. The role of NO–NOS system in the normal and pathological testis has been clearly demonstrated; however this system has not been explored with chronic inflammatory stimuli. The aim of this work was to study modulation of NOS isoform expression and NO production in the testis of rats with experimental orchitis and in the macrophage subsets.

Materials and methods

Animals

Male adult Sprague-Dawley rats 50–60 days old were purchased from Bioterio Central, Facultad de Farmacia y Bioquímica (Buenos Aires, Argentina). Animals were kept on a 22 °C and 14 h light, 10 h dark schedule and fed standard food pellets and water ad libitum. Animal handling and experimentation complied with the NIH Guide for the Care and Use of Experimental Animals.

Immunisation schedule

Rats of the EAO group were immunised with testicular homogenate (TH) prepared as previously described (Doncel et al. 1989). Briefly, rat testes were decapsulated, diluted in an equal volume of saline and disrupted in an Omni mixer for 30 s. The final concentration was 500 mg/ml wet weight. A total of 0.4 ml of TH emulsified with 0.4 ml of complete Freund's adjuvant (CFA) (Sigma–Aldrich, St. Louis, MO, USA) was injected intradermally in footpads and at multiple sites near ganglionar regions three times at 14 day intervals. The first two immunisations were followed by i.v. injection of 0.5 ml of Bordetella pertussis (Bp) (strain 10,536; Instituto Malbran, Buenos Aires, Argentina) containing 10^{10} microorganisms and the third by i.p. injection of 5×10^9 microorganisms. Rats in the control (C) group were injected with an emulsion of saline solution, CFA and Bp in the same conditions as EAO group. We also studied a group of untreated (non-immunised) normal (N) rats killed at the same time as rats from the other groups. EAO and C rats were killed 50–60 days after the first immunisation. Body weight was determined and testes were removed and weighed. One testis was fixed in Bouin's solution and embedded in paraffin for histopathology and immunohistochemistry and the other processed for different studies, except for determination of NO content in which both testes were used.

Seminiferous tubules and interstitial cell isolation

Testes were decapsulated and placed on a glass Petri dish containing phosphate-buffered saline (PBS). Seminiferous tubules (ST) were mechanically separated from interstitial cells (IC) with tweezers and IC collected and filtered through a fine stainless steel screen. IC were then centrifuged at $300 \times g$ 10 min and red blood cells depleted by osmotic lysis with ammonium chloride buffer (160 mM NH_4Cl, 170 mM Tris–HCl, pH 7.2).

Conditioned media of testicular macrophages

One testis of each rat was incubated with Type I collagenase (0.3 mg/ml; Worthington Biochemical Corporation, Freehold, NJ, USA) in PBS containing 0.1% bovine serum albumin (BSA) (Sigma–Aldrich) for 30 min at 34 °C in a Dubnoff shaking water bath. After adding cold PBS to inactivate collagenase, ST were allowed to settle at 4 °C. The supernatant containing IC was centrifuged at $300 \times g$ for 10 min at 4 °C. The pellet was resuspended in PBS, plated on 35 mm – diameter polystyrene Petri dishes (Nunc Inc., Naperville, IL, USA) and incubated for 2 h at 34 °C in a humidified atmosphere with 5% CO_2. Dishes were rinsed several times with PBS to remove unattached cells. In order to obtain conditioned media of testicular macrophages (CMTM), cells attached to dishes were cultured in Minimum Essential Medium Eagle (1:1; Sigma–Aldrich) without phenol red, supplemented with Hepes (Sigma–Aldrich), L-glutamine (2 mM; Sigma–Aldrich) and antibiotic–antimycotic solution (1×; Gibco-BRL, Rockville, MD, USA) for 20 h at 34 °C in a humidified atmosphere with 5% CO_2. CMTM were collected, centrifuged and stored at −70 °C until use. Adherent cells were harvested and counted to determine the number of macrophages; cell viability was determined by trypan blue dye exclusion test. The isolation procedure was performed in sterile and low endotoxin conditions as previously described (Yee and Hutson 1983).

NOS immunohistochemistry

The expression and localisation of iNOS, eNOS and nNOS in testis were examined by immunoperoxidase technique. Testis sections were deparaffinised and hydrated by successive series of ethanol. To avoid non-specific staining, unspecific sites were blocked with 5% BSA (Sigma–Aldrich) in PBS containing 20% of avidin blocking solution (Vector Laboratories, Burlingame, CA, USA). Then the sections were incubated with anti mouse iNOS (4.1 μg/ml), eNOS (0.5 μg/ml) and nNOS (0.5 μg/ml) rabbit polyclonal antibodies (BD Transduction Laboratories, San Diego, CA, USA) and diluted in PBS containing 20% biotin blocking solution (Vector Labs) for 2 h in a humidified chamber at room temperature (RT). A biotinylated rabbit anti-goat IgG (4 μg/ml; Vector Labs) was used as secondary antibody. Endogenous peroxidase was blocked with 1% H_2O_2 in

methanol for 30 min. The reaction was amplified with an ABC Peroxidase Elite Kit (Vector Labs) and the product visualised by adding diaminobenzidine substrate (Vector Labs). The sections were counterstained with haematoxylin. For negative controls the first antibodies were omitted and replaced by an irrelevant rabbit IgG (Vector Labs). Leydig cells were distinguished from other cells of the interstitial tissue by their prominent nuclei with distinct chromatin and conspicuous nucleolus (Christensen and Peacock 1980).

Western blotting

Decapsulated fresh testes were homogenised with a glass homogeniser in lysis buffer (1.5 ml/g of tissue) [50 mM Tris, 150 mM NaCl, 1% SDS, 2 mM EDTA, 1% sodium desoxycholate, 1% NP-40, pH 7.4] containing protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A and 10 μg/ml aprotinin, Sigma–Aldrich). Homogenates were centrifuged at 12,000 × *g* for 20 min at 4 °C. Protein concentration in lysates was determined by the Lowry method to assess equal loading. Before electrophoresis, samples were heated for 5 min at 95 °C, then 80 μg of protein were resolved in SDS-polyacrylamide gel electrophoresis (PAGE) 7.5% denaturing gel at 120 V for 75 min, then transferred to a PVDF membrane (Millipore, Bedford, MA, USA) for 1 h at 150 V at 4 °C. Molecular weight of immunoreactive bands was determined by comparison to a ladder of prestained protein standards with a molecular weight range of 250–10 kDa (Precision Plus Protein Standards All Blue) (Bio-Rad, Hercules, CA, USA) applied to a line in each gel. Protein transference and equal loading were monitored by staining membranes with Ponceau red. Membranes were then blocked with blocking solution (5%, w/v) of non-fat dry milk in Tris buffered saline tween-20 buffer (TBST, 10 mM Tris, 154 mM NaCl, 0.1% Tween-20 (v/v), pH 7.5) for 90 min. Blots were probed for 48 h with rabbit polyclonal antibodies against iNOS (2 μg/ml,), eNOS (4 μg/ml) and nNOS (2 μg/ml) from Santa Cruz Biotechnology, CA, USA. A rabbit polyclonal antibody anti-β-actin (0.175 μg/ml, Sigma–Aldrich) was used as internal loading control. After six washes (5 min each) in TBST, membranes were incubated with an anti-rabbit biotinylated antibody (0.25 μg/ml, Vector Labs). The reaction was enhanced with horseradish–streptavidin–peroxidase conjugates (0.33 μg/ml, Chemicon International, Temecula, CA, USA) and chemiluminescence was used to detect the horseradish–peroxidase-labelled protein. The bands were visualised in a G:Box Syngene system for imaging fluorescence and densitometrically quantified using Gene Tools software.

Nitric oxide determination

Nitrite, an indirect product of NO oxidation, was measured by the Griess reaction (Green et al. 1982). One rat testis was used to evaluate total nitrite production and the other to determine nitrite production by ST and IC. Decapsulated testes and ST were homogenised and sonicated in Tris–HCl buffer containing 0.05% Triton X-100 (100 μl/g of tissue) and IC were processed in three volumes (100 μl) of buffer. The homogenate was centrifuged at 12,000 × *g* for 30 min at 4 °C and supernatant obtained. An aliquot was then stored at −20 °C for protein determination by the Lowry method (Bio-Rad DC Protein Assay; Bio-Rad) and samples were deproteinised to prevent precipitates and turbidity by adding $ZnSO_4$ (0.15 M, 10 μl/ml) and NaOH (10 M, 0.6 ml/ml) in Tris–HCl buffer 0.05% Triton X-100. The solution was stirred, incubated in ice for 15 min, then centrifuged at 12,000 × *g* for 5 min at 4 °C; the supernatant was used to evaluate nitrite content. To determine nitrite content, 100 μl of CMTM, testes, ST and IC homogenate was applied to a 96-well culture plate, followed by 100 μl of Griess reagents (1% sulphanilamide in 5% phosphoric acid and 0.1% N-1-naphthylethylenediamine in equal parts, Sigma–Aldrich). After 15 min of colour development at RT, absorbance was measured in a microplate reader at 595 nm (Model 550, Bio-Rad). Nitrite concentration was determined using a sodium nitrite standards calibration curve ranging from 1.57 to 25 μM.

NOS activity determination

NOS activity in testis homogenates was assessed by measuring conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline as described previously with some modification (Hikim et al. 2005). Briefly, testes were homogenised in 500 μl of 20 mM Hepes buffer pH 7.4 (0.5 mM EDTA, 1 mM DTT, 5 mM L-valine, Sigma–Aldrich) containing protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A and 10 μg/ml aprotinin, Sigma–Aldrich). The homogenate was centrifuged at 13,000 × *g* for 20 min at 4 °C. The supernatant was passed through 500 μl of pre-washed Dowex resin (AG50W-X8, Na^+ form, Bio-Rad) to remove endogenous arginine and 50 μl were used to determine protein concentration by the Lowry method. Then total and Ca^{2+} independent NOS activity was determined by incubating 200 μl of supernatant in 20 mM Hepes buffer with 0.1 μCi of [^{3}H] L-arginine (specific activity: 43 Ci/mmol, Perkin Elmer, Boston, MA, USA), 1 mM NADPH, containing or not 0.45 mM Ca^{2+} at 37 °C for 30 min in a Dubnoff water shaking bath (80 cycles/min) with 5% CO_2. Aliquots of 200 μl were incubated in the presence of a NOS inhibitor, L-nitro arginine methyl ester (L-NAME, 2 mM, Cayman Chemical, MI, USA), in the same conditions as above. The reaction was stopped by incubating samples in ice for 15 min. Afterwards, samples were deproteinised to avoid turbidity by adding 120 μl 0.3 M $ZnSO_4$ and 4 μl 10 M NaOH diluted in 20 mM Hepes. Residual [^{3}H] L-arginine in the sample was eliminated by passing it through 1 ml pre-washed Dowex resin columns previously blocked with 20 μl L-citrulline (100 mM, Sigma–Aldrich); [^{3}H] L-citrulline was eluted with 1 ml of distilled water. Amount of [^{3}H] L-citrulline was determined with a liquid scintillation counter (Liquid Scintillation Analyser, TRI-CARB 2800TR, Perkin Elmer). Assays were run in duplicate. All values were corrected by substracting radioactivity determined in absence of testis homogenate.

ED1 and ED2 double immunofluorescence

To detect immunofluorescent (IF) patterns of different subsets of testicular macrophages, a double IF assay was performed on frozen testis cryostat sections (6–7 μm) fixed in acetone. Sections were first blocked with 3% normal horse serum in PBS for 40 min at RT, then incubated with a primary ED2 mouse monoclonal antibody (12.5 μg/ml, BD Trans Labs) for 1 h at RT followed by an anti-mouse FITC secondary antibody for 1 h (15 μg/ml, Vector Labs). Sections were then blocked with 3% BSA and incubated with ED1-AlexaFluor ®647 antibody (5 μg/ml, AbD Serotec, Oxford, UK) for 1 h at RT. Sections were mounted in PBS-glycerine and observed in a confocal laser microscope (FV300 Olympus). For negative controls, PBS was used instead of primary antibodies.

Flow cytometry

Testicular IC were obtained as described for macrophage isolation with some modifications. Once collagenase was inactivated and ST settled, the supernatant was washed with PBS and red blood cells were depleted by osmotic lyses with ammonium chloride buffer (160 mM NH_4Cl, 170 mM Tris–HCl, pH 7.2). Cells were washed, centrifuged at 300 × *g* for 10 min at 4 °C and counted in a Neubauer chamber by the trypan blue exclusion method. Then, 1×10^6 testicular IC were stained with a mouse monoclonal CD45-PECy5 antibody for 30 min at 4 °C (1.44 μg/ml, BD Trans Labs)

and with a mouse monoclonal ED2-AlexaFluor®647 antibody for 40 min at RT (5 μl direct, AbD Serotec). Cells were permeabilised with 0.1% saponin (MP Biomedicals Inc, Solon, USA) and intracellularly stained with a mouse monoclonal ED1-PE antibody for 40 min at RT (7 μl direct, AbD Serotec). Then cells were fixed with 0.5% paraformaldehyde for 10 min at 4 °C and incubated for 30 min at 4 °C with rabbit polyclonal antibodies against iNOS, eNOS or nNOS (41.67 μg/ml, BD Trans Labs) followed by an anti-rabbit FITC secondary antibody for 30 min at 4 °C (15 μg/ml, Vector Labs). Appropriate control isotypes were used. Evaluation was performed in a BD FACSCalibur cytometer acquiring 150,000 total events.

Statistical analysis

Statistical evaluations used Student's *t*-test and the Student–Newman–Keuls test. Differences were considered significant if $p < 0.05$.

Results

NOS localisation and content in the testis

Constitutive and inducible isoforms of NOS were expressed in testis of N, C and EAO rats. In testis sections of N and C rats mild staining was observed mainly in the interstitium where Leydig cells expressed the three NOS isoforms and endothelial cells expressed iNOS and eNOS (Fig. 1). In EAO rats, up-regulation of NOS isoforms was observed in cells of both compartments of the testis. In the ST degenerated germ cells, spermatocytes and spermatids expressed the three NOS whilst peritubular cells expressed eNOS and iNOS (Fig. 1). A very mild iNOS immunoreactivity in Sertoli cells was occasionally observed in the testis of C and EAO rats, being most of these cells negative.

We observed by Western blot that iNOS, eNOS and nNOS content were similar in the testis of N and C rats. An increase in the three isoform content was detected in the testis of rats with orchitis (Fig. 2). The increase of iNOS content was higher compared to that of constitutive NOS.

Nitric oxide production and NOS activity in testis

Since NOS was highly expressed in the interstitium and ST of EAO rats we were interested in determining whether this enzyme was active and able to generate NO. Interstitial and tubular cells from EAO rats produced significantly more NO than cells from C and N rats whose NO production was similar. Moreover, NO production by interstitial cells was higher than that of tubular cells in all groups studied (Fig. 3). When we measured NO production by the whole testis we found that EAO rats released three times more NO than C and N rats (% of NO_3 production, N: 97.213 ± 10.614, C: 100.000 ± 22.514, EAO: 414.979 ± 107.478 $p < 0.05$ mean ± SEM, Student–Newman–Keuls test, $n = 6$–9 rats per group). Tallying with data of NO production, we detected similar total NOS activity (iNOS plus eNOS plus nNOS) in testis of N and C rats (Fig. 4). NOS activity measured was specific since it was significantly reduced in the presence of the competitive inhibitor of NOS, L-NAME (2 mM) (fmol/min/mgprot N: 0.254 ± 0.055, N + L-NAME: 0.015 ± 0.011, $p < 0.001$, C: 0.476 ± 0.148, C + L-NAME: 0.037 ± 0.016 $p < 0.01$ mean ± SEM, Student's *t*-test, $n = 6$ rats per group). Total NOS activity in testis of EAO rats was significantly higher compared to C and N rats and was also lowered by L-NAME. iNOS activity was significantly higher in testis of EAO rats compared to C and N, comprising 71.22% of total activity in testis (Fig. 4).

NOS expression and nitric oxide production by testicular macrophages

We analysed the presence and distribution of macrophage subpopulations in testis by confocal microscopy. We observed that the three subsets of macrophages, $ED1^+ED2^-$, $ED1^-ED2^+$ and $ED1^+ED2^+$ were present in the testis and had a similar distribution pattern around ST in all groups of rats studied (Fig. 5). Numerous $ED1^+ED2^+$ cells were present in testis of EAO rats compared to C and N. Different fluorescence intensities observed in testicular macrophages by confocal microscopy showed the heterogeneous expression of antigens that characterises cells of each subset. By flow cytometry we confirmed the existence of the three subpopulations of testicular macrophages (Fig. 6). Analysis of NOS isoform expression in infiltrating, resident and double positive macrophages by flow cytometry showed expression of constitutive (eNOS, nNOS) and iNOS isoforms in the three testicular macrophage subpopulations in testis of N, C and EAO rats. In testis of EAO compared to C and N rats the percentage of macrophages expressing eNOS was significantly increased in $ED1^+ED2^-$ and $ED1^+ED2^+$subpopulations. In testis of C compared to N rats the percentage of macrophages expressing iNOS and nNOS was significantly increased in the $ED1^+ED2^-$ and $ED1^+ED2^+$ subpopulations. In $ED1^-ED2^+$ we observed no difference in NOS isoform expression between EAO and C rats (Fig. 6). However, in the testis of EAO rats we detected a higher percentage of $iNOS^+$ $ED1^-ED2^+$ than in N rats. Analysis of Gmean Index in macrophage subpopulations of N, C and EAO rats showed similar values for the three NOS isoforms (data not shown).

We analysed NO production in CMTM obtained from rat testis. Macrophages from EAO rats released significantly more NO than macrophages from C and N rats, whereas no difference in NO production was detected comparing C and N (Fig. 7). Since we determined the number of macrophages/dish we were able to calculate NO production/macrophage. This analysis showed that macrophages from EAO rats released significantly more NO than those from C and N rats. (NO μM per 10^5 macrophages, N: 1.052 ± 0.499, C: 1.292 ± 0.336, EAO: 5.046 ± 1.262 $p < 0.05$ mean ± SEM, Unpaired *t* test with Welch correction, $n = 5$–13 rats per group). Increased production of NO by EAO macrophages may be due both to the increased number of macrophages and to increased production per macrophage.

Discussion

We demonstrated that in testis of rats with chronic inflammatory stimuli NO production and NOS expression was up-regulated. Constitutive and inducible NOS isoforms were expressed by cells of the interstitium and ST. Whereas in testis of normal and control rats NOS isoform content was low and restricted to cells of the interstitium, in orchitis the content of the three isoforms increased being detected in degenerating spermatocytes, spermatids and peritubular cells.

NO production and NOS activity in testis of normal and control rats was similar, indicating that adjuvant has no effect on enzyme activity. In the inflammatory microenvironment of testis of EAO rats NOS content and activity increased concomitant with NO production, with selective up-regulation of iNOS isoform. *In vitro* combinations of cytokines (TNF-α, IFN-γ and IL-1α) and LPS were reported to up-regulate iNOS mRNA and increase NO production by Sertoli and peritubular cells, and IL-1β was able to stimulate iNOS mRNA expression and NO production by Leydig cells (Bauché et al. 1998; Tatsumi et al. 1997). *In vivo*, LPS treatment stimulated iNOS expression in germ cells, mainly in pachytene spermatocytes, and also in Sertoli and Leydig cells (O'Bryan et al. 2000).

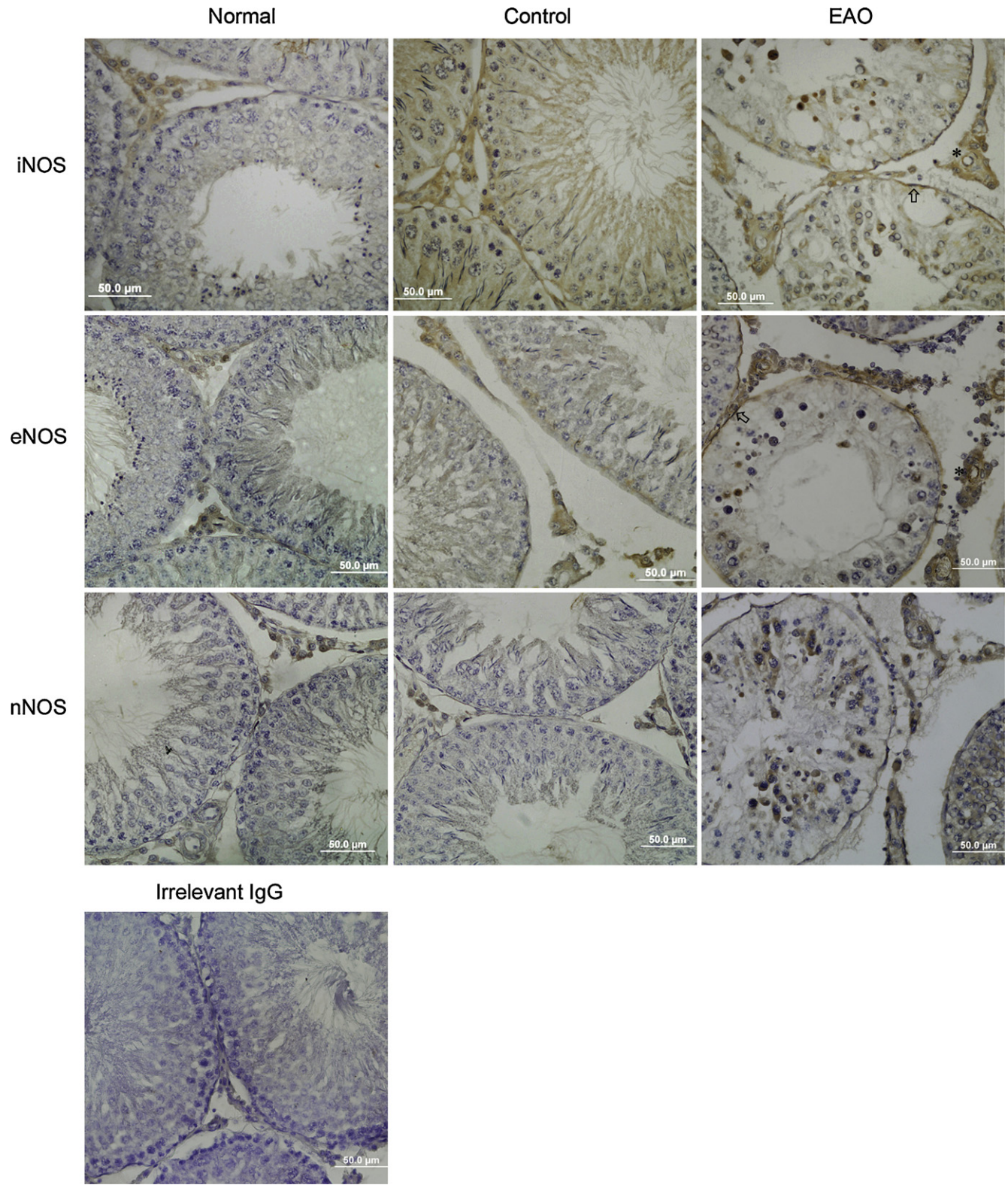

Fig. 1. Immunohistochemical localisation of NOS isoforms in testis sections from normal, control and EAO rats, by the immunoperoxidase technique. Note NOS expression in cells of the interstitium, Leydig cell (prominent nuclei with distinct chromatin), endothelial cell (asterisk). In the seminiferous tubules germ cells expressed iNOS, eNOS and nNOS in EAO testis. Peritubular cells expressed iNOS and eNOS in EAO rats (arrow). Rabbit IgG was used in the negative control.

Pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-6 produced in large amounts by interstitial lymphocytes and macrophages during EAO development might possibly be involved in iNOS up-regulation (Jacobo et al. 2011; Rival et al. 2008; Theas et al. 2008). Although eNOS and nNOS expression have not been studied in testis in response to pro-inflammatory factors these isoforms have been reported to be up-regulated in experimental torsion, in models of obstruction and in infertile men (Fujisawa et al. 2001; Moon et al. 2005; Oktem et al. 2009). Promoter regions of nNOS and eNOS contain consensus sequences for binding

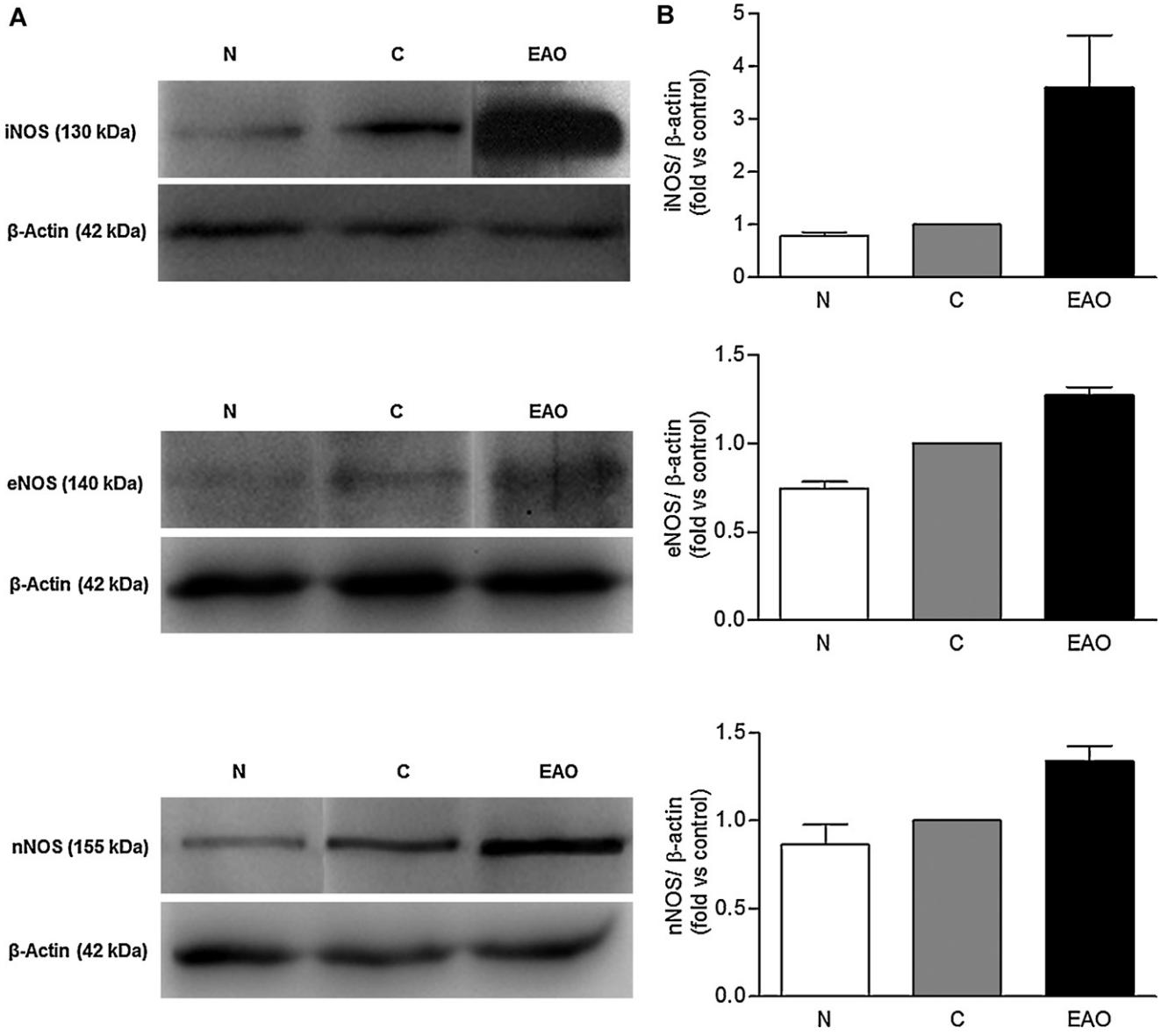

Fig. 2. Inducible, endothelial and neuronal NOS expression in the testis of normal (N), control (C) and EAO rats. (A) Representative Western blots of NOS content determined in the testicular lysates. (B) Semiquantitative results of NOS content obtained by densitometric analysis of Western blots. Data represent mean ± SEM of 3 rats per group. Data from C rats were arbitrary set at 1.

transcription factors AP-1, nuclear factor IL6, NF-kB and others, which could feasibly respond to cytokines; in fact eNOS is up-regulated by IFN-α, IFN-β and LPS in different tissues (Förstermann et al. 1998; Wang et al. 2002).

In normal testis we demonstrated by flow cytometry that $ED1^+ED2^-$, $ED1^+ED2^+$ and $ED1^-ED2^+$ macrophages expressed the inducible and constitutive NOS isoforms. In the inflamed testis of EAO rats we observed an increase in the percentage of $ED1^+$

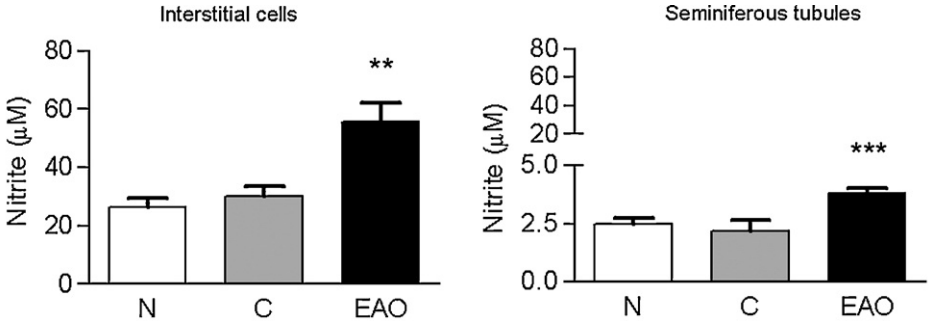

Fig. 3. Nitric oxide (NO) production by interstitial cells and seminiferous tubules of normal (N), control (C) and EAO rats. Nitrite content was evaluated by the Griess reaction. Each column represents the mean ± SEM of 6–9 rats per group. Data were analysed by Student–Newman–Keuls test, $^{**}p < 0.01$, $^{***}p < 0.001$ vs respective C.

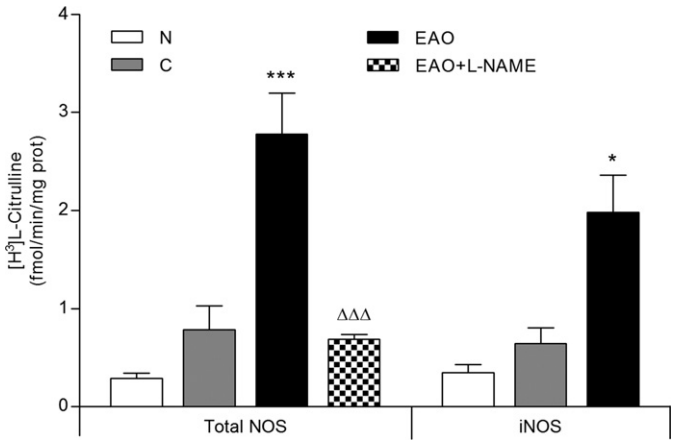

Fig. 4. Nitric oxide synthase activity determination in testis of normal (N), control (C) and EAO rats. Total NOS activity corresponding to constitutive (eNOS and nNOS) and inducible (iNOS) isoforms was determined in the presence of Ca^{2+}, whereas iNOS activity was determined in the same samples in the absence of Ca^{2+}. The inhibitor L-NAME (2 mM) was added to the incubation buffer to inhibit total NOS activity in the EAO group. Each column represents the mean ± SEM of 6–8 rats per group. Data were analysed by Student–Newman–Keuls test $^{*}p < 0.05$, $^{***}p < 0.001$ vs respective C, $^{\Delta\Delta\Delta}p < 0.001$ vs total activity of EAO group.

macrophages that expressed iNOS, eNOS and nNOS isoforms. Recently, Winnall et al. (2011) demonstrated that iNOS mRNA was up-regulated in $CD163^{-}$ testicular macrophages in response to LPS. In experimental autoimmune uveoretinitis (EAU) an increased number of $ED1^{+}$ macrophages expressing iNOS were observed, concomitant with increasing severity of retinal damage (Robertson et al. 2002; Zhang et al. 1999).

As evidenced by our results, $ED1^{+}ED2^{-}$, $ED1^{+}ED2^{+}$ macrophages reacted to adjuvant and to the inflammatory microenvironment by up-regulating the expression of inducible and constitutive NOS isoforms, whereas resident $ED1^{-}ED2^{+}$ population was less sensitive to these stimuli. Interstitial EAO macrophages showed a differential expression pattern for other markers in response to chronic inflammation: MCP-1 is expressed by all $ED1^{+}$ and only a few $ED2^{+}$ macrophages and IL-6 is expressed only by $ED1^{+}$ macrophages (Guazzone et al. 2003; Rival et al. 2006). These results concord with the concept that testicular macrophage population is heterogeneous in function and supports the pro-inflammatory role proposed for the $ED1^{+}$ macrophage subpopulation. During EAO, pro-inflammatory cytokines (TNF-α, IFN-γ, IL-6) present in the interstitium may preferentially modulate NOS expression in $ED1^{+}$ subpopulation. $ED1^{-}ED2^{+}$ macrophages seem to be more resistant to respond to testicular factors since they up-regulated iNOS only with the combined stimuli of adjuvants and testicular homogenate. Heterogeneity in macrophage subpopulation also seems to occur in retina where resident retinal macrophages and those at the earliest phase of EAU generated little NO spontaneously and were unable to release NO in response to IFN-γ and TNF-α *in vitro*. By contrast, macrophages present in the late phase of the disease released higher levels of NO in response to pro-inflammatory cytokines (Robertson et al. 2002).

Macrophages generate different levels of NO that serve different functions. Interestingly the amount of NO produced from activated macrophages is dependent on the manner and factor (LPS/cytokines) by which they are stimulated (Thomas et al. 2008). The number of $ED1^{+}ED2^{-}$ and $ED1^{+}ED2^{+}$ macrophages rises in the testis of EAO rats (Rival et al. 2008). In the present work we demonstrated that the percentage of these macrophage subsets expressing NOS also increased, suggesting that the high NO production is due mainly to the rise in the number of $ED1^{+}/NOS^{+}$ macrophages and to the NO production per macrophage. We speculate that regulation of NOS activity occurs in EAO macrophages to generate a five fold increase in NO levels since the degree of NOS isoform expression by these cells was similar to that of normal and control rats (data not shown). NOS activity may be regulated at transcriptional and posttranslational levels by stimulatory and inhibitory factors (Kone et al. 2003). In normal and control rats, inhibitory factors or lack of stimulatory proteins possibly keep NOS activity and NO production at basal levels. During the development of orchitis, inflammatory stimuli may remove inhibition and/or induce NOS activity. In the aqueous humour, calcitonin gene-related peptide suppresses NO production by activated macrophages through posttranslational regulation of iNOS (Taylor et al. 1998). In murine macrophages a protein denominated as the NOS-associated protein-110 kDa inhibits iNOS catalytic activity by preventing dimerisation (Su et al. 2005). Another important factor of NOS regulation is its location within the cell (Ratovitski et al. 1999). For instance, eNOS activity may be increased ten times by simply translocating to the membrane; translocation and association of iNOS with the submembranous actin cytoskeleton in activated macrophages may affect its activity and NO release from macrophages (Kone et al. 2003).

We demonstrated that in normal testis NO is produced at low levels whereas in chronically inflamed testes NO levels rose. Cells from interstitium and from ST release NO, the former being the major producers of NO in normal, control and inflamed testis. Multifaceted biological responses to NO are in function of concentration and duration of exposure, and therefore differential cellular responses result from specific incremental NO levels. At sustained NO levels lower than 1 μM NO actions are direct, for instance, NO can activate guanylate cyclase (GC) and MAP kinase signalling pathways. It is above 1 μM NO that nitrosation of critical proteins such as poly ADP ribose polymerase and caspases involved in apoptosis occurs (Lee and Cheng 2008; Thomas et al. 2008). In consonance with these concepts, a potential role for NO in the control of testicular function in health and disease is suggested. NO may behave as a survival factor at the physiologically low concentrations present in normal testis, and also as a pro-apoptotic, pro-inflammatory factor during pathological conditions of the organ.

In normal testis, a physiological role of iNOS in the determination of sperm number and testis size has been suggested, since in iNOS null mutant mice a reduced rate of spermatocyte apoptosis and increased sperm count was observed (Lue et al. 2003). Moreover, Lee and Cheng (2008) demonstrated that NOS/sGC/protein kinase G (PKG) participates in the regulation of the blood testis barrier.

During the development of orchitis, germ cell apoptosis takes place through mitochondrial apoptotic pathway activation; germ cell sloughing occurs concomitant with changes in adhesion molecule expression in cells of the seminiferous epithelium (Pérez et al. 2011; Theas et al., 2006). We speculated that the rise in NO levels, generated mainly by activated pro-inflammatory-type testicular macrophages ($ED1^{+}$), may contribute to control the above mentioned cellular functions and possibly others leading to the development of the inflammatory scenario causing germ cell loss and infertility.

Clinical and pathological evidence shows that chronic inflammatory conditions of the testes may disrupt spermatogenesis and irreversibly alter both sperm number and quality. Specifically, histopathology of testicular biopsies from infertile men indicates a high prevalence of inflammatory reactions. Characterisation of NO–NOS system regulation in EAO opens new approaches to understanding the pathogenesis of chronic testicular inflammation, thus providing a basis for improved diagnosis and the development of novel therapies.

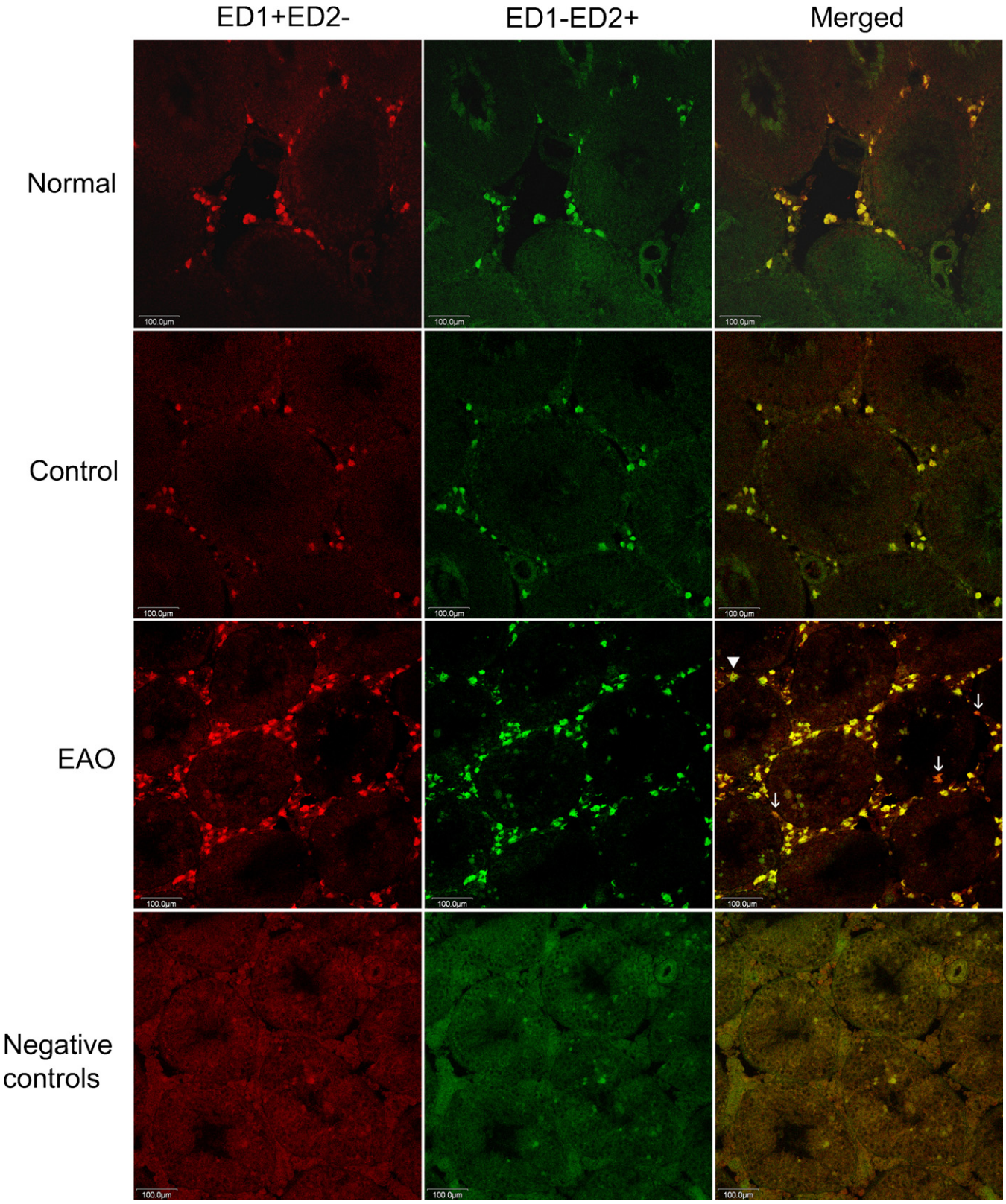

Fig. 5. Presence and distribution of testicular macrophage subpopulations. Immunostaining for ED1 (red) and ED2 (green) in frozen testicular sections from normal, control and EAO rats visualised by confocal microscopy. Note the increased number of macrophages in testis of EAO rats, the majority of them $ED1^{+}ED2^{+}$. Arrow $ED1^{+}ED2^{-}$ macrophages, arrowhead $ED1^{-}ED2^{+}$.

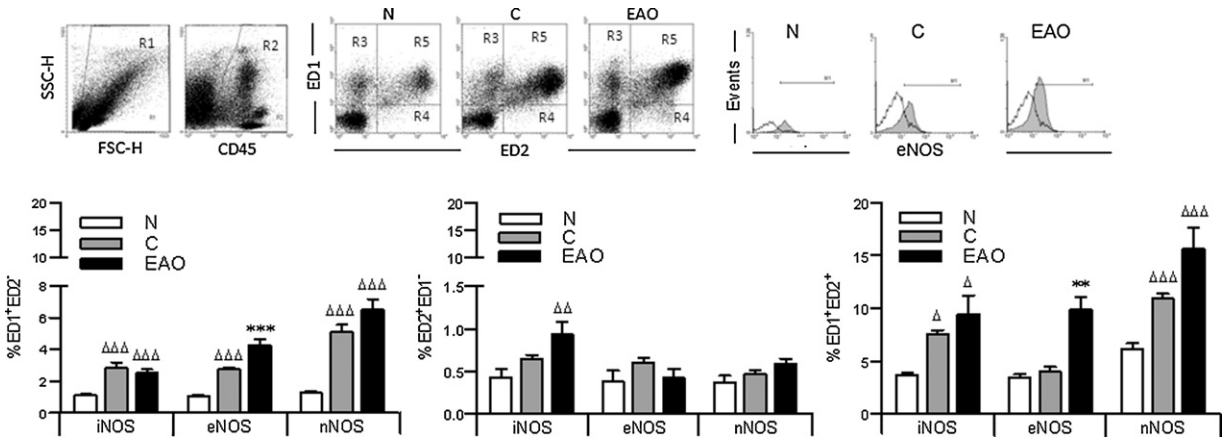

Fig. 6. Expression of NOS isoforms in testicular macrophage subsets of normal (N), control (C), and EAO rats. Testicular interstitial cells were stained for CD45/ED1/ED2 and NOS and analysed by flow cytometry. In the side vs forward scatter dot plot, a gate was made to select viable cells and exclude debris (R1). A gate was drawn selecting $CD45^+$ leucocytes based on the isotype control (R2). In the ED1 vs ED2 dot plot macrophage subpopulations were defined as $ED1^+ED2^-$ on R3; $ED2^+ED1^-$ on R4, and $ED2^+ED1^+$ on R5. NOS expression was analysed in R3, R4 and R5. Representative histograms showing eNOS expression in $ED1^+ED2^-$ macrophages. Each column represents the mean ± SEM of 6–9 rats per group. Data were analysed by Student–Newman–Keuls test $^{**}p < 0.001$, $^{***}p < 0.001$ EAO vs respective C; $^{\Delta}p < 0.05$ C, $^{\Delta\Delta}p < 0.01$ C, $^{\Delta\Delta\Delta}p < 0.001$ C vs respective N.

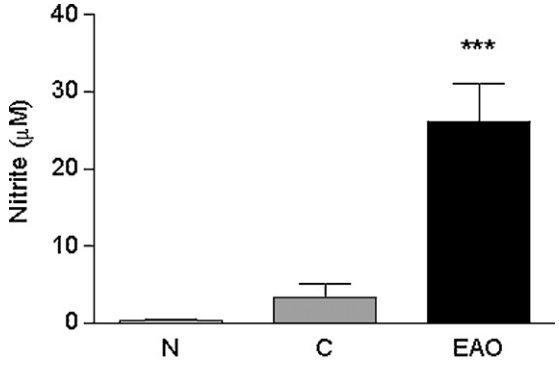

Fig. 7. Nitric oxide (NO) production by testicular macrophages. NO was measured in the conditioned media of testicular macrophages of normal (N), control (C) and EAO rats using the Griess reaction. Each column represents the mean ± SEM of 5–13 rats per group. Data were analysed by Student–Newman–Keuls test $^{***}p < 0.001$ vs C.

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