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Apocynin and raisanberine alleviate intermittent hypoxia induced abnormal StAR and 3β-HSD and low testosterone by suppressing endoplasmic reticulum stress and activated p66Shc in rat testes

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ABSTRACT

We hypothesized that hypoxia induced testicular damage is mediated by an activated NADPH oxidase (NOX), therefore, APO (apocynin) an inhibitor of NOX and raisanberine (RS), a calcium influx inhibitor were tested if they could attenuate hypoxic toxicity to the testis. Male Sprague-Dawley rats were exposed to hypoxia ($10 \pm 0.5\%$ O_2) for 17 d and intervened with APO and RS in the last 6 d. Histological changes and expression of pro-inflammation factors were evaluated *in vivo*. Biomarkers in isolated Leydig cells incubated with H_2O_2 were also assayed *in vitro*. Hypoxic rats displayed lower serum testosterone and higher LH and FSH. Upregulation of p22/p47^{phox}, NOX2, MMP9, PERK and p66Shc was associated with downregulation of StAR, 3β-HSD and Cx43 in the hypoxia testis, revealed by Western blot and immunohistochemical assay, respectively. APO and RS at least partially normalize hypoxia caused male hypogonadism by suppressing ER stress, and p66Shc in testes.

1. Introduction

Male hypogonadism is characterized by decreased serum testosterone and degenerative changes in the testis and its prevalence seems to be increased in modern society. Low testosterone in serum can be caused by stress responses [1] A declined serum testosterone may be consequence to many etiological factors including diabetes, hypertension and aging. The testis is predisposed to local hypoxic reactions implicated in many pathological changes in the testis including chronic exposure to hypoxia. Therefore, the hypoxic lesions in the testis may be the basic changes involved in the molecular aspects in pathology underlying testicular dysfunction. Emerging data reveal that male hypogonadism is frequently present in a status of oxidative stress in testes, as revealed in our previous findings, caused by adenine toxicity, hyperthyroidism, diabetes, and hypoxia [2–6].

Hypoxia exposure causes a series of changes characterized by sustained elevation in pulmonary vascular resistance and, therefore, raises pulmonary blood pressure, and the consequent hypoxemia causes testicular dysfunction and abnormalities of calcium modulating proteins in the heart [7,8]. However, hypoxia under some conditions may stimulate Leydig cells to secrete more testosterone *in vitro* [9]. Testicular dysfunction appears due to excessive production of reactive oxygen species (ROS) implicated in the molecular aspects in the testis disease [10,11]. An activation of NADPH oxidase (NOX) which has been considered as the main source of ROS production in the presence of activated endothelin receptor A (ET_A) [12]. Therefore, an excess of endothelin-1 (ET-1) which causes more ROS genesis may take an active part in pathological processes responding to hypoxia by inducing oxidative stress [7,12].

In the testis, ET-1 contributes to modulating the transport of sperms in seminiferous tubules toward the epididymis by

Abbreviations: APO, apocynin; Bip, immunoglobulin binding protein; CHOP, C/EBP homologous protein; CPU86017-RS, an optical isomer of CPU86017, a derivative of berberine; RS, raisanberine; Cx43, connexin 43; CXs, connexins; DAPI, 4′,6′ diamidino-2-phenylindole; ER, endoplasmic reticulum; ET, endothelin-1; ET_A, endothelin receptor A; FITC, fluorescein isothiocyanate; GSHPx, glutathione peroxidase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; HE, haematoxylin–eosin; HPG axis, hypothalamus–pituitary–gonad axis; MDA, malondialdehyde; MMP9, matrix metalloprotease 9; NOX, NADPH oxidase; Nox2, a catalytic subunit of NADPH oxidase; Nox4, a catalytic subunit of NADPH oxidase; p22^{phox}, a modulating subunit of NADPH oxidase located in the membrane; p47^{phox}, a modulating subunit of NADPH oxidase located in cytosol; p66Shc, a member of Shc family in controlling the oxidants in cells; PERK, double-strand RNA-activated protein kinase-like ER kinase; ROS, reactive oxygen species; StAR, steroidogenic acute regulatory protein.

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contracting the muscle-like cells around the tubules. There may be two types of abnormal ET pathway implicated in male hypogonadism: either downregulated or upregulated ET_A is present in male hypogonadism caused by adenine or stress (by isoproterenol medication), respectively [1,2]. In hypoxic testicular damage, the status of ET_A is upregulated [6]. An impairment of spermatogenesis is a direct consequence to an increase in oxidants in the testis, related to upregulated ET_A eventually activating NOX in the testis [4].

p66Shc, a cellular redox system signaling molecule, deteriorates oxidative stress by activating cytomembrane oxidase activity, inhibiting the ability of oxygen free radical scavenging and interfering mitochondrial electron transport chain [13,14]. Upregulated p66Shc is always accompanied with an over-activated ET-NOX pathway and will produce excessive ROS, so an activated p66Shc is likely involved in a vicious cycle of the ET-NOX pathway to exacerbate the production of oxidants, implicated in many cardiovascular diseases as well as testicular dysfunction and in inflammation [15].

Endoplasmic reticulum (ER), a complicated cytoplasm organelle is responsible for protein synthesis and calcium homeostasis. Environmental stress may impair ER function, causing unfolding protein responses (UPR) and an abnormal calcium balance promoting cell dysfunction and eventually leading to apoptosis. Oxidative stress is active to facilitate the appearance of ER stress which mediates a series of cellular abnormalities resulting in cardiovascular disease [16–18]. Moreover, ROS have been suggested in promoting the UPR toward pro-apoptotic changes [19]. Our previous study showed that the ER stress markers PERK, Bip, CHOP and abnormal StAR and 3β-HSD were significant in damaged testes that were positively responded to raisanberine (CPU86017-RS, RS) due to its calcium influx blocking activity [6,20].

MMPs are the most important proteases taking an active part in modulating extracellular matrix degradation and remodeling. MMP9 is important in maintaining the normal structure and function of the seminiferous tubules [21]. Intercellular gap junctional communication is performed by a family of proteins known as connexins. Cx43 is the most abundant and extensively distributed connexin and its downregulation adversely causes organ abnormalities in diabetic complications and cellular damage due to isoproterenol induced stress reactions [12,22]. An alteration of Cx43 is also involved in abnormal function and structure of the testis suffering from hypoxia exposure [20]. Testicular disorders by intermittent hypoxia exposure may be attributed to a deficiency of Cx43-mediated gap junctional signaling in the testis. Cx43 contributes to the intercellular signal communications and constitutes the blood–testis barrier (BTB). Reduced expression of Cx43 is found in the testes relating to male hypogonadism and infertility [20,23]. The abnormal Cx43 resulting in testicular damage on hypoxia may be modulated by NOX in the testis.

We hypothesize that intermittent hypoxia impairs the testes through damage to spermatogenesis in seminiferous tubules and testosterone synthesizing genes including StAR and 3β-HSD in the Leydig cells. The oxidants caused by hypoxia promote ER stress and activate p66Shc gene possibly to be mediated by activation of NADPH oxidase (NOX). Apocynin (APO) an inhibitor of NOX is likely to be effective in alleviating the hypoxia induced abnormalities in the testis. The effects of APO may be comparable to the calcium influx limiting effects of RS, a berberine derivative, which has been proved in relieving the testis disease caused by hypoxia [6,20].

2. Methods

2.1. Experimental animals and treatment

All procedures performed on the animals were conducted in accordance with the Animal Regulations of Jiangsu Province, China. Male Sprague-Dawley rats, weighing 220–250 g, were purchased from Zhejiang Experimental Animal Center [License Number: SCXK(Z)20080033] and housed under controlled conditioning (25 ± 1 °C constant temperature, 55% relative humidity, 12 h lighting cycle). The rats were allowed to restrained standard diet (15 g per day for each rat) beneficial to the diabetic untreated rats and all rats were free access to water.

Rats were randomly divided into 4 groups ($n = 6$): control (Con), intermittent hypoxia (Hyp), intermittent hypoxia + apocynin (H + APO, Sigma, USA) and intermittent hypoxia + RS (H + RS), RS is synthesized by the Department of Medicinal Chemistry, China Pharmaceutical University. Rats were exposed to hypoxia 8 h per day ($10 \pm 0.5\%$ O_2 in a normobaric chamber, controlled by driving N_2 into it, monitored by an Oxygen Analyzer CY-12C (MeiCheng Electrochemical Analytical Instrument Factory, Hangzhou, China) for 17 days except for control group (under normal condition). The rats were administrated with 0.5% CMC-Na (Sinopharm Chemical Reagent Co. Ltd., China) and the vehicle administered in both control and rats exposure to hypoxia. Interventions (p.o, mg kg^{-1} per day) with APO (80, dissolved in 0.5% CMC-Na) and RS (80, dissolved in 0.5% CMC-Na) were conducted for the last 6 days by gavage. Ten gram of soda lime and calcium chloride each were placed in the hypoxic chamber to absorb extra H_2O and CO_2 and were changed at 5 days interval. On the day 18, rats were sacrificed and the testes were harvested and stored in liquid nitrogen for RT-PCR and WB assay, and fixed by 10% formalin or 4% paraformaldehyde for histological examination (Sigma, St. Louis, MO, USA).

2.2. Sperm counting and motility rate

The spermatozoa were collected from the caudal epididymidis of each rat as previously described with some modifications [24,25]. Briefly, the caudal epididymidis were cut into small pieces in 2 ml preheated Ringer's solution and placed in a beaker to let sperms swim up for 10 min. After diluted with trypan blue solution, the sperm solution was transferred to a blood cell counting chamber for the counting of total number (density), and the motility rate and appearance of spermatozoa were observed under an inverted microscope (Nikon TE 2000-U, Japan).

2.3. Sexual behavior test

Sexual behaviors of all male rats were tested in accordance with the procedures described previously with some modifications [26]. Briefly, sexually receptive female rats (8 weeks old) whose vaginal smears showed proestrous features after consecutive repetitions of 2 cycles of the 4-day estrous cycle were selected. After male rats were placed in the chamber for 10 min in the night, a sexually receptive female rat was introduced into the chamber under low-level red-light illumination. Copulatory behavior was targeted within about 20 min in the night (21:00–21:20). The following parameters were recorded and counted: mounting latency, mounting frequency and ejaculation frequency [27,28].

2.4. Biochemical marker assay

A part of the testes were homogenized with normal saline after the rats were cervical dislocated. Activities of MDA, GSHpx in the testes were measured by biochemical kits (Nanjing Jiancheng Institute of Bio-engineering Company, Nanjing, China). All procedures were conducted in strict accordance with manufacturers' instructions. Serum testosterone, FSH and LH concentration were measured by radioimmunoassay (RIA) with kits purchased from the Institute of Jiuding Medical Bio-engineering company (Tianjin, China).

2.5. Leydig cell culture

Leydig cells were isolated from the testis of normal rats and cultured as described previously [29,30]. Except for control group, Leydig cells were incubated with H_2O_2 for 12 h, and APO and RS were added during the last 6 h. After incubation, Leydig cells were harvested for detection of biomarkers including $p22^{phox}$, $p47^{phox}$, StAR and 3β-HSD.

2.6. RT-PCR

The testes were extracted with Trizol solution for mRNA and cDNA were synthesized (Invitrogen, Carlsbad, CA, USA) as described previously [1]. Briefly, the sequences of primers (Invitrogen Trading Co., Ltd., Shanghai, China) and amplification (Eppendorf Mastercycler Personal PCR Cycler, Genmany) conditions were showed in Table 1. Finally, the density of band was detected and compared among groups.

2.7. Western blotting

After rats were sacrificed, the testis proteins were extracted (Biouniquer Biotechnology Co., Ltd., Hangzhou, China) as previously described [1]. Briefly, after determining the protein concentrations, the supernatant was stored at −20 °C before use. An aliquot of the sample was heated to 98 °C in loading buffer (Biouniquer Biotechnology Co., Ltd., Hangzhou, China) and fractionated using 10% SDS-PAGE. The disposed protein was transferred to BioTrace™ Pure Nitrocellulose Blotting Membrane (Pall Corporation, Pensacola, USA) and blocked with 5% (wt./vol.) nonfat milk. The blocked nitrocellulose membrane was incubated at 4 °C overnight with

Table 1
Primer sequences and amplification conditions used for RT-PCR.

Gene	Forward primer	Reverse primer	Length	Conditions
Cx43	5′-CTGGCTGCGAAAACGTCTGCTATG-3′	5′-CCACGGGAACGAAAATGAACACC-3′	1064 bp	94 °C, 50 s; 58 °C, 30 s; 72 °C, 60 s; 30 cycles
ET_A	5′-ATCGCTGACAATGCTGAGAG-3′	5′-CCACGATGAAAATGGTACAG -3′	226 bp	94 °C, 60 s; 56 °C, 40 s; 72 °C, 40 s; 30 cycles
MMP9	5′-CGTGGCCTACGTGACCTATG -3′	5′-GGATAGCTCGGTGGTGTCCT-3′	592 bp	94 °C, 40 s; 64 °C, 40 s; 72 °C, 90 s; 30 cycles
NOX2	5′-CCTATGACTTGGAAATGGAT-3′	5′-CAGAGCCAGTAGAAGTAGAT-3′	537 bp	94 °C, 60 s; 55 °C, 40 s; 72 °C, 40 s; 30 cycles
PERK	5′-GCCGATGGGATAGTGATG-3′	5′-GCAGCCTCTACAATGTCTTCT-3′	460 bp	94 °C, 45 s; 55 °C, 45 s; 72 °C, 45 s; 30 cycles
p22phox	5′-GACGCTTCACGCAGTGGTACT-3′	5′-CACGACCTCATCTGTCACTGG-3′	485 bp	94 °C, 45 s; 60 °C, 45 s; 72 °C, 60 s; 30 cycles
p47phox	5′-GGCCAAAGATGGCAAGAATA-3′	5′-TGTCAAGGGGCTCCAAATAG-3′	221 bp	94 °C, 45 s; 56 °C, 45 s; 72 °C, 45 s; 30 cycles
p66shc	5′-TACAACCCACTTCGGAATGGTCT-3	5′-ATGTACCGAACCAAGTAGG-3′	473 bp	94 °C, 45 s; 58 °C, 45 s; 72 °C, 45 s; 30 cycles
StAR	5′-CTCAACAACCAAGGAAGGCTGG-3′	5′-GCAGGTGGGGCCGTGTTCAGC-3′	404 bp	94 °C, 60 s; 56 °C, 40 s; 72 °C, 40 s; 30 cycles
GAPDH	5′-GCTGGGGCTCACCTGAAGG-3′	5′-GGATGACCTTGCCCACAGCC-3′	343 bp	94 °C, 45 s; 55 °C, 45 s; 72 °C, 45 s; 30 cycles

specific primary antibody. After 3 washes with PBS, blots were incubated with horseradish peroxidase (HRP)-conjugated goat secondary antibody IgG (1:1000) for 1 h at room temperature. Antigen were visualized by imaging acquisition and quantified by densitometry (Genegenus, Syngene, England). Relative abundance was obtained by normalizing the density of target protein against β-actin. The sources of antibodies were as below: polyclonal rabbit anti-ET_AR–IgG (1:200), polyclonal rabbit anti-StAR–IgG (1:200), polyclonal rabbit anti-PERK–IgG (1:200), polyclonal rabbit anti-p66Shc–IgG (1:500) and polyclonal rabbit anti-MMP9–IgG (1:500) were purchased from Santa Cruz Biotechnology Inc., USA; polyclonal rabbit anti-$p47^{phox}$–IgG (1:300) was purchased from Upstate, USA; polyclonal rabbit anti-$p22^{phox}$–IgG (1:500) was purchased from Uscnlife, USA; polyclonal rabbit anti-$p67^{phox}$–IgG (1:200) was purchased from Affinity Bioreagents, USA; polyclonal rabbit anti-Cx43–IgG (1:300), polyclonal rabbit anti-β-actin–IgG, horseradish peroxidase (HRP)-conjugated goat/rabbit secondary antibody IgG were purchased from Wuhan Boster Biological Technology, Wuhan, China.

2.8. Histological changes in the seminiferous tubules

The testes, fixed with neutral 10% formalin, were embedded in paraffin and sliced into 4-μm-thick pieces for histopathological examination. Then the slides were deparaffinized in xylene, dehydrated by ethyl alcohol in decreasing concentrations (100%, 95% and 70%) and stained with hematoxylin–eosin. All pictures were viewed under an inverted microscope (Nikon TE 2000-U, Japan) by a pathologist blinded to the experiment.

2.9. Immunohistochemistry (immunofluorescence) assay

For immunohistochemistry analysis, testes were fixed in 4% paraformaldehyde (Wuhan Boster Biological Technology, Wuhan, China). Testes embedded in paraffin were sliced into 4-μm-thick pieces. After dewaxing with xylene and hydration with decreasing concentrations of ethanol (100%, 95%, 90%, 80%, and 70%), the sections antigen retrieval were conducted by microwave. Then the slides were soaked in 3% H_2O_2–methanol solution for 15 min to inactivate endogenous peroxidase. The primary antibody of NOX2 (1:50), PERK (1:50), StAR or p66Shc were added and incubated, respectively, in wet box for 2 h after washing 3 times with PBS. Then FITC (TRITC)-labeled secondary antibodies (1:400, KeyGEN BioTECH, Nanjing, China) or HRP-conjugated secondary antibody were incubated for 1 h at 37 °C. After washing 3 times with PBS, the slides were stained with DAPI (KeyGEN BioTECH, Nanjing, China) at room temperature protected from light for 10 min. All pictures were viewed under fluorescence microscope (OLYMPUS IX51, Japan).

2.10. Statistical analysis

All data were presented as mean ± SE and analyzed with SPSS11.5 (USA) software. For statistical evaluation, one-way analysis of variance was used, following Dunnett's test. The Student Newman Keuls test was performed when the variance was equal, and the Games–Howell test was used when variance was not equal. A probability value $P < 0.05$ was considered statistically significant.

3. Results

3.1. Abnormal sperm density and sexual behaviors

The number and motility of sperm were examined under microscopy and were found to be decreased in hypoxia group ($P < 0.01$) compared with control (Table 2). On the other hand, the frequency of mounting and ejaculation of rats suffering from hypoxia was significantly decreased by 46.5% and 58.6% ($P < 0.01$) compared to control (Table 2). NADPH oxidase inhibitor APO and RS a calcium influx blocking agent alleviated these abnormalities significantly ($P < 0.01$).

3.2. Altered redox system

The amount of oxidants and activity of antioxidant enzyme were measured in the testis. After exposed to intermittent hypoxia, the lipid-peroxidation product MDA increased ($P < 0.01$) while the GSHpx decreased ($P < 0.01$) compared to control. Treatment with APO and RS alleviated these redox parameters significantly ($P < 0.01$) (Table 3).

3.3. Serum testosterone, FSH and LH

After hypoxia exposure, serum testosterone decreased by 61.9% ($P < 0.01$) (Table 3). At the meantime, hypoxia exposure caused an increase in serum levels of LH and FSH significantly ($P < 0.01$), compared with the control. These changes of three hormones in serum were partly suppressed by either APO or RS.

3.4. StAR and 3β-HSD in vivo

Intermittent hypoxia decreased expression of mRNA and protein of StAR by 50% and 57.6% ($P < 0.01$) and 3β-HSD protein by 60% in the hypoxia group ($P < 0.01$) *in vivo* (Fig. 1). Abnormalities of downregulated StAR and 3β-HSD in the hypoxic rats were attenuated significantly by medication of APO and RS.

The incubation of isolated Leydig cells with H_2O_2 was conducted and downregulation of StAR and 3β-HSD was significant ($P < 0.01$), compared to control *in vitro*. The abnormal StAR and 3β-HSD were alleviated by both APO and RS (Fig. 2A and B).

3.5. NADPH oxidase and ET_A

Under intermittent hypoxia exposure, ET_A expression appeared to be upregulated ($P < 0.01$), compared with the control.

In Leydig cells incubated with H_2O_2, an increase in $p22^{phox}$ and $p47^{phox}$ was significant ($P < 0.01$), relative to control. These changes were attenuated by APO and RS *in vitro*, respectively (Fig. 2C and D). Furthermore, an increased expression of NOX subunits of $p22^{phox}$, $p47^{phox}$ and NOX2 ($gp91^{phox}$) was significant, ($P < 0.01$) *in vivo*, compared to the control (Fig. 3C–H).

By using immunohistochemical approach an increase in catalytic subunit NOX2 was significant ($P < 0.01$), *in vivo* (Fig. 4B). As labeled by fluorescent dye, NOX2 protein was more in the cells located in the inter-tubular space, but less in the reproductive epithelium in the normal testes. Under intermittent hypoxia, the intensity of fluorescent dye was increased, and the cells surrounding the center of seminiferous tubules and located in the inter-tubular space were highly labeled with fluorescent dye which

Table 2
The latency and frequency of mounting, ejaculation frequency, and motility and density of sperm are monitored in male rats exposed to intermittent hypoxia for 17 days and interventions are conducted with either APO (apocynin) or RS (raisanberine) in the last 6 days of hypoxia exposure.

Group	Mounting latency (s)	Mounting number (20 min)	Ejaculation number (20 min)	Sperm motility (%)	Sperm density (10^6)
Control	28 ± 5.3	43 ± 3.2	5.8 ± 0.47	86 ± 1.7	5 ± 0.58
Hypoxia	34 ± 5.5	23 ± 2.7**	2.4 ± 0.4**	63 ± 3.2**	3.4 ± 0.5**
Hypoxia + APO	29 ± 5.8	40 ± 3.8##	4.4 ± 0.51#	80 ± 3.9##	4.5 ± 0.47##
Hypoxia + RS	32 ± 3.5	36 ± 2.3#	4.3 ± 0.42##	77 ± 3.2#	4.2 ± 0.19#

$n = 6$, mean ± SEM.
** $P < 0.01$ *vs.* control.
# $P < 0.05$.
$P < 0.01$ *vs.* Hypoxia.

Table 3
The measurements in plasma are abnormal in male rats exposed to hypoxia for 17 d and are attenuated by intervention with either APO or RS during the last 6 days of hypoxia.

Group	Testosterone (ng/ml)	LH (mIU/ml)	FSH (mIU/ml)	MDA (nmol/mgprot)	GSHPx (U/mgprot)
Control	4.2 ± 0.62	0.08 ± 0.02	0.41 ± 0.05	4.3 ± 0.45	1025 ± 69
Hypoxia	1.6 ± 0.15**	0.32 ± 0.05**	1.3 ± 0.2**	21.0 ± 2**	471 ± 52**
APO	3.1 ± 0.28#	0.19 ± 0.02#	0.84 ± 0.07#	8.1 ± 0.8##	822 ± 52##
RS	2.7 ± 0.19#	0.17 ± 0.02#	0.85 ± 0.05#	8.5 ± 0.8##	798 ± 53##

$n = 6$, mean ± SEM.
** $P < 0.01$ *vs.* control.
# $P < 0.05$.
$P < 0.01$ *vs.* Hypoxia.

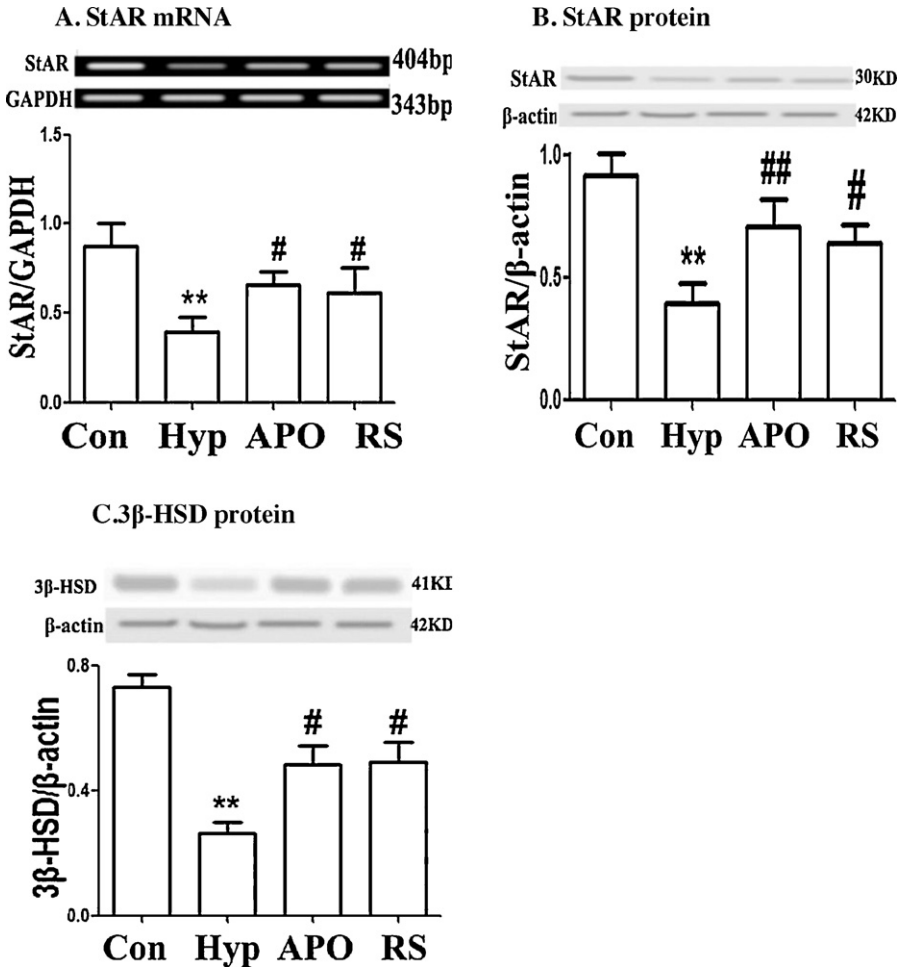

Fig. 1. Downregulation of StAR mRNA (A) and protein (B) and 3β-HSD protein (C) is significant in the testes in rats exposed to intermittent hypoxia for 17 days. These changes are significantly alleviated by medication of apocynin (APO) and raisanberine (CPU86017-RS, RS) during the last 6 days. $n = 6$, mean ± SEM. ** $P < 0.01$ *vs.* Control; # $P < 0.05$, ## $P < 0.01$ *vs.* hypoxia.

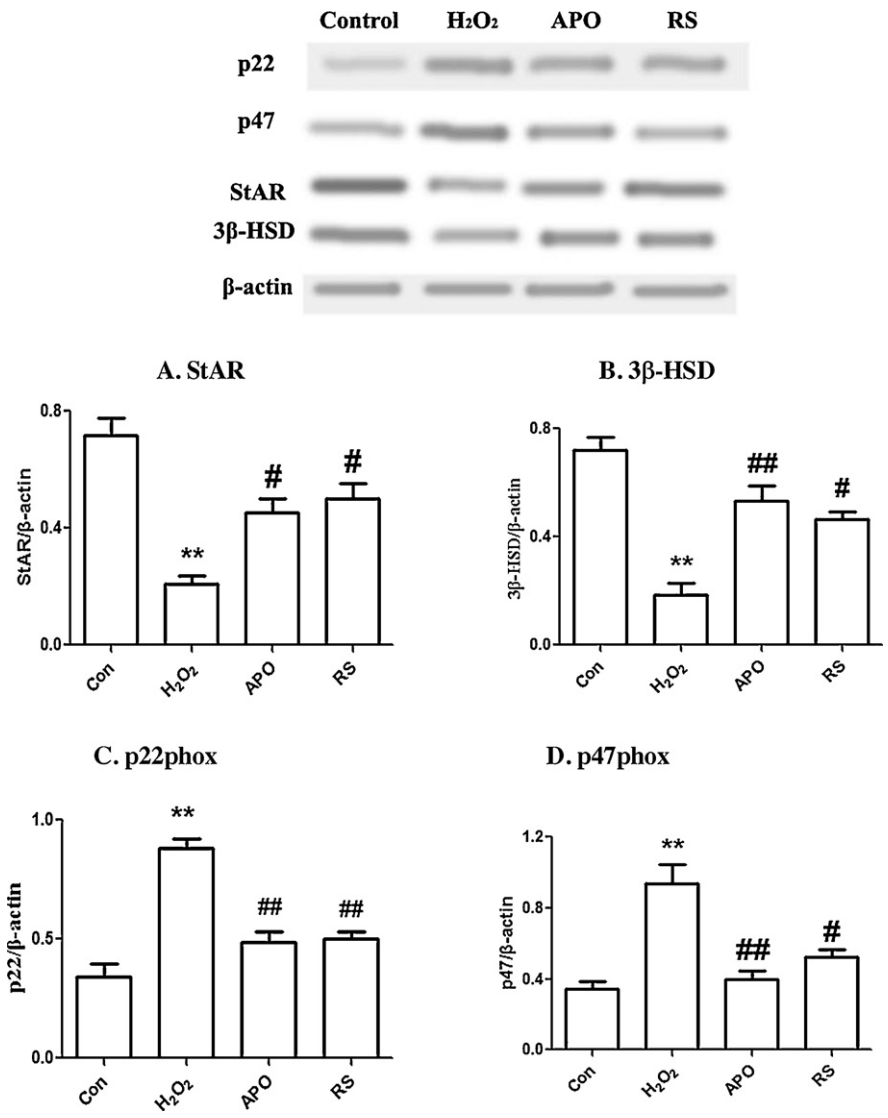

Fig. 2. Abnormal p22phox, p47phox, StAR, and 3β-HSD were found in isolated Leydig cells after incubation with H_2O_2 (10^{-6} M) for 12 h. An activation of p22phox (A) and p47phox (B) is significant ($P < 0.01$), in association with downregulated StAR (C) and 3β-HSD (D) ($P < 0.01$) in Leydig's cells as compared with the control. These changes are alleviated by either NADPH oxidase inhibitor APO or RS a blockade on calcium influx. $n = 3$, mean ± SEM. $^{**}P < 0.01$ *vs.* control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* hypoxia.

represented an increased activity of NOX there. The NOX2 positive cell ratio increased, compared to the control. The enhanced fluorescent intensity of NOX2 containing cells was suppressed by either NOX inhibitor APO or RS, respectively (Fig. 4B).

3.6. Histological changes

Microscopically, chronic exposure to intermittent hypoxia disrupted the normal appearance of the testes seriously. Reproductive cells mainly spermatogenic and Sertoli cells are in well organized in layers from the basement toward the lumen and a plenty of sperms can be seen at the center in the seminiferous tubules in control group. The layered cells are tightly attached to the basement of the tubules with Leydig cells rich in the space between the tubules (Fig. 4A). After hypoxia exposure for 17 d, the seminiferous tubules were significant damaged by distorting the histological arrangement in the tubules, with much fewer spermatozoa left at the lumen and an expanded gap seen between the reproductive epithelial cells and the basement. With the toxicity of hypoxia, the number of Leydig cells was greatly reduced leaving more space among the tubules. The appearance of histological structure of the testis was greatly improved toward the normal following the interventions with either APO or RS (Fig. 4A).

3.7. MMP9 and Cx43

Upregulated mRNA and protein abundance of MMP9 were found significantly ($P < 0.01$) in the hypoxia testes, relative to the control (Fig. 5A and B). At the meantime, Cx43 was downregulated by 48.9% ($P < 0.01$) and 43.9% ($P < 0.01$) in mRNA and protein abundance in the hypoxic testes, compared to the control (Fig. 5C and D). APO and RS ameliorated the abnormal expression of MMP9 and Cx43 significantly.

3.8. ER stress chaperone PERK

In the present study PERK labeled with mild fluorescence was in cytoplasm of cells located in the space among the seminiferous tubules (Fig. 4C). Following exposure to intermittent hypoxia an increase in the fluorescence was significant, in cells both located in the space among the tubules and surrounding the lumen of the seminiferous tubules. An increase in PERK containing cell ratio was

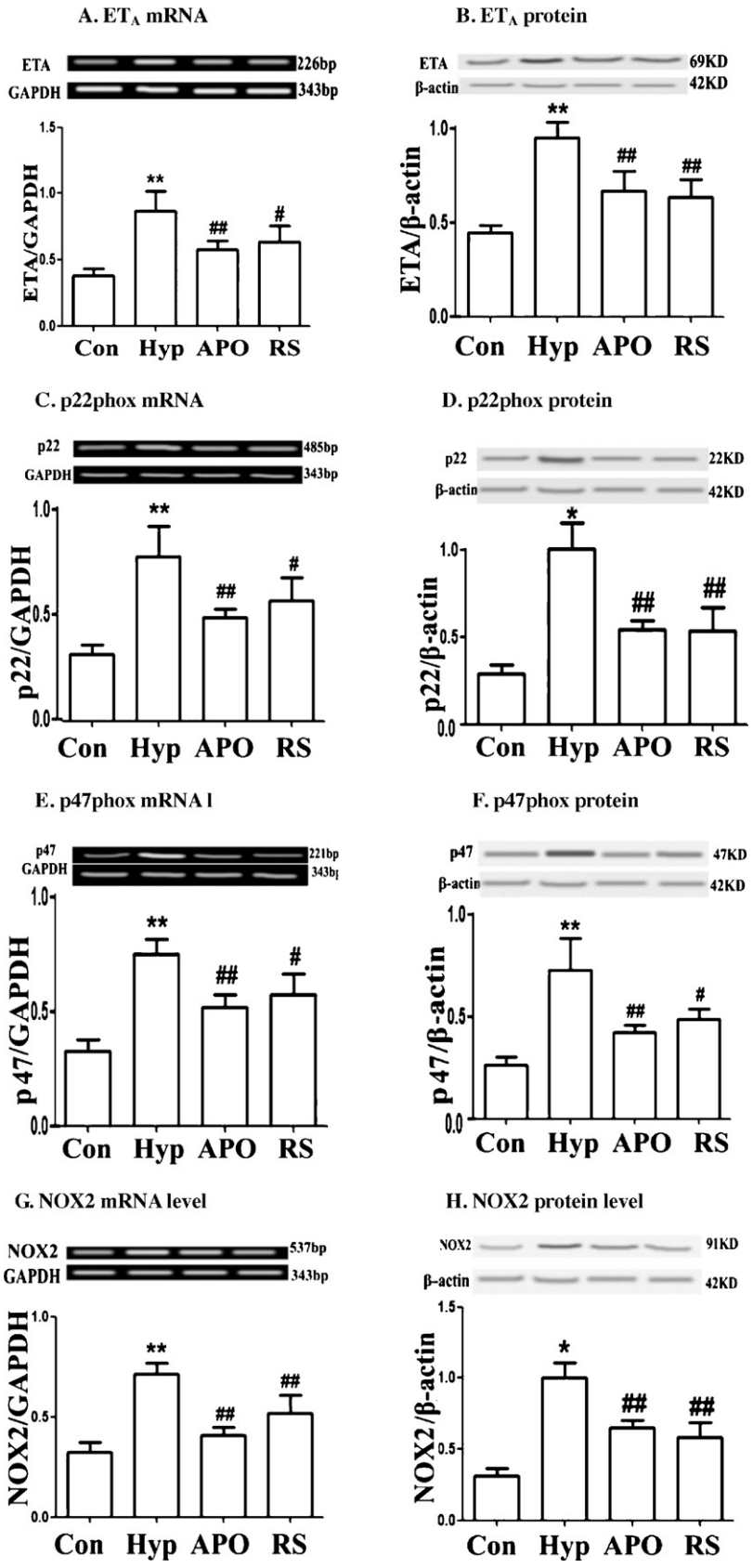

Fig. 3. Expression of mRNA and protein of ET_A (A and B) and NADPH oxidase subunits: p22phox (C and D), p47phox (E and F) and NOX2 (G and H) is upregulated significantly ($P < 0.01$), compared with the control and these abnormalities were attenuated by APO and RS, respectively. $n = 6$, mean ± SEM. $^{**}P < 0.01$ *vs.* Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* hypoxia.

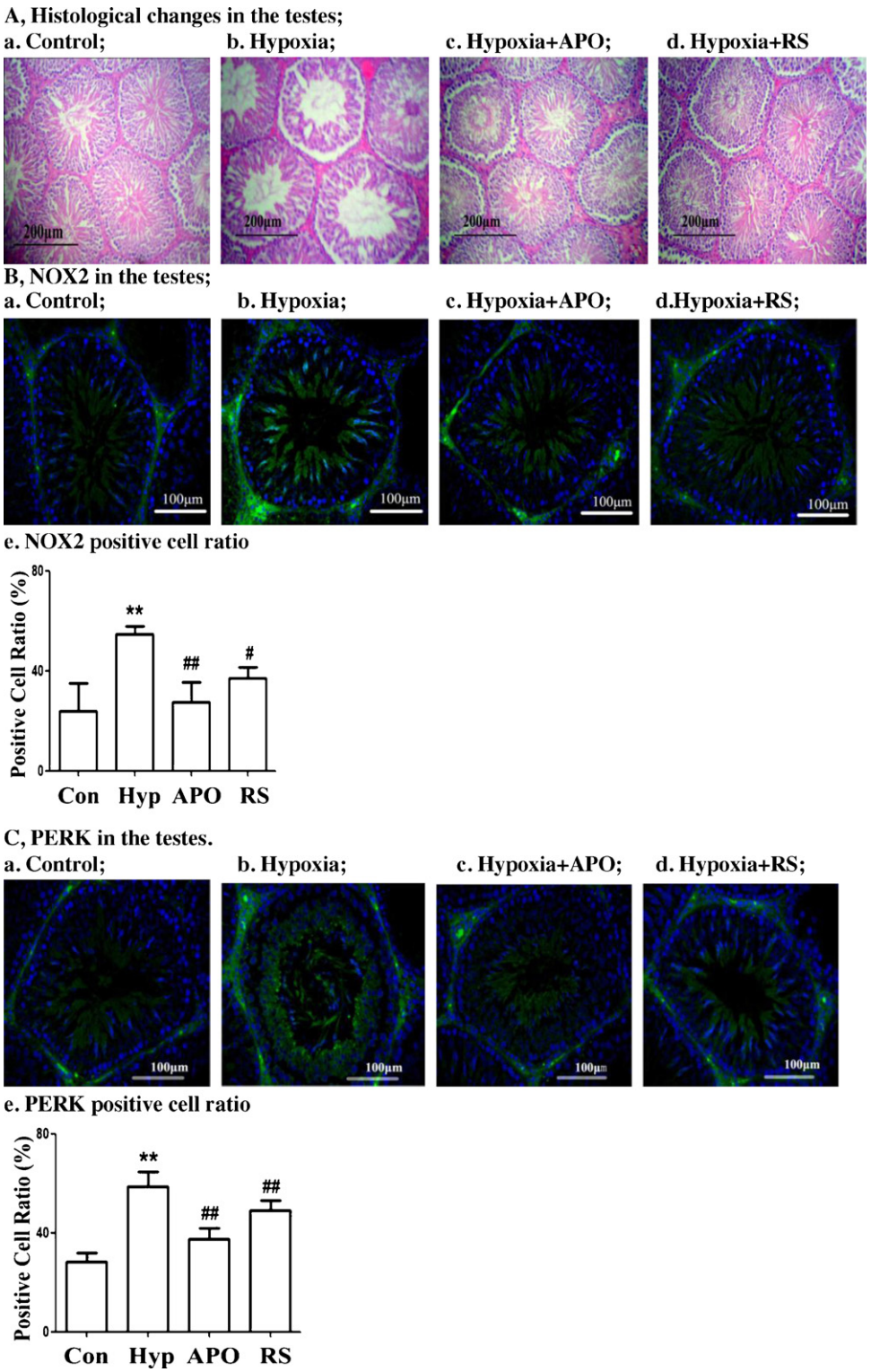

Fig. 4. (A) Representative histological changes are found in the testis induced by intermittent hypoxia and are alleviated by the treatment of APO and RS. Cross sections are stained with hematoxylin and eosin. The testis from control group is presented with typical features of seminiferous epithelium, while those from rats suffering from intermittent hypoxia exhibit impaired spermatogenesis and distorted appearance of seminiferous tubules. These changes are normalized by APO and RS. (a) Control, (b) hypoxia, (c) APO, and (d) RS. (B) The Immuno-fluorescence assay of NOX2 is conducted to show an increase in NOX2 fluorescent density in intermittent hypoxia group, while APO and CPU86017-RS are effective to alleviate these abnormalities significantly (a–d), respectively. Green fluorescence indicates the locations of NOX2 protein and blue color represents testicular sections counterstained by DAPI (4′,6′ diamidino-2-phenylindole). Hypoxia significantly increases NOX2 positive cell ratio (e) in testes. In contrast, APO and RS treatment inhibit the upregulated fluorescence dramatically. $n = 6$, mean ± SEM. $^{**}P < 0.01$ *vs.* Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* hypoxia. (C) Enhanced immunohistochemical fluorescence of PERK is found in Leydig's cells by intermittent hypoxia (a–d), and the PERK positive cell ratio (e) is increased. These abnormalities are suppressed by APO and RS, respectively. $n = 6$, mean ± SEM. $^{**}P < 0.01$ *vs.* Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* hypoxia. (D) The immunohistochemical assay of p66Shc is carried out (a–d) and the p66Shc positive cell ratio (e) is increased in the hypoxic testis. APO and RS attenuate these changes significantly. $n = 6$, mean ± SEM. $^{**}P < 0.01$ *vs.* Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* hypoxia. (E) The Leydig cells were incubated with H_2O_2 for 12 h, and apocynin (APO) and raisanberine (RS) are added at 10^{-6} M during the last 6 h. The immunofluorescent assay of 3β-HSD showed a declined protein in the H_2O_2 group and the fluorescence of 3β-HSD was recovered by APO and RS, respectively.

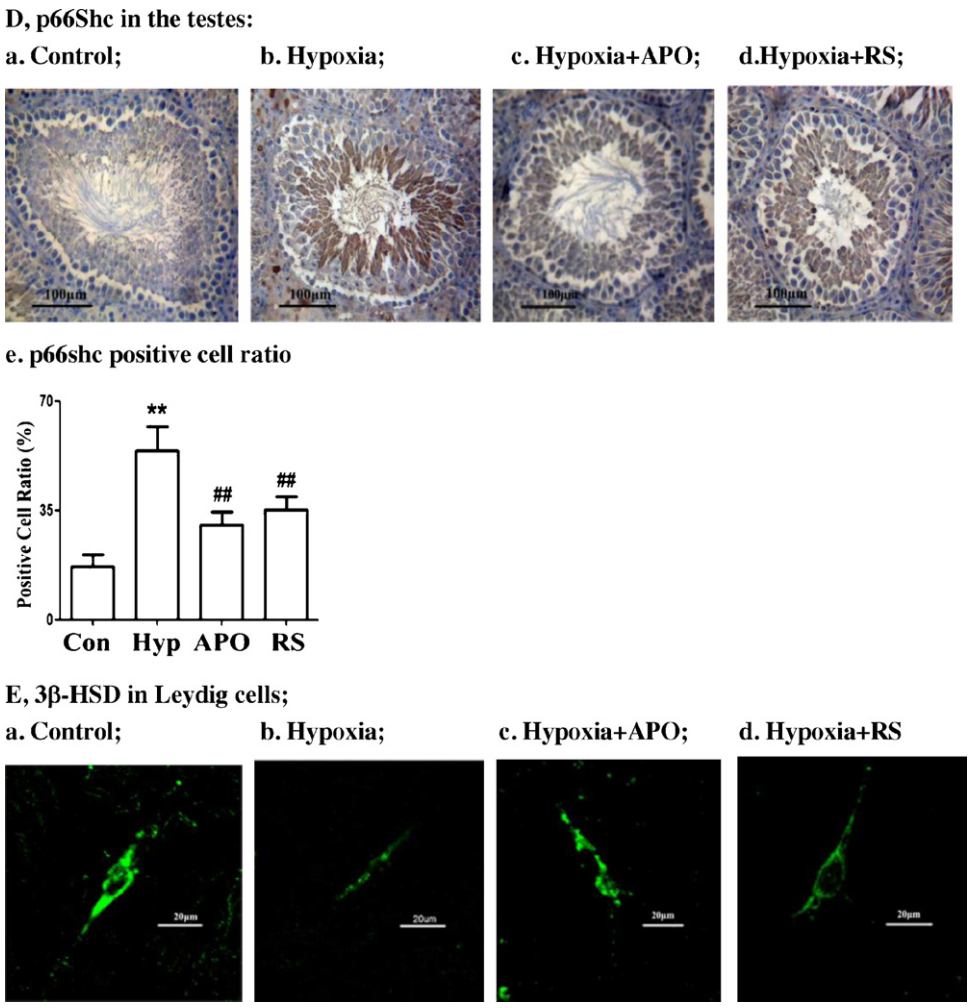

Fig. 4. (*Continued*)

remarkable in the hypoxic testes, $P < 0.01$, relative to control. Furthermore, revealed by Western blot, an increased expression of PERK by 56.9% and 40.6% in mRNA and protein levels was significant, $P < 0.01$, compared to the control (Fig. 5, E, F). APO and RS intervention alleviated significantly the abnormal PERK in testicular tissues. (Figs. 4C and 5E, F)

3.9. Redox signaling molecule p66Shc

The immunohistochemical assay of p66Shc of the testes was conducted and the p66Shc protein was labeled with brown color in cells surrounding lumen of the tubules. An increased deposits of p66Shc were found following exposure to intermittent hypoxia, and an increase in p66Shc positive cell ratio was also found, ($P < 0.01$), compared to the control. The brown color in cells surrounding the lumen was significantly declined by interventions with either APO or RS, respectively (Fig. 4D). By measuring mRNA and protein of p66Shc, we found that intermittent hypoxia upregulated the expression of p66Shc in testicular tissues significantly ($P < 0.01$), relative to the control (Fig. 5G and H). APO and RS at least partly attenuated the increment of expression of p66Shc due to hypoxic exposure respectively ($P < 0.01$) (Fig. 5G and H).

4. Discussion

Intermittent hypoxia causes a low oxygen tension in the blood stream leading to damage to cellular organelles. The reproductive epithelium is known to be one of the most vulnerable to hypoxemia by inhibiting spermatogenesis and declining the biosynthesis of testosterone. Our previous study has shown that NOX plays an important role in initiating diabetic testicular complications [4]. In the present study we found that intermittent hypoxia caused a reduction of serum testosterone, abnormal sexual behaviors and marked changes in morphology of the testes. The declined expression of StAR and 3β-HSD was also found in the affected testes consequent to an increase in oxidants due to hypoxia exposure. The production of MDA was increased while the activity of GSHPx was decreased in hypoxic testes. Hypoxia produces an increase in oxidants in the testes originally from the abnormal electron transport chain (ETC) in mitochondria. Superoxide anion from the leakage of ETC activates NOX to promote more ROS genesis in the testes. As shown in present study, the expression of NOX subunits was upregulated significantly, implying that oxidative stress occurs in the dysfunctional testes induced by hypoxia. Unregulated expression of PERK on exposing hypoxia indicates the occurrence of ER stress resulting from excessive oxidants [6,16]. NOX inhibitor APO alleviated the upregulation of PERK, verifying the participation of oxidative stress involved in ER stress.

Male hypogonadism is characterized by pathological changes of the testis and low serum testosterone. Testosterone, mainly produced in Leydig cells, is synthesized under the involvement of StAR and 3β-HSD. The first and rate-limiting step of steroidogenesis involves the transportation of cholesterol from the outer membrane to the inner membrane of mitochondria where it is

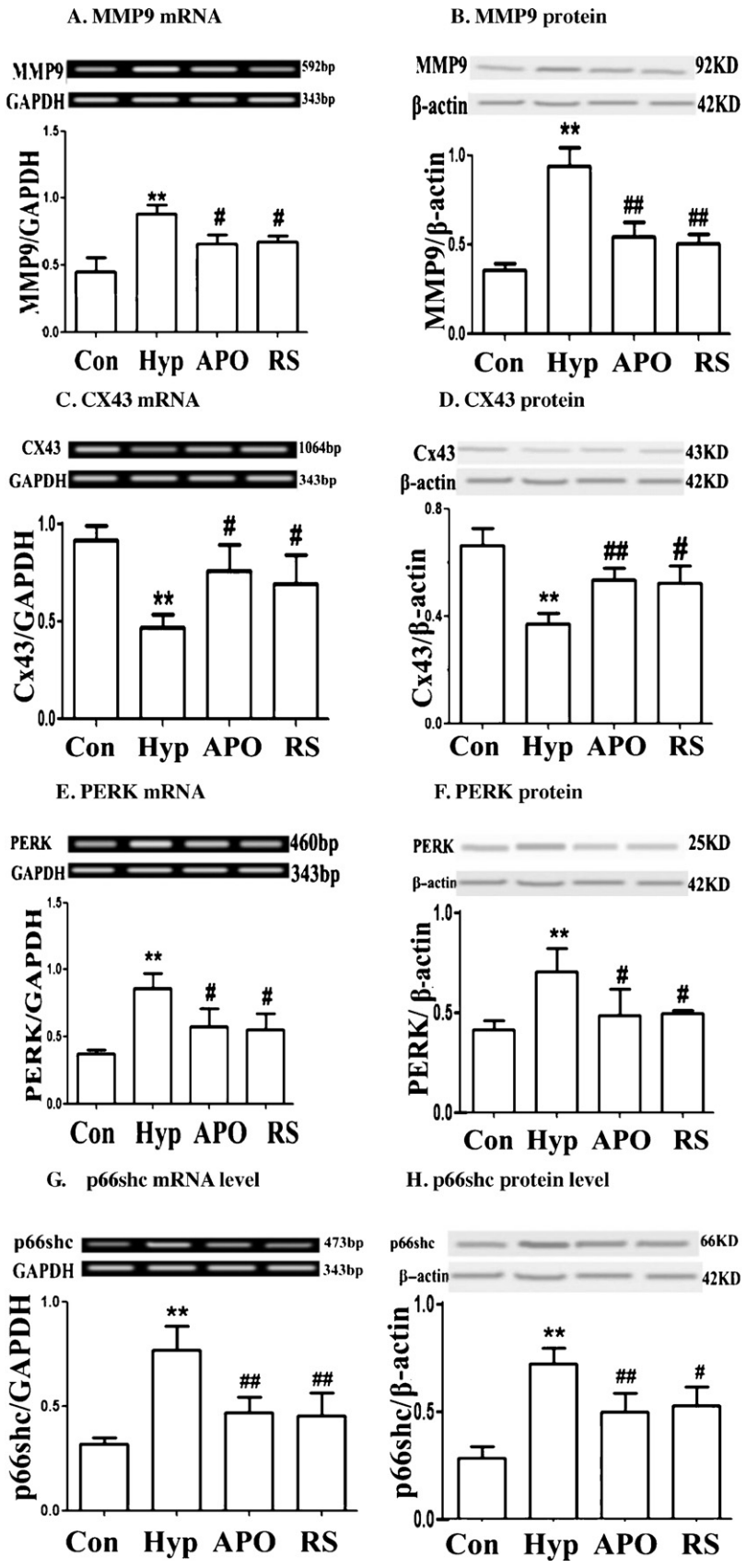

Fig. 5. An increased expression of MMP9 (A and B), decreased expression of Cx43 (C and D), and upregulation of PERK (E and F) and p66Shc (G and H) are significant in the hypoxic testes compared with the control and these changes are attenuated by APO and RS. $n = 6$, mean ± SEM. $^{**}P < 0.01$ *vs.* Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* hypoxia.

converted into pregnenolone by p450scc. The process of transportation by StAR is partly impaired by mitochondrial dysfunction due to hypoxia. Downregulated 3β-HSD is significantly implicated in hypoxia induced testicular dysfunction, in line with impaired production of testosterone on hypoxic exposure [6]. In the present study, the hypoxia-induced downregulation of these two genes was significantly attenuated by the NADPH oxidase inhibitor APO and RS.

LH acts on Leydig cells to stimulate testosterone production and FSH promotes spermatogenesis by coordinately with the activities of testosterone and local paracrines in the testis. The activity of FSH is likely mediated *via* several signaling pathways involving cAMP/PKA, MAPK pathway in proliferative Sertoli cells and PI3-K pathway. Regarding the levels of FSH and LH in serum, in our previous study, male hypogonadism caused by isoproterenol belongs to hypergonadotropic male hypogonadism, showing increased FSH and LH in serum [1]. In the present study, the property of damaged testes by intermittent hypoxia is hypergonadotrophic by sharing with some changes in the abnormal testes caused by isoproterenol. In contrast, hypogonadotrophic male hypogonadism, characterized by decreased FSH and LH, is seen in diabetes mellitus [4]. This is due to different response of hypothalamus–pituitary–gonad (HPG) axis to low serum testosterone concentration. The HPG axis regulates the development, reproductive and endocrine function of the gonads critically [31] and diabetic lesions in the neural network of hypothalamus are likely responsible for hypogonadotrophic hypogonadism.

Hypoxia is a ubiquitous process to activate ET system and NADPH oxidase, possibly relating to an activation of the calcium influx through the L-type calcium channels. Over-activated ET_A and NOX mediate a serious of cardiovascular disease, testicular complications of diabetes, diabetic nephropathy and hypoxia-induced pulmonary arterial hypertension [4,22,32]. The rhythmic contraction of seminiferous tubules depends on stimulation of ET which is secreted mainly by Sertoli cells as well as Leydig cells. However, under intermittent hypoxia state, both over-activated ET system and NOX may lead to impaired movement and spermatogenesis of the seminiferous tubules, therefore, impeding the final maturation of sperm and a decrease in the sperm density in the epididymis. NOX2 is a catalytic subunit of NOX participating in sequential post-translational modifications of $p22^{phox}$ and may contribute to producing the dominant superoxide anion affecting maturation of sperms and testosterone biosynthesis in Leydig cells [33].

p66Shc is a key protein modulating oxidative stress to adversely affect life span and its activation enhances oxidative damage to cells by producing more ROS in mitochondria [34–36]. $p66Shc^{-/-}$ cells show a potent antioxidant and anti-apoptotic ability in preventing cells from molecular changes due to oxidant damage. In the present study, intermittent hypoxia significantly increases the expression of p66Shc and NADPH oxidase inhibitor APO and RS ameliorate the testicular damage *via* suppression on abnormal p66Shc. Therefore, we may conclude that male hypogonadism by hypoxia may be attributed to activated p66Shc and NOX in the testis.

MMPs and CXs are essential for extracellular matrix degradation, recombination and intercellular signal communication in the testis, respectively. MMP9 is actively involved in the normal function and structure of the seminiferous tubules and Leydig cells. Cx43 is the most important protein located at testicular cellular gap junction affecting function and structure of the seminiferous tubules and biosynthesis of testosterone in Leydig cells. Spermatogenesis is a highly programmed process that requires concise regulation for degradation and remodeling of the extracellular matrix, providing high degree of coordination between cells in the testes necessary for spermatogenesis and sperm differentiation and proliferation, as well as the function of Leydig cells. In the testes, the BTB created by specialized junctions between Sertoli's cells near the basement membrane confers an immunological barrier by separating the events of meiotic division and post-meiotic germ cell development from the systemic circulation [23,37]. The BTB is constituted by coexisting gap junctions, tight junctions, desmosomes and basal ectoplasmic specializations. Cx43 is critical for tight junctions at the BTB of the seminiferous epithelial cells. Here we demonstrated that abnormal MMP9 and Cx43 and testicular pathology are modulated by a status of NOX that is in agreement with the previous findings in the cardiac fibroblasts incubated with isoproterenol [12]. These abnormalities were ameliorated significantly by NOX inhibitor APO, indicating an involvement of activated NOX in testicular damage at the molecular aspects. The activated NOX is also consequent to an increase in calcium influx through the L-type Ca^{2+} channels, a blockade on calcium influx by RS and nifedipine offers a relief to testicular damage by reducing NOX activation in the testis [20].

Endoplasmic reticulum (ER) is the organelle responsible for appropriate folding responses in intracellular protein synthesis and calcium homeostasis by regulating intracellular calcium release. Hypoxia and other environmental stresses cause maladaptive reactions for abnormal function of ER by interfering disulfide bound formation to induce unfold protein response (UPR). ER stress is an evolutionary highly conserved protective mechanism in cells that determines the outcome of cellular stress: repairing damage or leading to death by starting apoptotic changes of the nucleus, mitochondria, Golgi's apparatus and other subcellular organelles. Intermittent hypoxia exacerbates the transcription and translation of ER sensor PERK to produce more inflammatory/proinflammatory factors such as ET, NOX and p66Shc that facilitate oxidative stress by producing excessive ROS. The dysfunctional mitochondria resulting from hypoxia release ROS to stimulate NOX and p66Shc and to appear ER stress and disturbance in Ca^{2+} homeostasis in the testis. A blockade on either NOX or calcium influx may attenuate the hypoxic sufferings in the testis.

Emerging data showed that testicular damage caused by bisphenol or nonylphenol is through ER stress and apoptosis pathway [38,39]. In the present study, upregulation of expression of PERK is critical, associated with activated p66Shc in the testis, and we confirm that an increase in an ER sensor contributes to testis damage on exposure to hypoxia, in line with previous report [6,20]. RS and NOX inhibitor APO alleviate testicular dysfunction induced by hypoxia through suppressing abnormal ER stress chaperone PERK in the testis.

In conclusion, intermittent hypoxia produces excessive ROS to adversely affect testis function and structure by triggering oxidative stress and ER stress and p66Shc. Intermittent hypoxia induced male hypogonadism is possibly mediated by activated NADPH oxidase and an influx of calcium. Therefore, APO, a blocker for NOX and RS which blocks calcium influx are at least in part to alleviate testicular damage by inhibiting p66Shc and ER stress in the testis.

Conflict of interest

None.

Acknowledgements

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