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A synthetic steroid 5α-androst-3β,5,6β-triol blocks hypoxia/reoxygenation-induced neuronal injuries via protection of mitochondrial function ☆

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ABSTRACT

Ischemic stroke is a leading cause of death worldwide, yet therapies are limited. During periods of ischemia following reperfusion in ischemic stroke, not only loss of energy supply, but a few other factors including mitochondrial dysfunction and oxidative stress also make vital contribution to neuronal injury. Here we synthesized a steroid compound 5α-androst-3β,5,6β-triol by 3 steps starting from dehydroepiandrosterone and examined its effect on mitochondrial function and oxidative stress in primary cultured cortical neurons exposed to hypoxia followed by reoxygenation. 5α-Androst-3β,5,6β-triol dose-dependently protected cortical neurons from hypoxia/reoxygenation exposure. Rates of reduction in neuronal viability, loss of mitochondrial membrane potential, disruption of ATP production and oxidative stress were ameliorated in 5α-androst-3β,5,6β-triol pretreated cultures. In summary, these results suggest that 5α-androst-3β,5,6β-triol is neuroprotective against hypoxia/reoxygenation induced neuronal injuries through mediation of mitochondrial function and oxidative stress.

1. Introduction

Worldwide, stroke is the 2nd leading cause of death [1], yet therapies are very limited. Ischemic stroke accounts for 83% of all strokes [2], with the poor outcomes attributed mainly to neuronal injuries and death. Worse still, almost all identified neuroprotective compounds from preclinical research have failed to be translated from bench to bedside [3]. Understanding the process of neuronal injury resulting from exposure to the distinct environment during stroke - ischemia/reperfusion (I/R) is of critical importance in identifying useful targets for the development of pharmacological treatment of stroke.

Mitochondria comprise the central locus for energetic perturbations during I/R [4]. Besides, concomitant oxidative stress makes considerable contribution to neuronal injuries [5–7]. Previous evidence pointed to an increase of intracellular reactive oxygen species (ROS) accumulation during oxidative stress, with the source being mitochondria [8,9], logically, mitochondrial function is of central importance in ischemic/hypoxic and secondary insults. Meanwhile, excessive ROS in reverse causes damages to mitochondria and consequently exacerbates mitochondrial dysfunction [10]. Thereupon, that mitochondrial dysfunction acts with oxidative stress has been widely recognized to imperil neurons during acute ischemic/hypoxic insult.

Ever since Baulieu and colleagues reported dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) as the first neurosteroids in 1998 [11], there has been a growing interest in searching neuro-active steroids. Thereafter a trend has been set in the development of steroid drugs as treatments of both chronic neurodegenerative disease and acute brain injuries. A large number of steroids isolated and purified from marine sources were reported bioactive in the aspects of anti-tumor, anti-inflammation,

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anti-oxidation and so on, yet the low yields from extraction have been disconcerting considering the actual pharmacological values, which have, consequently, to a large degree promoted the preparations of purely synthetic compounds for preclinical studies and future possible clinical uses. Here we synthesized 5α-androst-3β,5,6β-triol (triol), a previously discovered steroid [12], from DHEA by 3 steps and obtained an overall yield of 76.0%. The neuroprotetive effect of triol was afterwards examined on a well-established cellular model of hypoxia/reoxygenation (H/R) injury [12] mimicking the I/R process during stroke focused on mediation of mitochondrial function and oxidative stress.

2. Experimental procedures

2.1. Chemistry

DHEA (Wuhan Yuancheng Technology, China) was used as a starting steroid. All chemicals and solvents were of analytical grade and purchased from Guangzhou Chemical Reagents Company China if not mentioned particularly. All melting points were measured on an X6 melting point apparatus and were uncorrected. All reactions were monitored by thin-layer chromatography (TLC) on silica gel G. Optical rotations were measured at 25 °C on Perkin Elmer Model 341 polarimeter. The IR spectra were recorded as KBr pellets on an EQUINOX 55 FT spectrophotometer (wave numbers in cm^{-1}). ^1H NMR and ^{13}C NMR spectra were recorded on a nuclear magnetic resonance spectrometer Bruker (Avancell, 400 MHz). Mass spectra were recorded on GC–MS (Agilent, USA) to confirm the structure of the compounds. All purity data were recorded on 7890A GC(Agilent, USA) with the GC column DB-5MS (Agilent, USA). The octanol–water partition coefficient (logP) was obtained as previously described [13,14] and concentrations of triol in the aqueous phase and organic phase were measured from 7890A GC system.

2.1.1. 5-En-androst-3β-ol (**2**)

Diethylene glycol (100 ml) was added into a four-necked flask with each neck used either as a stirrer, temperature controller, condenser pipe or inlet port. When reaching a temperature of 130 °C, DHEA 1 (10 g, 0.034 mol) and hydrazine hydrate (50 ml) were added into the flask. After heating and refluxing for 2 h, KOH (10 g, 0.178 mol) was added carefully into the mixture. The residual liquid and hydrazine hydrate were distilled until the temperature reached 210 °C, the mixture was then refluxed for 4 h and cooled down to room temperature [15]. 98% Sulfuric acid (10 ml) was added carefully to 1.5 L of water with a glass rod stirring synchronously, after well-distributed, the mixture from last reaction was poured into this acidic solution to counteract the residual alkali. Afterwards, the mixture was suction filtrated and the solid was dispersed into the water. Suction filtration was repeated twice following the vacuum drying to yield a white solid 2 (9.1 g, 95.8%), and the purity was 98.95%. $[\alpha]_D^t$: −67.18 (anhydrous ethanol, 10 mg/ml). Mp. 132–136 °C. IR (KBr) ν: 3242, 1453, 1058, 2933, 1058, 2933, 1058 cm^{-1}. ^1H NMR (DMSO-d6) δ: 0.71 (s, 3H, 19-CH_3), 0.88 (m, 1H, 14-H), 0.95 (m, 1H, 9-H), 1.00 (s, 3H, 18-CH_3), 1.38 (m,1H,8-H), 1.58 (m,1H, 7β-H), 1.62 (m, 1H, 2α-H), 1.84 (m, 1H, 2β-H), 2.0 (m, 1H, 7α-H), 2.25 (m, 2H, 4-CH_2), ^{13}C NMR (DMSO-d6) δ: 17.22 (CH_3), 19.39 (CH_3), 20.47 (CH_2), 21.11 (CH_2), 25.59 (CH_2), 31.63 (CH), 32.17 (CH_2), 32.21 (CH_2), 36.61 (C), 37.32 (CH_2), 38.69 (CH_2), 40.25 (CH_2), 40.54 (C), 42.28 (CH_2), 50.43 (CH), 54.83 (CH), 71.68 (CH), 121.62 (CH), 140.76 (C); 3.5 (m,1H,3α-H), 5.34 (t, 1H,6-H). EI-MS *m/z*: 274(M^+), 259 (M-CH_3), 256 (M-H_2O), 241 (M-CH_3–H_2O). Analysis calculated for $C_{19}H_{30}O$ C 83.50, H 11.02; found C 83.43, H 10.99.

2.1.2. 5α-3β,6β-Diformyloxyl-androst-5-ol (**3**)

Compound **2** (7 g, 0.026 mol) was added into 88% formic acid (140 ml) at room temperature, and heated to 65 °C with agitation at 400 rpm for 10 min. After cooling down to room temperature, 30% hydrogen peroxide (10 ml) was added with stirring. Reaction was maintained for 1 h until the solid dissolved completely and the suspension became pellucid. Next the mixture was heated to 60 °C for 1 h to resolve the rest hydrogen peroxide and poured into water (600 ml) with stirring. The granular white solids were collected by suction filtration after attaining room temperature. For dispersed solids, $KHCO_3$ was used for neutralizing the residual acid followed by vacuum drying. The resulting solids were further purified by column chromatography to afford compound **3** (7.9 g, 84.9%), and the purity was 94.62%. $[\alpha]_D^t$: −80.2 (dioxane, 10 mg/mL). IR (KBr) ν: 3259, 2954, 1375, 1720, 1692, 1058, 1058, 1034 cm^{-1}. UV (nm): 200–210, 225. ^1H NMR (DMSO-d6) δ: 0.72 (s, 3H, 18-CH_3), 1.00 (m, 1H, 14-H), 1.17 (s, 3H, 18-CH_3), 1.40 (d, 1H, 9-H), 1,54 (m, 1H, 8-H), 1.73 (s, 1H, 7α-H), 1.90 (m, 1H, 2β-H), 4.82 (s, 1H, 6-H), 5.29 (m, 1H, 3-H), 7.98 (s, 1H, 20-H), 8.00 (s, 1H, 21-H). ^{13}C NMR (DMSO-d6) δ: 16.39 (CH_3), 17.57 (CH_3), 20.45 (CH_2), 21.04 (CH_2), 25.36 (CH_2), 26.57 (CH_2), 30.97 (CH), 31.78 (CH_2, CH_2), 36.73 (CH_2), 38.55 (C), 38.68 (CH_2), 40.25 (CH_2), 40.95 (C), 45.37 (CH), 53.78 (CH), 70.64 (CH), 74.83 (C), 75.95 (CH), 160.28 (C), 160.74 (C). EI-MS m/z: 364 (M^+), 336 (M-CO), 318 (M-CO–H_2O), 300 (M-CO–2H_2O), 272 (M-2CO–2H_2O), 254 (M-2CO–3H_2O). Analysis calculated for $C_{21}H_{32}O_5$ C 69.20, H 8.85; found C 69.39, H 8.85.

2.1.3. 5α-Androst-3β,5,6β-triol (**4**)

25% Sodium hydroxide (20 ml) was added to a solution of compound **3** (7 g, 0.019 mol) in methanol (140 ml). The mixture solution was heated to 60 °C for 30 min and cooled down to room temperature. Then the mixture was diluted in water (650 ml) and acidified. The precipitates were filtered and washed with water, then thoroughly by vacuum drying to obtain a white crude solid 4 (5.5 g, 93.5%). The precipitates were filtered and washed with water, then thoroughly by vacuum drying to obtain a white crude solid 4 (5.5 g, 93.5%), the purity of which was 96.74%. The resulting solid was further purified by recrystallization with acetone/water (2:1) to afford compound **4** (3.6 g, 65.5%) with high purity (99.51%). $[\alpha]_D^t$: −27.7 (dioxane, 10 mg/mL). Mp. 216–221 °C. IR (KBr) *ν*: 3421, 2925, 2854, 1459, 1376, 719, 1043 cm^{-1}. ^1H NMR (DMSO-d6) δ: 0.67(s, 3H, 18-CH_3), 0.88 (m, 1H, 14-H), 1.034 (s, 3H, 19-CH_3), 1.86 (dd, J = 12.0, 1H, 4β-H), 3.30 (s, 1H, 6α-H), 3.65 (s, 1H, 5α-OH), 3.80 (m, 1H, 3α-H), 4.16 (d, J = 5.7, 1H, 3β-OH), 4.40 (d, j = 4.0, 1H, 6β-OH). ^{13}C NMR (DMSO-d6) δ: 16.30 (CH_3), 17.40 (CH_3), 20.14 (CH_2), 20.75 (CH_2), 25.20 (CH_2), 30.36 (CH), 31.12 (CH_2), 32.09 (CH_2), 34.85 (CH_2), 37.92 (CH_2), 38.68 (C), 40.05 (CH_2), 40.55 (CH_2), 40.91 (C), 44.88 (CH), 53.87 (CH), 65.75 (CH), 74.16 (CH), 74.30 (C). EI-MS m/z: 308 (M^+), 290 (M-H_2O) , 272 (M-2H_2O), 257 (M-2H_2O–CH_3). Analysis calculated for $C_{19}H_{32}O_3$ C 74.03, H 8.51; found C 74.09, H 10.54.

2.2. Biological assays

2.2.1. Primary cultures

New born Sprague–Dawley rats were provided by Experimental Animal Center of Sun Yat-sen University. Neurobasal A medium, fetal bovine serum (FBS), B27 Supplements, GlutaMAX, Trypsin solution, Penicillin–Streptomycin (P/S) was purchased from Gibco®, Life Technologies. Other chemicals, if not mentioned particularly, were all purchased from Sigma–Aldrich. Cortical neurons (CNs) were prepared from new born Sprague–Dawley rats as described previously [16] with little modification. Briefly, the cortex was dissected and placed in ice-cold aseptic dissection solution (DS). After mincing, place the tissue in DS containing 0.25%

trypsin for 15 min at 37 °C. Digestion was terminated by DS containing 10% FBS followed by centrifuging for 5 min at 1500 rpm and the supernatant was discarded. The cell pellet was resuspended in DS containing DNase I (8 mg/ml), Mg^{2+} and FBS, and homogenized by pipetting up and down for about 20 times. After sitting for 15 min, the supernatant was centrifuged for 5 min at 1500 rpm. The cell pellet was then collected and resuspended in Neurobasal A medium supplemented with 10% FBS, 2% B27, 2 mM GlutaMAX and 2 mM P/S. Cells were then seeded with density of 1×10^6 cells/ml into poly-l-lysine (0.5 mg/ml) coated dishes. Cells were incubated in a CO_2 chamber. Medium was replaced with non-serum formula 4 h after seeding, afterwards half medium was changed in every 3–4 days.

2.2.2. Hypoxia/reoxygenation exposure and drug treatment

Triol was dissolved in 20% hydroxypropyl-beta-cyclodextrin (HPBCD) solution (Xi'an Deli Biological Chemistry, China) in different concentrations as stock. At 8 day in vitro (DIV), cultures were pretreated with different concentrations of triol (0.1, 0.3, 1.0, 3.0 μM), or vehicle with a concentration equaling that in the highest triol dose group half an hour before hypoxia/reoxygenation exposure. For comparative study on the effects, cultures were pretreated with DHEA or triol at the concentrations of 0.1 and 3.0 μM or vehicle half an hour before H/R exposure. Cultures were then placed in a hypoxic chamber (CoyLab, USA) perfused with 1% O_2, 5% CO_2 and 94% N_2 for at 37 °C for 24 h then removed to the regular CO_2 chamber for 3 h for reoxygenation. Control cultures were incubated under normoxic condition in the regular incubator mentioned above for the corresponding duration.

2.2.3. Neuronal viability

Neuronal viability was measured by (3-(4,5-dimethylthiazolyll-2)-2,5-diphenyltetrazolium bromide) (MTT) assay. MTT reduction was measured in each group after H/R exposure and data was presented as percentage of normoxic control group. Fluorescein diacetate (FDA) and propidium iodide (PI) were used as indicators of living and dead cells, respectively. Images were acquired from a fluorescent microscope.

2.2.4. Mitochondrial membrane potential

5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1, Sigma–Aldrich) was loaded into cultured neurons as a MMP fluorescent probe. Briefly, primary cortical neurons were seeded onto 35 mm dishes for imaging and black-walled 96-well plates with flat bottom (Nunc) for fluorescent quantitation. After H/R treatment, cultures were incubated with 5 μM of JC-1 for 20 min at 37 °C then washed twice with phenol free Neurobasal A medium. Images were then acquired from a fluorescent microscope (Olympus) using a "dual-bandpass" filter. Fluorescent values were read on a microplate fluorescent reader (Molecular Devices, USA) at excitation 488 nm and emission 600 nm for red fluorescence or 530 nm for green fluorescence. Results were expressed as red/green ratio and the ratio was normalized by comparison with the normoxic control group.

2.2.5. ATP levels

Intracellular ATP levels were detected with an ATP Determination Kit (Molecular Probes™, Invitrogen) according to the manufacturer's instruction. Briefly, after H/R treatment, cultures in each group were treated with 100 μL cell lysis buffer (Thermo). Cell lysates were then moved to a white-walled 96-well plate (Nunc) with flat bottom, and incubated with luciferin and luciferase for 15 min in darkness. Luminescence was then read from a microplate luminometer (TECAN) with an emission 560 nm. Results were expressed as reduction percentage of normoxic controls.

2.2.6. ROS formation

Intracellular levels of reactive oxygen species (ROS) was detected by a fluorescent marker 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, Molecular Probes™, Invitrogen). Cells were cultured on 96-well plates with black-wall flat bottom for fluorescent quantitation. After H/R treatment, cells were incubated with 25 μM of carboxy-H_2DCFDA for 30 min at 37 °C in darkness. The microplate was gently washed for three times in warm HBSS/Ca^{2+}/Mg^{2+} and mounted with equivalent warm buffer. Fluorescence with excitation 495 nm and emission 529 nm was then read on a microplate fluorescence reader (TECAN). Results were expressed as percentage of normoxic controls.

2.3. Statistic analysis

ANOVA followed by Holm–Sidak post hoc tests were used for MTT reduction, MMP, ATP levels and ROS formation analysis. Differences at $P < 0.05$ were considered statistically significant.

3. Results

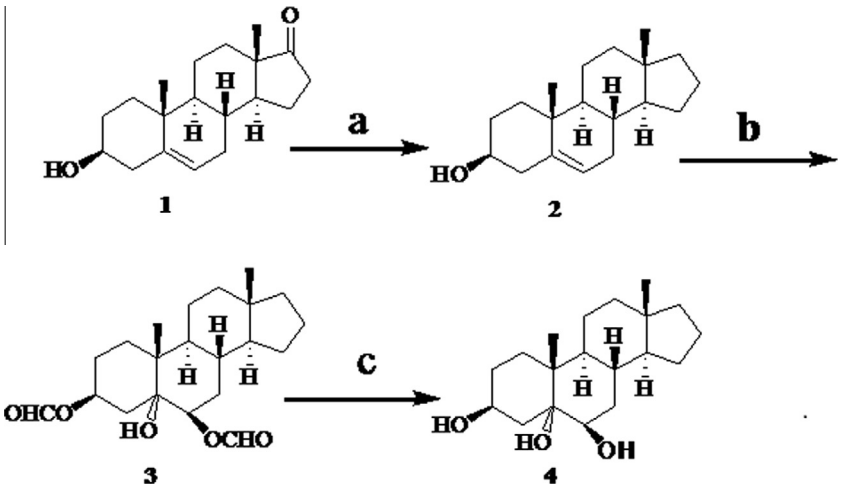
3.1. Chemistry

The synthetic route from DHEA (**1**) to the target compound androst-3β,5α,6β-triol (**4**) is shown in Scheme 1. The first reaction known as Wolf–Kishner–Huang Reaction, was optimized to achieve better yields as high as 95.8%. The double bond of compound **2** was oxidized to form 5α,6β-dihydroxy by H_2O_2/formic acid, meanwhile 3β-hydroxy and 6β-hydroxy were esterified to form 3β,6β-diformate which were eliminated after by alkaline reaction. The 5α-hydroxy was not esterified for steric hindrance. Ultimately the target compound **4** with high purity (99.51%) was obtained by recrystallization. The characteristic features of DHEA include the appearance of the ^{13}C NMR peak at 220.08 (17-C), 71.76 (3-CH), 121.95 (6-CH), 140.93 (5-C). The disappearance of ^{13}C NMR peak of compound **2** at 220.08 (17-C), together with the appearance of ^{13}C NMR peak at 40.54 (17-CH_2) implied that 17-carbonyl was reduced to methylene. The disappearance of the ^{13}C NMR peak at 121.95 (6-CH), 140.93 (5-C) and the appearance of ^{13}C NMR peak at 74.83 (5-C), 75.95 (6-CH) illustrated that the 5,6-double bonds formed 5α,6β-dihydroxy; the appearance of ^{13}C NMR peak at 160.28 (20-C), 160.74 (21-C) indicated the formation of 3,6-diformate which was not detected in compound **4** and the appearance of 65.75 (3-CH), 74.16 (6-CH), 74.30 (5-C) and 308 (M^+) from MS analysis proved the formation of the target compound 5α-androst-3β,5,6β-triol (**4**). The Partition Coefficient (logP) of triol is 3.31, indicating that triol is lipophilic.

3.2. Neuroprotective effect of 5α-androst-3β,5,6β-triol (triol)

3.2.1. Triol was neuroprotective against H/R exposure

Twenty-four hours of hypoxia followed by 3 h of reoxygenation resulted in visible morphological changes and cell death in primary cortical cultures (Fig. 1). In the normoxic control group, cortical neurons were showing well-rounded cell bodies with intact neurites forming a complete network, after H/R exposure, fractured neurites were seen. Live/dead staining by FDA/PI showed a decrease in intact living cells (green) and an increase in dead cells (red). Pretreatment with triol improved neuronal survival (increased FDA stained cell number and decreased PI stained cell number), and protected the morphology of cultured neurons from H/R exposure, with the maximum effect at a concentration of 3 μM. Consistently 24 h of hypoxia followed by 3 h of reoxygenation resulted in a decline in neuronal viability by about 40.0%



Scheme 1. (a) KOH, diethylene glycol, hydrazine hydrate, 210 °C. (b) H_2O_2, HCOOH, RT. (c) NaOH, MeOH, 60 °C.

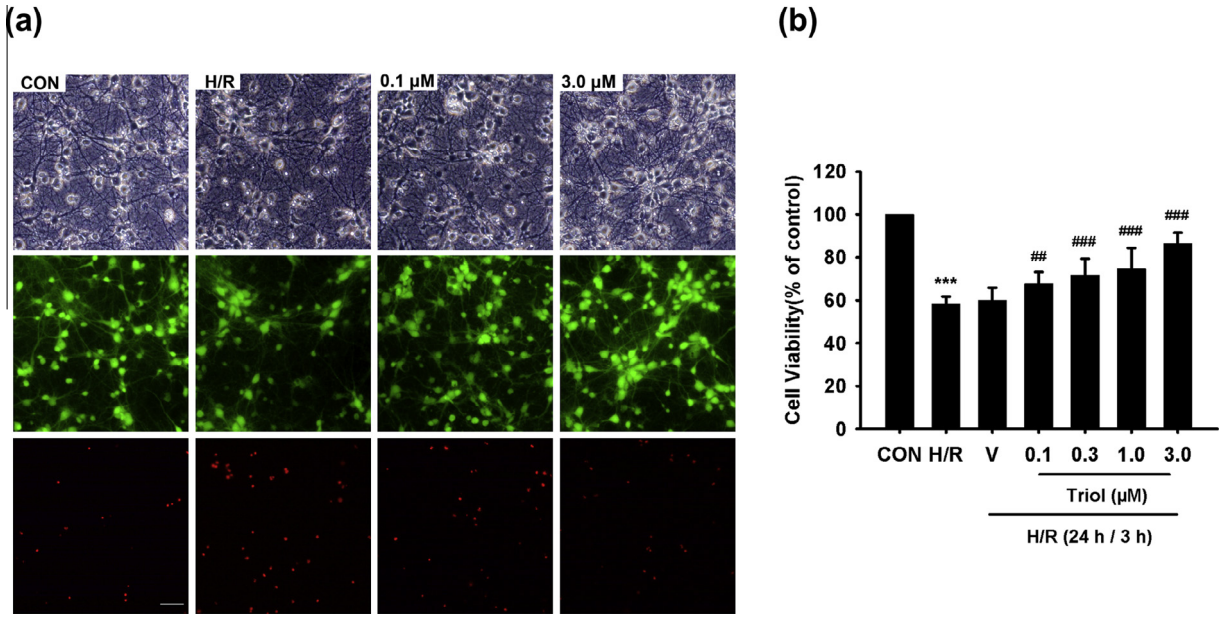


Fig. 1. Protective effects of triol on hypoxia/reoxygenation (H/R)-induced neuronal injuries in primary cultured cortical neurons. (a) Representative phase contrast and FDA/PI stained images. 24 h Hypoxia followed by 3 h reoxygenation (H/R) induced morphological alterations including mild shrinkage of cell bodies and fracture of neurites. FDA/PI staining showed a decrease in live cells (green) and increase in dead cells (red). Treatment with triol alleviated morphological damages. FDA/PI staining revealed a raised neuronal survival rate in triol treated groups. (b) The statistic graph of neuronal viability by MTT assay. H/R significantly reduced neuronal viability and treatment with triol enhanced neuronal viability in a dose-dependent manner. Data was presented as mean ± S.D. from seven independent experiments. $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ $^{\#\#\#}P < 0.001$ triol pretreated groups vs. H/R group. Bar = 20 μm. (For interpretation of color in this Figure, the reader is referred to the web version of this article).

compared with normoxic control. In contrast, neurons pretreated with triol were showing better cell viability in a dose-dependent manner with the maximum effect reaching 90.0% of normal control.

3.2.2. Triol exhbited stronger neuroprotection compared to DHEA against H/R-induced injuries

DHEA is a well known neurosteroid with various neurobiological activities [17]. We next compared the neuroprotective effects of triol and DHEA in the above-mentioned H/R injury model. We observed better cell morphology and higher survival rate in the cultures pretreated with triol compared with cultures pretreated with DHEA at the same concentration level (Fig. 2a). Cell viability obtained by MTT assay revealed that DHEA was neurprotective against H/R exposure at the concentration of 3.0 μM, however this effect was much weaker compared to triol (Fig. 2b).

3.2.3. Triol ameliorated mitochondrial dysfunction

3.2.3.1. Triol alleviated loss of MMP after H/R. MMP is an early surrogate biomarker and a commonly used indicator for mitochondrial function. JC-1 used in this study is a voltage sensitive probe to monitor MMP, when MMP polarizes, JC-1 accumulates in the mitochondrial matrix as a polymer (red), while if MMP depolarizes, JC-1 becomes a monomer and locates in the cytoplasm (green). As shown in Fig. 3a, in normoxic control group, red fluorescence markedly dominated green fluorescence, cells were showing a bright orange color. After H/R exposure, there was a significant increase in green fluorescence (orange turned partly green), suggesting loss of MMP, while cultures pretreated with triol were showing an enhancement of orange fluorescence consistent with the increase of triol doses, indicating an alleviation of MMP loss in these cultures. Similar effect was not seen in the cultures pretreated with DHEA.

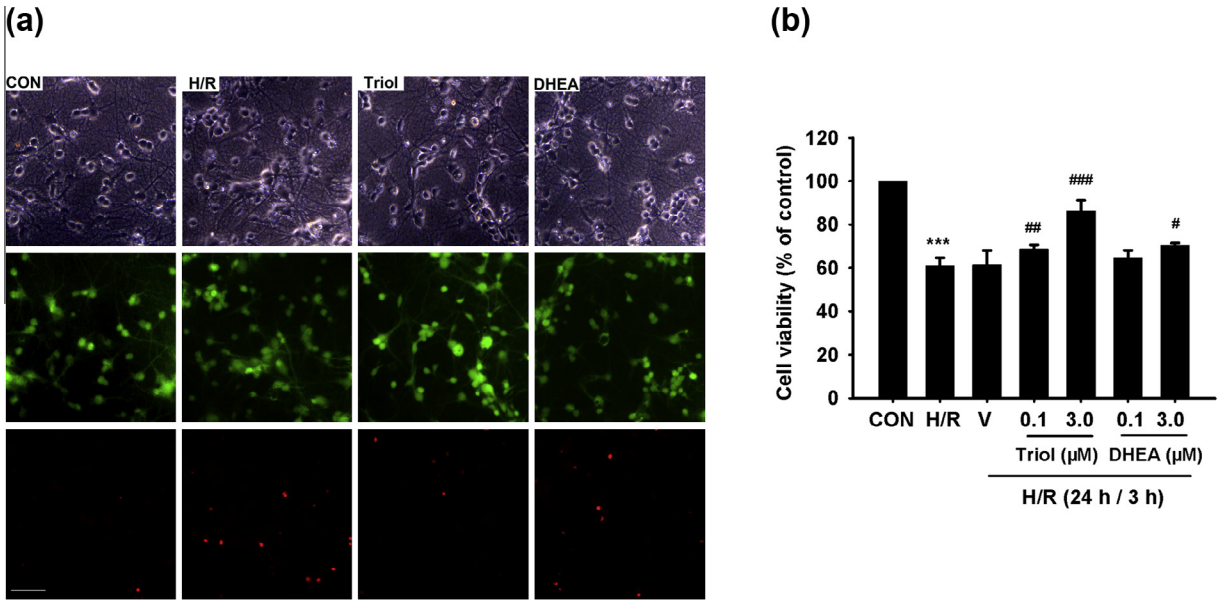


Fig. 2. Comparison of neuroprotective effects between triol and DHEA against H/R exposure (a) Representative phase contrast and FDA/PI stained images. Notably, cultures treated with triol are showing better morphology (phase contrast and FDA staining) and fewer dead cells (PI staining) compared to cultures treated with the same concentration of DHEA (3.0 μM). (b) The statistic graph of neuronal viability by MTT assay. H/R significantly reduced neuronal viability, which was improved by both low and high doses of triol, but only high dose of DHEA with weaker effect. Data was presented as mean ± S.D. from four independent experiments. $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ triol/DHEA pretreated groups vs. H/R group. Bar = 50 μm.

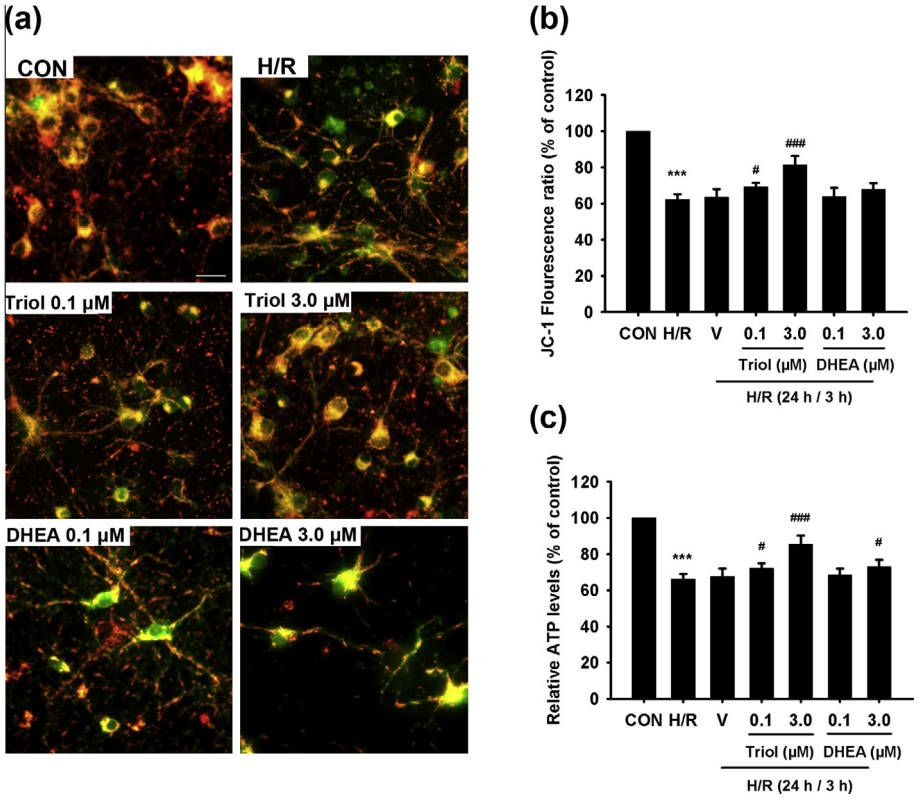


Fig. 3. Amelioration of mitochondrial dysfunction in triol and DHEA pretreated neurons after H/R. (a) Representative images of JC-1 stained neurons. Mitochondrial membrane potential (MMP) was detected by a fluorescent probe JC-1 and images were acquired from a fluorescent microscope with a "dual-bandpass" filter. In the control group, cells were showing a bright orange color and H/R exposure resulted in a turn from orange partly to green, indicating loss of MMP, while in triol pretreated neurons, red fluorescence was enhanced, indicating an improvement of MMP loss. Similar effect was not seen in DHEA pretreated cultures. (b) Statistic graph of MMP. Fluorescence values of JC-1 were obtained from fluorescent microplate reader, then calculated as red/green fluorescence ratio and normalized as percentage of the control group. Data was presented as mean ± S.D. from three independent experiments. (c) Intracellular ATP levels. ATP levels were detected by a luciferin and luciferase assay. Data was presented as mean ± S.D. from four independent experiments. $^{***}P < 0.001$, control vs. H/R group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ triol/DHEA pretreated groups vs. H/R group. Bar = 20 μm. (For interpretation of color in this Figure, the reader is referred to the web version of this article).

Quantification performed in the fluorescent microplate reader presented consistent results (Fig. 3b). H/R exposure reduced red/green fluorescence ratio by almost 40.0%, while treatment with triol improved loss of MMP in a dose-dependent manner. In the DHEA pretreated groups, no statistical significance was shown.

3.2.3.2. Triol preserved intracellular ATP levels. Mitochondria are the main source of ATP production, which reflects directly mitochondrial respiratory function. Therefore we next examined the intracellular ATP levels. In the H/R challenged cortical neurons, intracellular ATP levels were reduced by almost 40% (Fig. 3c) compared to normoxic controls. Intracellular ATP levels in the cultures were increased significantly during H/R when both treated with low (0.1 μM) and high (3.0 μM) of triol. At the concentration of 3.0 μM, DHEA also statistically increased the intracellular ATP level, however this effect was much weaker compared to that of triol.

3.2.4. Triol inhibited accumulation of ROS

Intracellular ROS level is a commonly used indicator of oxidative stress. Here we incubated cortical neurons with carboxy-H_2-DCFDA and fluorescent intensities were read and normalized as percentage of control. As shown in Fig. 4, oxidative stress in H/R treated neurons was evident, ROS level enhanced to almost 1.7-fold of control group, and treatment with triol reduced ROS accumulation in a dose-dependent manner. At the concentration of 3.0 μM, intracellular ROS level reduced to almost 1.2-fold of control group in the triol pretreated group. Notably, treatment with 3.0 μM of DHEA also significantly reduced the intracellular ROS accumulation during H/R, however, with a much weaker effect.

4. Discussion

Development of novel neuroprotectants has been indispensable in the treatment of ischemic stroke for the limitations of current therapies. In the present study DHEA was used as a starting drug to obtain the target compound 5α-androst-3β,5,6β-triol (triol) from 3 steps of reaction with an overall yield as high as 76.0%. We herein for the first time report the bioactivity of triol in a well established H/R injury model which has been used an in vitro ischemic stroke model, and triol exhibited robust neuroprotective effect in primary cultured cortical neurons against H/R exposure. As a starting material, DHEA itself is known to exert neuroprotective effects although its effect on H/R-induced neuronal injury is controversial [17]. In our model, we found that DHEA was neuroprotective at the concentration of 3.0 μM however, the effect was much weaker compared to that of triol. Meanwhile, there was a consistent improvement in triol pretreated groups in H/R-induced MMP loss and disruption of ATP production, indicating that regulation of mitochondrial function may be involved in the mechanism of action. Intracellular ROS formation, a commonly used indicator of oxidative stress, was also reduced in triol pretreated neurons, suggesting that triol also acts by reducing oxidative stress during H/R exposure. Also we found that both in terms of protection of mitochondrial function and inhibition of oxidative stress, triol exhibited notable advantages over DHEA.

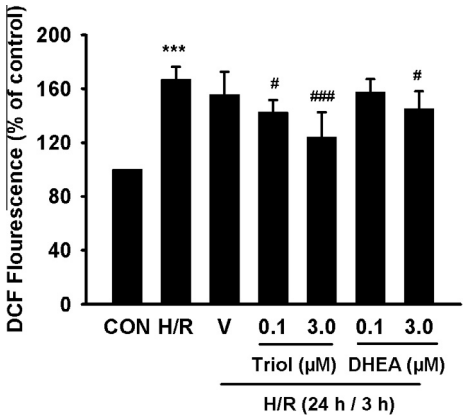


Fig. 4. Reduction of intracellular ROS formation in triol and DHEA pretreated neurons after H/R exposure. ROS levels were detected by a fluorescent probe H_2DCF-DA (DCF). Data were from three independent experiments as mean ± S.D. $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ triol/DHEA pretreated groups vs. H/R group.

It has been well demonstrated that H/R leads to massive disturbance in energy metabolism involving a series of cellular events and causes oxidative stress which is also very complicated process. In our study, particular attention was given to mitochondria in virtue of its irreplaceable role in energy metabolism. It is very hard to directly and quantitatively measure mitochondrial function, for this reason, served as a surrogate marker, MMP has been commonly used to indicate mitochondrial functional alterations. Loss of MMP during H/R was evident suggesting mitochondrial dysfunction, whereas in the triol pretreated groups MMP was retrieved, that is, mitochondrial dysfunction was alleviated. Meanwhile it is documented that hypoxic insult depolarizes mitochondria and consequently impacts ATP production by inhibition of oxidative phosphorylation [18]. In our study there was an enhancement of intracellular ATP levels in triol pretreated cultured neurons, showing that energy supply was preserved. Noteworthily, intracellular ATP levels reflect a combination of production and consumption. It needs further investigation to determine whether triol acts directly by promoting mitochondrial respiration. Furthermore, mitochondrial dysfunction is linked to oxidative stress, together they act as determinants of cell death or survival in stroke [19]. Oxidative stress can be defined as an imbalance between ROS production and/or impaired ROS metabolism that leads to an excessive intracellular ROS accumulation [20]. Consistent with previous reports, the intracellular ROS level was remarkably increased after H/R exposure whereas a significant reduction was seen in triol pretreated groups, which means that triol also reduces oxidative stress during H/R. Ulteriorly, it will be interesting to determine the specific role of triol in attenuating oxidative stress during H/R.

There is another concern that although it has been well established that inhibition of mitochondrial respiration causes electron leak to generate ROS in response to hypoxia [8,21,22], during different phases of H/R, ROS generation or oxidative stress is of considerable complexity [5,23–26], thus a more specific look into this issue seems necessary in further ascertaining that triol protects neurons against H/R by targeting specific mitochondrial sites. Notwithstanding, maintenance of MMP, ATP production and ROS generation are closely linked events with mitochondria being headquarters, and our experimental data overall supported that triol protected cultured neurons against H/R injury by blocking or ameliorating the occurrence of detrimental mitochondrial events, yet the exact molecular mechanisms especially the direct target of triol need further investigation.

Finally, logP is a crucial parameter in the mathematical prediction of membrane structure penetration of chemicals, and the logP value of triol is 3.31, which indicates that triol is lipophilic in nature and is expected to across the blood–brain barrier and other membrane structures. Taken together, triol is conclusively of great potential as a pharmaceutical candidate in the treatment of acute ischemic stroke.

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