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Contraceptive drug, Nestorone, enhances stem cell-mediated remodeling of the stroke brain by dampening inflammation and rescuing mitochondria

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ABSTRACT

Ischemic stroke remains a significant unmet need causing massive mortality and morbidity due to few treatment options with limited therapeutic window. The progestin Nestorone® (segesterone acetate) displays high affinity for the progesterone receptor in exerting its potent birth control and hormone replacement therapy. Accumulating evidence implicates a new utility of Nestorone in affording neuroprotection in a variety of central nervous system diseases, including stroke. However, the mechanism of action mediating Nestorone's neuroprotection in stroke remains unknown. Here, we showed that stand-alone treatments of Nestorone or human amniotic fluid-derived stem cells (hAFSc), but more pronounced with their combined treatment, led to significant improvements in behavioral function and reductions in infarction and peri-infarct cell loss in adult rats with ischemic stroke. We detected significantly lower levels of pro-inflammatory signals (OX6 and IBA1) coupled with enhanced levels of stem cell proliferation (Ki67) and differentiation (DCX and MAP2) in both brain and spleen of stroke rats that received stand-alone or combined treatments of Nestorone and hAFSc. In concert, the *in vitro* oxygen-glucose deprivation stroke model revealed that neural stem cells treated with Nestorone exhibited increased stem cell proliferation and differentiation that was accompanied by rescue of the mitochondrial respiratory activity characterized by reduced mitochondrial reactive oxygen species, increased ATP, elevated mitochondrial deacetylase Sirtuin 3 (SIRT3), and a normalized ratio of acetyl-superoxide dismutase 2 (Ac-SOD2)/SOD2, suggesting the key role of mitochondrial metabolism and oxidative protection in Nestorone's therapeutic effects in stroke.

1. Introduction

Stroke is a major cause of death and disability in the United States and around the world. Treatment options are few and limited to a narrow therapeutic window necessitating the need to find better approaches to address the significant unmet clinical need of stroke patients. Recognizing this urgent stroke demand, novel neuroprotective treatments have been explored in stroke models.

The contribution of progesterone (PROG)-dependent signaling pathways to the cell death cascade of stroke damage and the ability of PROG treatments to alleviate stroke deficits may represent relevant therapeutic targets in stroke [1–3]. Unfortunately, endogenous PROG only exerts neuroprotective effects in $PR^{+/+}$ mice during the first 24 h post-stroke [4], warranting an improved PROG-like drug to afford robust and long-term efficacy. To this end, Nestorone® (segesterone acetate), a synthetic progestin that selectively binds to the progesterone receptor (PR) and circumvents androgenic, estrogenic, and glucocorticoid activities associated with other synthetic progestins, stands as an ideal candidate for hormone replacement therapy and contraception [5,6]. Equally important, Nestorone also exerts neuroprotection against a myriad of neurological diseases characterized by demyelinating, motor neuron cell death, and inflammatory pathological pathways such as multiple sclerosis, amyotrophic lateral sclerosis, spinal cord injury, and stroke [1,5,6]. Notably, $PR^{+/+}$ mice exogenously treated with Nestorone over 24h continue to demonstrate improvements on the rotarod test and decreased infarct volume at 48h after stroke [5] and even up to a month

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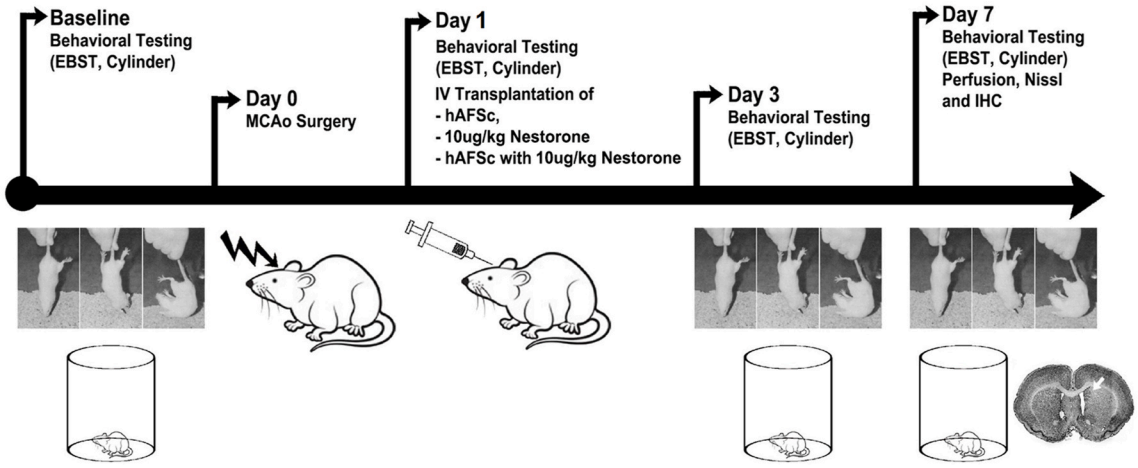

Fig. 1. Schematic diagram of *in vivo* animal procedures.

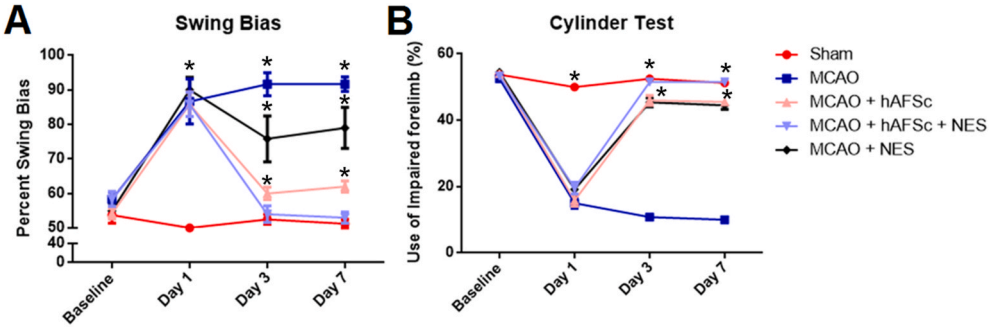

Fig. 2. Nestorone ameliorates stroke-induced behavioral deficits. Panel A, EBST. Panel B, cylinder test. Baseline data prior to stroke showed normal swing and paw use in animals. Following MCAO surgery on day 1, stroke animals displayed biased swing and paw use. At days 3 and 7 post-MCAO, stroke animals continued to exhibit biased swing and paw use, while stand-alone treatments with hAFSc (MCAO + hAFSc) or Nestorone (MCAO + NES) and combined treatment with hAFSc and Nestorone (MCAO + hAFSc + NES), but more pronounced with the combination therapy, resulted in significant attenuations of impaired swing and paw use compared to MCAO (p's < 0.05). Stand-alone and combined treatments did not significantly differ from sham animals (p's > 0.05).

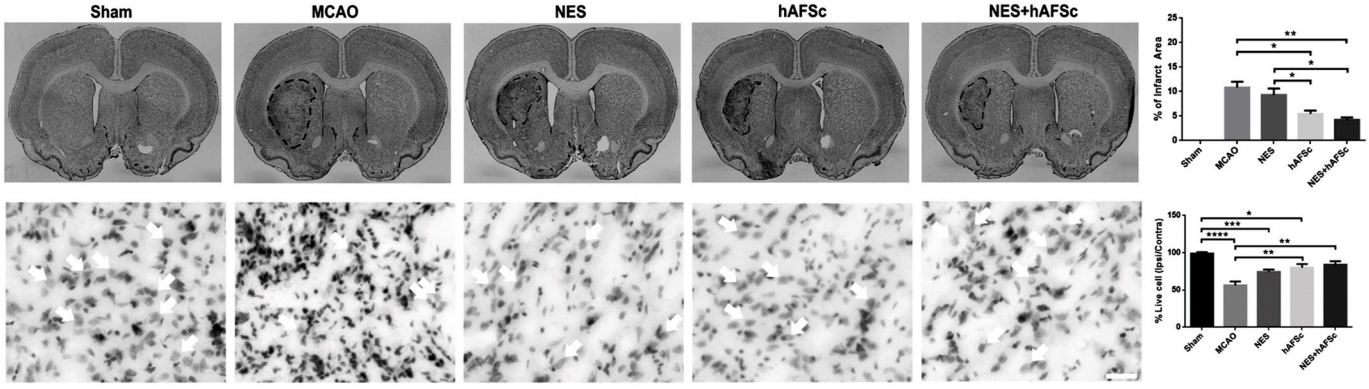

Fig. 3. Nestorone reduces infarct size and peri-infarct cell loss. At day 7, histological analyses using Nissl staining revealed typical MCAO-induced striatal brain infarction with the accompanying peri-infarct cell loss. Stand-alone treatments with NES or hAFSc reduced infarct area and peri-infarct cell loss, which were further decreased by combined NES and hAFSc compared to MCAo only (*p's < 0.05; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). Bar = 50um.

after stroke in rats [7], demonstrating that Nestorone provides neuroprotection reminiscent of PROG (i.e., dependent specifically upon PR signaling pathways) but promotes therapeutic effects beyond those seen with PROG [2,5,8]. These findings advance Nestorone as a potent stroke therapeutic.

The mechanisms underlying Nestorone neuroprotection may involve multi-pronged brain repair processes. Nestorone reduces glutamate-associated excitotoxic damage to rat neural progenitor cells (rNPC) and increases rNPC proliferation *in vitro* via enhanced expression of cell cycle proteins such as proliferating cell nuclear antigen and cell division cycle 2, and increased cell viability through upregulated expression of the mitochondrial ATP synthase-complex V alpha-subunit [9]. Nestorone may also be mediated by the extracellular signal-regulated kinase (ERK) signaling pathway because mitogen-activated protein kinase (MAPK) inhibitor UO126 prevents Nestorone-induced cell proliferation [9]. Additionally, Nestorone may promote neurogenesis, oligodendrogenesis, and myelination via insulin-like growth factor-1 (IGF-1) and the IGF-1 receptor signaling pathway [10]. Furthermore, Nestorone

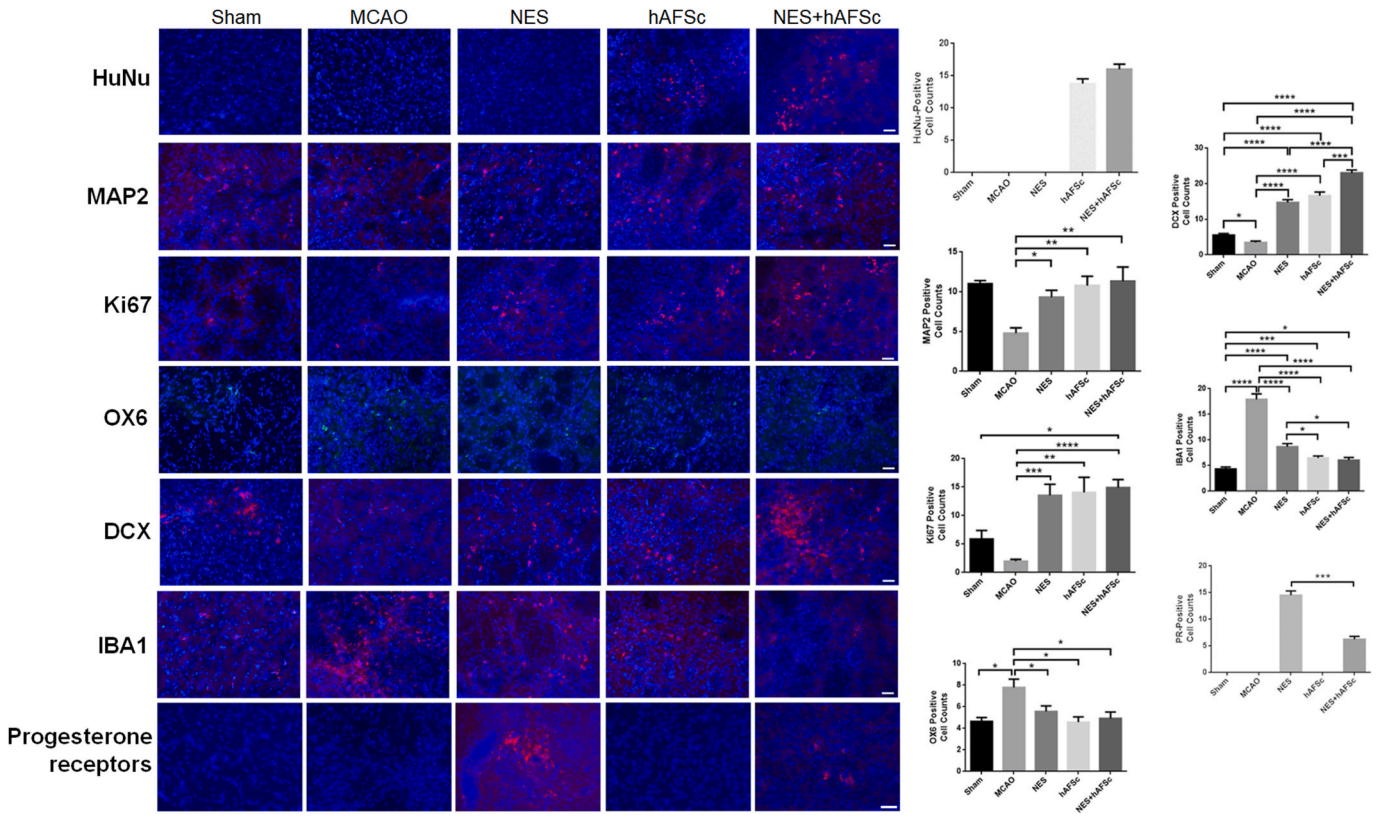

Fig. 4. Nestorone increases cell proliferation and differentiation, and dampens inflammation in the brain. MCAO decreased cell proliferation (Ki67) and differentiation (DCX and MAP2) but elevated inflammation (OX6 and IBA1) within the ischemic striatum. Stand-alone treatments with Nestorone or hAFSc increased proliferative Ki67-positive cells and neural differentiating DCX- and MAP2-positive cells, coupled with reduced inflammatory OX6- and IBA1-positive cells compared to those stroke animals that received the vehicle alone. Moreover, the combined Nestorone and hAFSc appeared to further enhance cell proliferation and differentiation and decrease inflammation compared to other treatments. The addition of Nestorone also seemed to increase hAFSc graft survival compared to those stroke animals that received hAFSc alone but did not reach statistically significant values. The treatment of Nestorone also showed activation of PR compared to those stroke animals that did not receive Nestorone (*p's < 0.05; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). Bar = 100um.

prompts remyelination to abrogate severe demyelination lesions in cuprizone animal models of chronic demyelination [6,11]. Downstream, Nestorone also dampens inflammation by decreasing astrogliosis, microgliosis, and pro-inflammatory molecules such as TNF-α, iNOS, NFκB, IκB and TLR4 [6,12]. A common denominator of these therapeutic effects shows Nestorone's selectivity for PR activation [6,11], with negligible effects on Nestorone metabolites, such as 3α and 5α-tetrahydronestorone [13,14].

To date, the aforementioned mechanisms have been mostly implicated in Nestorone's neuroprotection against the demyelinating disease of multiple sclerosis. Elucidating whether Nestorone functions via similar neuroprotective mechanisms in stroke remains to be determined. Here, we focused on assessing the role of Nestorone in aiding the stem cell-mediated remodeling of the stroke brain. In particular, while we attempted to replicate the PR-specific targeted action and inflammatory signaling pathway previously ascribed to Nestorone, we explored the innovative contribution of Nestorone in the proliferation and differentiation of stem cells by investigating its effects on mitochondrial metabolism and oxidative protection. We explored stand-alone treatments of Nestorone or human amniotic fluid-derived stem cells (hAFSc) and the combined treatment of Nestorone and AFS, initially assessing the standard behavioral and histological stroke outcomes, then determining pro-inflammatory signals and eventually probing stemness properties but more importantly the stem cells' mitochondrial respiration produced by Nestorone treatment using *in vivo* and *in vitro* stroke models.

2. Methods

2.1. Ethics statement

All experiments were conducted in accordance with the National Institute of Health Guide and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of South Florida, Morsani College of Medicine. The article adheres to the Transparency and Openness Promotion Guidelines, and all data supporting the findings of this study are available from the corresponding authors on reasonable request.

2.2. MCAO model

Adult male Sprague-Dawley rats (≈250 g) were subjected to transient intraluminal MCAO procedure (n = 32) or sham surgery (n = 8) (Fig. 1). Adult male Sprague-Dawley rats (approximately 250g) were anesthetized by a mixture of 1–2% isoflurane in nitrous oxide/oxygen (69%/30%) via face mask. Body temperature was maintained at 37 ± 0.3 °C during the surgical procedures. The midline skin incision was made in the neck with subsequent exploration of the right common carotid artery (CCA), the external carotid artery, and internal carotid artery. A 4-0 monofilament nylon suture (27.0–28.0 mm) was advanced from the CCA bifurcation until it blocked the origin of the middle cerebral artery (MCAO). The contralateral CCA was temporally ligated for 15 min to ensure a consistent blood flow occlusion (including collaterals) to the brain. Animals were allowed to recover from anesthesia during MCAO. After 60 min of transient MCAO, animals were re-

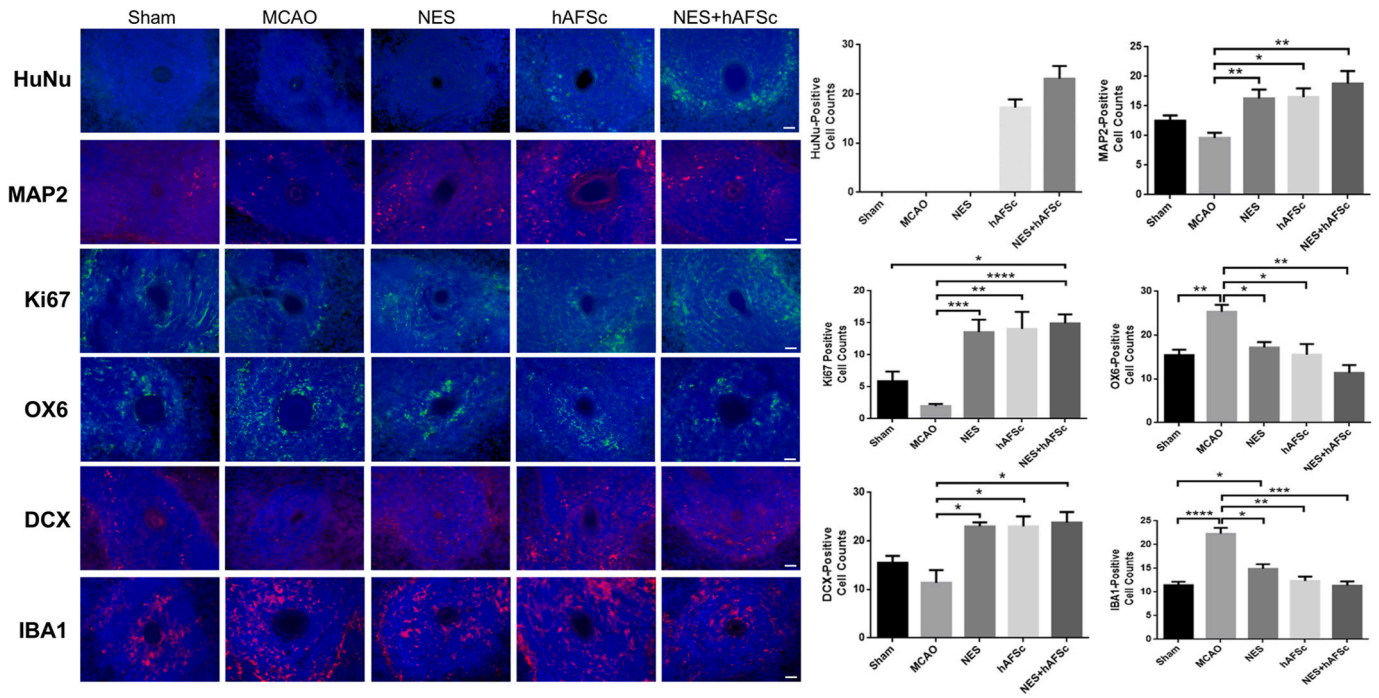

Fig. 5. Nestorone elevates cell proliferation and differentiation, and sequesters inflammation in the spleen. The spleen resembled the brain's response to Nestorone, in that MCAO reduced cell proliferation (Ki67) and differentiation (DCX and MAP2) but with upregulated inflammation (OX6 and IBA1). Stand-alone treatments with Nestorone or hAFSc elevated proliferative Ki67-positive cells and differentiating DCX- and MAP2-positive cells, and decreased the inflammatory OX6- and IBA1-positive cells compared to those stroke animals that received the vehicle alone. Moreover, the combined Nestorone and hAFSc showed a trend of further increasing cell proliferation and differentiation and reducing inflammation compared to other treatments. Nestorone also exhibited a trend of enhancing hAFSc graft survival compared to those stroke animals that received hAFSc alone but did not reach statistically significant values (*p's < 0.05; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). Bar = 100um.

anesthetized with 1–2% isoflurane in nitrous oxide/oxygen (69%/30%) using a face mask and reperfused by withdrawal of the nylon thread. Animals receiving the sham operation were anesthetized with 1–2% isoflurane in nitrous oxide/oxygen (69%/30%) via face mask. A midline incision was made in the neck and the right CCA was isolated. The animals were then suture closed and allowed to recover from anesthesia.

2.3. Behavioral tests

All investigators testing the animals were blinded to the treatment condition. Each rat was subjected to a series of behavioral tests to reveal motor performances at different time points before and after stroke, Nestorone treatment and hAFSc transplantation. The tests included the Elevated Body Swing Test (EBST) and the cylinder test. EBST is a measure of asymmetrical motor behavior that does not require animal training or drug injection. The rats were held, in the vertical axis, approximately an inch from the base of its tail and then elevated to an inch above the surface on which it has been resting. The frequency and direction of the swing behavior were recorded for over 20 tail elevations. A swing was counted when the head of the rat moved more than 10° from the vertical axis to either side. Normally, intact rats display a 50% swing bias, that is, the same number of swings to the left and to the right. A 75% swing bias toward one direction was used as criterion of motor deficit. The total number of swings made to the biased side was added per group and divided by 20, providing the average number of swings per treatment group. The cylinder test is an easy and sensitive tool to evaluate a rodent's spontaneous forelimb use, which has been used in a number of motor system injury models of stroke [15]. Specifically, about 1 h after EBST, the cylinder test was conducted placing the animal in a cylinder that allowed movement and encouraged wall exploration. The rat was allowed to explore the space by rearing and placing one or both forelimbs on the cylinder wall, and then using one or both forelimbs to land back on the floor. The forepaw contact was recorded once the forepaw touched the floor after the initial placement on the wall. All the forepaw contacts were counted for over a 1-min period. Decreased use of the impaired forelimb has been interpreted as a motor deficit [15].

2.4. Laser Doppler blood flow measurement

Brain blood flow measurements were measured using laser Doppler (Perimed, Periflux System 5000) at baseline, during MCAO, and 5 min after reperfusion to ascertain successful MCAO. The animal was under deep anesthesia during the measurement and the animal's head was shaved for brain measurement. For brain perfusion, the laser doppler probe was placed over the right frontoparietal cortical area supplied by the MCA. Ophthalmic ointment was applied and the animals were allowed to recover from anesthesia.

2.5. Transplantation and Nestorone treatment

At day 1 post-MCAO, animals (n = 8 per group) were anesthetized and randomly transplanted intravenously via the jugular vein with either PBS only (MCAO), hAFSc (4×10^6 cells/500 μL of sterile PBS; MCAO + hAFSc) or hAFc plus Nestorone (MCAO + hAFSc + NES), or NES alone (MCAO + NES). Sham animals did not receive any transplantation. Nestorone was administered at a dose of 10 ug/kg also intravenously delivered in conjunction with the transplantation.

2.6. Immunohistochemistry

At day 7 post-MCAO, the animals were euthanized by CO_2 and perfused with 0.9% saline and 4% paraformaldehyde. The brain and spleen were harvested and processed for HuNu (a marker for human

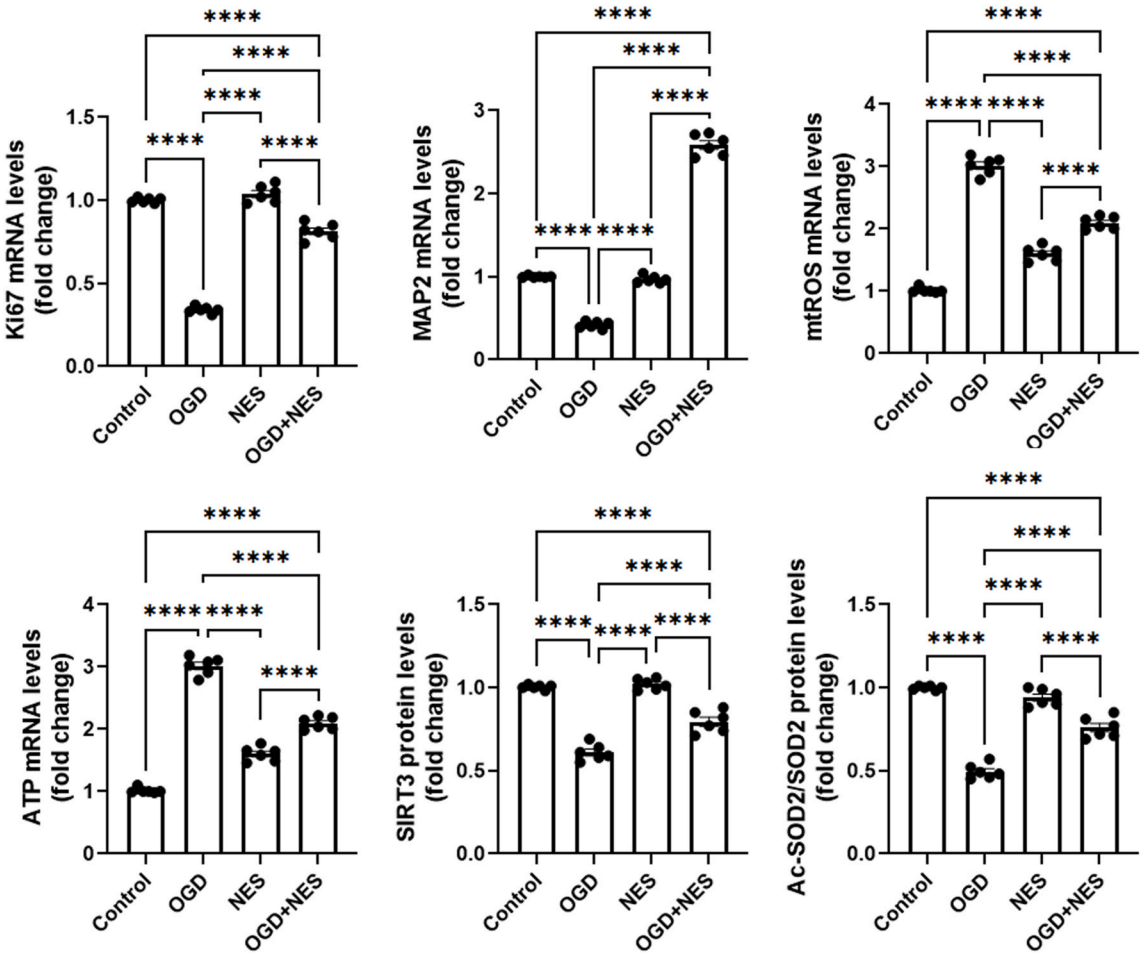

Fig. 6. Nestorone increases cell proliferation and differentiation, lowers mitochondrial ROS, and elevates ATP, SIRT3, and Ac-SOD2/SOD2 ratio levels in cultured NSC. OGD-treated NSC displayed a significant reduction in cell proliferation (Ki67) and differentiation (MAP2) coupled with increased mitochondrial ROS (mtROS) and reduced ATP levels compared to control. Nestorone significantly restored the number of cell proliferative Ki-67 positive cells and neurally differentiating MAP-2 positive cells, which were accompanied by reduction in mtROS and increased ATP levels compared to OGD-treated NSC (p's < 0.05). Furthermore, Nestorone restored the levels of the antioxidant mitochondrial protein, SIRT3, and the ratio of acetyl-SOD2/SOD2, which were downregulated by OGD (p's < 0.05).

nuclei; 1:500; ab191181, Abcam, Cambridge, UK), Ki67 (a marker of cell proliferation; 1:500; ab15580, Abcam), MAP2 and Doublecortin (DCX, markers of cell differentiation; 1:5000; ab5392, Abcam and 1:1000; ab18723, Abcam, respectively), and OX6 and IBA1 (markers of inflammation; 1:750; BD Bioscience, NJ, USA and 1:1000; 019–19741, Wako, Osaka, Japan, respectively). The brain and spleen were sectioned with a cryostat at 20 μm thickness, then incubated in blocking buffer (5% normal goat serum and 0.1% Tween 20 in PBS) for 3 h at 4°C, and with the specific antibody in blocking buffer at 4°C overnight. Then the tissues were washed three times with PBS followed by incubation with Alexa 488 secondary antibody (1:500) for 4 h at 4°C. The tissues were washed three times with PBS and mounted with anti-fade mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (H-1500; Vector Laboratories, Burlingame, CA, USA).

2.7. Isolation of hAFSs

Amniotic fluid samples were obtained from "G. d'Annunzio" University, Chieti. The study has been approved by the Ethics Committee for Biomedical Research of the "G. d'Annunzio" University, Chieti. For each sample, 2–3 ml of amniotic fluid, corresponding to a cell number ranging from 2×10^3 to 2×10^6 were centrifuged for 10 min at 1800 rpm. Pellets were resuspended in Iscove's modified Dulbecco's medium supplemented with 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich, Missouri, USA), 2 mM L-glutamine, 5 ng/ml basic fibroblast growth factor (FGF2) and incubated at 37°C with 5% humidified CO2. After 7 days, non-adherent cells were removed and the adherent cells allowed to growth in the same medium, which was changed every 4 days. When culture reached confluency (about 20 days after the primary culture), cells were treated with 0,05% trypsin and 0,02% EDTA, then counted and replaced in 25 cm^2 culture flasks.

2.8. Oxygen-glucose deprivation stroke model

The oxygen-glucose deprivation (OGD) model was slightly modified. Human neural stem cells (NSC; Neuromics, MD, USA) were thawed and (4×10^4 cells/well) seeded in 96-well plate coated by poly-L-lysine in Neurobasal media (GIBCO, CA, USA) containing 2 mM L-glutamine, 2% B27 (GIBCO) and 50 U/mL penicillin and streptomycin for 7–10 days at 37°C in humidified atmosphere containing 5% CO_2. On the day of experiment, the media of the NSC was changed to Dulbecco's phosphate-buffered saline (DPBS; 14040133; Gibco). The cells were placed in a sealed hypoxia incubator chamber (27310; StemCell Technologies) containing 95% N2 and 5% CO2) for 3 h, mimicking the ischemic stroke. We chose 3 h because based on our experience that 3 h OGD provided 50–60% cell death. After the 3-h period, fresh media was reintroduced and the cells were incubated in normoxia condition (37°C humidified atmosphere containing 5% CO2) for 24 h, which simulated the reperfusion in clinical setting. NSC were seeded at 0.5 x 105 cells/insert and separately cultured in cell culture inserts (353493, Falcon). After

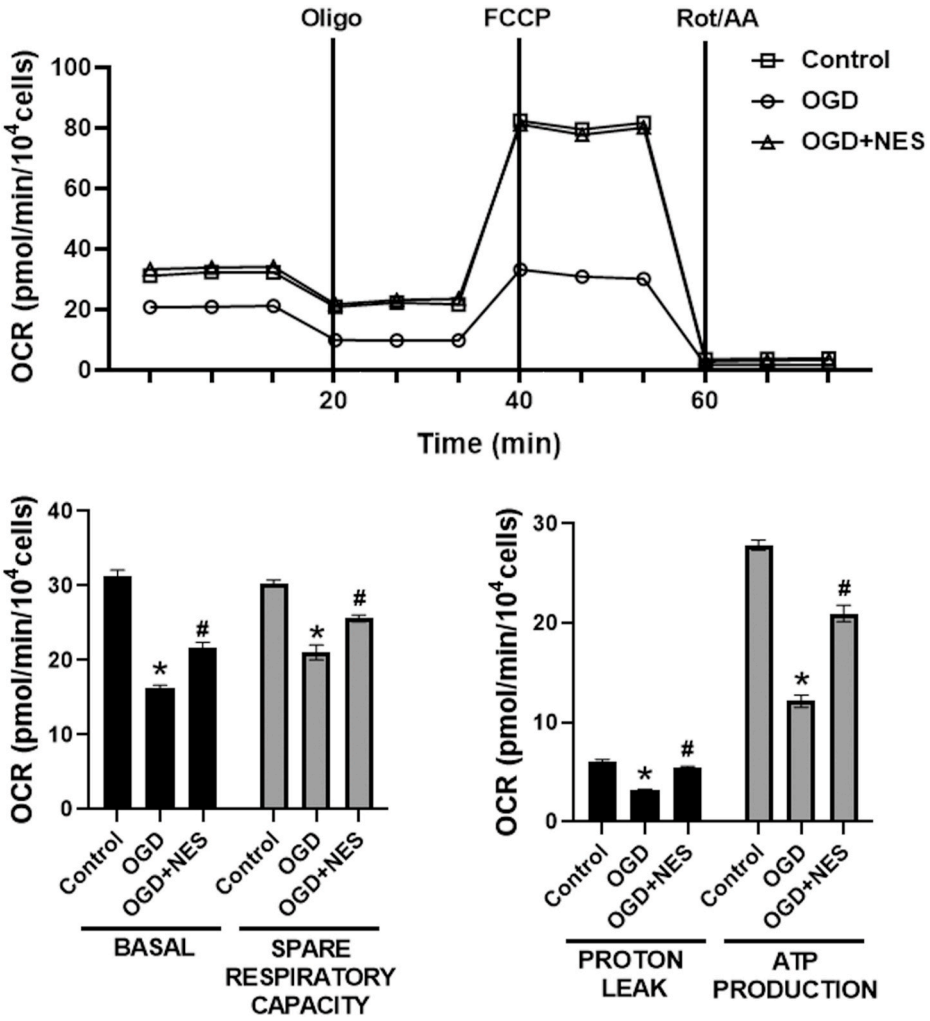

Fig. 7. Nestorone attenuates OGD-induced mitochondrial respiration deficits. The Seahorse XFe96 extracellular flux analyzer revealed that OGD significantly reduced the NSC's mitochondrial respiration compared to control as evidenced by decreased basal respiration, reduced spare respiratory capacity, lower proton leak, and diminished ATP production ($p < 0.05$). Treatment with Nestorone significantly rescued mitochondrial respiration across all indices compared to OGD treatment as revealed by increments in basal respiration, spare respiratory capacity, proton leak, and ATP production ($p < 0.05$).

exposing NSC to OGD, the NSC were treated with Nestorone (1 nM) by placing the inserts into the wells for 24 h then processed for mitochondrial respiration assay, qRT-PCR analysis of cell proliferation and differentiation (Ki67 and MAP2), detection of mitochondrial reactive oxygen species (mtROS) levels measured using MitoSOX™ Red mitochondrial superoxide indicator (M36008; Invitrogen™, San Diego, CA, USA) and ATP (adenosine triphosphate) content assessed by performing the Mitochondrial ToxGlo™ assay (G8001; Promega Co., Madison, WI, USA), and immunoblotting and densitometry of deacetylase Sirtuin 3 (SIRT3; (1:1000; D22A3; Cell Signaling Technology,Danvers, MA, USA), which is a central player in mitochondrial metabolism and oxidative protection, superoxide dismutase 2 (SOD2; (1:200; sc-30080; Santa Cruz Biotechnology Inc., Dallas, TX, USA), a key scavenger enzyme against reactive oxygen species (ROS), and acetyl-SOD2 (Ac-SOD2; 1:1000; acetyl-K68; ab137037; Abcam, Cambridge, MA, USA) since SIRT3-induced SOD2 activation results from SOD2 deacetylation promoted by SIRT3.

2.9. Mitochondrial respiration assay

To determine cellular oxygen consumption rate, the Seahorse extracellular flux analyzer XFe96 (102416; Agilent, Santa Clara, CA, USA) was used in combination with sequential injection of various compounds. Oxygen consumption rate measurements were performed following the manufacturer's protocol. On the day of experiments, NSC were detached from cell culture plates and seeded to a Seahorse 96-well plate (101085–004; Agilent) coated with Poly-D-lysine (100 μg/ml; P7886; Sigma, Springdale, AZ, USA) at 5.0 x 104 cells/well. The cells were immobilized by centrifugation method. Briefly, the Seahorse 96-well plate was centrifuged in swing bucket rotator with slow acceleration (4 on a scale of 9) to a max speed of 450 rpm with 0 brake. Then, the plate orientation was reversed and centrifuged again to max speed of 650 rpm with 0 brake. To determine cellular oxygen consumption rate (OCR), the Seahorse extracellular flux analyzer XFe96 (102416; Agilent) was used in combination with sequential injection of various compounds (1 μmol/L oligomycin, 1 μmol/L carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), 0.5 μmol/L Rotenone and Antimycin A). OCR measurements were performed by the manufacturer's protocol.

2.10. Statistical analysis

The data were evaluated using ANOVA followed by post hoc Bonferroni tests. Statistical significance was preset at $P < 0.05$. Data are presented as mean ± SD.

3. Results

3.1. Nestorone enhances stem cell-induced amelioration of stroke behavioral deficits

Animals that underwent the MCAO stroke insult displayed the typical impairments in motor functions as revealed by significant bias in swing response (EBST) and paw use (cylinder test) (Fig. 2). ANOVA revealed significant treatment effects with stand-alone treatments with

Nestorone or hAFSc resulting in significant attenuations of impaired swing and paw use, which were further improved by combined Nestorone and hAFSc compared to those stroke animals that received the vehicle alone (p's < 0.05). These behavioral improvements produced by stand-alone and combined Nestorone and hAFSc, which did not significantly differ from sham animals (p's > 0.05), occurred in both test dates of days 3 and 7 post-MCAO.

3.2. Nestorone aids in stem cell-induced reduction in infarct size and peri-infarct cell loss

At the conclusion of the behavioral tests at day 7, histological analyses revealed that stroke animals had the expected MCAO-induced striatal brain infarction with the accompanying peri-infarct cell loss (Fig. 3). ANOVA revealed significant treatment effects with stand-alone treatments with Nestorone or hAFSc reducing both infarct area and peri-infarct cell loss, which were further decreased by combined Nestorone and hAFSc compared to those stroke animals that received the vehicle alone (p's < 0.05).

3.3. Nestorone increases cell proliferation and differentiation, and dampens inflammation in the brain

Immunohistochemical analyses revealed that stroke animals had minimal cell proliferation (Ki67) and differentiation (DCX and MAP2) but with elevated inflammation (OX6 and IBA1) within the ischemic striatum (Fig. 4). ANOVA revealed significant treatment effects with stand-alone treatments with Nestorone or hAFSc increasing proliferative Ki67-positive cells and neuralyl differentiating DCX- and MAP2-positive cells, while reducing the inflammatory OX6- and IBA1-positive cells compared to those stroke animals that received the vehicle alone (p's $<$ 0.05). Moreover, combined Nestorone and hAFSc appeared to further enhance cell proliferation and differentiation and decrease inflammation compared to other treatments (p's < 0.05). The addition of Nestorone also seemed to increase hAFSc graft survival compared to those stroke animals that received hAFSc alone but did not reach statistically significant values (p's > 0.05). The treatment of Nestorone also showed activation of PR compared to those stroke animals that did not receive Nestorone (p's < 0.05).

3.4. Nestorone elevates cell proliferation and differentiation, and sequesters inflammation in the spleen

Reflecting the brain's response to Nestorone, the spleen in stroke animals displayed reduced cell proliferation (Ki67) and differentiation (DCX and MAP2) but with upregulated inflammation (OX6 and IBA1) (Fig. 5). ANOVA revealed significant treatment effects with stand-alone treatments with Nestorone or hAFSc elevating proliferative Ki67-positive cells and differentiating DCX- and MAP2-positive cells, while decreasing the inflammatory OX6- and IBA1-positive cells compared to those stroke animals that received the vehicle alone (p's < 0.05). Additionally, the combined Nestorone and hAFSc showed a trend of further increasing cell proliferation and differentiation and reducing inflammation compared to other treatments (p's > 0.05). The addition of Nestorone also exhibited a trend of enhancing hAFSc graft survival compared to those stroke animals that received hAFSc alone but did not reach statistically significant values (p's > 0.05).

3.5. Nestorone increases cell proliferation and differentiation, lowers mitochondrial ROS, and elevates ATP, SIRT3, and Ac-SOD2/SOD2 ratio levels in cultured NSC

To investigate the mechanism underlying Nestorone's neuroprotection via the stem cell remodeling process, cultured NSC were exposed to ambient condition (control) or to OGD. OGD-treated NSC demonstrated significant reduction in cell proliferation (Ki67) and differentiation (MAP2) coupled with increased mitochondrial ROS (mtROS) and reduced ATP levels compared to control (p's < 0.05) (Fig. 6). In contrast, the treatment of Nestorone significantly restored the number of cell proliferative Ki-67 positive cells and neurally differentiating MAP-2 positive cells, which were accompanied by reduction in mtROS and increased ATP levels compared to OGD-treated NSC (p's $<$ 0.05). Interestingly, the antioxidant mitochondrial protein, SIRT3, which was significantly downregulated by OGD was rescued by Nestorone (p's < 0.05). Moreover, whereas OGD significantly reduced the ratio of acetyl-SOD2/SOD2 levels compared to control (p's < 0.05), Nestorone normalized the ratio of acetyl-SOD2/SOD2 compared to OGD (p's < 0.05) Nestorone treatment alone in non-OGD NSC generally resembled NSC grown in ambient condition.

3.6. Nestorone attenuates OGD-induced mitochondrial respiration deficits

Next, we examined the effect of Nestorone on correcting the OGD-induced mitochondrial dysfunction. NSC's mitochondrial respiration was analyzed using Seahorse XFe96 extracellular flux analyzer (Fig. 7). OGD significantly reduced the overall NSC's mitochondrial respiration compared with control characterized by decreased basal respiration, reduced spare respiratory capacity, lower proton leak,and diminished ATP production ($p < 0.05$). Treatment with Nestorone significantly rescued the overall mitochondrial respiration across all indices compared with OGD treatment as revealed by increments in basal respiration, spare respiratory capacity, proton leak, and ATP production ($p < 0.05$).

4. Discussion

The present study demonstrated that Nestorone afforded neuroprotective effects in *in vivo* and *in vitro* stroke models. In stroke animals, Nestorone ameliorated behavioral and histological deficits, dampened pro-inflammatory signals, and enhanced levels of stem cell proliferation and differentiation (DCX and MAP2) in both brain and spleen. Stand-alone treatments of Nestorone and hAFSc were effective, but the combination of Nestorone and hAFSc produced more pronounced therapeutic effects. The *in vivo* functional benefits were replicated in the *in vitro* OGD stroke model, in that NSC treated with Nestorone displayed increased stem cell proliferation and differentiation. Equally important, we revealed for the first time that Nestorone rescued mitochondrial respiration of NSC by upregulating SIRT3 and restoring the Ac-SOD2/SOD2 ratio, implicating the pivotal role of maintaining mitochondrial metabolism and oxidative protection in affording stroke neuroprotection.

Targeting PROG-dependent signaling pathways presents as an appealing therapeutic pathway in mitigating stroke damage. PROG treatments alleviate stroke deficits but may confer only short-term effect [1–3,5]. The advent of Nestorone, which also selectively binds to PR, circumvents many androgenic, estrogenic, and glucocorticoid activities (albeit adverse effects) associated with other synthetic progestins, thereby Nestorone possesses improved therapeutic, as well as safety profiles [16,17]. Nestorone exerts neuroprotection in demyelinating, motor neuron cell death, and inflammatory pathological conditions [2,5,6], with a much more effective and long-lasting outcomes [5,7]. Several mechanisms have been postulated to mediate Nestorone neuroprotection such as enhancing cell proliferation [9] including neurogenesis and oligodendrogenesis [10], and dampening inflammation [6,12], altogether acting primarily through its selective PR activation [6,11,13,14]. Unfortunately, these postulated neuroprotective mechanism have so far been widely studied in the demyelinating disease of multiple sclerosis [6,11,18].

Here, we specifically probed Nestorone's ability to facilitate the stem cell-mediated remodeling of the stroke brain. We were able to replicate Nestorone's PR-selective targeted action in reducing stroke-inflammation and in increasing cell proliferation and differentiation *in*

vitro and *in vivo*. Importantly, we detected that Nestorone rescued mitochondrial metabolism and oxidative protection via SIRT3 upregulation and Ac-SOD2/SOD2 ratio restoration. SIRT3 is the main positive regulator of SOD2, which serves as the most efficient scavenger of mitochondrial ROS in mammalian cells [19–21]. SOD2 deacetylation induced by SIRT3 leads to SIRT3-mediated SOD2 activation [22,23]. Thus, the restoration of protein levels of acetyl-SOD2, SOD2, and mtROS in NSC under pathological conditions, such as stroke, implicates the direct role of SIRT3 in abrogating mitochondrial oxidative stress. The present results provide evidence that Nestorone afforded neuroprotection by rescuing mitochondrial metabolism and increasing bioenergetics level through SIRT3 and SOD2 activation pathway.

Nestorone's currently approved clinical applications as a contraceptive may expedite its transition from the laboratory to the clinic, especially as a stroke therapeutic. Transdermal gel-based Nestorone shows good tolerability, minimal side effects, and effectively quells ovulation in female patients [24]. In tandem with testosterone, Nestorone transdermal gel decreases spermatogenesis in males without severe adverse outcomes, indicating its safe and effective use in males as contraceptive [25]. On the other hand, in women, subdermal Nestorone implants suppresses ovulation for two years [25] and in combination with estrogen via a vaginal contraceptive system has gained recent FDA approval [26,27]. Although these clinical trials support Nestorone's utility as a contraceptive, they show the drug's safety profile under different delivery platforms in humans, guiding a lab-to-clinic pathway for advancing its use as a neuroprotective drug for stroke patients [24,26].

The present observations of Nestorone-mediated rescue of mitochondrial metabolism were conducted *in vitro*, thus whether similar restorations of bioenergetics were achieved *in vivo* warrant further investigations. The mitochondrial assays also used cultured NSC, while the *in vivo* setting included hAFSc and primary neural cells, thereby Nestorone targeted cells differed between the two set-ups also requiring additional investigations of testing Nestorone in hAFSc and primary neural cells. Finally, the animal studies were performed over 7 days post-stroke, whereas the cell culture set-up was conducted at 24 h post-stroke, which begs the question of whether the neuroprotective mechanism of Nestorone's rescue of mitochondrial respiration persists from acute to chronic phases of stroke. Despite these limitations, the present data offer novel pathways of neuroprotection on which to build upon strategies to abrogate mitochondrial dysfunction via stem cell-based and drug-aided (i.e., Nestorone) stroke therapeutics.

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