Video Article

New Application of an Atmospheric Pressure Plasma Jet as a Neuro-protective Agent Against Glucose Deprivation-induced Injury of SH-SY5Y Cells

Xu Yan*[1,3,4,5], Zhaozhong Meng*[2], Jiting Ouyang[2], Yajun Qiao[2], Fang Yuan[1,3,4,5]

[1]Department of Pathophysiology, Beijing Neurosurgical Institute/Beijing Tiantan Hospital, Capital Medical University

[2]School of Physics, Beijing Institute of Technology

[3]Beijing Key Laboratory of Central Nervous System Injury, Beijing Neurosurgical Institute, Beijing Tiantan Hospital, Capital Medical University

[4]Beijing Key Laboratory of Translational Medicine for Cerebrovascular Disease, Beijing Tiantan Hospital, Capital Medical University

[5]China National Clinical Research Center for Neurological Diseases, Beijing Tiantan Hospital, Capital Medical University

*These authors contributed equally

Correspondence to: Xu Yan at devil-yx@163.com, Jiting Ouyang at jtouyang@bit.edu.cn, Fang Yuan at florayuan@vip.sina.com

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Abstract

The atmospheric pressure plasma jet (APPJ) has attracted the attention of many researchers from multiple disciplines in recent years because its emissions include multiple types of reactive nitrogen species (RNS) and reactive oxygen species (ROS). Our previous study has shown the cytoprotective effect of the APPJ against oxidative stress-induced injuries. The aim of the present study is to provide a detailed *in vitro* treatment protocol regarding the neuroprotective applications of helium APPJs on glucose deprivation-induced injury in SH-SY5Y cells. The SH-SY5Y human neuroblastoma-derived cell line was maintained in RPMI 1640 medium supplemented with 15% fetal calf serum. The culture medium was then changed to RPMI 1640 without glucose before APPJ treatment. After a 1 h incubation in a cell incubator, cell viability was determined using Cell Counting Kit 8. The results showed that, compared to the glucose deprivation group, cells treated with APPJ exhibited significantly increased cell viability in a dose-dependent manner, with 8 s/well observed as an optimal dose. Meanwhile, helium flow had no effect on the glucose deprivation-induced cell impairment. Our results indicated that APPJ could be potentially used as a treatment method for the diseases in the central nervous system related to glucose deprivation. This protocol could also be used as a cytoprotective application for other cells with different impairments, but the cell culture and APPJ treatment conditions should be readjusted, and the treatment dose must be relatively low.

Video Link

The video component of this article can be found at https://www.jove.com/video/56323/

Introduction

The adult brain almost exclusively uses glucose as a substrate for energy metabolism under normal physiological conditions. The human brain constitutes only 2% of the body weight but consumes approximately 25% of the total glucose within the body[1]. It is well documented that glucose metabolism dysfunction is one of the major pathological changes during ischemic stroke and various neurodegenerative diseases, including Alzheimer's disease (AD), Huntington's disease (HD), and Parkinson's disease (PD)[2,3]. The lack of glucose and either impaired glucose uptake or oxidative phosphorylation can directly impact ATP production and further induce neural cell death, which may increase the risk of neuronal dysfunction, suggesting that maintaining cell viability or delaying cell injury after glucose deprivation might be a reasonable approach for treating these diseases. The investigation of neuroprotective effects via glucose modulation, focusing on anti-inflammatory agents, ion channel modulators, free radical scavengers, neurotrophic factors, *etc.* has been of interest. However, translation of these neuroprotective approaches from bench to clinical practice has not been successful[4].

Atmospheric pressure plasma jets (APPJs) are a new kind of atmospheric low temperature gas discharge technology that has attracted the attention of many researchers from multiple disciplines in recent years. APPJs have been used for decades in various biomedical applications such as cancer cell treatment, bacterial inactivation, blood coagulation, wound healing, oral medicine, *etc.*[5,6], due to its emissions of multiple types of reactive nitrogen species (RNS) and reactive oxygen species (ROS) (**Figure 1**)[7]. Previous plasma bio-medicine applications mainly focused on the oxidative and/or nitrative stress on bacteria, cells, and tissues[8]. However, the APPJ could also be a "double-edged sword" since RNS and ROS are important intracellular signaling molecules related to many physiological and pathophysiological processes[9]. Nitrous oxide (NO) controls a wide range of biological processes and plays a dual role in the human body, especially in central nervous system (CNS). Low levels of NO have shown their neuroprotective activities both *in vitro* and *in vivo* via multiple signal pathways[10]. Our previous study first reported that helium APPJ-induced NO production was involved in the neuroprotective effect of APPJ against oxidative stress-induced injuries[11]. However, the effects of APPJs on other injuries have not been reported. Therefore, the aim of the present study is to provide an *in vitro* treatment

protocol regarding the neuroprotective applications of helium APPJ on glucose deprivation-induced injury in SH-SY5Y cells. Different from previous studies, our protocol used low-dose plasma treatment for neuroprotective applications without the consequences of excessive plasma-induced injuries, indicating that the APPJ treatment could be potentially used as a novel "NO donor drug" for future research and even for clinical translation. This protocol was also suggested to be used as a cytoprotective application for other cell types with different impairments, but the APPJ treatment conditions should be re-adjusted and the treatment dose must be relatively low.

Protocol

1. Preparation of the APPJ device

CAUTION: Please consult all relevant material safety data sheets (MSDS) before use. Please use appropriate safety practices when performing all the experiments, including the use of a fume hood and personal protective equipment (safety glasses, protective gloves, lab coat, *etc.*). The protocol requires standard cell handling techniques (sterilizing, cell recovery, cell passaging, cell freezing, cell staining, *etc.*).

1. Choose a quartz tube with an internal diameter of 1 mm and an external diameter of 3 mm. Smooth the cross-sections at both ends using a polishing foil.
2. Use a Nickel-plated stainless-steel needle with a diameter of 1.0 mm as the high voltage electrode. Grind its tip to a radius of curvature of 0.05 mm.
3. Wrap aluminum foil (2 mm in width) around the quartz tube at 1 cm from the quartz tube nozzle. Fix the stainless-steel needle point at 1 cm from the other end of the aluminum foil. Use the aluminum foil ring as the low voltage electrode.

2. Acquisition of Jets

1. To provide an AC signal, connect the high-voltage power amplifier to the function signal generator that serves as a power supply. To record the waveforms of the applied voltage to the high voltage electrode, connect one end of the high-voltage probe to the digital oscilloscope and connect the other end to the power supply. To protect the circuit, use a 2 kΩ resistor as a protective resistor. Connect the circuit as shown in **Figure 2**.
   CAUTION: Do not touch the high voltage lines.
2. Continuously pass helium (volume fraction, 99.999%) over the quartz tube and control the gas flow rate at a stable 1.4 Standard Liter per Minute (SLM).
   NOTE: We do not use a filter before treating cell cultures. The volume fraction of helium used in the experiment is 99.999%, and most microorganisms cannot live under these conditions.
3. Turn on the power of the oscilloscope, signal generator, and high-voltage power amplifier. Rotate the frequency adjustment knob to 5 kHz. Gradually increase the voltage to a peak-to-peak value of 6 kV.
   NOTE: The jet is long enough (approximately 3 cm) when the peak-to-peak value of the voltage applied in the needle is approximately 6 kV.

3. Preparation of SH-SY5Y Cells

1. Grow the SH-SY5Y human neuroblastoma-derived cell line in a 25 cm^2 flask in RPMI 1640 medium supplemented with 15% fetal calf serum (FBS). Maintain the cells in a humidified incubator containing 5% CO_2 and 95% air at 37 °C.
2. When the cells reach 85% confluence, carefully aspirate the culture media and add 1 mL 0.25% trypsin + 0.1% EDTA to the cells.
3. After 15 s incubation at room temperature, carefully aspirate the trypsin and add 2 mL RPMI 1640 containing 15% FBS to neutralize.
4. Gently pipette up and down, washing the bottom of the well, until the SH-SY5Y monolayer is completely detached.
5. Count the cells by means of the hemocytometer and adjust the cell concentration to 2 x 10^5 cells/mL by adding RPMI 1640 medium +15% FBS, and then transfer 100 µL of cell suspension to each well of a 96-well plate.
6. Allow the cells to attach for 12 h in a cell incubator before APPJ treatment.

4. APPJ treatment of SH-SY5Y

1. Adjust the distance between the nozzle of the quartz tube and the platform where the 96-well plate will be placed to 3 cm. Ensure that the beam can touch the surface of the culture medium.
   NOTE: The distance is not measured from the bottom of the plate. It is first adjusted to 3 cm between the nozzle of the quartz tube and the platform used to place 96-well plate.
2. Before the APPJ treatment, change the culture medium in each well except the control wells to RPMI 1640 without glucose medium.
3. Place the plate under the APPJ nozzle and ensure that the jets can shoot vertically into each well.
4. Treat cells in separate wells with APPJ for 0 s, 1 s, 2 s, 4 s, 8 s, and 12 s.
   NOTE: APPJ is generated by ionizing helium (**Figure 1**). The cells injured by glucose deprivation are treated by 4 s and 8 s helium flow to eliminate the effects of helium on cells. All the treatments should be performed in triplicate.

5. Cell Viability Assay

NOTE: Do not change the medium in this step.

1. After the APPJ treatment, incubate the cells for 1 h in a cell incubator.
2. Add 10 µL of Cell Counting Kit-8 (CCK-8) solution to each well.
3. Incubate the cells at 37 °C for 4 h.

NOTE: The SH-SY5Y cell line is sensitive to glucose deprivation conditions[12]. Cell viability decreases to nearly 50% after 1 h glucose deprivation, which is the optimum cell viability condition for pharmacodynamics studies. CCK-8 has no cytotoxicity to cells and cells are incubated with CCK-8 reagent for another 4 h in the glucose deprivation conditions after APPJ treatment to check cell viability. After 8 h glucose deprivation and APPJ treatment, the protective effect of APPJ was significantly reduced because the long-duration of glucose deprivation led to severe damage of the SH-SY5Y cells. There was no evidence of living cells after 24 h glucose deprivation[11].

4. Measure the absorbance at 450 nm with a microplate reader.

Representative Results

Data are expressed as the mean ± SD of at least three independent experiments. Group results were analyzed for variance using ANOVA. All analyses were performed using statistical analysis software Prism and $p<0.05$ was the threshold for statistical significance.

Cell viability was measured after 4 h of CCK-8 incubation. As shown in **Figure 3**, glucose deprivation reduced the viability of SH-SY5Y cells to 44.1 ± 2.6% compared to that of the control group (cells normally cultured in RPMI 1640 medium containing 15% FBS). The APPJ treatment significantly increased cell viability in a dose-dependent manner at an optimal dose of 8 s/well, and the cell viability reached to 62.27 ± 3.1%. Gas flow had no effect on the glucose deprivation-induced cell impairment (**Table 1**).

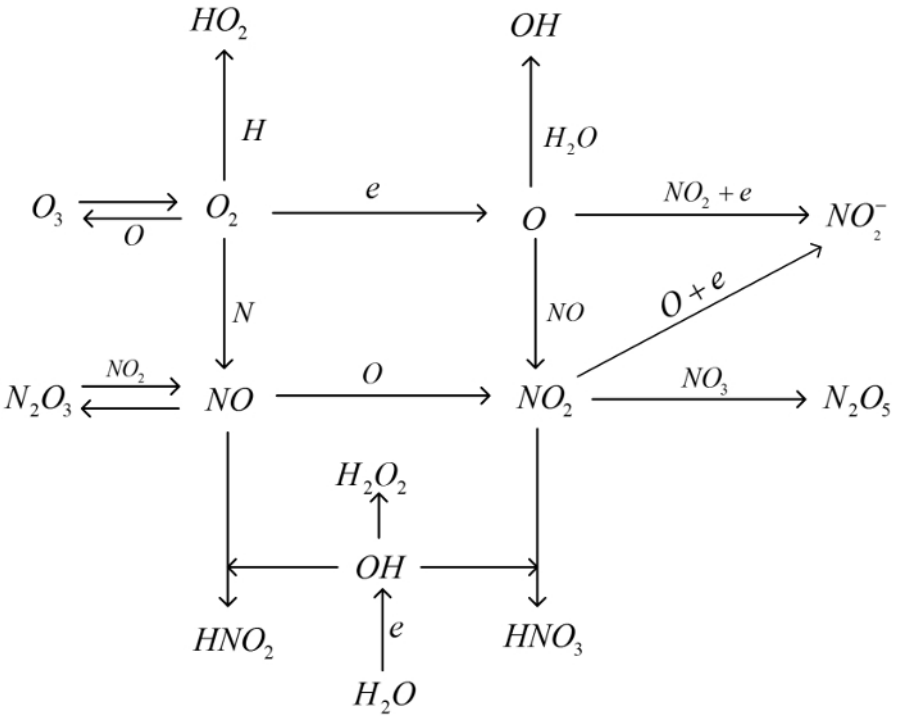

Figure 1: Typical RNS and ROS reactions in APPJ emissions. Please click here to view a larger version of this figure.

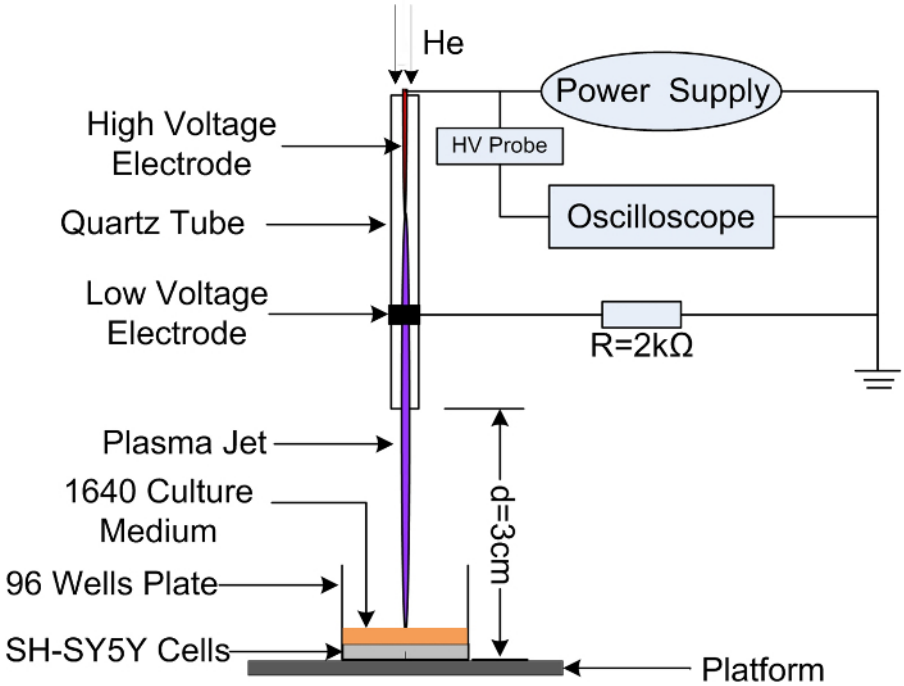

Figure 2: Schematic of the experimental setup. Please click here to view a larger version of this figure.

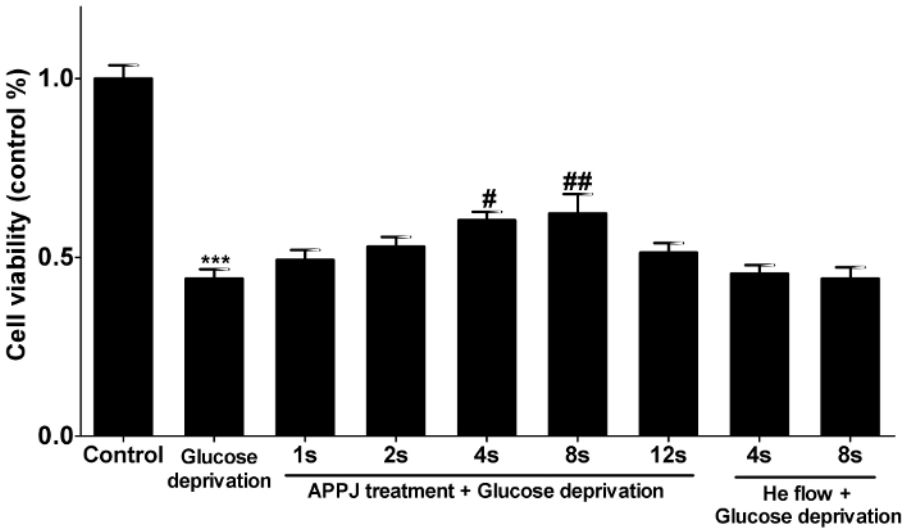

Figure 3: Protective effect of APPJ on glucose deprivation-induced injury of SH-SY5Y cells. Cells were treated with APPJ and subjected to glucose deprivation for 1 h, after which the cell viability was determined using the CCK-8 assay. Error bars represent mean ± SD. ***P <0.001 versus control; #P <0.05 and ##P <0.01 versus the glucose deprivation group (*n* = 3). Please click here to view a larger version of this figure.

Groups		Cell viability (control %)
Control		100 ± 3.7%
Glucose deprivation		44.1 ± 2.6%***
APPJ treatment + Glucose deprivation	1 s	49.3 ± 2.8%
	2 s	53.0 ± 2.7%
	4 s	60.4 ± 2.3%#
	8 s	62.3 ± 3.1%##
	12 s	51.3 ± 2.7%
He flow + Glucose deprivation	4 s	45.4 ± 2.4%
	8 s	44.1 ± 3.1%

Table 1: Percent viability data of SH-SY5Y cells after glucose deprivation with or without APPJ treatment. ***P <0.001 versus control; #P <0.05 and ##P <0.01 versus the glucose deprivation group (*n* = 3).

Discussion

SH-SY5Y cells are a human neuroblastoma-derived cell line and are widely used as an appropriate cell model for *in vitro* studies on neurotoxicity or neuroprotection[12]. The SH-SY5Y cell line was sensitive to glucose deprivation conditions. Cell viability decreased to nearly 50% after 1 h glucose deprivation, which is the optimal cell viability condition for studies of pharmacodynamics. Furthermore, CCK-8 reagent has no cytotoxicity to cells and cells were incubated with CCK-8 reagent for another 4 h in the glucose deprivation conditions after APPJ treatment to check cell viability. In the current study, we provide a detailed *in vitro* treatment protocol regarding the neuroprotective applications of APPJ on the glucose deprivation-induced injury of SH-SY5Y cells.

Modifications and troubleshooting
The CCK-8 incubation time could be shorter if the color in each well significantly changed. But if SH-SY5Y cell density is below 1 x 10^4 cells per well, cells will be dead after glucose deprivation and APPJ treatment. It is also recommended to reduce the gas flow rate, while ensuring that the plasma beam can touch the surface of the culture medium. APPJ could also be used as a cytoprotective agent for other neuronal related cell lines (HT-22, neuro-2A, or even primary neurons) with different impairments (hypoxia, oxidative stress, *etc.*), but the cell culture and APPJ treatment conditions should be readjusted, and the treatment dose must be relatively low. We have tried to reduce distance in this APPJ generation parameter, and we found that the plasma jet could directly affect the attachment of SH-SY5Y cells which could result in cell injuries (SH-SY5Y cells were easily detached from their adherent state). We believe that the treatment distance should be based on the cell characteristics and the tolerance to the plasma jet treatment.

Limitations of the technique
The current protocol only focused on the *in vitro* neuroprotective effect of APPJ on glucose deprivation-injured SH-SY5Y cells. Previous research has shown that inhalation of plasma could improve cardiac functions in a rat myocardial infarction model[13]. More work is still needed to investigate the *in vivo* treatment method for the brain protection.

Significance with respect to existing methods
Previous research on plasma medicine paid more attention to inactivation capacities in bacteria, cancer cells, and tissues because of the oxidative and/or nitrative stress induced by APPJ treatment[14]. Our protocol used low-dose plasma treatment for neuroprotective applications without the consequences of excessive plasma-induced injuries, indicating that the APPJ treatment could be potentially used as a novel "NO donor drug" for the future research and even for clinical translation.

Critical steps within the protocol
The most critical step in this protocol is to make sure the APPJ treatment dose is relatively low, since over treatment with APPJ will aggravate cell injuries and directly induce cell death. Another critical step is to control the glucose deprivation duration or the cells will die and the cytoprotective effect of APPJ will be significantly reduced. Pure helium, rather than helium mixed with a small amount of O_2 or air, was used. When helium mixed with a small amount of O_2 or air is used, the amount of ROS in the plasma will increase. It is very difficult to make a diagnosis when complicated plasma chemical reactions occur.

Future applications
It is also worthwhile to note that the APPJ treatment was applied after induction of glucose deprivation on SH-SY5Y cells, indicating that APPJ could be potentially used as a treatment method for glucose deprivation-related diseases in the central nervous system, especially ischemic stroke. Therefore, it is necessary for future studies to evaluate the treatment conditions of the neuroprotective effect of APPJ both alone and in combination with other neuroprotective agents at different periods after glucose deprivation.

Disclosures

No conflicts of interest were declared in relation to this paper.

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