Video Article

Screening Assay for Oxidative Stress in a Feline Astrocyte Cell Line, G355-5

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Abstract

An often-suggested mechanism of virus induced neuronal damage is oxidative stress. Astrocytes have an important role in controlling oxidative stress of the Central Nervous System (CNS). Astrocytes help maintain a homeostatic environment for neurons as well as protecting neurons from Reactive Oxygen Species (ROS). CM-H2DCFDA is a cell-permeable indicator for the presence of ROS. CM-H_2DCFDA enters the cell as a non-fluorescent compound, and becomes fluorescent after cellular esterases remove the acetate groups, and the compound is oxidized. The number of cells, measured by flow cytometry, that are found to be green fluorescing is an indication of the number of cells that are in an oxidative state. CM-H_2DCFDA is susceptible to oxidation by a large number of different ROS. This lack of specificity, regarding which ROS can oxidize CM-H_2DCFDA, makes this compound a valuable regent for use in the early stages of a pathogenesis investigation, as this assay can be used to screen for an oxidative cellular environment regardless of which oxygen radical or combination of ROS are responsible for the cellular conditions. Once it has been established that ROS are present by oxidation of CM-H_2DCFDA, then additional experiments can be performed to determine which ROS or combination of ROSs are involved in the particular pathogenesis process. The results of this study demonstrate that with the addition of hydrogen peroxide an increase in CM-H_2DCFDA fluoresce was detected relative to the saline controls, indicating that this assay is a valuable test for detecting an oxidative environment within G355-5 cells, a feline astrocyte cell line.

Protocol

1. Cell culture of feline astrocytes and treatment with H_2O_2

1. Culture cell line G355-5 in a 75 cm^2 flask with DMEM media plus nutrients at 37°C and 5% CO_2 until 60% confluent (˜ 5 days). Replace the media every 1-2 days or as needed to maintain a healthy culture.
2. Remove media and add 2 ml of 0.25% trypsin. Lift the cells by pipetting for 45 s - 1 min at room temperature (RT).
3. Quickly transfer the suspension to a 15 ml conical tube containing 10 ml of media. Mix gently.
4. Centrifuge at 300 x g, 23°C for 3 min to obtain a pellet. Discard the supernatant without disturbing the pellet.
5. Add 6-7 ml of fresh media and resuspend the cells.
6. Add ˜ 1 ml of cells to each well of a 6-well plate. Add 2-3 ml of media to each well and incubate until 80-90% confluent.
7. Prepare a 100µM solution of peroxide (H_2O_2) in media.
8. Remove media from each well and replace with either 2 ml fresh media (control), 2 ml of H_2O_2 (treatment) or 2ml of DPBS. Incubate at 37°C, 5% CO_2 for 3 h.
9. Remove solutions and add 1 ml of trypsin to lift the cells. Transfer cells to a 1.7 ml Eppendorf tube containing 0.5 ml DPBS. This step should be performed on each well individually in order to prevent cell death from prolonged trypsin incubation.
10. Spin at 300 x g for 3 min, discard the supernatant and resuspend in 1 ml of DPBS.

2. Staining for ROS

1. All steps for staining should be carried out with minimal light exposure to prevent bleaching.
2. Add 1 µl of 50 mM CCCP to the positive control. Incubate at 37°C, 5% CO_2 for 5 min.
3. Add 5 µl of a 10 mM CM-H_2DCFDA solution to the H_2O_2 group. Incubate at 37°C, 5% CO_2 for 15-30 min. No stain is added to the saline sample.
4. Wash samples with 2 ml of DPBS to remove residual stain.
5. Spin at 300 x g for 3 min to obtain a pellet.
6. Resuspend cells in 0.5 ml of DPBS. Add 1 µl PI to each sample, except the saline.
7. Transfer samples to a 5ml flow cytometer tube.
8. Run on the flow cytometer.

3. Flow cytometry

1. Run samples on a Beckman Coulter FC500 (or equivalent) equipped with a 488 nm argon laser and the following band passes: 525, 775 and 620 nm, all ± 20 nm.
2. Prepare the appropriate controls: unstained cells, CM-H_2DCFDA stained only, PI only, double stained (negative control).
3. Make a protocol that has the following histoplots: FS vs. SS, PI vs. CM-H_2DCFDA. It should also have the following histograms: cell number vs. FL1, FL2 and FL3, which corresponds to the stains.
4. Before running the samples, check equipment, fluids and waste container. Warm up the equipment for at least 20 min.
5. Clean the equipment and perform a calibration with the appropriate beads according to the manufacturers standards.

6. Select your protocol and run the samples.
7. Clean the equipment as done previously.
8. Clean the vacuum line according to the manufacturers standards.
9. Turn off the flow and clean the head/vacuum line.
10. Turn off the software and the computer.

4. Representative Results:

Flow cytometry results were analyzed using FlowJo 7.6 and the stain controls were used to objectively set the gating. Based on this, healthy cells appear on the left of the histogram and ROS (oxidative stress) was detected as a shift of cells to the right. Figure 1 shows the results of the effect of hydrogen peroxide on healthy feline astrocytes. Data from FL1 was used to measure the intensity of CM-H_2DCFDA, which indicates the presence of ROS. As expected, a higher amount of ROS was detected in the sample treated with H_2O_2. This is displayed on the histogram as a shift in fluorescent intensity from left to right. When comparing healthy cells treated with DMEM versus DPBS, there was no significant change in the amount of ROS (Fig 2).

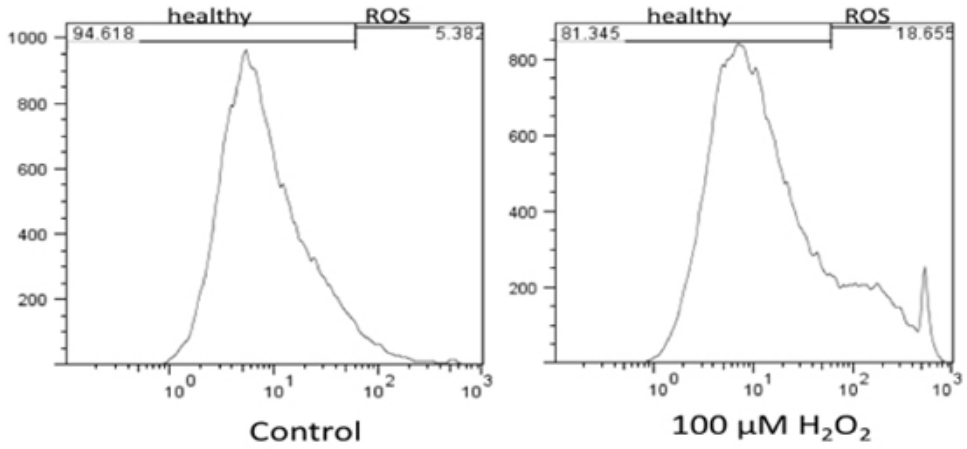

Figure 1. Flow cytometry results of levels of oxidative stress in healthy feline astrocytes and the effect of H_2O_2 on healthy cells. The results indicate that H_2O_2 increases levels of ROS in healthy cells as compared to cells with no treatment (DMEM).

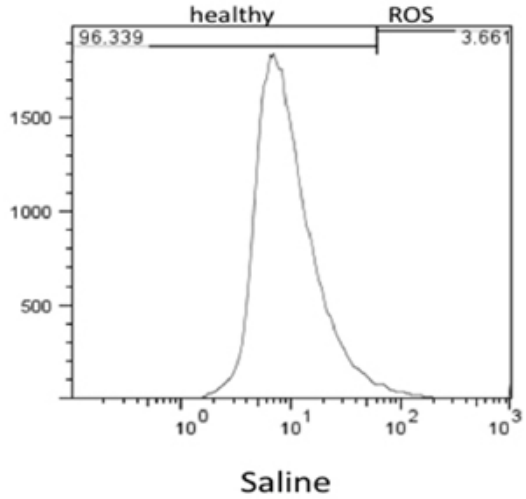

Figure 2. Flow Cytometry results of levels of ROS detected in healthy cells incubated for 3hrs with DPBS.

Discussion

An often suggested mechanism of virus induced neuronal damage is oxidative stress [1, 4, 7, 9, 13, 15, 16, 18-22, 27, 31]. Basically, it is proposed that through viral exposure the glia (astrocytes and microglia) release reactive oxygen radicals such as, hydroxide, superoxide anion, nitric oxide, and/or hydrogen peroxide, which are toxic to neurons. Similarly, oxidative stress has also been suggested as a major pathologic mechanism in methamphetamine-induced neurotoxicity [2, 6, 17]. Since our group is interested in the effects of both virus and drugs of abuse on the CNS, we elected to use a quick screening test to determine the present of excess ROS in astrocytes. The majority of neuronal glutathione is produced by the astrocytes and then delivered to the neurons [5, 8, 10-12, 24, 26, 29, 30]. Thus, the astrocytes role in oxidative stress is important in maintaining a homeostatic environment for neurons as well as protecting neurons from ROS, and is the reason that astrocyte cell cultures were chosen for this study.

CM-H_2DCFDA is also known as 2',7'-dichlorofluorescein and H2DCF. CM-H_2DCFDA is a cell-permeable indicator for presence of reactive oxygen species. CM-H_2DCFDA enters the cell as a non-fluorescent compound, which becomes fluorescent after cellular esterases remove the acetate groups, and the compound becomes oxidized. Once inside the cell and the acetate groups are removed, the CM-H_2DCFDA can be oxidized by a number of ROS species, including nitric oxide, peroxynitrite anions, hydrogen peroxide, and organic hydroperoxides [3, 14, 25], resulting in a green fluorescent product [23, 28]. This lack of specify in the ROS, which can oxidize CM-H_2DCFDA, makes it a valuable reagent in the early stages of a pathogenesis investigation, in which an oxidative stress mechanism is believed to be playing a role, but it is unknown which oxygen radical might

be involved, rather than test for each ROS separately. Once it has been established that ROS are present by oxidation of CM-H_2DCFDA, then additional experiments can be performed to determine, which ROS or combination of ROSs are involved in the particular pathogenesis process. The results of this present study demonstrate that with the addition of hydrogen peroxide an increase in CM-H_2DCFDA fluoresce was detected when compared to the saline control, indicating that that this assay is valuable test for detecting an oxidative environment within the G355-5 cells, a feline astrocyte cell line.

Disclosures

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