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Real-Time Assessment of BBB Dysfunction Induced by Oxidative Stress and D-Galactose–Mediated Senescence in Cerebral Microvascular Endothelial Cells

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Abstract - Blood–brain barrier (BBB) dysfunction contributes to aging-related neurological disorders, including Alzheimer's disease, stroke, and vascular dementia. Oxidative stress and cellular senescence are major drivers of endothelial injury and BBB disruption. We evaluated the effects of hydrogen peroxide (H_2O_2)-induced oxidative stress and D-galactose–induced senescence on human cerebral microvascular endothelial cell (hCMEC) barrier function. hCMECs were exposed to increasing concentrations of H_2O_2 (25–750 μM) or D-galactose (12.5–400 mM). Barrier integrity was continuously monitored using the Agilent xCELLigence RTCA eSight platform, which combines impedance-based measurements with live-cell imaging. Intercellular gap formation was quantified using ImageJ, and EC_{50} values were calculated from dose–response curves. Both H_2O_2 and D-galactose induced dose-dependent barrier dysfunction. H_2O_2 reduced barrier integrity with an EC_{50} of 189 μM, whereas D-galactose exhibited an EC_{50} of 95 mM and produced measurable impairment even at the lowest concentration tested. Both treatments reduced barrier integrity to approximately 52% of baseline. Live-cell imaging demonstrated preserved cell viability across all treatment groups. Quantitative gap analysis revealed dose-dependent increases in intercellular spacing following high-dose H_2O_2 exposure that closely paralleled impedance changes. In contrast, D-galactose treatment produced significant reductions in impedance without corresponding increases in gap formation across all dose groups, suggesting that impedance measurements are more sensitive for detecting early senescence-associated barrier dysfunction. In conclusion, oxidative stress and cellular senescence significantly impair hCMEC barrier function. The RTCA eSight impedance platform provides a sensitive real-time approach for studying BBB dysfunction and neurovascular aging and may detect subtle barrier alterations before they become detectable by conventional imaging-based analyses.

Keywords: Blood-brain barrier, Oxidative stress, Senescence, live cell imaging, neurodegeneration, cellular integrity

1. Introduction

The blood–brain barrier (BBB) is a specialized vascular interface that maintains central nervous system homeostasis by regulating the exchange of molecules and cells between the circulation and the brain [1]. Human cerebral microvascular endothelial cells (hCMECs) form the structural and functional basis of the BBB through tightly regulated cell-cell junctions. BBB dysfunction is a hallmark of numerous neurological and neurodegenerative disorders, including Alzheimer's disease, vascular dementia, stroke, and traumatic brain injury [2, 3].

Oxidative stress and cellular senescence are increasingly recognized as major contributors to neurovascular aging and BBB breakdown [4, 5]. Excessive reactive oxygen species can disrupt endothelial tight junctions, increase vascular permeability, and promote neuroinflammation. Likewise, senescent endothelial cells exhibit impaired barrier function and altered cellular metabolism that may accelerate age-related neurodegeneration [6, 7, 8]. Despite their importance, quantitative assessment of BBB dysfunction under oxidative and senescent conditions remains challenging using conventional endpoint assays.

The Agilent xCELLigence Real-Time Cell Analysis (RTCA) eSight platform combines impedance-based barrier assessment with live-cell imaging, enabling continuous monitoring of endothelial barrier integrity [9]. In this study, we used

this platform to compare the effects of H_2O_2-induced oxidative stress and D-galactose–induced cellular senescence on hCMEC barrier function.

2. Materials and Methods

2.1 Cell Culture and Treatments

Primary hCMECs were cultured under standard conditions and seeded onto RTCA eSight E-plates until confluent monolayers were established. Oxidative stress was induced using hydrogen peroxide (H_2O_2; 25–750 µM), while cellular senescence was induced using D-galactose (12.5–400 mM). Cells were monitored for a total of 2 hours following treatment.

2.2 β-galactosidase staining

D-galactose–induced cellular senescence in hCMECs was confirmed using senescence-associated β-galactosidase staining according to the manufacturer's instructions (Cell Signaling, Senescence β-Galactosidase Staining Kit, #9860).

2.3 Real-Time Barrier Function Assessment

Barrier integrity was continuously monitored using the Agilent RTCA eSight platform. Electrical impedance measurements were recorded in real time and normalized to pre-treatment baseline values. Dose-response curves were generated and EC_{50} values were calculated.

2.4 Live-Cell Imaging and Gap Analysis

Bright-field images were acquired throughout the experiment using the eSight imaging module. Images were analyzed using ImageJ to quantify intercellular gap formation as an indicator of barrier disruption.

2.5 Statistical Analysis

Data are presented as mean ± SEM. Dose-response relationships were analyzed using nonlinear regression. EC_{50} values are reported with 95% confidence intervals.

3. Results

3.1 H_2O_2 Induces Dose-Dependent CMEC Barrier Dysfunction

As shown in Figure 1, exposure to H_2O_2 resulted in progressive reductions in hCMEC barrier integrity across concentrations ranging from 25 to 750 µM. Impedance measurements demonstrated a clear dose-dependent response, with an EC_{50} of 189 µM (95% CI: 160–218 µM; n = 3 per concentration). Higher concentrations produced substantial reductions in barrier integrity, indicating increased endothelial permeability and junctional disruption.

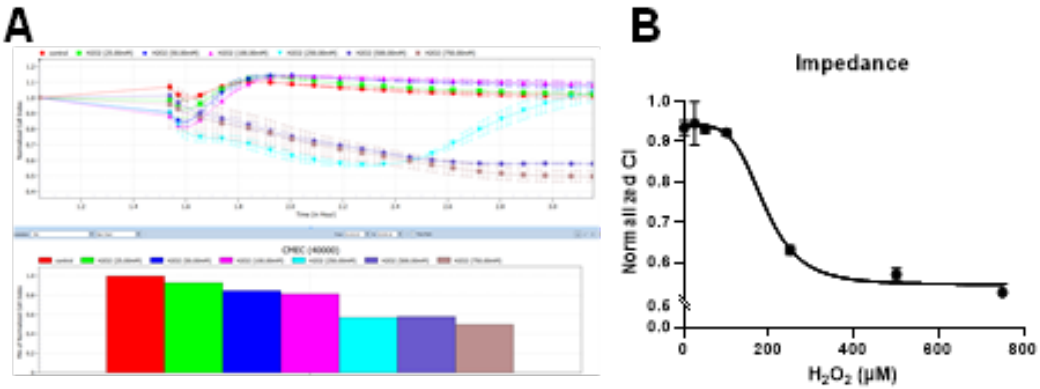

Figure 1. H_2O_2 induces dose-dependent hCMEC barrier dysfunction. Cultured CMECs were exposed to H_2O_2 (25–750 µM) and impedance reflecting barrier integrity was continuously monitored for 2 hours using the Agilent xCELLigence RTCA eSight platform. (**A**) Representative normalized cell index (CI) curves. (**B**) Quantification of barrier impairment using the lowest normalized CI value observed during the 2-hour monitoring period. Dose–response analysis was used to calculate the EC_{50} value for H_2O_2-induced barrier dysfunction. N=3 in each treatment.

3.2 D-Galactose-Induced Senescence Impairs CMEC Barrier Function

We first confirmed D-galactose treatment induced CMEC senescence with increased β-galactosidase staining (Figure 2A). D-Galactose exposure at concentration range 12.5–400 mM also induced dose-dependent barrier dysfunction (Figure 2B). The calculated EC_{50} was 95 mM (95% CI: 0–514 mM; n = 3 per concentration). Notably, measurable reductions in impedance were observed at the lowest concentration tested (12.5 mM), suggesting that CMEC barrier function is highly sensitive to senescence-associated metabolic stress.

Despite differences in EC_{50} values, the maximal effects of H_2O_2 and D-galactose were similar. Both treatments reduced barrier integrity to approximately 52% of baseline levels, indicating that oxidative stress and cellular senescence can induce comparable degrees of endothelial dysfunction.

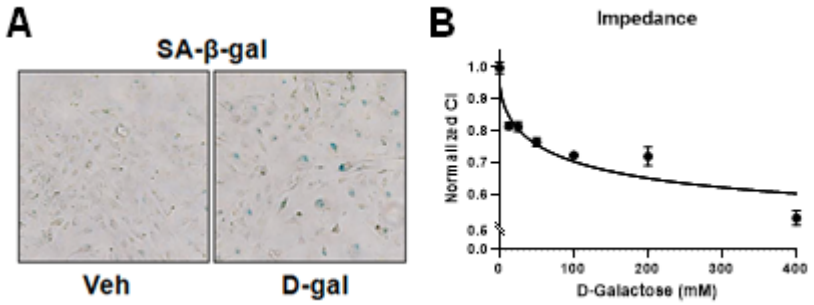

Figure 2. D-Galactose-Induced Senescence Impairs CMEC Barrier Function. (**A**) β-galactosidase staining confirmed D-Galactose induced CMEC senescence. (**B**) Cultured CMECs were exposed to D-galactose 12.5–400 mM and impedance reflecting barrier integrity was continuously monitored for 2 hours. Quantification of barrier impairment using the lowest normalized CI value observed during the 2-hour monitoring period. Dose–response analysis was used to calculate the EC_{50} value for D-Galactose-induced barrier dysfunction. (N=3 in each concentration.)

3.3 Live-Cell Imaging Analysis for Barrier Disruption Detection

As shown in Figure 3, live-cell imaging demonstrated distinct morphological responses of hCMEC monolayers following H_2O_2- and D-galactose exposure. While intercellular gap analysis was relatively insensitive at low and moderate H_2O_2 concentrations, treatment with higher concentrations of H_2O_2 (500 and 750 µM) produced significant increases in gap formation. These structural changes paralleled the H_2O_2-induced reductions in impedance, especially in high concentrations.

In contrast, quantitative gap analysis revealed no significant increases in intercellular gap formation in D-galactose treatment across all the concentrations, suggesting that conventional imaging-based gap measurements are less sensitive for detecting early senescence-associated barrier dysfunction. Importantly, hCMECs remained largely attached and viable throughout both H_2O_2 and D-galactose treatments, indicating that barrier impairment occurred prior to overt cell death.

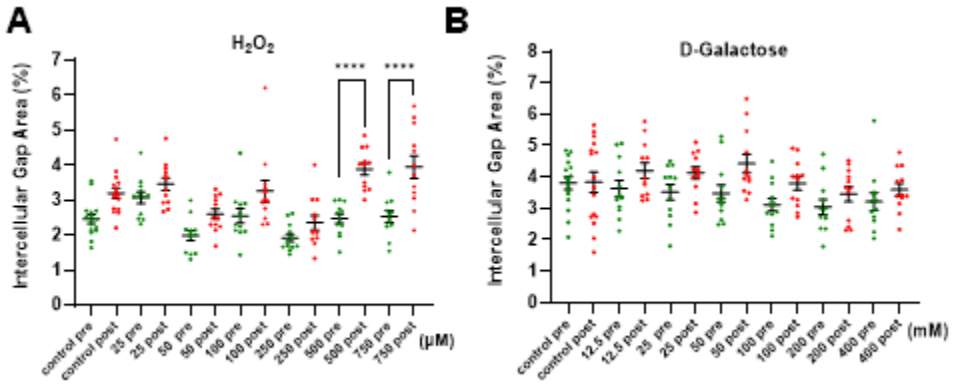

Figure 3: Live-cell imaging analysis to detect inter-cellular gap formation in hCMECs treated with H_2O_2 (A) and D-Galactose (B). Bright-field images were acquired at pre-treatment and post-treatment conditions using the integrated eSight imaging module.

Images were processed using ImageJ following cell-border enhancement procedures. Intercellular gap areas were quantified and expressed relative to the total field area as an indicator of barrier disruption. N=12-16 in each condition (4 images per well for 3-4 wells). Data are presented as mean ± SEM. ****: $p < 0.0001$.

4. Discussion

This study demonstrates that both oxidative stress and cellular senescence impair BBB endothelial function in a dose-dependent manner, as evidenced by progressive reductions in impedance measured using the RTCA eSight platform. H_2O_2-induced oxidative stress produced a robust and concentration-dependent loss of barrier integrity, whereas D-galactose–induced senescence resulted in a more gradual but substantial decline in endothelial barrier function.

The differential responses observed by live-cell imaging provide insight into the mechanisms underlying barrier dysfunction. High concentrations of H_2O_2 (500–750 μM) induced significant intercellular gap formation that closely paralleled impedance reductions, suggesting that oxidative stress directly disrupts endothelial cell-cell junctions and monolayer integrity. In contrast, D-galactose treatment caused marked reductions in impedance without significant increases in intercellular gap area. These findings indicate that senescence-associated barrier dysfunction may occur through more subtle alterations in endothelial physiology, such as impaired tight-junction function, cytoskeletal remodeling, or changes in cell adhesion, which are not readily detected by conventional gap analysis.

Importantly, hCMECs remained largely viable throughout both treatments, indicating that barrier dysfunction preceded overt cell death. The discordance between impedance measurements and gap analysis in the D-galactose model highlights a key advantage of impedance-based monitoring, which appears more sensitive for detecting early functional changes in barrier integrity than morphology-based image analysis alone.

A major strength of the RTCA eSight platform is its ability to continuously monitor barrier function while simultaneously acquiring live-cell images from the same cell population. This multimodal approach provides complementary functional and structural information and enables detection of early barrier impairment before the development of prominent morphological changes.

Several limitations should be acknowledged. The current study examined short-term responses and did not directly investigate molecular mechanisms of barrier disruption. Future studies incorporating tight-junction proteins (ZO-1, occludin, and claudin-5), inflammatory signaling pathways, mitochondrial dysfunction, and permeability assays will help elucidate the mechanisms by which oxidative stress and cellular senescence contribute to BBB dysfunction and neurovascular aging.

5. Conclusions

Oxidative stress and D-galactose–induced cellular senescence cause significant, dose-dependent impairment of hCMEC barrier integrity. Real-time impedance monitoring enables sensitive detection of BBB dysfunction before overt cell death occurs. These findings highlight the importance of oxidative stress and senescence in neurovascular aging and establish the RTCA eSight platform as a valuable tool for investigating BBB dysfunction.

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