Blast-overpressure induced modulation of PARP-SIRT-NRF2 axis in stress signaling of astrocytes and microglia.

VIJAYA PRAKASH KRISHNAN MUTHAIAH[1], Kathiravan Kaliyappan[1], Supriya Mahajan[2], Ramkumar Thiyagarajan[3], and Krishnamoorthy Gunasekaran[4]

[1]University at Buffalo School of Public Health and Health Professions
[2]University at Buffalo School of Medicine and Biomedical Sciences
[3]The University of Kansas Medical Center
[4]Dambi Dollo University

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Abstract

Background: The pathomechanism of blast TBI and blunt TBI is different. In blast injury, evidence indicate that a single blast exposure can often manifest long-term neurological impairments. However, its pathomechanism is still elusive and treatments were symptomatic. Poly ADP Ribose Polymerase-1 (PARP1) is being implicated in the parthanatos and secondary neuroinflammation. Animal studies indicate the over-activation of as a major downstream event underlying the neurological sequalae of several traumatic and neurodegenerative disorders irrespective of the mode of cell death. PARP over-activation forms ADP polymers on several nuclear proteins known as trans-PARylation by consuming NAD+ and ATP. As NAD+ is a substrate for sirtuins, it too has been implicated in the oxidative stress underlying TBI pathology. Hypothesis: We recently established the implication of PARP1 following blast overpressure (BOP) and its differential response on astrocytes and microglial cells. We found that the inhibition of PARP is proven to be beneficial by attenuating oxidative stress. In this study, we hypothesized the involvement of the PARP1-SIRT-NRF2 axis following Blast induced PARP over-activation in glial cells for the manifestation of oxidative stress in BOP insults. Objective: Our objective is to determine the downstream modulation of the PARP-Sirt-NRF2 axis and changes in ATP levels following blast exposure in astrocytes and microglia cell lines. Results: As a result of NAD+ being a common substrate for PARP1 and Sirtuins, we found the decreased expression of SIRT1, SIRT3 and NRF2, a major transcriptional regulator for the expression of antioxidant genes. We found that ATP levels were elevated post-BOP from both glycolysis and oxidative phosphorylation (OXPHOS), an increase of ATP by glycolysis more significant than OXPHOS source indicating the pro-inflammation post-BOP. Conclusion: This result shows that blast-induced PARP over-activation impacts the deacetylation activity of sirtuins and consequently impacts the regulation of antioxidant levels in astrocytes and microglia.

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[1]Vijaya Prakash Krishnan Muthaiah[*], [1]Kathiravan Kaliyappan,[2]Ramkumar Thiayagarajan, [3]Supriya Mahajan, [4]Krishnamoorthy Gunasekaran

[1]Department of Rehabilitation Sciences, School of Public Health and Health Professions, University at Buffalo, Buffalo, NY 14215, USA.

[2]Department of Geriatric Medicine, Kansas University Medical Center, The University of Kansas, Kansas City, KS, 66160, USA.

[3]Department of Medicine, Division of Allergy, Immunology & Rheumatology, SUNY University at Buffalo, 6074 Clinical and Translational Research Center, 875 Ellicott St, Buffalo, NY,

14203, USA.

[4]**Department of** Medical Biochemistry, Institute of Health, Dambi Dollo University, Dambi Dolo, Oromia Region, Ethiopia.

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Corresponding Author:

Department of Medical Biochemistry,

Institute of Health,

Dambi Dolo University,

Dambi Dolo, Oromia Region,

Ethiopia.

Email: krishnapdf2011@gmail.com

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Abstract:

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Introduction.

The combat deployed veterans suffering blast concussive injury manifest neurological impairments even after years of mild insult (Mac Donald et al., 2017). These manifestations even lead to the consideration of mental health as a TBI marker of prognosis for blast injury (Mac Donald et al., 2019; Mac Donald et al., 2021). It is evidenced that a single blast could lead to the manifestation of early onset of neurodegenerative disorders. Hence, it is essential to investigate the cellular mechanism of blast TBI. Cell death mechanism and molecular pathways following TBI have been investigated (Bao et al., 2019; Sater et al., 2018; Zhou et al., 2012) by several experimental invitro and invivo models of TBI. These in vivo and in vitro models were investigated using controlled cortical impact injury which translates to the impact or inertial TBI. Given the nature of the insult, the TBI pathomechanism of impact injury (Blunt trauma) is different from non-impact injury (Blast trauma). However, even the blast overpressure specifications of previously reported in vitro models of primary blast injury ((Arun et al., 2011; Campos-Pires et al., 2018; Canchi et al., 2017; Chen et al., 2009; Effgen et al., 2012; Hue, 2014; Sory et al., 2020; Zander et al., 2016) were of high frequencies in nature. In the present study, conforming to the ideal Friedlander equation of blast wave (with a duration of impulse wave <2 ms followed by a negative pressure phase), we used a blast set-up with a power spectral density of blast overpressure (BOP) energy lying within several kHz below 10 kHz (Kaliyappan et al., 2021; Schmitt et al., 2021) to have a better mechanistic insight on cellular pathomechanism post-blast TBI to translate to semi-quasi real-world IED conditions.

Poly ADP Ribose Polymerase-1 (PARP), a nuclear enzyme, being a key mediator of cell death, its over-activation, has been reported in both*in vivo* and *in vitro* models of several neurodegenerative disorders and traumatic insults. PARP-induced PAR (Poly ADP Ribose) polymer formation results in NAD and ATP depletion mediating cell death through release of Apoptosis-Inducing Factor (AIF), often referred to as Parthanatos. However, evidence is confounding in terms of mode of cell death as its over-activation is implicated in both apoptosis (Chiarugi and Moskowitz, 2002) and necrosis (Ha and Snyder, 1999) with overlapping signaling cascades. In addition, several proteases (such as caspase, cathepsin, and calpains) induced differential cleavage of PARP is reported following insults (Boulares et al., 1999; Chaitanya et al., 2010). Evidence indicates that PARP cleavage inhibits the necrotic mode and paves way for cell death by apoptosis (Los et al., 2002). As NAD availability determines the level of Sirtuin (Silent information regulator) activation, it was reported that PARP1 activation reduces Sirtuin (SIRT1) activity and influences oxidative metabolism (Bai, Péter, et al., 2011). Sirtuins are predominantly NAD-dependent lysine deacetylases (SIRT1,2,3 & 5) and ADP ribosylases (SIRT4, 6 & 7) (Balcerczyk and Pirola, 2010) in addition to the function of various post-translational modifications on a spectrum of targets, that are localized in various cellular compartments such as nucleus (SIRT1, 6 and 7), mitochondria (SIRT3, 4 & 5) and cytoplasm (SIRT2)(Dai et al., 2018; Houtkooper et al., 2012). Sirtuins influence the levels of reactive oxygen species and antioxidant enzymes by regulating the mitochondrial electron transport complexes (Ahn et al., 2008) through the Foxo3a transcription factor (Rangarajan et al., 2015). NRF2 (Nuclear Factor erythroid-derived 2 like -related factor 2) is one of the major targets of Sirtuins, NAD-dependent class 3 histone deacetylases. NRF2 is normally labeled for proteasomal degradation by coupling with CUL3 and KEAP1 (Dodson et al., 2019). However, deacetylation of NRF2 by sirtuins frees them which in turn serves as a co-transcription factor for mRNA expression of several antioxidant enzymes by binding to Antioxidant Response Element (ARE) regions of DNA. Zander et al., 2015 studied the outcome of single blast and repeated blast on dissociated neurons. As we demonstrated PARP overactivation post-blast exposure, in the present study, we investigated the cellular pathomechanism of blast injury on the PARP-Sirt-NRF2 axis using in vitro model of low-frequency blast injury **using astrocytes (C6) and microglial cells (HTHU).**

Materials and Methods.

Cell culture and Blast Injury:

The Rat C6 (ATCC CCL-107^{TM}) and HTHU cells (From Jonathan Karn, CWRU, Cleveland, OH) were cultured at DMEM (Corning, 10-017-CV)/F12 (Corning, 10-080-CV) 114 medium

supplemented with 10% fetal bovine serum (Sigma, F2442) and 1% penicillin-streptomycin (Corning, 30-002 Cl). The acoustic shock tube generates blast over-pressure (BC Precision Tools Inc) using compressed air at 23 psi is used to create an in-vitro model of blast injury similar to our previous reports (Kaliyappan et al., 2021; Schmitt et al., 2021). The frequency spectrum of the impulse waveform is represented by fig 1. Cells cultured at 12 well plates and 100 mm Petri dishes were exposed to single blast over-pressure at ˜172 dB SPL. Post-injury, the cells were maintained at a humidified air incubator (Fisher Scientific 610) at 37 ° C and 5% $CO_2$for 6 h, 12 h, and 24 h for various assays to investigate the effect of blast PARP-Siirt-NRF2 axis.

CM-H2DCFDA staining for ROS detection.

Reactive oxygen species generation in C6 & HTHU cells after BOP was visualized using the method described earlier (Surma et al., 2014). To visualize the ROS in live cells, the cultured cells were stained using the CM-H2DCFDA (LifeTechnologies) with 4',6-diamidino-2-phenylindole (DAPI). In brief, the C6 & HTHU cells (20000cells/plate) were seeded in a 15mm glass-bottom cell culture dish (NEST, Cat# 801002) exposed to BOP. Post 24hours, the cells were incubated with 13uM CM-H2DCFDA in a culture medium at 37oC for 10 minutes. After incubation, the cells were carefully washed thrice with warm PBS, then the cells were counterstained with DAPI (10ug/ml) in PBS for 10 minutes and immediately imaged. The ROS in the cells were observed as a fluorescent emission of CM-H2DCFDA (excitation/emission: 945/530 nm) with DAPI (excitation/emission: 358/461 nm).

Analysis of Mitochondrial membrane potential (JC10 assay)

The mitochondrion membrane potential after BOP was assessed by the Cell Meter JC-10 Assay Kit (AAT Bioquest). The cells were seeded (1 X 104/well) in 96 well plates, cultured for 12 hours, and exposed to the BOP. After 24 hours post-BOP, the treated cells were added with 50µL/well of JC-10 dye-working solution (1 part of Component A + 100 parts of Component B) and incubated at 37oC in dark. After 60 minutes, 50 µL of Component C was added to each well before reading the fluorescence intensity. The fluorescence intensities were measured on a microplate reader (BioTek, USA) using dual fluorescence (Ex/Em= 490/525 nm, Cutoff=515 nm, and 540/590nm, Cutoff=570 nm) with bottom read mode. The ratio of green/red was calculated to denote the extent of mitochondrial depolarization.

Cellular and Mitochondrial ATP production.

Non-mitochondrial cellular ATP production (glycoATP) through glycolytic pathway and mitochondrial ATP production (mitoATP) through oxidative phosphorylation (OXPHOS) was quantified using XFp Real-Time ATP rate assay (Agilent Technologies, Santa Clara, USA). Proton efflux rate (PER) through the glycolytic pathway is used to measure cellular ATP whereas the difference in oxygen consumption (Oxygen consumption rate, OCR) after addition of mitochondrial inhibitors (oligomycin, inhibits Complex V and Rotenone/Antimycin mix inhibits Complex I/III) are used to measure mitochondrial ATP. The sum of glycoATP and mitoATP provides the total cellular ATP production. In brief, 5x105 cells were seeded into 100 mm Petri-dish 12h before the BOP. Both sham control and BOP exposed C6 and HTHU cells were subjected for real-time ATP rate assay after different time-points (6,12 and 24 h) by transferring them to a calibrated seahorse XF tissue-culture plate (5 x 104 cells/well). The basal extracellular acidification rate (ECAR) and OCR were analyzed in real-time (Sea horse XFp analyzer) in the Seahorse assay medium supplemented with 10mM glucose, 2mM L-glutamine, and 1mM pyruvate. Following the addition of 1 µM oligomycin and a mix of 0.5 µM rotenone/antimycin-A, mitochondrial OCR was measured. The sample triplicate was used in all experiments.

mRNA expression of Sirt1, Sirt3, NRF2, and GCLC

The expressions levels of Sirt1, Sirt3, Nrf2, and GCLC genes following BOP were analyzed by quantitative polymerase chain reaction (qPCR). The total RNA from the C6 and HTHU cells were extracted using TRIzol

Reagent (Cat# 15596026; Thermo Fisher Scientific, USA) and assessed in NanoDrop One (Thermo Scientific, USA) for its purity and concentration. The High-Capacity cDNA Reverse Transcription Kit (Cat# 4368814; Thermo Fisher Scientific, USA) was used to synthesis cDNA from the extracted RNA samples, according to the manufacturer's guide. The qPCR was performed in CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). The thermal cycler reaction conditions were set as follows: Polymerase Activation and DNA Denaturation 30 sec at 95oC; Denaturation for 15 sec at 95°C; Annealing/Extension for 30 sec at 60oC. The reaction mixture was prepared by adding the cDNA template, forward and reverse primers (Integrated DNA Technologies Inc, USA), and 2X SsoAdvanced Universal SYBR(r) Green Supermix (Bio-Rad, USA). The primer details were listed in Table 1. The GAPDH was used as the internal control for the assessment. After 40 reaction cycles, the relative gene expression was calculated using the standard curve of cycle thresholds (CT). In brief, the results were analyzed by the relative quantification method (2-ΔΔCT) using CFX Manager software (Bio-Rad, USA). The specific products were confirmed by analyzing the melt curves. The experiment was carried out using sample triplicates.

Table 1. List of primers

Name	Primer details	Primer sequence
SIRT1	Forward primer	5'GAAACCCTCAATTTCTGTTCTGCT3'
	Reverse primer	3'AATGCGATGCTGACTTCCTTCT5'
SIRT3	Forward primer	5'GGCACTACAGGCCCAATGTC3'
	Reverse primer	3'TCTCTCAAGCCCGTCGATG5'
NRF2	Forward primer	5'CCCAGCACATCCAGACAGAC3'
	Reverse primer	3'TATCCAGGGCAAGCGACTC5'
GCLC	Forward primer	5'TGCACATCTACCACGCAGTC3'
	Reverse primer	3'ACCAACATGTACTCCACCTCG5'
GAPDH	Forward primer	5'CCGCATCTTCTTGTGCAGTG3'
	Reverse primer	3'CGATACGGCCAAATCCGTTC5'

Western Blotting - Protein expression of Sirt1, Sirt3

The western blotting procedure was performed to assess the protein levels of Sirt1 and Sirt3 in C6 and HTHU cells. In brief, the BOP exposed C6 and HTHU cells were washed twice with PBS and added with RIPA lysis buffer (Cat# sc-24948; Santa Cruz Biotechnology, USA) at desired time points (6h, 12h & 24h) and the cell lysate was collected. The total protein levels in the samples were measured using the Pierce BCA Protein Assay kit (Cat# 23227; Thermo Fisher Scientific, USA). the proteins were separated by SDS-PAGE by adding 30µg of proteins in each well of 10% precast Tris-glycine gels with the protein Standards (Cat# 1610374; BioRad, USA). Then, the separated proteins were transferred into PVDF Transfer Membrane (Cat# 88518; Thermo Fisher Scientific, USA) using Wet transfer method. The blots were incubated in 5% BSA for one hour at room temperature to reduce the non-specific binding. Following the blocking procedure, the blots were incubated in Anti-Sirt1 Rabbit mAB (1:2000) (Cat# 9542; Cell Signaling Technology, USA), Anti-Sirt3 Rabbit mAB (1:2000) (Cat# 83732; Cell Signaling Technology, USA) in 4°C for overnight. Then, the blots were washed and incubated in pre-diluted HRP conjugated Anti-Rabbit secondary antibodies (1:5000) (Cat# 7074; Cell Signaling Technology, USA) for 1 hour at room temperature. The blots were imaged in ChemiDoc MP Imaging System using SuperSignal West Femto Maximum Sensitivity Substrate (Cat# 34095; Thermo Fisher Scientific, USA). The protein band intensities were measured using Image Lab Software (BioRad, USA) and normalized against the internal control protein, βActin (Cat# sc-47778; Santa Cruz Biotechnology, USA).

Results

BOP increases reactive oxygen species.

The reactive oxygen species levels in the C6 and HTHU cells were qualitatively analyzed using CM-H2DCFDA staining. It is a useful fluorescent indicator, which passively diffuses into the cells and is cleaved by the intracellular esterases and oxidation. Increased ROS levels in the cells were identified by the fluorescent signal (Em: 517-527nm) upon excitation with appropriate wavelength light (Ex: 492-495nm). The results showed increased fluorescence (arrows) in C6 and HTHU cells at post-BOP 24h (Figure 1). The increased fluorescence indicates the increased oxidation in the cells following BOP exposure (Kalyanaraman et al., 2012).

Mitochondrial membrane potential (JC10 assay)

The mitochondrial membrane potential in BOP exposed C6 and HTHU cells were measured using spectrophotometric analysis by measuring the JC-10 aggregates because of membrane polarization. The results indicate a significant increase in the membrane polarisation following blast exposure (Figure 2). The JC-10 aggregates were significantly increased ($F(1, 12) = 79.56$; $p<0.001$) in BOP 6h and BOP 12h exposed C6 cells when compared with the sham group. Among BOP groups, the BOP 6h ($F(2, 8) = 22.06$; $p<0.01$) & BOP 12h ($F(2, 8) = 22.06$; $p<0.001$) group C6 cells have significantly higher membrane polarization when compared to post-BOP 24h group. Similarly, the HTHU cells also showed a significant increase ($F(1, 12) = 217.2$; $p<0.001$) in membrane polarization at BOP 6h and BOP 12h groups when compared to the sham-exposed cells. The HTHU cells showed a time-dependent decrease in membrane polarization in BOP exposed cells. The 6h post-BOP exposed HTHU cells showed significantly increased ($F(2, 12) = 104.3$; $p<0.001$) membrane polarization when compared to BOP 12h & BOP 24h groups.

Changes in ATP production following BOP in C6 and HTHU cells

The mitochondrial cellular ATP and non-mitochondrial cellular ATP production were measured using the Seahorse XFp Real-Time ATP rate assay (Agilent Technologies, Santa Clara, USA). The results indicated the significant increase in the mitochondrial ATP ($F(5, 12) = 5.992$; $^*p<0.05$) levels in the BOP 24 h group of C6 cells when compared to sham exposed C6 cells (Figure 3). The non-mitochondrial glycolytic ATP and total ATP were found to be significant in C6 cells when compared to sham cells. Among BOP groups, the total ATP was significantly increased in 12h post-BOP ($F(5, 12) = 6.340$; $^*p<0.05$) when compared to the 6h post-BOP group. However, the BOP 6h HTHU cells showed a significant increase in mitochondrial ATP ($F(5, 12) = 12.02$; $p<0.01$), glycolytic ATP ($F(5, 12) = 19.57$; $p<0.001$) and total ATP ($F(5, 12) = 16.16$; $p<0.001$) when compared with the sham-exposed cells. These ATP levels were found to be non-significant in BOP 12h & BOP 24h group when compared to sham cells. Moreover, the total ATP ($F(5, 12) = 16.16$; $p<0.001$), mitochondrial ATP ($F(5, 12) = 12.02$; $p<0.01$) and the glycolytic ATP ($F(5, 12) = 19.57$; $p<0.001$) were significantly reduced in BOP 12h & BOP 24h groups when compared to BOP 6h group.

Western blotting analysis of Sirt1 & Sirt3

The western blotting demonstrated the changes in protein expression levels of Sirt1 & Sirt3 after BOP at various time points (Figure 4). The Sirt1 protein levels showed a significant change in BOP 6h & BOP 24h ($F(5, 12) = 3.298$; $p<0.05$) groups when compared with sham-exposed cells. Moreover, the Sirt1 protein level was reduced in BOP exposed cells in a time-dependent manner. The Sirt1 level was significantly reduced in BOP 12h & BOP 24h ($F(5, 12) = 3.298$; $p<0.001$) when compared to BOP 6h group C6 cells. However, the Sirt3 expression was significantly increased only in BOP 24h ($F(5, 12) = 2.034$; $p<0.05$) cells when compared to sham C6 cells. Like Sirt1 expression, the Sirt3 protein levels also reduced in a time-dependent manner. In C6 cells, the Sirt3 expression was found to be significantly reduced in BOP 24h when compared to BOP 6h ($F(5, 12) = 2.034$; $p<0.001$) & BOP 12h cells ($F(5, 12) = 2.034$; $p<0.005$). Similarly, the Sirt1 & Sirt3 protein levels in the BOP exposed HTHU cells showed a time-dependent reduction over various timepoints. The Sirt1 was found to be significantly reduced in BOP 12h ($F(5, 12) = 3.790$; $p<0.05$) & BOP 24h ($F(5, 12) = 3.790$; $p<0.001$) group HTHU cells when compared to sham group. Moreover, the Sirt1 level was significantly reduced in BOP 24h ($F(5, 12) = 3.790$; $p<0.005$) when compared to BOP 6h HTHU cells. The Sirt3 level was found to be significantly increased in BOP 6h ($F(5, 12) = 4.013$; $p<0.001$) & the same was significantly decreased in BOP 24h ($F(5, 12) = 4.013$; $p<0.005$) HTHU cells when compared to

sham exposed group. When comparing the BOP exposed groups, the BOP 12h (F (5, 12) = 4.013; p<0.001) & BOP 24h (F (5, 12) = 4.013; p<0.001) groups showed a significant reduction in the Sirt3 levels when compared to BOP 6h HTHU cells.

BOP exposure results in increased PAR expression

The expression level of PAR was visualized by the qualitative immunofluorescence staining procedure. The results display the increased PAR expression in C6 and HTHU cells at post-BOP 24h time point when compared to sham exposed cells (Figure 5).

BOP modulates the mRNA expressions of Sirt1, Sirt3, NRF2 & GCLC in C6 & HTHU cells

The changes in mRNA expression levels of Sirt1, Sirt3, NRF2 & GCLC after BOP was analysed using qPCR procedure in C6 and HTHU cells at various timepoints (Figure 6). The qPCR results have clearly demonstrated the significant reduction in Sirt1 (F (5, 30) = 11.00; p<0.005), Sirt3 (F (5, 30) = 6.644; p<0.05) and NRF2 (F (5, 30) = 12.99; p<0.001) levels in post-BOP 6h group when compared to sham in C6 cells. But the expression was not significantly changed in post-BOP 12h group when compared to sham exposed C6 cells. Moreover, the mRNA expressions of Sirt1 (F (5, 30) = 11.00; p<0.05) and NRF2 (F (5, 30) = 12.99; p<0.005) were significantly reduced with a significant increase in GCLC expression levels (F (5, 30) = 6.995; p<0.005) in post-BOP 24h cells when compared to sham group. Among BOP exposed group C6 cells, the Sirt1 (F (5, 30) = 11.00; p<0.001), Sirt3 (F (5, 30) = 6.644; p<0.001) & NRF2 (F (5, 30) = 12.99; p<0.001) levels were significantly increased in post-BOP 12h when compared to post-BOP 6h group. Surprisingly, the Sirt1 (F (5, 30) = 11.00; p<0.001), Sirt3 (F (5, 30) = 6.644; p<0.005) & NRF2 (F (5, 30) = 12.99; p<0.005) expression were significantly reduced in post-BOP 24h group when compared to BOP 12h C6 cells. Additionally, the GCLC expression was increased in time dependent manner after BOP. The GCLC was found to be increased significantly in post-BOP 24h cells when compared to BOP 6h (F (5, 30) = 6.995; p<0.001) and BOP 12h group (F (5, 30) = 6.995; p<0.05) C6 cells. In HTHU cells, the mRNA expression levels of Sirt1 (F (5, 30) = 21.98; p<0.001) and NRF2 (F (5, 30) = 11.10; p<0.05) was found to be significantly increased in post-BOP 12h group. These Sirt1 (F (5, 30) = 21.98; p<0.005) and NRF2 (F (5, 30) = 11.10; p<0.005) expression were reduced significantly in post-Bop 24h group when compared sham exposed HTHU cells. Among the BOP exposed HTHU cells, the Sirt1 level was significantly increased in post-BOP 12h timepoint (F (5, 30) = 21.98; p<0.001) when compared to BOP 6h group. But the expression levels of Sirt1 (F (5, 30) = 21.98; p<0.001) & NRF2 (F (5, 30) = 11.10; p<0.001) was reduced significantly in BOP 24h group when compared to BOP 12h cells. But the Sirt3 expression was significantly reduced in BOP 24h HTHU cells (F (5, 30) = 4.821; p<0.001) compared to BOP 6h group. Additionally, the NRF2 and GCLC expression levels was found to be significantly decreased in post-BOP 24h timepoint when compared to BOP 6h and BOP 12h group HTHU cells.

Discussion:

The cell death mechanisms of TBI, especially the impact injury (blunt) were investigated widely ((Ng and Lee, 2019; Stoica and Faden, 2010). However, the cellular mechanism of neurodegeneration post-blast (non-impact) injury remains elusive. Miller et al., 2015 demonstrated the blast-induced activation of microglia and astrocyte organotypic hippocampal cultures (Miller et al., 2015). Bricker-Antony and Rex 2014 showed that cell death after blast trauma in eyes was primarily non-apoptotic (Bricker-Anthony et al., 2014). In our recent study on*in vitro* model (astrocytes and microglia) of low-frequency Blast-overpressure, PARP over-activation was evidenced with perturbation of mitochondrial energetics in microglia and astrocytes. **We found that low-frequency BOP-induced trauma results in PARP over-activation resulting in trans-PARylation with increased expression of mitochondrial electron transport complexes with reduced basal mitochondrial respiration resulting in** increased apoptosis with marginal necrosis. **In another report of impact trauma, we found that inhibition of PARP using a known potent inhibitor 3-aminobenzamide (3AB) attenuated the trauma-induced oxidative stress by increasing the master redox regulator glutathione through increasing the expression of the rate-limiting enzyme, glutamate-cysteine ligase catalytic (GCLC) unit in the process of glutathione**

synthesis (Krishnan Muthaiah et al., 2019). NAD^+ (nicotinamide adenine dinucleotide) is a common substrate for both PARP and Sirtuins, with NRF2 being one of the common deacetylation targets of Sirtuins, here, we investigated the effect of blast injury and its modulation on the PARP-Sirt-NRF2 axis. Zander et al., 2015 studied the effect of blast injury on neuronal populations (Zander et al., 2016). However, non-neuronal glial cells play a critical role in mitochondrial dysfunction, neuroinflammation, oxidative stress, and blood-brain barrier disintegrity post-TBI (Mira et al., 2021). Hence, to further probe the cellular mechanism of blast injury, here we investigated the impact of the blast-overpressure on modulation of the antioxidant defense system in in-vitro cultures of microglia (HTHU) and astrocytes (C6).

Post-BOP, we found that reactive oxygen species increased significantly in both astrocytes and microglia which is due to the loss of mitochondrial membrane potential ($[?]\Psi_m$) as evident from the increased ratio of JC10 aggregates to JC10 monomers (Sivandzade et al., 2019). This loss or reduced $[?]\Psi_m$ is attributed to the perturbated mitochondrial permeability transition leading to the release of cytochrome c, AIF, and other factors of cell death induction (Cheng et al., 2012). As MPT lays the ground for ATP synthesis, we determined the ATP generation of both cells post-BOP from both mitochondria and glycolysis. In astrocytes, post-BOP, though, the mitochondrial ATP and glycolytic ATP levels reduced abruptly, the ATP levels were recuperated at 24 hours. Similar to this differential response of astrocytes in recuperating the mitochondrial ATP, Almeida et al., 2001 reported the differential response of astrocytes in bioenergetic recuperation through glycolytic ATP. However, under BOP insults, we found that the recuperation was observed in both sources of mitochondria and glycolysis. It has been postulated that ATP release from injured tissue establishes the long-range extracellular ATP gradient for the chemotaxis of the remote microglia to reach the injured site to clear debris (Dou et al., 2012) in addition to ATP being a mediator of calcium signaling between astrocytes and microglia (Verderio and Matteoli, 2001). On the contrary, in microglia, the ATP levels were found to be increased in the acute phase of post-BOP, but the ATP levels were reduced or found to be the same as controls. Evidence indicates that at pro-inflammatory conditions, microglia use glycolysis for energy production, and at anti-inflammatory conditions, microglia prefer oxidative phosphorylation (OXPHOS) for energy production (Yang et al., 2021). At present, though, ATP levels from both sources were elevated post-BOP, an increase of ATP by glycolysis more significant than OXPHOS source indicating the pro-inflammation post-BOP.

Like our earlier report, we confirmed the BOP-induced PARP over-activation through increased formation of PAR polymers on several proteins in both astrocytes and microglia post-BOP. The trans-PARylation process utilizes NAD^+ (Kamaletdinova et al., 2019). Reports indicate that under the conditions of glucose withdrawal, cells rely on mitochondria for ATP through activation of Sirt1 and Sirt3 (Hirschey et al., 2010; Song and Hwang, 2018). However, NAD^+ being a substrate for sirtuins to deacetylate several targets of transcriptional regulators (Canto et al., 2013), PARP over-activation directly affects the Sirtuins activity which was reflected from the reduced gene and protein expression of Sirt1 and Sirt3 post-BOP. This finding is corroborated with (Figure 7) earlier evidence of inhibition of capability of Sirt1 to deacetylate its targets as a result of rapid depletion of NAD+ levels by PARP overactivation (Furukawa et al., 2007; Luna et al., 2013) and increased Sirt1 activation through PARP1 inhibition (Bai, P. et al., 2011). As the transcriptional activity of NRF2 is regulated by Sirtuins (Kratz et al., 2021), the downregulation of Sirt1 and Sirt3 is reflected in the gene expression of NRF2 as evidenced by the decreased levels post-BOP. Similar findings were observed in-vivo study as well (Ding et al., 2016). As NRF2 binds to the cis-acting element of ARE, this downregulated SIRT/NRF2 pathway seems to suppress the NRF2/ARE (Anti-oxidant response element genes) (Mani et al., 2013) which was evidenced by the downregulated expression of the rate-limiting enzyme of glutathione synthesis, glutathione cysteine ligase catalytic unit (GCLC) enzyme in microglia. NRF2 is a key regulator of Glutathione synthesis through glutathione cysteine ligase enzyme in which GCLC is part of the heterodimer along with GCLM (modifier unit) (Erickson et al., 2002; Zhong et al., 2013). These results were corroborated with another study in which GCLC was downregulated by siRNA specific for Sirtuins (Cao et al., 2016). However, the GCLC gene expressions were found to be upregulated in astrocytes which agreed with increased Total ATP levels. It is unclear how GCLC levels and ATP levels were increased

post-BOP in astrocytes in contrary to microglia. However, the differential expression of GCLC in microglia and astrocytes needs to be confirmed with GCL activity (K_{cat}) (Lee et al., 2006).

Overall, this differential response of astrocytes and microglia in response to BOP warrants further investigation in terms of other effectors and under different parameters of trauma specifications such as blast repetition, intensity, inter-blast intervals. Thus, the study of the effect of BOP on the modulation of the PARP-Sirt-NRF2 axis provides mechanistic insights on blast-induced glial pathomechanism.

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Statement of Ethics: This study utilizes in vitro cell lines as an experimental platform to investigate the signaling mechanism following blast injury. Hence, this study does not warrant approval from Institutional Animal Care and Use Committee and Institutional Review Board.

Conflict of Interest Statement. The authors have no conflicts of interest to declare.

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Author Contributions. Vijaya Prakash Krishnan Muthaiah has conceptualized and administered the project in addition to resource support and manuscript preparation. Kathiravan Kaliyappan has performed most of the in vitro experiments such as cell viability, flow cytometry, western blotting, mitochondrial respiration, and quantitative PCR. Krishnamoorthy Gunasekaran has provided technical consultation and helped with the data analysis. Supriya Mahajan has helped in the data curation, manuscript preparation, and critical reading of the manuscript.

Data Availability Statement. All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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FIGURES

Figure 1. Qualitative assessment of CM-H2DCFDA expressions in C6 & HTHU cells after BOP exposure. The images display the cells expressing the nuclear stain DAPI (i & iv), ROS marker CM-H2DCFDA (ii &v), and merged images (iii & vi) in control (i-iii) and 24h (iv-vi) post-BOP A) astrocytes (C6) and B) microglia (HTHU) cells. The white arrows indicate the cells expressing the CM-H2DCFDA with the nuclear stain DAPI in C6 (iii and vi) and HTHU (iii and vi) cells.

Figure 2. Mitochondrial membrane potential after blast overpressure. The histograms show the mitochondrial membrane potential (JC10) in a) Astrocytes (C6 cells) and b) Microglia (HTHU cells) at various time points (6, 12, and 24h) following BOP. Statistical difference across different time-points in BOP group (*p<0.05 vs 6h; **p<0.005 vs 12h; ***p<0.001 vs 24h) and from different groups were indicated (###p<0.001 vs Sham). Values are represented as Mean ± SEM (n=3).

Figure 3. Effect of BOP on the cellular energetics of Astrocytes (C6) and Microglia (HTHU cells) after blast overpressure in various time-points (6h, 12h & 24h) using Seahorse XF 24 Analyzer. The histogram shows the mitoATP; GlycoATP & TotalATP in C6 (A-C) and HTHU (D-F) cells. Statistical difference between the cell groups were (*p<0.05; **p<0.005; ***p<0.001) represented as Mean ± SEM (n=3).

Figure 4 . Assessment of SIRT1 & SIRT3 expressions by western blotting analysis in Astrocytes (C6) & Microglia (HTHU) cells after BOP exposure. The immunoblots show the protein expressions of SIRT1 & SIRT3 levels in A) C6 and D) HTHU cells normalized against the internal control β-Actin. The histogram shows the semi-quantitative protein expression of SIRT1 (B & E) & SIRT3 (C & F) levels in C6 and HTHU cells respectively. Statistical difference between the cell groups were (*p<0.05; **p<0.005; ***p<0.001) represented as Mean ± SEM (n=3).

Figure 5. Qualitative immunofluorescence assessment of PAR expressions in Astrocytes (C6) & Microglia (HTHU) cells after BOP exposure. The images display the cells expressing the nuclear stain DAPI (i & iv), PAR polymer (ii &v) and merged images (iii & vi) in control (i-iii) and 24h post-BOP (iv-vi) in A) C6 and B) HTHU cells. The white arrows indicate the cells expressing the PAR polymer with nuclear stain DAPI in C6 and HTHU cells.

Figure.6 Reverse transcription-quantitative polymerase chain reaction: SIRT1 (A&E), SIRT3 (B&F), NRF2 (C&G) & GCLC (D&H) mRNA expressions in a) C6 cells b) HTHU cells in various time-points (6hrs, 12h & 24h). The values in the histogram represents the mean ± SEM (n=3). Statistical difference between the

various timepoint BOP groups (*p<0.05; **p<0.005; ***p<0.001) and compared with sham group (#p<0.05; ##p<0.005; ###p<0.001 vs Sham) were represented as Mean ± SEM (n=3).

Figure 7. Potential interaction between PARP1, NFE2L2 and Sirt1 as determined from Genemania.

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