Neuroprotective effects of arbutin against oxygen and glucose deprivation-induced oxidative stress and neuroinflammation in rat cortical neurons

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In this study, the neuroprotective potential of arbutin (100 $\mu mol\ L^{-1}$) pre-treatment and post-treatment against oxygen/glucose deprivation (OGD) and reoxygenation (R) induced ischemic injury in cultured rat cortical neurons was explored. The OGD (60 min) and reoxygenation (24 h) treatment significantly ($p < 0.001$) compromised the antioxidant defence in cultured neurons. Subsequently, an increase ($p < 0.001$) in lipid peroxidation and inflammatory cytokines (tumour necrosis factor-α and nuclear factor kappa-B) declined neuron survival. In pre- and post-condition experiments, treatment with arbutin enhanced both survival ($p < 0.01$) and integrity ($p < 0.05$) of cultured neurons. Results showed that arbutin protects ($p < 0.05$) against peroxidative changes, inflammation, and enhanced the antioxidant activity (*e.g.,* glutathione, superoxide dismutase and catalase) in cultured neurons subjected to OGD/R. It can be inferred that arbutin could protect against ischemic injuries and stroke. The anti-ischemic activity of arbutin can arrest post-stroke damage to the brain.

Keywords: arbutin, cerebral ischemia, inflammation, oxidative stress

Oxidative stress and underlying neuroinflammation increase the risk of cerebral ischemia manyfold (1). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can modify protein structure resulting in vascular remodelling and neuronal injury (2, 3). Peroxidation of lipids gives rise to highly reactive aldehydes that form neurotoxic aggregates with biological substrates such as advanced-glycation end products, DNA, and methylglyoxal (3). Free radicals enhance the preponderance of ischemic attack and are also implicated in post-ischemia complications (4, 5). Under ischemic conditions, excessive excitatory signals in the brain intensify redox-imbalance and intracellular calcium overload that impedes aerobic respiration and ATP formation. Lack of blood supply creates an ischemic zone populated by pro-inflammatory cytokines, adhesion factors, neutrophils, macrophages, and lymphocytes (3, 4). Furthermore, upregulation of transcription activity of

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nuclear factor kappa-B (NFκB) augments the expression of inflammatory genes and encoding of cytokines and chemokines (6). These sequences of events lead to hemodynamic disturbances that precede an ischemic attack. Within a few hours after ischemia, pro-inflammatory cytokines instigate the expression of chemotactic factors that further aggravate the brain injury (7). Brain autopsy of ischemic patients revealed a pathogenic increase in pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and chemokines (8). Several lines of evidence indicate that phytoproducts might avert or even modify the pathogenesis of cerebral ischemic injury (2).

Arbutin (4-hydroxyphenyl-β-D-glucopyranoside) is a natural hydroquinone abundantly present in plants like *Origanum majorana* (Lamiaceae), *Arctostaphylos uva-ursi* (Ericaceae), *Pyrus communis* (Rosaceae) and *Bergenia crassifolia* (Saxifragaceae) (9). Earlier findings support antihypertensive, antidiabetic, antiseizure, antimicrobial, gastroprotective, hepatoprotective, and antihyperlipidaemic properties of arbutin (10–14). Arbutin inhibits several enzymes (*e.g.*, tyrosinase, α-amylase and α-glucosidase), free radicals, and downregulates the transcription of many pro-inflammatory cytokines (10, 15). Previous studies indicated improvement in motor-functions and behavioural parameters by arbutin that emphasized its neuroprotective action (16–18). Experimental evidence shows that arbutin can alleviate the excitotoxic and autophagic mechanisms of neurodegeneration (14, 17). However, there is a need to explore the neuroprotective potential of arbutin to sufficient depth. In the present study, we aimed to establish the anti-ischemia and neuroprotective effects of arbutin using *in vitro* protocols.

EXPERIMENTAL

Materials

Arbutin (purity > 98 %) was procured from Sigma-Aldrich (USA) and prepared in 0.01 % dimethylsulfoxide (DMSO) (Himedia, India). Dulbecco's Modified Eagle's medium (DMEM), ethylenediaminetetraacetic acid (EDTA), foetal bovine serum (FBS), hydrogen peroxide, 3-(4,5-dimethylthiazole-2-yl)-2,5-dipenyltetrazolium bromide (MTT), nicotinamide adenine dinucleotide (NAD$^+$ and NADH) and trypsin were from Himedia; 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), glacial metaphosphoric acid, disodium hydrogen phosphate, manganese chloride (MnCl$_2$) and 2-mercaptoethanol were from SRL (India). Thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were procured from Loba Chemie (India).

Animals and primary cortical neurons culture

Forty Wistar rats (either sex, days 0–1, weight range 7–9 g) were used in this study. The guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi (India), were followed. The research protocol was duly approved by the institutional animal ethics committee (IAEC). The rat pups were euthanized and brains were harvested in a sterile environment. After separation of the meninges, the cortex was isolated and rinsed with sterile phosphate buffer saline (PBS, pH 7.4). The cortical tissue was pulverized into smaller sizes, triturated and digested with 0.25 % trypsin for 20 min at 37 °C, which was then inactivated by 10 % decomplemented foetal bovine serum (FBS). The mixture was triturated through a pipette to make a homogenous

preparation, filtered through 70-µm sterilized filters and the filtrate was centrifuged (CPR-30 Remi Compufuge, Vasai, India) for 10 min at 300×*g*. The sediment was washed once with PBS and suspended in the plating medium, DMEM, containing 0.225 % sodium bicarbonate, 1 mmol L^{-1} sodium pyruvate, 2 mmol L^{-1} L-glutamine, 0.1 % dextrose, 100 U mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin and 5 % FBS. The pellet was washed twice in this medium. Neurons are then plated on poly-L-lysine pre-coated plates or glass coverslips at the density of 2×10^6 cells cm^{-2}. Neurons are grown in a plating medium consisting of 89 % high-glucose DMEM, 10 % foetal bovine serum, 100 U mL^{-1} penicillin, 0.1 mg mL^{-1} of streptomycin. After 24 h, the medium was changed to a fresh neurobasal medium supplemented with 2 % B27 (neuron supplement, Invitrogen, Thermo Fisher Scientific, USA) and 100 U mL^{-1} penicillin, 0.1 mg mL^{-1} of streptomycin, and then refreshed every 2–3 days. Cultures were incubated at 37 °C in a 95 % air/5 % CO$_2$ in a humidified incubator. Experiments were performed at 5–7 days of neuron culture growth *in vitro* (19, 20).

Oxygen-glucose deprivation (OGD) and arbutin treatment

The culture medium was changed to glucose-free DMEM in order to deprive the rat cortical neurons of O$_2$ and glucose (OGD). The cultured neurons were washed twice with glucose-free DMEM, and thereafter incubated in a hypoxic chamber at 37 °C for 1 h, equilibrated with 94 % N$_2$, 1 % O$_2$ and 5 % CO$_2$. After completion of 1-h OGD, the medium was
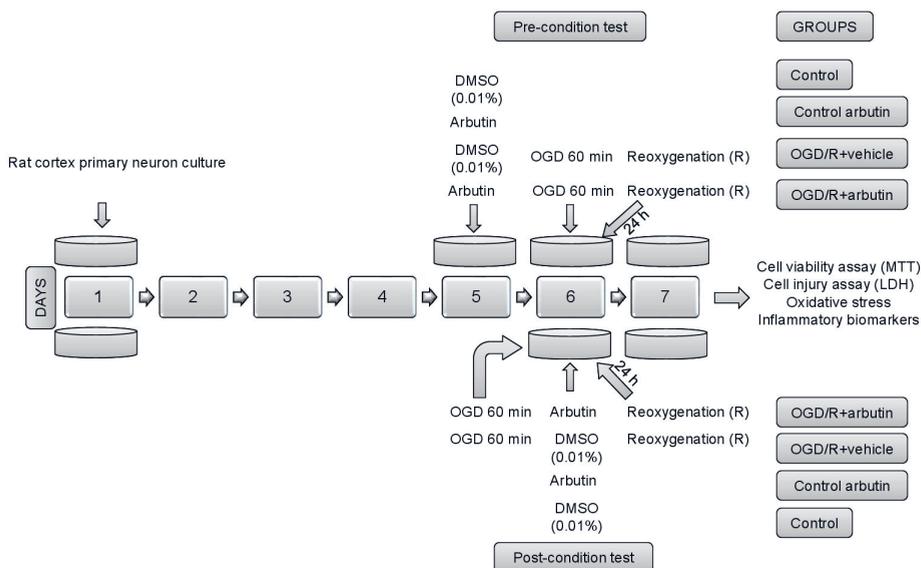
Fig. 1. Experimental protocol used in the present study. In the precondition experiment, cultured rat cortical neurons were pre-treated with the dimethylsulfoxide (DMSO) vehicle or arbutin (100 µmol L^{-1}) for 24 h followed or not by oxygen and glucose deprivation (OGD) and reoxygenation (R). In the post-condition experiment, rat primary neurons were subjected to OGD in a glucose-free medium, and, thereafter, treated with vehicle or arbutin (100 µmol L^{-1}) before reoxygenation.

replaced with neurobasal medium supplemented with 2 % B27 and neurons were incubated for 24 h under normoxic conditions (95 % air and 5 % CO_2 at 37 °C). For measurements of MTT and enzyme activity, rat cortical neurons were cultured in a neurobasal medium supplemented with 2 % B27, 100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin (21, 22). A total of eight groups (n = 5 in each group) were undertaken for pre- and post-conditioning experiments (Fig. 1). For control experiments, the neuron cultures were subjected to identical conditions and treated with either 0.01 % DMSO vehicle (control) or arbutin (100 µmol L^{-1}) (control arbutin) for 24 h, except 60 min of OGD and 24 h of reoxygenation (R) (OGD/R). OGD/R+vehicle group was treated with 0.01 % DMSO for 24 h before OGD/R (pre-condition experiment) or immediately after OGD before reoxygenation (post-condition experiment). OGD/R+arbutin group was treated with arbutin (100 µmol L^{-1}) for 24 h before OGD/R (pre-condition experiment) or immediately after OGD before reoxygenation (post-condition experiment) (23).

Cell viability assay

The viability of cells is examined by 3-(4,5-dimethylthiazole-2-yl)-2,5-dipenyltetrazolium bromide (MTT) assay. This test determines the metabolically active mitochondria in the cells. Cells were seeded in 96-well plates at 5 × 10^3 cells per 100 µL. After 5 days of growth, cells were subjected to OGD and reoxygenation, with arbutin added into the medium. After 24 h of reoxygenation, MTT (yellow color salt) was added (2 mL) to the cells at the final concentration of 0.5 mg mL^{-1} and the plates were incubated for 24 h at 37 °C. During incubation, living cells converted (*via* mitochondrial dehydrogenase) the tetrazolium component of the dye solution into a formazan product (purple color). The insoluble formazan product was then precipitated by centrifugation, the supernatant is removed and 150 µL DMSO is added for 20 min to solubilize the formazan product. Absorbance which is directly proportional to the number of surviving cells is measured on a microplate reader (Bio-Rad, USA) at 570 nm (24).

Cell injury assay

Cell injury is detected by measuring lactate dehydrogenase (LDH). The assay is based on the reduction of NAD$^+$ by LDH. The resulting NADH is utilized in the stoichiometric conversion of a tetrazolium dye. LDH release is measured in the culture medium using the LDH assay kit (Sigma-Aldrich). Culture medium (100 µL) is transferred from culture wells to 96-well plates and mixed with the 100 µL reaction solution provided in the kit. Absorbance is measured at 490 nm 30 min later using a microplate reader (25). The background absorbance of the multi-well plates measured at 690 nm is subtracted.

Oxidative stress

The cultured neurons were harvested, sonicated for 3 min in an ice-cold water bath, proteins were precipitated with 4.5 % trichloroacetic acid (TCA) and centrifuged (10 min at 3000×*g*, 4 °C) to collect the supernatant. Lipid peroxidation was calculated using the TBARS (thiobarbiturate reactive substances) assay. Quantification of TBARS corresponds to the MDA level that shows the extent of lipid peroxidation. Briefly, the assay mixture consisted of 1 mL above prepared supernatant, TCA (20 %, 1.5 mL), 1 mL of freshly prepared

TBA (0.67 %) and absorbance was read at 535 nm. Lipid peroxidation values were expressed as nmol TBARS mg^{-1} protein (26, 27).

For reduced glutathione (GSH) assay, the mixture consisted of 5,5′-dithio-bis-2-nitrobenzoic acid (DNTB) (0.4 % in 1 % sodium citrate) and double-distilled water. Absorbance was read at 412 nm against a blank. Values were expressed as micromoles of reduced glutathione per mg protein (26).

For reduced glutathione (GSH) assay, the mixture consisted of 0.3 mL supernatant, 2 mL Na$_2$HPO$_4$ (0.3 mol L^{-1}), 0.25 mL of 5,5′-dithio-bis-2-nitrobenzoic acid (DNTB) (0.4 % in 1 % sodium citrate) and double-distilled water. Absorbance was read at 412 nm against a blank. Values were expressed as micromoles of reduced glutathione per mg protein (26).

For catalase (CAT) assay, the reaction mixture consisted of 20 µL supernatant, 1.2 mL of H$_2$O$_2$ (10 mmol L^{-1}) and 50 mmol L^{-1} PBS (pH 7.0). The decrease in absorbance was noted at 240 nm for 5 min. One unit of CAT activity was defined as one micromole of H$_2$O$_2$ decomposed per minute at 25 °C using the molar absorption coefficient of H$_2$O$_2$ (43.6 L mol^{-1} cm^{-1} at λ_{max} = 240 nm). The results were expressed as CAT U mg^{-1} protein (28).

To determine the superoxide dismutase (SOD) activity, a decrease of absorbance was measured using the microplate reader (and UV plates) at 340 nm in a reaction mixture containing 3 mmol L^{-1} NADH, 25 mmol L^{-1}/12.5 mmol L^{-1} EDTA/MnCl$_2$, 10 mmol L^{-1} 2-mercaptoethanol, and 50 mL of the cell lysate in 300 mL of triethanolamine-diethanolamine buffer (pH 7.4), after 20 min. One SOD activity unit is defined as the amount of SOD capable of inhibiting 50 % rate of NADH oxidation observed in the control. The results were expressed as SOD U mg^{-1} protein (28).

TNF-α and NFκB

Double antibody sandwich ELISA was used to determine the nuclear factor kappa-B (NFκB) and tumor necrosis factor (TNF-α) levels. Standard methods provided in kits of TNF-α (Krishgen Biosystems, India) and NFκB (KinesisDx, USA) were followed. The sonicated culture sample was centrifuged at 2500×*g* for 20 min and the supernatant was used for the estimation of NFκB and TNF-α content. This supernatant was added to rat monoclonal antibody pre-coated wells (96 wells) and treated with secondary antibodies labeled with biotin followed by incubation for 1.5 h (37 °C). Afterward, streptavidin-horseradish peroxidase was added to the wells and again incubated at 37 °C for 1 h after covering the plate. After incubation, chromogenic solution A and B or TMB substrate was added that produced bluish color. A stop solution was added to stop the reaction and absorbance was noted at 450 nm (λ_{max}) in ELISA microplate reader (iMARK, Bio-Rad, USA) within 15 min after the reaction has been stopped. The concentration of NFκB and TNF-α in an unknown sample was calculated from the standard curve. The amount of NFκB is expressed in ng mL^{-1} and TNF-**α** level in pg mL^{-1}.

Statistical analysis

All the data are expressed as mean ± standard error of the mean (SEM). Statistical differences between the groups were assessed with a one-way ANOVA followed by Tukey's *post-hoc* test. A value of $p < 0.05$ is considered statistically significant.

RESULTS AND DISCUSSION

Arbutin protects neurons against OGD triggered injury

In pre-/post-condition paradigms, arbutin treatment in rat cortical culture without OGD (control arbutin) showed neuron viability akin to the control neurons that received DMSO vehicle only. Exposure to OGD (60 min) and reoxygenation (24 h) decreased the mean absorbance ($\lambda_{max} = 570$ nm) for MTT that measures cell viability. MTT assay results indicated that the viability of cultured rat cortical neurons that were subjected to OGD/R was significantly decreased ($p < 0.001$) in comparison to vehicle-treated controls that were excluded from OGD/R treatment in pre- (Fig. 2a) and post-condition paradigms (Fig. 2b). The LDH activity was significantly ($p < 0.001$) enhanced when the cultured neurons were subjected to OGD/R in pre- (Fig. 2c) and post-condition experiments (Fig. 2d). These findings showed that OGD/R significantly compromised neuron integrity. Interruption of metabolic fuel and oxygen delivery hampers cellular oxidative metabolism and neuron survival. Restoration of oxygen supply further perpetuates the injury cascade in neurons (29). In this study, results of MTT assay revealed that arbutin (100 μmol L^{-1}) pre-treatment [$F_{(3,19)} = 24.33$; $p < 0.001$] or post-treatment [$F_{(3,19)} = 27.85$; $p < 0.001$] significantly ($p < 0.01$) enhanced the viability of rat primary cortical neurons against OGD/R injury in comparison to neurons subjected to OGD/R and vehicle treatments only. Furthermore, a significant decrease ($p < 0.05$) in the LDH activity in response to arbutin pre-treatment [$F_{(3,19)} = 16.67$; $p < 0.001$] and post-treat-
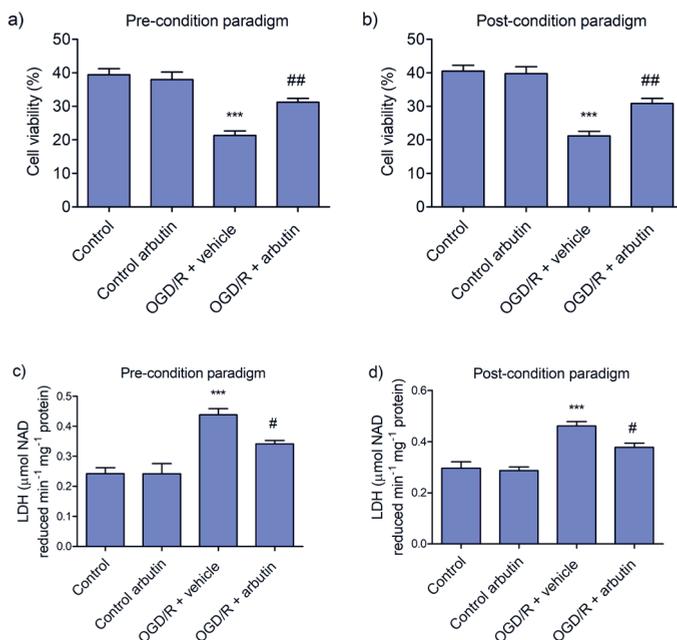
Fig. 2. Effects of arbutin on cell viability (MTT assay) and cell death (LDH assay) in rat primary neuron culture in: a) and c) pre- and b) and d) post-condition paradigms. Mean ± SEM ($n = 5$). Significant difference: ***$p < 0.001$ *vs.* control group, #$p < 0.05$, ##$p < 0.01$ *vs.* OGD/R+vehicle group.
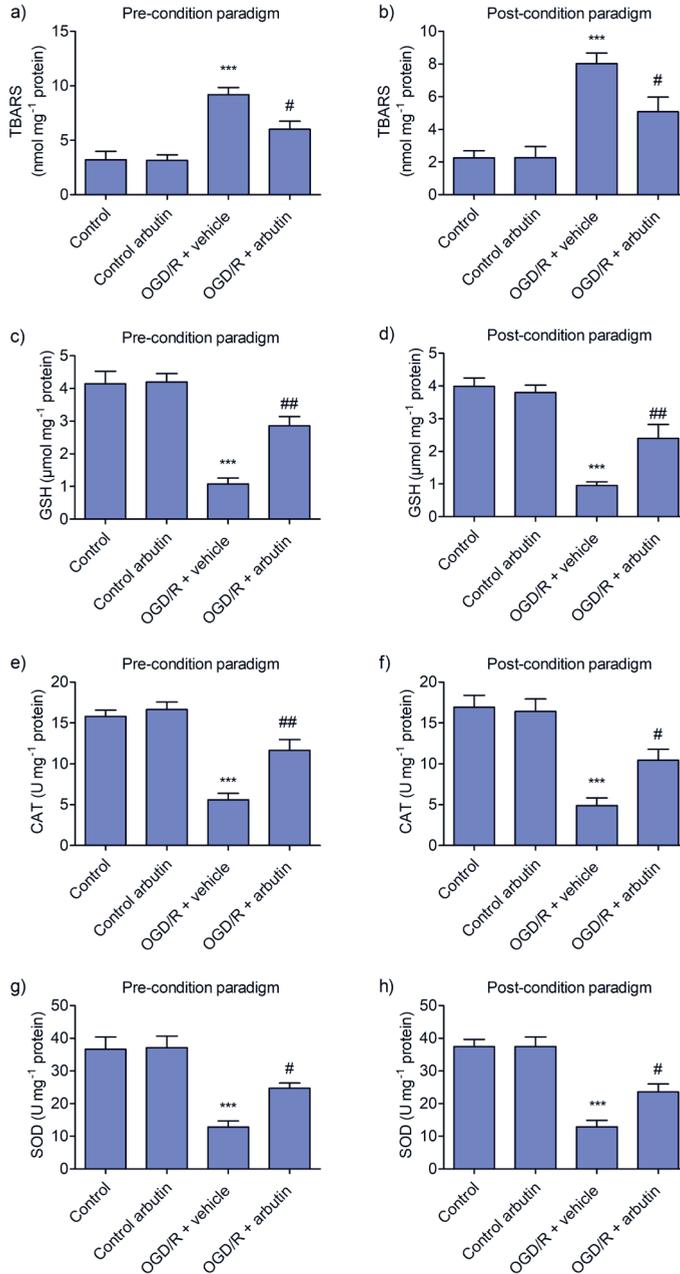
Fig. 3. Effects of arbutin on the parameters of oxidative stress in rat primary neuron culture in: a), c), e), and g) pre- and b), d) f) and h) post-condition paradigms. Mean ± SEM ($n = 5$). Significant difference: ***$p < 0.001$ *vs.* control group, #$p < 0.05$, ##$p < 0.01$ *vs.* OGD/R+vehicle group.

ments [$F_{(3,19)}$ = 19.01; $p < 0.001$] was observed in the cultured neurons subjected to OGD/R injury. In both, pre-/post-condition experiments, the LDH activity of arbutin treated neurons and vehicle-treated neurons were found to be similar.

Arbutin reduces the oxidative stress in rat cortical neurons against OGD

Vehicle (control) or arbutin (control arbutin) treated OGD deprived cultured neurons showed a similar TBARS content. In both, pre-/post-condition experiments, exposure to OGD markedly increased the lipid peroxidation (TBARS) in the rat cortical neurons as shown by a significant ($p < 0.001$) rise in TBARS content in comparison to the control neurons which received DMSO vehicle only. Hypoxia and lack of metabolic fuel (glucose) supply trigger ROS that causes aggregation of oxidized lipids. The extent of lipid peroxidation directly correlates with the severity of brain injury and inflammation (30). However, treatment of neurons with arbutin (100 µmol L^{-1}) before [$F_{(3,19)}$ = 17.89; $p < 0.001$] (Fig. 3a) or after [$F_{(3,19)}$ = 16.11; $p < 0.001$] OGD (Fig. 3b) caused a significant ($p < 0.05$) decrease in the TBARS in comparison to the cultured neurons that were subjected to OGD and vehicle treatments only.

The endogenous antioxidants (*e.g.*, GSH, catalase and SOD) maintain redox homeostasis within neurons by detoxifying several types of free radicals (*e.g.*, ROS, RNS, H$_2$O$_2$) and peroxidation products. In previous studies, a conspicuous decrease in sulfhydryl group
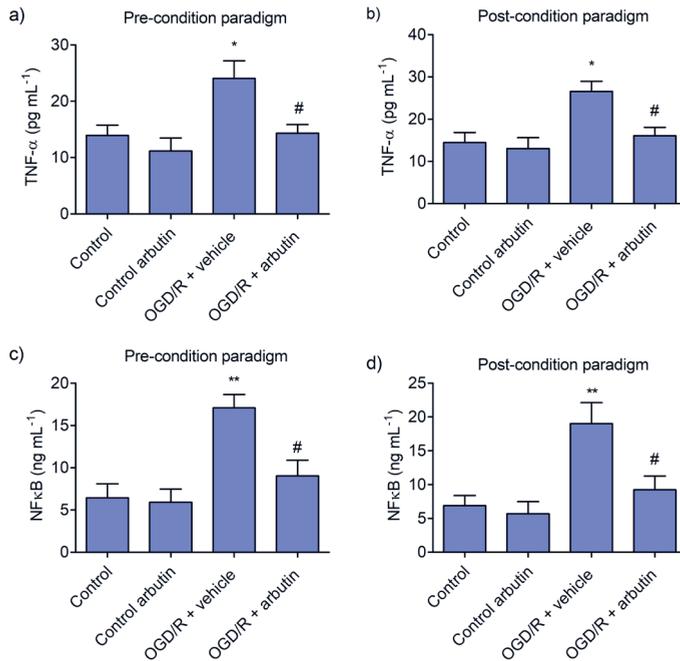
Fig. 4. Effects of arbutin on biomarkers of neuroinflammation (TNF-α and NFκB) in rat primary neuron culture in: a) and c) pre- and b) and d) post-condition paradigms. Mean ± SEM (*n* = 5). Significant difference: *$p < 0.05$, **$p < 0.01$ *vs.* control group, #$p < 0.05$ *vs.* OGD/R+vehicle group.

(-SH) antioxidants has been observed in cerebral ischemia (1, 31). Results of the present study revealed analogous antioxidant status (GSH, CAT and SOD) of neuron culture that received vehicle (control) or arbutin (control arbutin). Exposure of rat primary neuron culture to OGD/R caused a marked ($p < 0.001$) decrease in the GSH content, and CAT and SOD activities in comparison to vehicle treated control that were excluded from OGD/R treatment in pre- and post-condition paradigms. However, arbutin (100 µmol L^{-1}) treatment before OGD prevented the reduction of GSH ($p < 0.01$) [$F_{(3,19)}$ = 26.13; $p < 0.001$] (Fig. 3c), CAT ($p < 0.01$) [$F_{(3,19)}$ = 27.25; $p < 0.001$] (Fig. 3e) and SOD activity ($p < 0.05$) [$F_{(3,19)}$ = 16.40; $p < 0.001$] (Fig. 3g) in comparison to the neurons subjected to OGD and vehicle treatments only. Furthermore, post-ischemia arbutin treatment caused a significant rise in the GSH ($p < 0.01$) [$F_{(3,19)}$ = 26.06; $p < 0.001$] (Fig. 3d), CAT ($p < 0.05$) [$F_{(3,19)}$ = 18.12; $p < 0.001$] (Fig. 3f) and SOD activity ($p < 0.05$) [$F_{(3,19)}$ = 24.61; $p < 0.001$] (Fig. 3h) in rat cortical neurons compared to neurons that received OGD and vehicle treatments.

Arbutin decreases the inflammation in rat cortical neurons against OGD

In vehicle- or arbutin-treated OGD deprived cultured neurons we observed a similar TNF-α and NFκB level. In agreement with previous reports (1, 7), the present findings showed that in pre-condition [$F_{(3,19)}$ = 6.166; $p < 0.001$] (Fig. 4a) and post-condition experiments [$F_{(3,19)}$ = 6.863; $p < 0.001$] (Fig. 4b), the rat cortical neurons subjected to OGD caused marked increase in the TNF-α ($p < 0.05$) in comparison to the control neurons deprived of OGD. The NFκB level was also significantly enhanced ($p < 0.01$) in cultured neurons exposed to OGD in both pre-condition [$F_{(3,19)}$ = 9.575; $p < 0.001$] (Fig. 4c) and post-condition paradigms
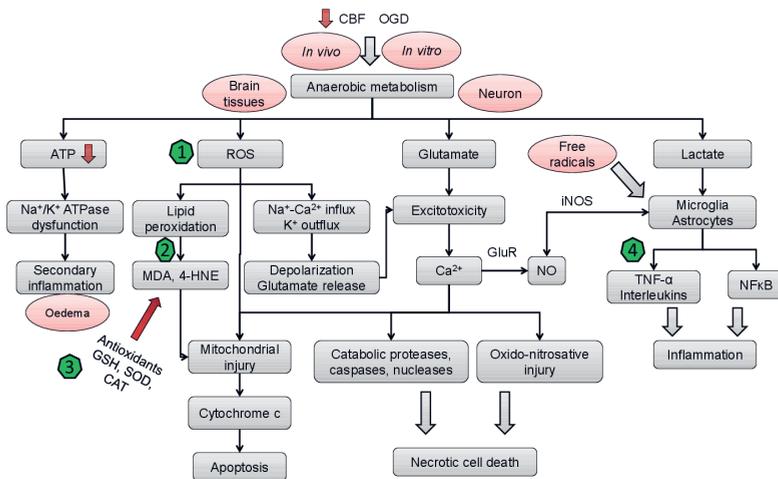
Fig. 5. Putative neuroprotective mechanism of arbutin against oxygen and glucose deprivation triggered ischemic injury to cultured neurons. 4-HNE – 4-hydroxy 2-nonenal, ATP – adenosine triphosphate, CAT – catalase, CBF – cerebral blood flow, GluR – glutamate receptors, GSH – glutathione, iNOS – inducible nitric oxide synthase, MDA – malondialdehyde, NFκB – nuclear factor kappa-light-chain-enhancer of activated B cells, NO – nitric oxide, OGD – oxygen-glucose deprivation, ROS – reactive oxygen species, SOD – superoxide dismutase, TNF-α – tumour necrosis factor-alpha.

[$F_{(3,19)}$ = 7.564; $p < 0.001$] (Fig. 4d) when compared with the control neuron culture that were excluded from exposure to OGD. NFκB is a transcription factor that upregulates the expression of inflammatory cytokines (*e.g.*, interleukins, inducible nitric oxide synthase, cyclooxygenase and TNF-α), apoptotic factors (*e.g.*, Bcl-2) and IκB (6). Hyperactive NFκB is the key feature after post-ischemic reperfusion in the brain (31). However, treatment of neurons with arbutin before OGD prevented the increase in TNF-α ($p < 0.05$) and NFκB ($p < 0.05$) in comparison with the vehicle-treated OGD/R neurons. Neuron culture treated with arbutin post OGD triggered ischemia showed a significant reduction in TNF-α ($p < 0.05$) and NFκB ($p < 0.05$) as compared to the neuron culture that was subjected to OGD and vehicle treatments only. Results of the present study showed that treatment of cultured neurons with arbutin after or before OGD/R injury significantly attenuated the TNF-α and NFκB expressions. These findings amply indicate the anti-inflammatory activity of arbutin against OGD/R triggered ischemic injury in rat primary neuron culture (Fig. 5).

CONCLUSIONS

Exposure of primary neuron culture to OGD/R triggered oxidative stress and neuroinflammation while decreasing the neuron viability. Treatment with arbutin before or after OGD/R protected the neurons owing to control over oxidized lipids, inflammation, and an increase in antioxidants. The present findings indicate that arbutin can prevent the sequence of events that lead to ischemia. Furthermore, it can alleviate the progression of the neurodegenerative cascade after ischemic injury.

Abbreviations, acronyms, symbols. – Bcl-2 – B-cell lymphoma 2, CAT – catalase, CBF – cerebral blood flow, ELISA – enzyme-linked immunosorbent assay, GluR – glutamate receptors, GSH – glutathione, 4-HNE – 4-hydroxy 2-nonenal, IκB – inhibitor kappa B, iNOS – inducible nitric oxide synthase, LDH – lactate dehydrogenase, NAD – nicotinamide adenine dinucleotide, NFκB – nuclear factor kappa-light-chain-enhancer of activated B cells, OGD – oxygen-glucose deprivation, PBS – phosphate buffer saline, R – reoxygenation, RNS – reactive nitrogen species, ROS – reactive oxygen species, SOD – superoxide dismutase, TBARS – thiobarbituric acid reactive substances, TNF-α – tumour necrosis factor-α.

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