

Original Research Article

Protective effect of punicalagin against intracerebral haemorrhage via improvement in blood-brain barrier integrity, and suppression of reactive oxygen species and NF- κ B-mediated NLRP3 inflammasome

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Abstract

Purpose: To study the effect of punicalagin in a rodent model of intracerebral haemorrhage (ICH).

Methods: Collagenase type IV (0.2 U) was used to induce ICH in Sprague-Dawley rats. The animals were randomly assigned to 5 treatment groups ($n = 24$) as follows: group I (normal control animals), group II (ICH control group administered saline), and groups III, IV and V (ICH + punicalagin at doses of 12.5, 25, 50 mg/kg, respectively).

Results: Punicalagin treatment improved brain tissue architecture and blood-brain barrier integrity, and reduced ICH-mediated oxidative stress. It effectively upregulated the expression of Nrf-2/HO-1, and enhanced the activation of NF- κ B and NLRP3 inflammasome ($p < 0.05$). Furthermore, punicalagin significantly down-regulated ICH injury-induced increase in IL-1 β and IL-18 ($p < 0.05$).

Conclusion: These results suggest that punicalagin is a potential therapeutic candidate for the management of ICH.

Keywords: Blood brain barrier, Inflammation, Intracerebral haemorrhage, Nuclear factor-kappa B, Punicalagin, Reactive oxygen species

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INTRODUCTION

Intracerebral haemorrhage (ICH) is the most common cerebrovascular disease with increasing incidence and high mortality and morbidity. It is responsible for about 15-20 % of all strokes [1]. It has been shown that ICH-induced primary or early brain injury results within the first few hours following ICH, causing hematoma and brain oedema which compress the adjacent tissues [2].

Secondary injuries arise from accumulation of extravasated blood and degraded products of blood cells following injury [3]. This leads to damage of the blood brain barrier (BBB) integrity, neuronal degeneration/loss, and neurobehavioral impairments [4].

Numerous studies have shown that excitotoxicity, oxidative stress, inflammation and cytotoxicity are implicated in brain injury post-ICH. Free

radicals derived from the breakdown of RBC and autooxidation of haemoglobin induce oxidative stress which is involved in the etiology of ICH-induced brain injury [5]. Studies have shown increased levels of oxidative stress in ICH patients [6].

Secondary brain injuries in ICH are caused by inflammatory responses mediated by leukocytes, microglia and inflammatory mediators including chemokines, cytokines, and prostaglandins [7]. Activated microglial cells which release pro-inflammatory cytokines are recruited to the site of injury within minutes following ICH. The activated microglial cells also induce transcription of nuclear factor kappa B (NF- κ B)-related signaling after ICH, leading to the expressions of genes of the inflammatory pathway [8].

The NLRP3 is a member of the nucleotide-binding domain and leucine-rich repeat containing (NLR) proteins, with pyrin domain containing 3 inflammasome. It is a major component of the innate defence mechanisms [9]. Studies have demonstrated the critical roles of NLRP3 in ICH-induced brain injury [10]. Activated NLRP3 regulates processing of caspase-1 and interleukins (IL) i.e. IL-1 β and IL-18 to their mature forms which subsequently promote recruitment of inflammatory cells, resulting in further amplification of inflammatory responses.

Recent studies have demonstrated that nuclear factor erythroid-2 related factor 2 (Nrf2) inhibits activation of NLRP3 inflammasome induced by ROS [11]. As a major transcription factor, Nrf2 regulates the expressions of key antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GPx), NAD(P)H:quinone oxidoreductase-1 (NQO1), and heme oxygenase-1 (HO-1) [12]. Moreover, Nrf2 exerts neuroprotective effects following ICH by mitigating neurological impairments and reducing brain oedema and oxidative stress [13]. Thus, therapeutic agents that inhibit ROS and reduce inflammatory responses could effectively protect tissues against ICH-induced injury [9,10].

Plant-derived compounds have been shown to exert neuroprotective effects [14]. Punicalagin, a polyphenol widely present in pomegranates has been reported to exhibit potent anti-inflammatory, antioxidant, and anti-proliferative effects [15].

The present study investigated the effects of systemic administration of punicalagin in collagenase type IV-induced ICH in rats.

EXPERIMENTAL

Study design

Adult male Sprague-Dawley rats aged 8 to 10 weeks, and weighing 290-320 g, were obtained from the Laboratory Animal Care Centre of our institution. The study design and the protocols were approved by the Institutional Ethical Committee and were conducted in line with the guidelines of the National Institutes of Health on the care and use of experimental animals [16].

The animals were kept in sterile cages (3 rats/cage) under standard controlled environmental conditions (12-h light/12-h dark cycle, temperature of 23 \pm 2 $^{\circ}$ C, and 55 - 60 % relative humidity), and were provided with standard pelleted diet and water. The rats were acclimatised to the animal house environment for five days before initiation of the study.

Animal grouping and ICH induction

The study was performed on collagenase type IV-induced ICH in rats as previously described by Rosenberg *et al* [17] and Feng *et al* [10], but with minor modifications. The animals were randomly assigned to 6 treatment groups (24 animals per group) as follows: group I (normal control animals), group II (ICH control group administered saline), and groups III, IV and V (ICH + punicalagin (Sigma-Aldrich, St.Louis, MO, USA) at doses of 12.5, 25, 50 mg/kg, respectively). Rats in group VI were given punicalagin at a dose of 50 mg/kg. Punicalagin was administered via oral gavage every day for 14 days prior ICH induction, and at 1 h prior to surgery on the day of ICH induction.

In the induction of ICH, the animals were anaesthetised with pentobarbital sodium (45 mg/kg b.wt., Sigma-Aldrich) via intraperitoneal injection. The rats were maintained at 37 $^{\circ}$ C with an insulation board connected to a water bath circulation system. The rats were in prone position in a rat brain stereotaxic apparatus. A midline incision was made on the scalp of each rat, in order to expose the skull and bregma. In the right part of the brain, 1 mm burr hole was bored and a 5- μ L micro-syringe with a needle was inserted through the hole at stereotaxic coordinates of 0.1 mm anterior, 3.5 mm lateral, and 6.0 mm ventral to the bregma. Collagenase type IV (Sigma-Aldrich) was used for the induction of ICH. The collagenase (0.2 U in 1 μ L sterile normal saline) was administered via stereotaxic intra-striatal injection. The needle was retained *in situ* for 10 min after injection to avoid back-flow. Subsequently, it was carefully

removed from the position and the hole was sealed using bone wax. All rats were sacrificed 24h following ICH, and the brains were excised immediately and used for analysis.

Determination of brain water content

The excised brains were separated along the midline to 2 hemispheres. The cerebellum was removed and used as the internal control. To determine the wet weight, each hemisphere was weighed separately on an electronic analytical balance. The tissue samples were then dried at 100 °C for 24 h in an electric oven, and the dry weight was measured. Brain oedema (B) was calculated using Eq 1.

$$\text{Brain oedema (\%)} = \{(Ww - Dw)/Ww\} \dots\dots (1)$$

where Ww and Dw are the weight of wet and dry brain, respectively.

Assessment of blood brain barrier (BBB) permeability

The permeability of BBB is a measure of membrane integrity. It was assessed by quantifying the extent of extravasation of Evan Blue (EB). The procedure was done as described earlier by Belayev *et al* [18] and Yang *et al* [19] with minor modifications. Rats from each experimental group were anesthetized and injected with EB solution (2 % in saline; 4 mL/kg body weight) intravenously into the femoral vein. Following 2 h of circulation, the rats were anesthetized and sacrificed by intracardial perfusion of 250 mL of PBS (0.01M) to clear EB from cerebral circulation. The brains were excised and separated into 2 hemispheres along the midline. The brain tissues were incubated with 2 mL of 50 % trichloroacetic acid (TCA) and homogenised. The homogenates were centrifuged at 15,000 rpm for 20 min at room temperature, and the supernatant obtained was collected and diluted with ethanol in a 1:3 ratio. The fluorescence intensity of the solution was measured at excitation wavelength of 620 nm, and emission wavelength of 680 nm. The extravasation of EB was expressed as mg/g of brain weight.

Histological examination of brain tissues

The excised brains were post-fixed in paraformaldehyde solution (4 %) at 4 °C for 24-48 h. Following dehydration, the brain tissue samples were paraffin-embedded, and coronal sections of 4-µm thickness were made. The tissue sections were treated with xylene. Following rehydration with graded ethanol

concentrations and with deionized water, the sections were treated with hematoxylin and eosin (H&E), and were visualised and analysed under a light microscope (Leica-DM2500, Germany).

Determination of ROS, lipid peroxidation and antioxidant enzymes

Brain tissues (n = 6) were homogenized in ice-cold PBS and centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant collected was used for assay of lipid peroxidation, ROS, SOD, CAT and GSH levels. Total protein content of the supernatant obtained was determined with BCA method using protein assay kit from BioRad (Hercules, CA, USA). Lipid peroxidation levels as a measure of malondialdehyde (MDA) content, were determined using lipid peroxidation assay kit (Sigma-Aldrich) in line with instructions specified by the kit manufacturer. The MDA levels were expressed as nM MDA/mg of protein.

The ROS levels were determined with DCF ROS/RNS Assay Kit (Abcam). Dichlorodihydrofluorescein DiOxyQ (DCFH-DiOxyQ), a fluorogenic probe specific for ROS/RNS was used in the assay. The fluorescence intensity reflects the levels of ROS/RNS in the sample. The fluorescence was measured at 480 nm (excitation) and 530 nm (emission) in Synergy™ 2 Multi-function Microplate Reader.

The activities of CAT and SOD, and GSH content were determined using their appropriate assay kits from Sigma-Aldrich.

Immunoblotting analysis

The brain tissues were homogenised using cell lysis buffer (20 mM Tris-HCl, pH 7.5, containing 1 mM Na₂EDTA, 1 mM EGTA, 1 % Triton, 150 mM NaCl, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 2.5 mM sodium pyrophosphate, and 1 µg/mL leupeptin) from Cell Signalling Technology (USA). The cell extracts obtained were centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant collected was used for analysis. ReadyPrep™ Protein Extraction Kit (Cytoplasmic/Nuclear) from Bio-Rad (CA, USA) was used for cytosol and nuclear protein extraction. Protein content of the extract and the cytosol and nuclear fractions were determined with the Bradford method using kits from Bio-Rad. Protein samples of equal concentration (60 µg) obtained from whole cell, cytoplasmic and nuclear extracts were electrophoretically separated in 12 % SDS-PAGE gels.

The separated, size-fractionated protein bands were transferred onto PVDF membranes (Thermo Fischer Scientific). The membranes were then blocked in 5 % skimmed milk for 60 min to rule out any endogenous peroxidase, and then washed with Tris-buffered saline and Tween-20 (TBST). The PVDF membranes were then incubated overnight at 4 °C with primary antibodies for NF- κ B-p65, caspase-1, NLRP3, PYCARD (ASC), HO-1, cleaved-IL-1 β , Nrf-2 (1:1000 dilution; Cell Signaling Technology); β -actin, I κ B α , IKK β , IKK α , p-I κ B α , p-IKK β , p-IKK α and TNF- α (1:1000 dilution; Santa Cruz Biotechnology, USA). Thereafter, the membranes were again washed with TBST and further treated with secondary antibody (horse radish peroxidase-conjugated; 1:2000 dilution; Santa Cruz Biotechnology) for 60 min at room temperature. Following incubation, the membranes were washed with TBST and visualized with enhanced chemiluminescence system (Millipore, USA). The positive bands were scanned and analysed using Image J software (SuperSignal, Pierce, IL, USA).

Determination of cytokines

The serum levels of cytokines IL-1 β and IL-18 were assayed at 24 h after ICH. Serum samples from whole blood were kept at -80 °C until used. Kits from R & D systems (MN, USA) were used in line with the instructions specified by the kit manufacturers.

Statistical analysis

The results of the study were statistically analysed with one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) using Statistical Package for Social Sciences (SPSS software, version 21.0, IBM Corporation, USA). Values of $p < 0.05$ were regarded as statistically significant.

RESULTS

Punicalagin reduced brain oedema

Following 24 h of ICH induction, the brain water content was determined. The results indicated significant ($p < 0.05$) brain oedema (Figure 1 A) in ICH control group. The water content was 67.32 % in normal control, relative to 89.8 % in ICH control group. Brain oedema associated with ICH was reduced significantly ($p < 0.05$) in punicalagin-treated animals in a dose-dependent manner, relative to ICH control. Rats treated with punicalagin alone at a dose of 50 mg/kg b.wt. did not present significant changes in brain water content.

Punicalagin treatment improved BBB integrity

Disruption of the BBB was assessed by measuring EB dye extravasation in rat brain. Extravasation of EB dye was significantly ($p < 0.05$) increased following ICH induction, when compared with normal control (Figure 1 B). However, administration of punicalagin significantly reduced the EB content ($p < 0.05$), relative to the ICH control group. The observed decrease in extravasation of EB indicates improved BBB integrity. The administration of punicalagin (50 mg/kg) alone did not alter the integrity of the BBB, when compared to normal control animals.

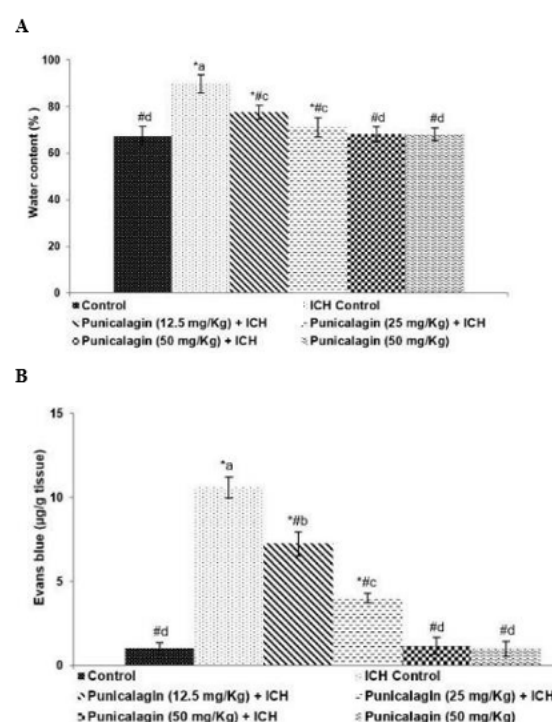


Figure 1: Effect of punicalagin on brain oedema and BBB barrier integrity. (A) Punicalagin effectively reduced brain water content (B), decreased extravasation of EB dye, and improved BBB integrity. Values are presented as mean \pm SD, $n = 6$; $p < 0.05$ as determined by one-way ANOVA followed by DMRT analysis; * $p < 0.05$ vs normal control; # $p < 0.05$ vs ICH control; a - d are mean values of the various treatment groups that differ from each other at $p < 0.05$

Punicalagin improved the histology of brain tissue

The effect of punicalagin on brain tissue architecture was determined following induction of ICH. Straining of brain tissues with H&E revealed the presence of inflammatory cells, severe neuronal degeneration and cell damage (Figure 2). However, treatment of the rats with punicalagin prior to ICH significantly improved the histology of the brain tissues as evidenced by

reduction in neuronal damage, when compared to ICH control animals.

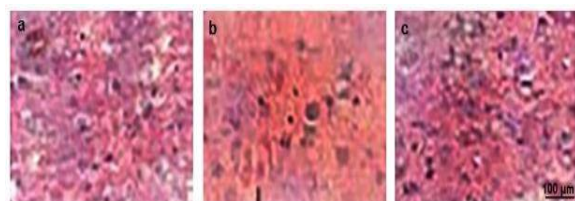


Figure 2: Effect of punicalagin on brain tissue histology following ICH. Punicalagin treatment reduced inflammatory cell infiltration and reduced neuronal degeneration. a: normal Control; b: punicalagin (50 mg/kg) + ICH; c: punicalagin (50 mg/kg)

Punicalagin reduced oxidative stress and improved antioxidant status

Oxidative stress has been associated with brain injury post-ICH [6]. The ROS and MDA levels were measured in brain tissues at 24 h following induction of ICH. Figure 3 A and B revealed multi-fold increases in ROS and MDA levels following ICH. The ROS generation increased to 420.60 % in ICH control, relative to normal control group, while MDA content increased to 19.02 nM/mg protein following ICH. Interestingly, treatment with punicalagin significantly decreased the ROS levels ($p < 0.05$). Rats treated with punicalagin at the dose of 50 mg/kg had maximal decreases in ROS levels, when compared to rats treated with lower doses of 12.5 and 25 mg/kg. The ROS levels decreased to 145.81 %, relative to 420.60 % in ICH control animals. In line with ROS results, MDA levels were also significantly ($p < 0.05$) reduced by punicalagin treatment, when compared to ICH control. The MDA levels were reduced to 8.91, 3.67 and 1.31 nM on treatment with punicalagin at doses of 12.5, 25 and 50 mg/kg, respectively.

To further assess the neuroprotective effects of punicalagin against ICH-induced oxidative stress, the activity of the antioxidant enzymes SOD and CAT were assayed. Figures 3 C and D indicate decreased activities of CAT and SOD in ICH control rats, when compared with the corresponding activities seen in normal control rats. Moreover, a marked decline in the level of GSH was seen in ICH group, relative to normal control group (Figure 3 E). The GSH levels decreased to 17.25 nM/mg protein from 36.5 nM/mg protein. Punicalagin at a dose of 50 mg/kg significantly ($p < 0.05$) raised GSH content to 38.81 nM/mg protein, when compared with 17.25 nM/mg protein in ICH control. Furthermore, SOD and CAT activities were increased significantly in the punicalagin-administered animals. The SOD activity increased to 73.32,

92.76 and 108.78 U/mg protein, while CAT activity was enhanced to 19.20, 24.67 and 28.78 U/mg protein in animals treated with punicalagin at doses of 12.5, 25 and 50 mg/kg, respectively.

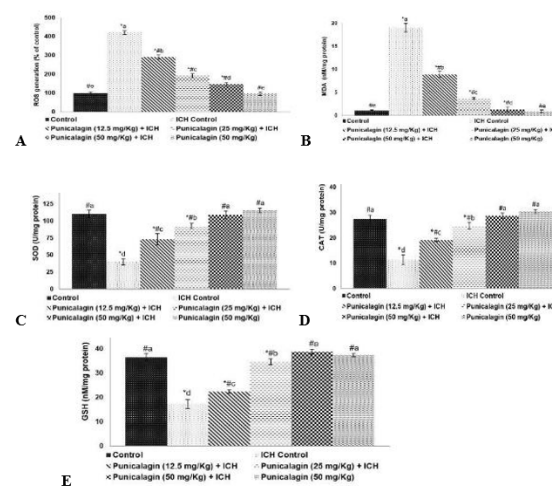


Figure 3: Effect of punicalagin on oxidative stress following ICH. Punicalagin treatment effectively reduced ROS generation (A), and MDA levels (B), and increased the activities of antioxidant enzymes SOD (C) and CAT (D), and GSH levels (E)

Punicalagin promoted Nrf2 and HO-1 expressions

Figures 4 A and B show that the results of expressions of Nrf2 and HO-1 assayed 24 h after ICH induction using western blot analysis. There were significant upregulations in Nrf2 expressions in the nuclear fraction following ICH ($p < 0.05$), while Nrf2 expressions were decreased in the cytosol. The increase in the nuclear translocation of Nrf2 was further enhanced from 144 % in ICH control to 179.98 % in rats treated with punicalagin at the dose of 50 mg/kg. In addition, HO-1 expressions were significantly and dose-dependently increased by punicalagin, when compared with the ICH control group.

Punicalagin down-regulated NLRP3 inflammasome

Activation of NLRP3 inflammasome has been observed in ICH induced brain injury [9]. There were significant ($p < 0.05$) enhancements in the expressions of NLRP3, ASC and caspase-1 following ICH induction in rats, relative to normal control (Figures 5A and 5B). Activation of NLRP3 inflammasome was further seen in significant ($p < 0.05$) elevations in the expressions of cleaved IL-1 β (Figure 5 A to Figure 5 C) and also in increased serum levels of cytokines (IL-1 β and IL-18).

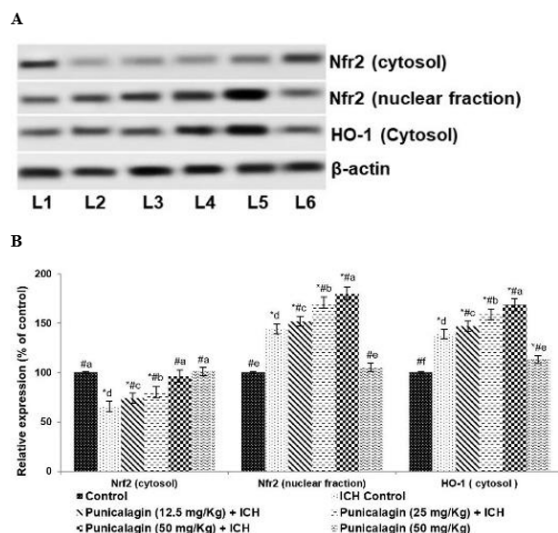


Figure 4: Effect of punicalagin on antioxidant regulators. (A) Representative blot revealing punicalagin-induced improvement in the expressions of Nrf2 and HO-1. (B) Relative expressions of proteins from different treatment groups, with control expressions set at 100 %. The results are presented as mean \pm SD ($n = 6$); $p < 0.05$ as determined using one-way ANOVA, followed by post hoc analysis by DMRT; * $p < 0.05$ vs normal control; # $p < 0.05$ vs ICH control; a-e represent mean values from treatment groups that differ at $p < 0.05$. L1: normal control; L2: ICH control; L3: punicalagin (12.5 mg/kg) + ICH; L4: punicalagin (25 mg/kg) + ICH; L5: punicalagin (50 mg/kg) + ICH; L6: punicalagin (50 mg/kg)

Punicalagin administration resulted in significant downregulations of caspase-1, NLRP3 protein, ASC and IL-1 β , and reduced levels of serum IL-1 β and IL-18 ($p < 0.05$). The results showed that punicalagin at a dose of 50 mg/kg b.wt. was most effective in reducing the expressions of NLRP3 inflammasome components to near normal levels, when compared to the lower doses of 12.5 and 50 mg/kg. Treatment with 50 mg/kg punicalagin at the dose of mg/kg reduced caspase-1 and ASC expressions to 102 and 106.2 % from 157.31 and 176.05 %, respectively, while the serum levels of IL-1 β and IL-18 decreased from 76 and 118.50 pg/mL to 31.2 and 43 pg/mL, respectively. These results suggest that punicalagin effectively down-regulated NLRP3 inflammasome.

Punicalagin regulated NF- κ B signalling cascade

Activation of NF- κ B signalling is essential for activation of NLRP3 inflammasome. The NF- κ B/NLRP3 signalling route is one of the major pathways that regulate the expressions of inflammatory mediators such as interleukins, e.g., IL-1 β [20]. At 24 h after ICH induction, there were marked increases in nuclear NF- κ B (p65)

expressions, and substantial decreases in its cytosolic levels ($p < 0.05$).

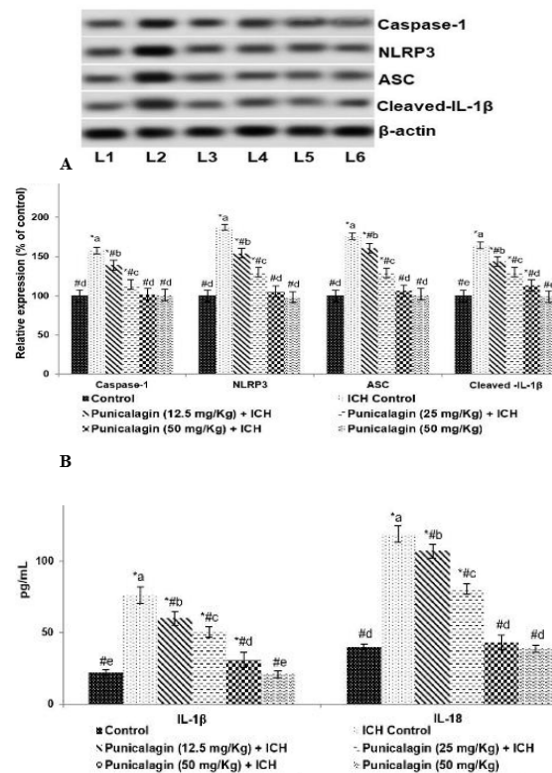


Figure 5: Effect of punicalagin on NLRP3 inflammasome and pro-inflammatory cytokines. (A) Representative blot revealing the down-regulation of NLRP3 inflammasome components and IL-1 β by punicalagin treatment. (B) Relative protein expressions from different treatment groups, with control protein expressions set at 100 %. (C) Punicalagin administration reduced serum levels of IL-1 β and IL-18. The results are presented as mean \pm SD, $n = 6$. $p < 0.05$, as determined using one-way ANOVA, followed by post-hoc analysis with DMRT; * $p < 0.05$ vs normal control; # $p < 0.05$ vs ICH control; a-e are mean values from different treatment groups that differ at $p < 0.05$. L1: normal control; L2: ICH control; L3: punicalagin (12.5 mg/kg) + ICH; L4: punicalagin (25 mg/kg) + ICH; L5: punicalagin (50 mg/kg) + ICH; L6: punicalagin (50 mg/kg)

These results, which indicate the stimulation of the NF- κ B, are shown in Figure 6 A and B. Furthermore, elevated expressions of TNF- α , and phosphorylation of kinases i.e. IKK α , IKK β , and I κ B α were observed at 24 h following ICH (Figures 6 A to Figure 6 C).

Punicalagin administration resulted in a significant suppression of NF- κ B p65 (nuclear fraction) expressions, when compared with ICH control ($p < 0.05$). At a dose of 50 mg/kg, punicalagin significantly suppressed the expression of NF- κ B p65 in the nuclear fraction from 177.08 to 103.20 % ($p < 0.05$).

Furthermore, punicalagin at doses 12.5, 25 and 50 mg/kg significantly down-regulated the levels of p-IKK α , p-IKK β and p-I κ B α , relative to ICH control group ($p < 0.05$). The ICH-induced enhancement in TNF- α expressions was reduced from 198.05 to 121.74 % on treatment with punicalagin at the dose of 50 mg/kg ($p < 0.05$). These observations suggest the inhibition of activation of NF- κ B/NLRP3 inflammasome by punicalagin.

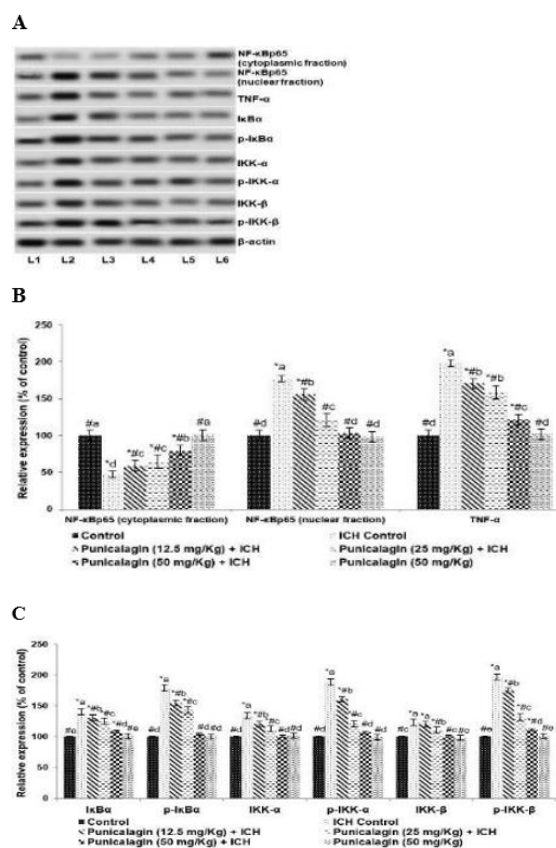


Figure 6: Effect of punicalagin on activation of NF- κ B signalling following ICH. (A) Representative blot revealing the down-regulation of regulatory proteins of the NF- κ B signalling pathway and inhibition of NF- κ B activation by punicalagin treatment. (B and C) Relative protein expressions from different treatment groups, with control protein expressions set at 100 %. The results are presented as mean \pm SD ($n = 6$); $p < 0.05$ as determined with one-way ANOVA, followed by post-hoc analysis with DMRT; * $p < 0.05$ vs normal control; # $p < 0.05$ vs ICH control; a-e represent mean values from different treatment groups that differ at $p < 0.05$. L1: normal control; L2: ICH control; L3: punicalagin (12.5 mg/kg) + ICH; L4: punicalagin (25 mg/kg) + ICH; L5: punicalagin (50 mg/kg) + ICH; L6: punicalagin (50 mg/kg)

DISCUSSION

Several studies have suggested that inflammation and oxidative stress are critically associated with the pathology of ICH, a

devastating haemorrhagic stroke [5]. Inhibitions of inflammatory responses and ROS are reported to be beneficial in ICH [21]. In this investigation, punicalagin treatment at doses 12.5, 25 and 50 mg/kg effectively improved BBB integrity and brain tissue histology, and reduced brain oedema 24h following ICH induction. Studies have demonstrated that oxidative stress is a major contributor to ICH-induced brain injury [22]. Following ICH-induced injury, the breakdown products of various cells including RBCs generate ROS, subsequently leading to oxidative stress and depletion of antioxidant reserves [2,3].

Low levels of antioxidant defences, along with high metabolic rate and abundant lipids make the brain cells highly sensitive to lipid peroxidation and oxidative damage [23]. Interestingly, in this study, up-regulated Nrf2 in the nuclear fraction, and raised HO-1 in the cytosol were noticed in the ICH control rats at 24 h following ICH. This could be a measure of the innate defence mechanisms under oxidative stress conditions. However, punicalagin treatment significantly activated Nrf2-mediated antioxidative signalling, as evidenced by multi-fold enhancement in the expressions of Nrf2 and HO-1. Studies have shown that Nrf2, a major transcription factor, is a chief regulator of innate antioxidative responses in the brain, and it also protects cells by regulating inflammatory responses [11].

Under regular physiological conditions, the Nrf2 protein in the cytoplasm remains bound to Keap1 protein [24]. However, stressful conditions stimulate the phosphorylation of Nrf2. The phosphorylated Nrf2 separates from Keap1 and gets translocated to the nucleus and regulates the expressions of downstream target genes [25]. Heme oxygenase-1 (HO-1) exerts antioxidant effects against ROS-induced oxidative stress. The transcription of HO-1 is stimulated by Nrf2 in neuronal cells. Elevated HO-1 expression significantly reduces cell membrane damage and also reduces ROS production [24]. Thus, the significantly increased Nrf2 and HO-1 expressions following ICH injury reflect the protective effects of punicalagin. Punicalagin-mediated increases in Nrf2 and HO-1 expressions were consistent with decreased ROS and MDA levels, indicating decreased oxidative stress levels. Moreover, the activities of SOD and CAT, and levels of glutathione which were markedly reduced following ICH, but were significantly enhanced on punicalagin treatment. These results indicate that the punicalagin-mediated decreases in ROS and MDA levels, and the enhanced antioxidant status could be due to its direct antioxidant effects or via increase in Nrf2 /HO-1 signalling.

Neuroinflammation is well known as a major contributor to the pathogenesis of ICH-induced brain injury [2]. Several lines of evidence suggest that NF- κ B, a major transcription factor that controls the expressions of several inflammatory cytokines, is activated within minutes after ICH injury [22]. NF- κ B remains inactive in the cytosol under normal physiological conditions, and is bound to inhibitory proteins i.e. inhibitors of NF- κ B (I κ Bs). Once stimulated, the inhibitor subunit I κ B gets activated through phosphorylation by the I κ B kinase (IKK) complex which consists of IKK α and IKK β [26]. The I κ B, upon activation gets rapidly degraded, leading to the dissociation of NF- κ Bp65 complex [27]. Once dissociated, NF- κ Bp65 gets translocated to the nucleus. In the nucleus, NF- κ Bp65 initiates transcription of key down-stream genes including TNF- α , and the cytokines IL-1 β and IL-6 [26].

In this study, the enhanced expression of nuclear NF- κ Bp65, along with up-regulated TNF- α and IL-1 β expressions indicate the activation of NF- κ B signalling at 24h after induction of ICH. In addition, the raised serum levels of IL-1 β reflect the activation of the pathway. It is also known that activation of NF- κ B signalling is essential for NLRP3 inflammasome activation [20]. NLRP3 belongs to Nod-like receptor family, and it is involved in ICH-induced early brain injury [10]. NLRP3 inflammasome is composed of NLRP3 protein (sensor protein), ASC protein (adaptor), and pro-caspase-1 (effector).

It has been reported that oxidative stress conditions are triggers for the activation of the NLRP3 inflammasome [20]. Once activated, NF- κ B initiates the expressions of NLRP3 protein. Activated NLRP3 inflammasome induces caspase-1-mediated activations of IL-1 β and IL-18 [20]. These cytokines promote and regulate neutrophil infiltration, and also induce inflammatory responses [28]. The elevated expressions of NLRP3 inflammasome components (NLRP3 protein, caspase-1 and ASC protein, along with IL-1 β and IL-18 levels) reflect activation of NLRP3 inflammasome following ICH. This activation also demonstrates activation of NF- κ B signalling and the activation of NLRP3 inflammasome, along with increased levels of ROS production observed at 24 h after ICH.

In this study, punicalagin administration led to significant down-regulations of phosphorylation of the NF- κ B pathway regulatory proteins (I κ B α , IKK α and IKK β), suggesting inhibition of the pathway. This downregulation could have contributed to marked decrease in the activation

of NF- κ B as indicated by reduced nuclear levels of NF- κ Bp65 and TNF- α . Interestingly, in line with down-regulation of NF- κ B activation and ROS production, it was observed that punicalagin effectively blocked the activation of NLRP3 inflammasome, as shown in marked declines in the expressions of NLRP3, caspase-1, and ASC. Furthermore, the levels of the cytokines IL-1 β and IL-18 were significantly decreased by punicalagin treatment. These results suggest that punicalagin caused down-regulations of inflammatory responses and oxidative stress, which demonstrate its neuroprotective effects against ICH injury.

CONCLUSION

The results of this study demonstrate that punicalagin administration significantly protects brain cells following ICH induction by reducing oxidative stress, and regulating ROS levels and NF- κ B/ROS-mediated activation of NLRP3 inflammasome. Punicalagin also improves the antioxidant status of the cells via activation of Nrf2 activity and enhancement of the activities of the antioxidant enzymes SOD and CAT. These observations suggest that punicalagin has a promising potential for use as a therapeutic option for ICH.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Both the authors have equally contributed to this study. Darong Wu and Bo Li designed this study, collected and analysed the data, and also prepared the manuscript.

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