Original Research Article

Punicalagin alleviates brain injury and inflammatory responses, and regulates HO-1/Nrf-2/ARE signaling in rats after experimental intracerebral haemorrhage

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Abstract

Purpose: To investigate the effect of punicalagin, an ellagitannin present in pomegranates, on intracerebral haemorrhage (ICH)-induced inflammatory responses and oxidative stress, and also unravel the underlying mechanism(s) of action.

Methods: Collagenase type IV (0.2 U) was used to induce ICH in adult male Sprague-Dawley rats. Punicalagin was given to the rats at doses of 25, 50, and 75 mg/kg body weight via oral gavage for 15 days before ICH induction. The animals were sacrificed 24h following induction of ICH, and their brains were excised immediately and used for analysis. Histological changes were determined with Haematoxylin and Eosin (H&E) staining. Permeability to blood-brain barrier (BBB) was determined by quantifying the extent of extravasation of Evan Blue (EB). Protein expressions of HO-1/Nrf-2/ARE and NF-κB signaling were assayed using immunoblotting and RT-PCR. Levels of reactive oxygen species (ROS) and serum levels of cytokines were also determined.

Results: Punicalagin treatment reduced inflammatory cell infiltration and cell damage, improved brain tissue architecture and BBB integrity. The punicalagin treatment increased the activities of antioxidant enzymes, and enhanced antioxidant status via activation of Nrf-2/ARE/HO-1 signaling pathway (p < 0.05). The treatment upregulated the expressions of HO-1 to 174 %, relative to 127 % in ICH control rats. Furthermore, it enhanced NF-κB levels and reversed the ICH injury-induced upregulations of IL-6, IL-18 and IL-1β.

Conclusion: These findings indicate that punicalagin exerts neuroprotective effect in rats after experimental ICH through regulation of theHO-1/Nrf-2/ARE signaling pathway. Thus, punicalagin has therapeutic potential for ICH.

Keywords: Brain injury, Haemoxygenase-1, Intracerebral haemorrhage, Inflammatory responses, Nrf2/ARE signalling, Punicalagin

INTRODUCTION

Spontaneous intracerebral haemorrhage (ICH) is a type of stroke with increasing incidence and high mortality and morbidity [1]. It accounts for approximately 10-15% of all types of strokes [2]. An ICH-induced primary/early brain injury occurs within a few hours following ICH, causing

hematoma and brain oedema. Extravasation and accumulation of blood lead to raised intracranial pressure, resulting in cell injury and compression on the adjacent tissues [3]. Secondary injuries due to extravasated blood and degradation products of red blood cells within the hematoma further exacerbate brain injury, leading to oxidative stress, disruption of the integrity of BBB, neuronal degeneration and neurobehavioral deterioration [4-6].

Researchers have revealed that oxidative stress, inflammation, and cytotoxicity as essential factors involved in post-ICH brain injury, where excess free radicals derived from lysis of red blood cells and autooxidation of haemoglobin contribute to cell injury [6]. Following ICH, hemin released from red blood cells contributes to the depletion of glutathione stores and cellular NADPH levels, thereby resulting in cellular damage [7]. Studies have suggested that promoting hemin catabolism aids in clearing hematoma [8]. Heme oxygenase (HO), the rate-limiting enzyme in heme catabolism, induces HO-1, the inducible form of HO, improves the integrity of BBB, and reduces neurological impairments [9].

Inflammatory mediators such as chemokines, cytokines, and prostaglandins released at the perifocal tissues by activated inflammatory cells following ICH, trigger secondary brain injuries [10]. The activated inflammatory cells induce activation of the nuclear transcription factor-κB (NF-κB)-mediated signal. It is known that NF-κB is a chief regulator of the inflammatory process, the activation of which triggers and magnifies the inflammatory response in ICH [5].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a chief transcription factor that critically regulates anti-oxidative responses via the antioxidant response elements (ARE) of the target genes. It is present in all types of brain cells, including microglia and neurons [11]. The activation of Nrf2 triggers the expressions of several antioxidant enzymes such as glutathione-metabolizing enzymes i.e. glutathione-S-transferase (GST) and glutathione peroxidase (GPx); catalase (CAT), superoxide dismutase (SOD), NAD(P)H: quinone oxidoreductase-1 (NQO1) and HO-1 [12]. The nuclear factor erythroid 2-related factor 2 has been shown to exert neuroprotective effects following ICH by mitigating neurological impairments, reducing brain oedema, and relieving oxidative stress [13].

Studies have revealed that plant-derived compounds exert neuroprotective effects in rodent models of ICH [14]. Punicalagin(2,3-hexahydroxydiphenoyl-gallagyl-D-glucose) is an ellagitannin present in pomegranates. It exhibits potent anti-inflammatory [15], anti-proliferative and antioxidative effects [16]. The present investigation was designed to determine the effect of oral administration of punicalagin on ICH-induced brain injury in rats.

EXPERIMENTAL

Animal grouping and study design

Adult male Sprague-Dawley rats (8 to 10 weeks old, weighing 290 - 320g) were obtained from the Laboratory Animal Centre of The Wuhan University. The handling of animals, grouping, study design, and the experimental protocols were approved by the Ethical Committee of Wuhan University (approval no. TXW15744). The study was carried out in line with the guidelines of National Institutes of Health on the care and use of experimental animals [17].

The animals were housed in sterile cages (n = 3/cage) under standard controlled conditions (12-h light/dark cycle, temperature of 23 ± 2 ℃, and relative humidity of 55 - 60 %). They were provided unlimited access to commercially available standard rat pelleted diet and water. The rats were acclimatized to the animal house environment for five days before the initiation of the study. The study was conducted in a collagenase type IV-induced ICH rat model. The induction was performed as described earlier by Rosenberg *et al* [18], but with minor changes. The animals were randomly assigned to 6 treatment groups (n = 24 / group): group I (normal control); group II (ICH control group administered equivalent volume of saline in place of punicalagin); groups III, IV and V (ICH + punicalagin (Sigma-Aldrich, St.Louis, MO, USA) at doses of 25, 50 and 75 mg/kg bodyweight (bwt), respectively. Group VI rats received punicalagin at a dose of 75 mg alone. Punicalagin was administered via oral gavage every day for two weeks before ICH induction. The drug was administered 1 h before surgery on the day of ICH induction.

ICH induction

The animals were administered pentobarbital sodium anaesthesia (45 mg/ kg bwt, Sigma-Aldrich) via intraperitoneal injection, and were maintained at 37 ℃ with an insulation board linked to a water bath circulation system. Each rat was positioned in a prone position in a rat brain stereotaxic apparatus, and a midline incision was made on the scalp to expose the skull and bregma. In the right part of the brain, a 1-mm burr hole was drilled and a micro-syringe

(5 μL) with a needle was inserted through the hole at stereotaxic coordinates of 3.5 mm lateral, 6.0 mm ventral and 0.1 mm anterior to the bregma. Collagenase type IV (Sigma-Aldrich) was used for ICH induction. Collagenase (0.2 U in 1 μL sterile normal saline) was administered via stereotaxic intra-striatal injection. The needle was retained *in situ* for about 10 min in the same position after collagenase injection to avoid back-flow. Thereafter, the needle was carefully removed and the hole was sealed using bone wax. All rats were sacrificed 24 h following induction of ICH, and their brains were excised immediately and were used for analysis.

Determination of brain oedema

Brain oedema was measured 24 h following ICH. The brains were separated along the midline into two hemispheres. The cerebellum was isolated and removed and used as internal control. To measure the wet weight, each hemisphere was weighed separately on an electronic analytical balance. The tissue samples were then kept at 100 ℃ in an electric oven for 24 h and the dry weight was recorded. Brain water content (BWC) was calculated using Eq 1.

$$BWC\ (\%) = \{(Ww - Dw)/Ww\}100 \ldots\ldots\ldots (1)$$

where Ww and Dw are the weight of the weight of the fresh brain and the dry after drying, respectively.

Histological analysis

The excised brains were post-fixed in 4 % paraformaldehyde solution for 24 - 48 at 4 °C. Following dehydration, the brain tissue samples were paraffin-embedded and coronal sections of 4-μm thickness were cut. The tissue sections were treated with xylene. Following rehydration with graded ethanol and deionized water, the sections were stained with H&E. The stained sections were visualized and analysed under a microscope (Leica-DM2500, Germany).

Assessment of BBB permeability

The permeability of BBB as a measure of membrane integrity was evaluated by quantifying the extent of extravasation of Evan Blue (EB). The protocol as described by Belayev *et al*. [19] was followed, with minor alterations. Following anaesthesia, rats from each experimental group were injected intravenously with EB solution (2 % in saline) at a dose of 4 mL/ kg bwt in the femoral vein. Two hours following injection, the rats were anesthetized and sacrificed by intracardial perfusion of 250 mL of PBS (0.01 M) in order to clear Evan Blue in the cerebral circulation. The brains were then excised and separated into 2 hemispheres along the midline.

The tissues were incubated with 2 mL of 50 % trichloroacetic acid (TCA) solution, and homogenized using a glass Teflon homogenizer (Thomas Scientific). The homogenates were subjected to centrifugation at 15,000 rpm for 20 min at room temperature. The supernatant obtained was diluted with ethanol in a 1:3 (v : v) ratio. The extravasation of EB was measured in terms of its fluorescence intensity (excitation at 620 nm and emission at 680 nm) and expressed as mg/ g of brain weight.

Determination of ROS, lipid peroxidation, and antioxidant enzymes

Brain tissues (n = 6/ group) were homogenized in ice-cold phosphate-buffered saline (PBS) and centrifuged at 3000 rpm for 15 min at 4 ℃. The resultant supernatant was used for assay of activities of catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD, lipid peroxidation, ROS production and total glutathione (GSH) levels. The total protein content of the supernatant was determined with bicinchoninic acid assay (BCA) using commercial kits (Thermo Fischer Scientific, USA).

The activities of antioxidant enzymes were assayed with ELISA in line with the instructions specified by the manufacturer. Activity of SOD was determined using assay kits from Rand D systems (MN, USA), while CAT and GPx were assayed with kits from Abcam. Levels of GSH were determined using glutathione assay kit from Sigma-Aldrich, as per instructions. Lipid peroxidation levels were measured in terms of malondialdehyde (MDA) content using lipid peroxidation assay kit (Abcam, USA), and expressed as nmoles MDA/mg of protein.

The ROS contents were measured with DCF ROS/RNS Assay Kit (Abcam ab238535) using dichlorodihydrofluoresc in DiOxyQ (DCFH-DiOxyQ), a fluorogenic probe specific for ROS/RNS. The assay is based on the principle that fluorescence intensity reflects the levels of ROS/RNS present in the sample. Intensity was measured at excitation wavelength of 480 nm and emission wavelength of 530 nm using Synergy™ 2 Multi-function Microplate Reader.

Immunoblotting

Protein expression analysis was performed at 24 h following ICH. The excised brain tissues were homogenised using cell lysis buffer from Cell Signalling Technology, USA (20 mM Tris-HCl

(pH 7.5) 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 150 mM NaCl,1 mM Na_3VO_4,1 μg/mL leupeptin, 1 mM beta-glycerophosphate). The whole-cell extracts obtained were subjected to centrifugation at 3000 rpm; 15 min) at 4 °C. Cytosol and nuclear fractions were separated from the whole-cell homogenate using ReadyPrep™ Protein Extraction Kit (cytoplasmic/nuclear) from Bio-Rad, CA, USA. The total protein content of the whole-cell extracts, cytosol and nuclear fractions were quantified using kits from Thermo Fischer Scientific. Protein samples of equal concentration (60 μg) from whole-cell, cytoplasmic and nuclear extracts were loaded on 12 % SDS-PAGE gels and were electrophoretically separated and blot-transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo Fischer Scientific). The blotted membranes were blocked using 5 % skimmed milk for 60 min to exclude any non-specific interactions. The membranes were then washed with Tris-buffered saline and Tween- 20 (TBST) and incubated overnight at 4 °C with primary antibodies against NF-κb-p65, HO-1, Nrf-2, NAD(P)H quinone dehydrogenase 1(NQO1), and inducible nitric oxide synthase (iNOS), TNF-α (1:1000, Cell Signaling Technology), as well as β-actin, COX-2, IκBα, IKKβ, IKKα, p-IκBα, p-IKKβ and p-IKKα (1:1000, Santa Cruz Biotechnology, USA).Following incubation, the membranes were washed with TBST and further incubated with secondary antibody (horseradish peroxidase-conjugated; 1:2000, Santa Cruz Biotechnology) for 60 min at room temperature. The membranes were washed again with TBST and the bands were visualized using an enhanced chemiluminescence system (Millipore, USA). The positive bands obtained were scanned and analysed using Image J software (SuperSignal; Pierce, IL, USA).

Real-time (RT)-qPCR

Quantitative real-time RT-PCR was performed using Prime Script™ RT-PCR kits (Takara, Japan). The RT PCR cycle steps were at 95 °C for 30 sec, 40 cycles of 95 °C for 3 sec, 60 °C for 34 sec. The primer sequences used are shown in Table 1.

Table 1: Primer sequences used for PCR

Gene	Forward	Reverse
iNOS	GTGCTAATGCG GAAGGTCATG	GCTTCCGACTTT CCTGTCTCAGTA
NQO1	GTTTGCCTGGC TTGCTTTCA	ACAGCCGTGGC AGAACTATC
HO-1	GGTGATGGCCT CCTTGTACC	GTGGGGCATAG ACTGGGTTC
GAPDH	CCAGCCTCGTC TCATAGACA	GTAACCAGGCG TCCGATACG

GAPDH mRNA expression was used as the internal control to assess expressions of the test genes.

Determination of cytokines

The serum samples separated from whole blood were kept at -80 °C until used. Serum levels of cytokines IL-1β, IL-6 and IL-18following 24 h post-ICH were measured using kits from R&D Systems (MN, USA).

Statistical analysis

Data were analysed using a one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT). All statistical analyses were done using the SPSS package (Version 21.0, IBM Corporation, USA). Values of $p < 0.05$ were regarded as statistically significant.

RESULTS

Punicalagin reduced brain oedema

Brain water content measured at 24 h following ICH induction revealed significant ($p < 0.05$) brain oedema (Figure 1) in the ICH control group, relative to normal control. The water content was 68.74 % in normal control, as against 88.58 % in the ICH control group. However, the ICH-induced brain oedema was reduced to75.5, 69.1 and 67.24 % on punicalagin treatment at doses of 25, 50 and 75 mg/kg, respectively. Punicalagin at doses of 50 and 75 mg/kg effectively prevented increases in brain oedema, and the brain water content was maintained at near normal values. Treatment with 75 mg punicalagin alone caused no significant changes in brain water content.
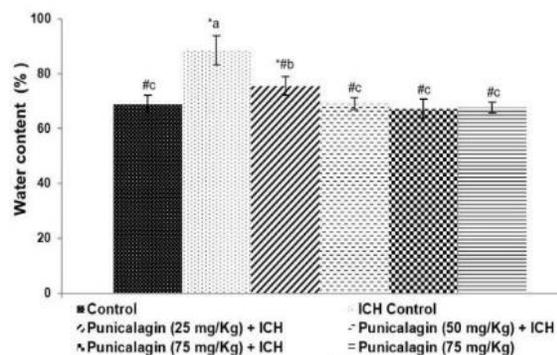
Figure 1: Effect of punicalagin on brain oedema. Values are presented as mean ± SD, n = 6. $p < 0.05$ as determined with one-way ANOVA followed by DMRT analysis. * $p < 0.05$ vs control; # $p < 0.05$ vs ICH control; a-c represent mean values that differ from each other at $p < 0.05$

Punicalagin reduced ICH-induced brain tissue alterations and improved the integrity of BBB

The effect of systemic administration of punicalagin on brain tissue architecture were assessed following ICH induction. Hematoxylin and Eosin (H&E) staining of brain tissues revealed inflammatory cell infiltration in the tissues, severe neuronal degeneration and cellular injury (Figure 2). Punicalagin administration prior to induction of ICH significantly reduced inflammatory cell infiltration and prevented marked histological alterations observed following ICH. The neuronal degeneration and cell damage were lower, when compared to ICH control animals.

The ICH-induced disruption of the BBB was evaluated by determining the extravasation of EB dye in the brain tissues. In this study, there was a significant ($p < 0.05$) increase in EB dye extravasation following ICH, when compared with normal control. Administration of punicalagin at all 3 tested doses clearly reduced the EB content, when compared with the ICH control group. The reduction in extravasation of EB indicated improvement in BBB integrity. At doses of 50 mg and 75 mg/ kg, punicalagin exerted higher protective effects, when compared to the effect produced by a dose of 25 mg/kg. Furthermore, a dose of 75 mg/ kg, when administered alone, did not alter the integrity of the BBB, when compared to normal control animals.
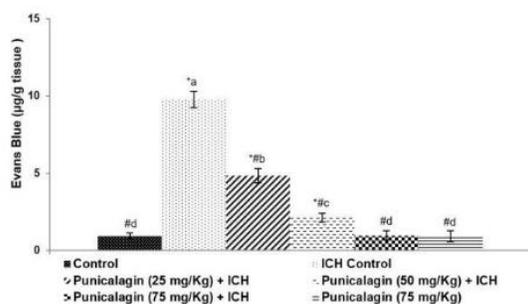
Figure 2: Effect of punicalagin on blood brain barrier permeability. Values are presented as mean ± SD, n = 6. $p < 0.05$ as determined by one-way ANOVA followed by DMRT analysis; *$p < 0.05$, vs control; #$p < 0.05$, vs ICH control; a-d represent mean of different experimental groups that differ from each other at $p < 0.05$

Punicalagin reduced oxidative stress

Levels of ROS and lipid peroxidation were measured in brain tissues at 24 h following induction of ICH. Multi-fold increases in ROS and MDA levels following ICH were observed, as

shown in Figures 3a and 3b. The generation of ROS increased to 405.58 % in the ICH control, relative to normal control group. The MDA content rose to 18.05 nmoles/ mg protein following ICH, while MDA level in normal control was 1.06 nmoles/ mg protein.

Interestingly, treatment with the tested doses of punicalagin significantly decreased ROS levels, with doses of 50 and 75 mg/kg being more effective in decreasing ROS levels than a lower dose of 25 mg/ kg. Treatment with a dose of 75 mg/ kg decreased the ROS levels to 115.09 %, relative to 405.58% in ICH control. Similar to ROS trend, MDA levels were also significantly ($p < 0.05$) reduced on punicalagin treatment, when compared to ICH control. The MDA levels were reduced to 6.12, 2.07 and 1.1 nmoles/ mg protein on treatment with punicalagin at doses of 25, 50 and 75 mg, respectively.
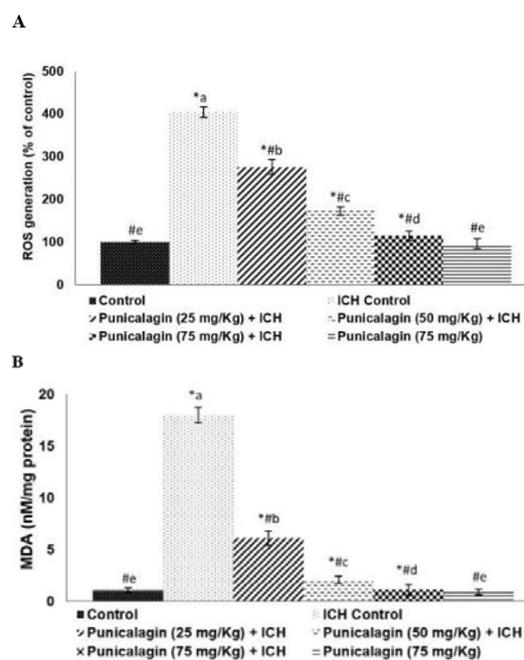
Figure 3: Effect of punicalagin on ICH-induced oxidative stress. Punicalagin reduced ROS generation (a) and MDA levels (b), following intra-cerebral haemorrhage. Values are presented as mean ± SD (n = 6). *$p < 0.05$, vs control; # $p < 0.05$ vs ICH control; a-e represent significant differences ($p < 0.05$) amongst different experimental groups

Punicalagin promoted Nrf2/ARE signaling and HO-1 expressions

The protein expressions of Nrf2, NQO1, and HO-1 were assayed 24h after ICH induction. The expression levels of Nrf2 were significantly higher ($p < 0.05$) in the nuclear fraction (Figures 4 a and b) than in cytosol following ICH

induction. The increase in the nuclear translocation of Nrf2 was further enhanced from 139 % in ICH control to162, 179.07 and 181.98 % in animals treated with 25, 50 and 75 mg punicalagin, respectively. Moreover, mRNA expression levels of HO-1 and NQO1 were significantly upregulated ($p < 0.05$). The elevated protein levels are in line with the mRNA expressions of HO-1 and NQO1. The mRNA levels of HO-1 and NQO1 increased 2.1 and 1.8 folds, respectively, on treatment with 75 mg punicalagin (Figures 5 a and b). The results indicate activation of Nrf2 signaling.

To investigate if ICH-induced activated Nrf2/ARE signal following ICH-induced oxidative stress, the activity of antioxidant enzymes i.e. CAT, GPx and SOD were assayed. Figure 6 a and b show significantly decreased activities of CAT, GPx and SOD in ICH control rats, relative to normal control rats ($p < 0.05$). A marked decline in GSH levels was observed in the ICH group, when compared with the normal control group (Figure 6d).
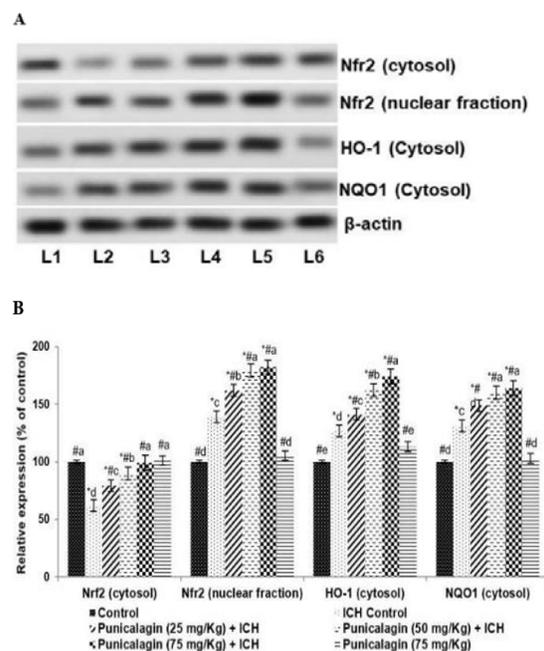
Figure 4: Effect of punicalagin on Nrf2/ARE signaling. Punicalagin regulated protein expressions of antioxidant regulators. (a and b): representative protein blot gel; (a) proteins with respect to control. Values are presented as mean ± SD (n = 6). *$p < 0.05$, vs control; # $p < 0.05$, vs ICH control; a-e represent significant differences ($p < 0.05$) amongst different experimental groups. L1: Control; L2: ICH Control; L3: punicalagin (25 mg/kg) + ICH; L4: punicalagin (50 mg/kg) + ICH; L5: punicalagin (75 mg/kg) + ICH; L6: punicalagin (75 mg/kg)
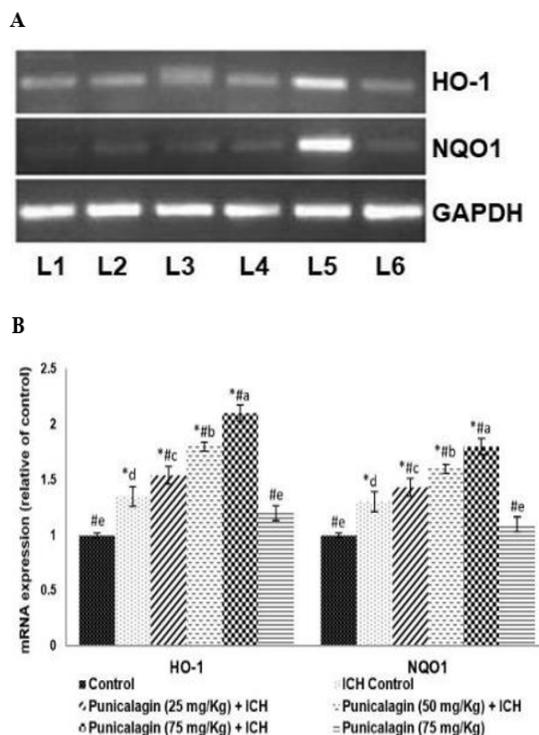
Figure 5: Effect of punicalagin on mRNA levels of HO-1 and NQO1. Punicalagin enhanced mRNA levels of HO-1 and NQO1 (a-b). Representative gel (c) mRNA expression relative to control. Values are presented as mean ± SD (n = 6); *$p < 0.05$, vs control; # $p < 0.05$, vs ICH control; a-e represent significant differences ($p < 0.05$) amongst different experimental groups. L1: Control; L2: ICH Control; L3: punicalagin (25 mg/kg) + ICH; L4: punicalagin (50 mg/kg) + ICH; L5: punicalagin (75 mg/kg) + ICH; L6: punicalagin (75 mg/kg)

The activities of SOD decreased to 46.11 U/ mg protein in the ICH control group, while SOD activity in normal control was 110.18 U/mg protein. The activities of CAT and GPx were reduced to 10.44 and 12.38 U/ mg protein, respectively, while GSH levels decreased to 13.09 nmoles/mg protein from 37.25 nmoles/mg protein. However, punicalagin at a dose of 75 mg/ kg markedly ($p < 0.05$) raised GSH content to 39.85 nmoles/mg protein, as against 13.09 nmoles/mg protein in ICH control. Furthermore, SOD and CAT activities were increased significantly in punicalagin-treated rats: SOD activity increased to 70.32, 99.08 and 112.9 U/mg protein, while GPx activity increased to 23.19, 36.07 and 38.14 U/mg protein, due to treatment with punicalagin at doses of 25, 50 and 75 mg/kg, respectively. At these doses, the corresponding increased activities of CAT were 17.42, 23.67 and 30.55 U/mg protein, respectively.
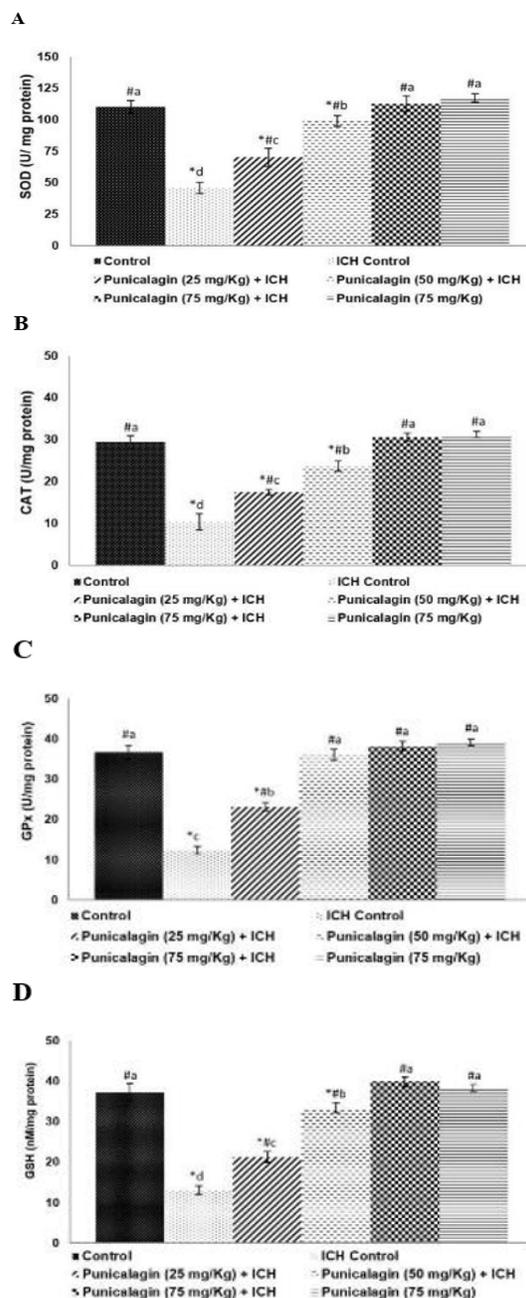
Figure 6: Effect of punicalagin on antioxidant status following ICH. Punicalagin increased the activities of antioxidant enzymes SOD (a), CAT (b) and GPx (c); and enhanced GSH levels (d). Values are presented as mean ± SD (n = 6). *$p < 0.05$, vs. control; #$p < 0.05$, vs ICH control; a-d represent significant differences ($p < 0.05$) amongst different experimental groups

Punicalagin regulated NF-κB signal cascade

The NF-κB signaling is activated under oxidative stress. In this study, ICH induction caused marked upregulation in nuclear NF-κB (p65) expressions, resulting insubstantially lower cytosolic levels of NF-κB (p65) ($p < 0.05$; Figures

7 a and b). These observations indicate the activation of NF-κB. Furthermore, there were up-regulated expressions of TNF-α, and increased levels of phosphorylation of the regulatory kinases (IKKα, IKKβ, and IκBα) (Figures 7 a – c). However, punicalagin supplementation resulted in a significant downregulation NF-κB p65 (nuclear fraction) expression, when compared to ICH control ($p < 0.05$). At a dose of 50 mg/ kg, punicalagin reduced the expression of NF-κB p65 in the nuclear fraction from 188.21 to 110.23 %, while a dose of 75 mg/ kg reduced it to 99.7 %. Punicalagin at the tested doses of 25, 50 and 75 mg/ kg significantly ($p < 0.05$) down-regulated p- IKKα, p- IKKβ and p-IκBα expressions, relative to ICH control group. The enhanced TNF-α observed in ICH was reduced to 122.34 % from 192.05 % ($p < 0.05$) in 50 mg/ kg punicalagin group, when compared with ICH control. There were significant up-regulations in mRNA and protein expressions of COX-2 and iNOS following ICH induction (Figures 8 a - d). Interestingly, punicalagin caused marked suppression of mRNA and protein expressions. These observations suggest that NF-κB signaling was inhibited by punicalagin.

Punicalagin reduced serum cytokine levels

Activation of NF-κB signaling following ICH was further supported by significant elevations in levels of inflammatory cytokines (Figure 9). There were marked increases in serum levels of IL-1β, IL-6 and IL-18 ($p < 0.05$). Punicalagin-mediated down-regulation of NF-κB signaling was reflected in decreased serum levels of IL-1β, IL-6, and IL-18.The IL-1β levels decreased to 23.2pg/ mL from 84 pg/ mL; IL-6 decreased to 9.31 pg/ mL from 46.78 pg/ mL, while IL-18 was reduced from 123 pg/ mL to 42 pg/ mL on treatment with punicalagin at a dose of 75 mg/ kg. These results suggest effective down-regulation of the inflammatory mediators by punicalagin.

DISCUSSION

Several studies on ICH have suggested that oxidative stress and inflammatory responses critically contribute to brain oedema and tissue injury [5]. Accumulating data show that inhibition of neuroinflammation and free radical-induced oxidative stress are beneficial in ICH [6]. In the present study, it was found that systemic administration of punicalagin at doses of 25, 50 and 75 mg/kg prior to induction of experimental ICH effectively improved BBB integrity and brain tissue histology, and reduced brain oedema and tissue injury.
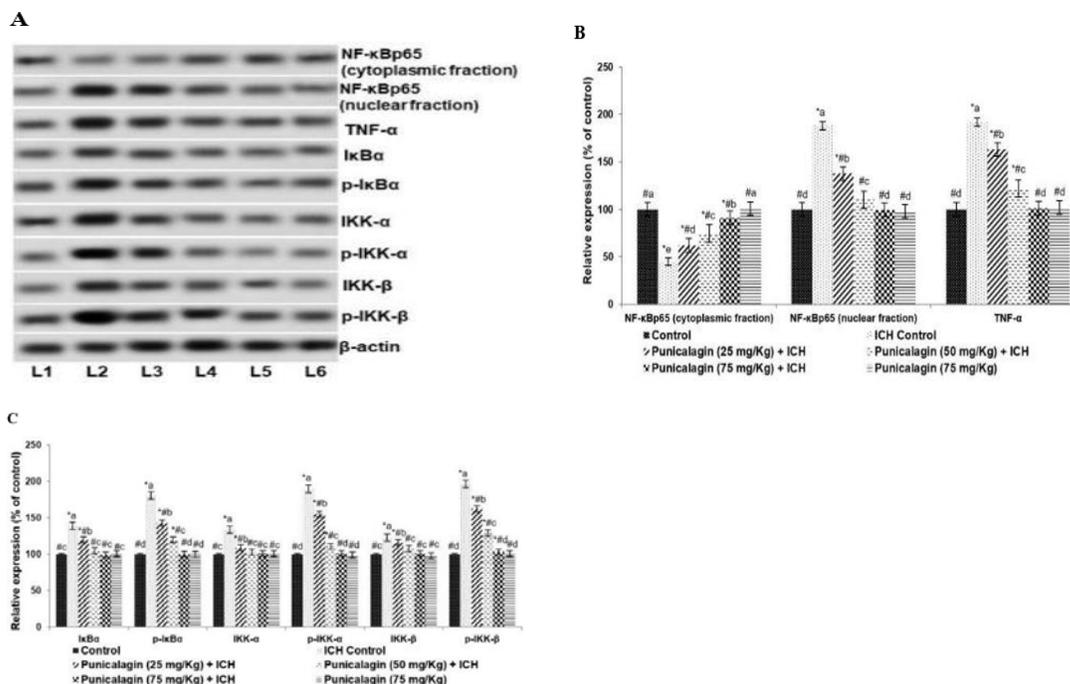
Figure 7: Effect of punicalagin on NF-κB- mediated inflammatory responses. Representative protein blot of test proteins (a). Expressions of proteins relative to control (b and c), with control expressions set at 100 %. Values are presented as mean ± SD (n = 6); *$p < 0.05$, vs control; #$p < 0$ vs ICH control; a-e represent significant differences ($p < 0.05$) amongst different experimental groups. L1: Control; L2: ICH Control; L3: punicalagin (25 mg/kg) + ICH; L4: punicalagin (50 mg/kg) + ICH; L5: punicalagin (75 mg/kg) + ICH; L6: punicalagin (75 mg/kg)
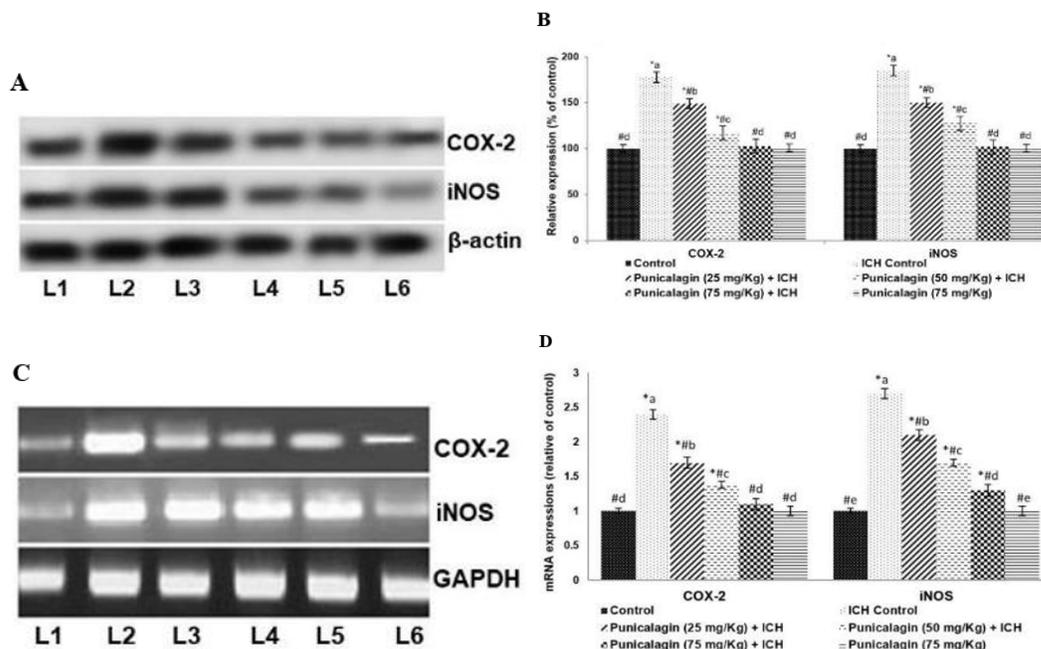
Figure 8: Effect of punicalagin on expressions of COX-2 and iNOS. (a) Representative immunoblot; (b) relative expressions of proteins; (c) mRNA expressions of COX-2 and iNOS; (d) relative mRNA expressions with respect to control. Values are presented as mean ± SD, n = 6; *$p < 0.05$, vs control; #$p < 0.05$, vs ICH control; a-e represent significant differences ($p < 0.05$) amongst different experimental groups. L1: Control; L2: ICH control; L3: punicalagin (25 mg/kg) + ICH; L4: punicalagin (50 mg/kg) + ICH; L5: punicalagin (75 mg/kg) + ICH; L6: punicalagin (75 mg/kg).
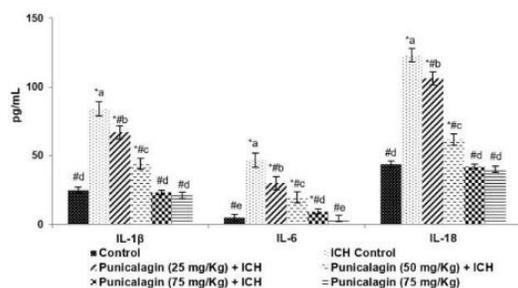
Figure 9: Effect of punicalagin on the levels of inflammatory cytokines. Values are presented as mean ± SD, n = 6. $p < 0.05$ as determined by one-way ANOVA followed by DMRT analysis; *$p < 0.05$, vs control; # $p < 0.05$, vs ICH control; a-e represent significant differences ($p < 0.05$) amongst different experimental groups. L1: Control; L2: ICH Control; L3: punicalagin (25 mg/kg) + ICH; L4: punicalagin (50 mg/kg) + ICH; L5: punicalagin (75 mg/kg) + ICH; L6: punicalagin (75 mg/kg)

The integrity of the BBB is pivotal in maintenance of homeostasis in the central nervous system. Disruption of the BBB integrity is regarded as a major event leading to ICH-associated brain injury [20]. The observed improvement in BBB integrity and reduction in brain oedema in the study indicate the protective effects of punicalagin.

Oxidative stress has been well documented as a major contributor to brain injury in ICH [21]. Moreover, ICH-induced injury causes the breakdown of various cells including RBCs, resulting in increased generation of ROS. Excessive production of ROS subsequently leads to oxidative stress and depletion of antioxidant reserves including glutathione. Reactive oxygen species-induced oxidative stress promotes inflammatory responses that further aggravate brain injury [3].

There were significantly up-regulated Nrf2 expression in the nuclear fraction, along with enhanced HO-1 and NQO1 expressions in the cytosol 24h following ICH. This could be a measure of the innate defence mechanisms for combating oxidative stress. One of the major regulators of the cellular antioxidative responses is Nrf2. The Nrf2/ARE signaling exerts a wide range of cytoprotective effects including antiapoptotic and anti-inflammatory responses [22]. Enzymes such as COX-2 and iNOS are regulated by Nrf2/ARE signaling. Under various environmental stress conditions such as oxidative stress and inflammation, Nrf2 is activated [23]. Thus, the increases in the expressions of Nrf2 observed in ICH could be attributed to the multi-fold increases in ROS levels following ICH.

Under stress conditions, Nrf2 gets activated, separates from Keap1 protein in the cytosol, and gets translocated to the nucleus where it regulates the expressions of downstream target genes [24]. Heme oxygenase-1 (HO-1) protein is an important protein downstream of Nrf2 [13]. It exerts antioxidant effects against ROS-induced oxidative stress. Punicalagin treatment resulted in a substantial increase in the expressions of Nrf2, NQO1 and HO-1. The activities of Nrf2/ARE-regulated antioxidant enzymes (SOD, CAT and GPx), and levels of glutathione which were reduced markedly following ICH, were significantly enhanced on punicalagin treatment. Punicalagin-mediated increase in antioxidant status was consistent with decreased ROS and MDA levels, indicating decreased oxidative stress. These observations suggest that punicalagin-mediated decrease in oxidative stress and enhanced antioxidant status could be due to its direct antioxidant effects, or due to an increase in Nrf2 /ARE/ HO-1 signaling. Induction of HO-1 has been found to improve the integrity of BBB and also reduce neurological impairments [9].

Neuroinflammation is involved in the pathogenesis of ICH-induced brain injury which causes cerebral oedema and tissue damage [3]. It is known that ICH-induced neuroinflammation is associated with raised levels of various pro-inflammatory cytokines including IL-1β, IL-6 and IL-18. The activation of neutrophils, macrophages and microglia which occurs following ICH synergistically contributes to the secretion of large amounts of inflammatory mediators such as cytokines and chemokines (TNF-α, IL-1β and IL-8). These pro-inflammatory factors further aggravate secondary brain tissue injury [4].

Several lines of evidence suggest that activation of NF-κB, a key transcription factor that controls the expression of several inflammatory cytokines, occurs within minutes after the ICH injury. Nuclear factor-κB remains inactive in the cytosol under normal physiological conditions, and it is bound to inhibitors of NF-κB- Inhibitor kinase (IκBs). However, once stimulated, the inhibitor subunit of IκB gets activated upon phosphorylation by IκB kinase (IKK) complex which comprises IKK-α and β kinases [25]. Inhibitor kinase (IκB), on activation gets rapidly degraded [26], leading to dissociation of NF-κBp65 which, in its free state, is translocated to the nucleus. Nuclear Factor-κBp65(NF-κBp65) then initiates the transcription of key down-stream genes including TNF-αand cytokines IL-1β and IL-6 [25].

The enhanced expression of nuclear NF-κBp65 levels along with up-regulated TNF-α COX-2 and iNOS expressions as observed 24h after induction of ICH indicate the activation of NF-κB signaling. The raised serum levels of cytokines (IL-1β, IL-6 and IL-18) reflect activation of NF-κB signaling. In this study, systemic administration of punicalagin resulted in marked down-regulation of the phosphorylation levels of regulatory proteins of NF-κB pathway i.e. IκBα, IKKα and IKK-β, signifying inhibition of the pathway. This suppression of regulatory kinases by punicalagin could have positively contributed to the down-regulation of NF-kB activation as reflected in reduced nuclear levels of NF-κBp65. Tumor necrosis factor (TNF)-α, iNOS and COX-2 expressions were suppressed by punicalagin, which further suggests inhibition of NF-κB signaling. Furthermore, serum levels of the cytokines IL-1β, IL-6 and IL-18 were significantly decreased on punicalagin treatment.

CONCLUSION

Punicalagin administration regulates HO-1/Nrf2/ARE signaling and NF-κB signaling-mediated inflammatory responses. These findings suggest that punicalagin may have beneficial effect as a potent drug candidate for the management of ICH. However, more investigations at the molecular level are needed on the effects of punicalagin.

DECLARATIONS

Acknowledgement

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. All the authors have equally contributed to this study. Fuchi Zhang, Kang Wu, and Jincao Chen designed this study, carried out laboratory works, collected and analyzed the data, and also prepared the manuscript. Xiaolin Wu and Can Xin also contributed to collecting and analyzing the data. Minghui Zhou and Jin Lei, also contributed to laboratory studies and in revising this manuscript.

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