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Research Article
Neuroprotective Activity of Icariin Against Hypoxic-ischemic Brain Injury in Neonatal Rats

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Abstract

Background and Objective: Hypoxic-ischemic brain damage (HIBD) results in various neurological disorders including cerebral palsy, epilepsy as well as stroke. The current study was undertaken to investigate the neuroprotective activity of icariin (IC) in hypoxic-ischemic brain damage (HIBD) in a neonatal rat model. **Materials and Methods:** Totally 327 day (P7) old rat pups were randomly chosen and divided into 4 groups as sham control (no ligation nor hypoxia), hypoxic-ischemia (HI) model in which pups are subjected to unilateral (left) carotid artery ligation and followed by exposure to hypoxia (8% O_2 and 92% N_2). Whereas, IC 100 or 200+HI group Pups received 100 or 200 mg kg^{-1} of IC orally (o.p) for 7 days prior to HI induction. **Results:** Pre-treatment with IC for 7 days significantly abolished the increased cerebral infarct size, edema, apoptosis, lipid peroxidation and inflammatory markers as well as improved the antioxidant status. Moreover, the expression of a protein involved in an inflammatory cascade like nuclear factor kappa b p65 subunit (NF-κB p65) and tumor necrosis factor alpha (TNF-α) are markedly down-regulated on administration with 100 or 200 mg of IC. **Conclusion:** Taking together that IC (200 mg) can effectively protect the brain damage and could be used as neuroprotective agent against HIBD patients along with standard neuroprotective drug to enhance the neuroprotection.

Key words: Neurological disorders, icariin, neuroprotective drug, cerebral palsy, hypoxic-ischemic, neuroprotective agent, brain damage

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hypoxic-ischemic brain damage (HIBD) is a common complication induced by perinatal as phyxia in infants and kids which often results in disabilities and even death[1]. Epidemiological studies have shown that one in every 500 infant born is affected by HIBD[2], which also trigger series of irreversible brain damage and results in various neurological disorders including cerebral palsy, epilepsy[3]. Pathophysiology of HIBD is very complicated and is still not fully uncovered. However, excitotoxicity, energy failure, oxidative stress, apoptosis and inflammation are reported to be a key contributor to HIBD[4,5]. Recently, many scientists are focused on the direct relationship between oxidative stress, inflammatory response against HIBD[1,6]. Moreover, currently the usage of anti-convulsants, stem cell transplantation and hypothermic treatments are recommended for treating HIBD. Nevertheless, the above treatment did not show better results in all HIBD patients[7]. Hence, the need of potent anti-ischemia/reperfusion agents are in high demand owing to increased mortality and morbidity related to cerebrovascular diseases as well as lack of effective therapies against cerebrovascular diseases[8].

Icariin (IC) is a naturally occurring prenylflavonoid (glycoside) is the major active phycomponent of Genus *Epimedium* (herba or brevicornum) which belong to family Berberidaceae. Plants/herbs of Epimedium is commonly used in traditional medicine to improve bone and muscle strength, sexual strength and reverse numbness especially in China and Korea[9]. The IC is reported as phytoestrogen that can easily pass through the blood-brain barrier (BBB) and can effectively act as a neuroprotective agent[10,11]. Since it's a phytoestrogen it can also improve bone density and act as anti-osteoporotic agent[12]. The IC exhibit a wide range of pharmacological properties including antioxidant, anti-inflammatory, immune-modulatory, aphrodisiac and cardioprotective properties[13,14].

Recently, icariin is reported to enhance motor and cognitive function in a Traumatic Brain injury rat model[15]. Another study conducted by Xiong and others have highlighted that icariin can significantly attenuate cerebral-reperfusion injury by inhibiting inflammatory response and thus showcase its neuroprotective property[16]. However, till date, no studies were conducted to explore the neuroprotective activity in neonatal rats against hypoxic-ischemic brain damage (HIBD). Based on the above-mentioned antioxidant, anti-inflammatory properties of IC, so the present study was designed to explore the neuroprotective activity of icariin (IC) in hypoxic-ischemic brain damage (HIBD) in a neonatal rat model by evaluating the cerebral infarct area/size, edema, apoptosis, lipid peroxidation and inflammatory markers as well as antioxidant status.

MATERIALS AND METHODS

Experimental animals: A total of 32 neonatal rat (pups-SD) of mixed sex were purchased (with mother rat) from Laboratory animal center (Lab animal, Hebei, China) and maintained at Animal house (Hebei Medical University). Neonatal rats (pups) were maintained at 23-24°C (55-60% humidity) with 12/12 light and dark cycle and allow to feed on rat pellet and water (*ad libitum*). All the animal experimental protocols were approved by an Ethical committee member board of Hebei General Hospital affiliated to Hebei Medical University (ECB/HGH-0112/002/18) and conducted by adhering the guidelines put forth by the National Science Academy Guidelines of China for the Use and Care of experimental animals. This study was carried out the animal house/laboratory at Hebei Medical University (Hebei General Hospital) from March, 2017-April, 2017.

HIBD induction or HI insult: After one week of pre-treatment (Saline or IC) with respective drugs, all the neonatal rats (P15) were subjected to HI insult (except the sham group) as indicated previously by Zhu *et al.*[17] with slight modifications. All the neonatal rats (n = 32) were initially anesthetized with diethyl-ether or isoflurane. A small incision made at the left neck to expose the left carotid artery and dissected between the double ligatures and sutured using 4-0 silk sutures. After the recovery period (2 h), all the pups were exposed to hypoxia by placing pups in a hypoxia chamber (8% oxygen and 92% Nitrogen) for 2 h and maintained at 37°C using a water bath. Finally, all the neonatal rats were transferred back to their respective dams and immediately treated with either saline or IC as one-time post-treatment regimen. In case of sham-control neonatal rats undergone midline, incision and exposed to carotid artery but not ligated nor exposed to hypoxia, they were only exposed to normal atmospheric condition.

Experimental grouping: A total of 32 pups were maintained at laboratory condition for 7 days and utilized for this study. All the 7 days old neonatal rats were divided into 4 groups with 8 rats in each. During HI insult 2 rats in the HI group were dead (since no pre-treatment). Sham-control neonatal rats received only saline without any ligation nor hypoxia. While HI group neonatal rats were pups are subjected to unilateral (left) carotid artery ligation and followed by exposure to hypoxia (8% O_2 and 92% N_2) and administrated with only saline. Whereas, IC 100 or 200+HI group neonatal

rats received either 100 or 200 mg kg^{-1} of IC orally (o.p; gastric intubation) for 7 days prior to HI induction as well as one post-treatment dose after HI induction.

Sample preparation: Next day (P16) after HI induction, all the neonatal rats were euthanized using sodium pentobarbitone and the skull was opened to remove the whole brain (weighed bot dry and wet) and cerebral cortex were gently removed and portion of the cerebral cortex were stored at -80°C for morphological analysis (infarct area) and the remaining portion are homogenized using Tris-HCl buffer and followed by followed by centrifugation at 1000 g for 10 min and the resultant supernatant (stored at -80°C until use) were used for various biochemical and molecular analysis.

Morphological analysis

Cerebral infarct size (Neuronal deficit): As mentioned before the portion of the cerebral cortex were dissected into 2 mm coronal slice using ultra-microtome and stained in a 0.1% triphenyl tetrazolium chloride (TTC) staining solution for 10 min at 37°C in a dark condition and then incubated with 4% paraformaldehyde for overnight[17]. The coronal sectional was picturized using a digital camera (Nikon, Japan) and the infarct size/area is quantified using image-analyzing software (Image-Pro-Plus 6; Media Cybernetics: MD, USA).

Cerebral edema: The degree of cerebral edema was evaluated using a gravimetric method based on brain water content as indicated previously[18]. Based on the dry and wet weight the water content (edema) can be calculated using below equation:

$$\text{Cerebral edema (water content)} = \frac{\text{Wet weight} - \text{dry weight}}{\text{Wet weight}} \times 100\%$$

Biochemical parameters

Cerebral antioxidant and lipid peroxidation products: The activity of cerebral antioxidants like catalase (CAT), glutathione peroxidase (Gpx) and superoxide dismutase (SOD) and the levels of lipid peroxidation products like malondialdehyde (MDA) were measured using a commercial kit (Shanghai Yantuo Biotechnology, Shanghai, China) based on supplier's specification.

Apoptotic markers: The activity of cerebral caspase-3 and caspase-9 was assayed using commercial rat specific ELISA kit from Beyotime International., Biotechnology (Jiangsu, China) based on manufacturers procedure.

Cerebral inflammatory markers: The supernatant of cerebral tissue was used to extract the nuclear and cytosolic fraction using nuclear and cytosolic extraction kit from Guge Biotechnology (Wuhan, China). Then the levels of various cerebral inflammatory markers like Interleukin one beta (IL-1β), Interleukin six (IL-6) and Tumor necrosis factor alpha (TNF-α) were measured in cerebral cytosolic fraction using commercial ELISA kit provided by NeoBioscience Technology Co., (Beijing, China). Whereas, the concentration of nuclear factor kappa b p65 subunit (NF-κb p65) an active NF-κb subunit was measure in cerebral nuclear fraction using from ELISA NF-κb p65 transcription factor assay kit from Abcam (Cambridge, UK).

Immunoblot: The protein levels were estimated using BCA protein assay kit from Bio-Rad laboratories (CA, USA) after treating with lysis buffer solution. An equal volume of protein (50 μg in each well) was uniformly separated using 12% SDS-PAGE apparatus and electro-transferred onto polyvinylidene difluoride (PVDF) membrane. Followed by incubation of membrane with primary antibodies for overnight at 4°C. Primary antibodies: Rabbit polyclonal anti-NF-κb p65 (1:1000), rabbit monoclonal anti-TNF-α (1:800 dilution) and rabbit monoclonal anti-β actin (1:1200 dilution) a standard/control. The unbound antibodies are removed by washing with PBS (twice) and followed by the addition of secondary antibody-rabbit polyclonal anti-horseradish peroxidase (HRP) antibody (1:10000 dilution) and incubated for 1 h at 37°C. The protein bands in the PVDF membrane was developed using enhanced Chemiluminescence kit from Thermo Fisher Scientific Inc., (MA, USA). The developed protein bands were picturized and analyzed using image-analyzing software (Image-Pro-Plus 6; Media Cybernetics: MD, USA).

Statistical analysis: Values are presented as the mean±standard error of the mean (SEM) for 8 rats in each experimental group. Comparison between the experimental group was conducted by one-way analysis of variance (ANOVA) followed by Tukey's test using GraphPad Prism (ver:5; Graphpad Software Inc., CA, USA). A p-value less than 0.05 is deemed as the statistical different.

RESULTS

Efficacy of IC on cerebral cortex infarct area/size and edema: The cerebral infarct size (28%) and edema (80%) were significantly increased ($p<0.001$) in HIBD model (HI insult)

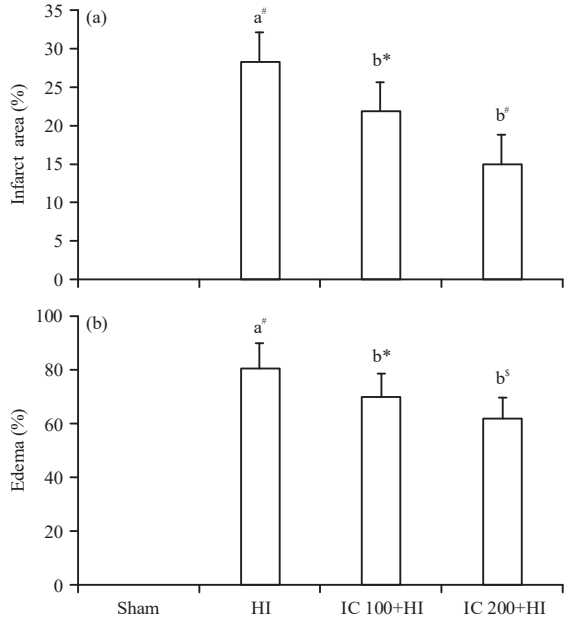

Fig. 1(a,b): (a) Efficacy of IC on cerebral cortex infarct area/size and (b) edema in sham control and experimental pubs

Values are expressed as the mean±standard error of the mean (SEM) for 8 rats in each experimental group, Probability (P) value: *p<0.05, $p<0.01, #p<0.001, where 'a' represents the comparison between control and HI model (Sham vs. HI), while 'b' represents the comparison between icariin (IC) and HI model (IC 100/200 vs. HI), HI: Hypoxic-ischemia model, IC: Icariin

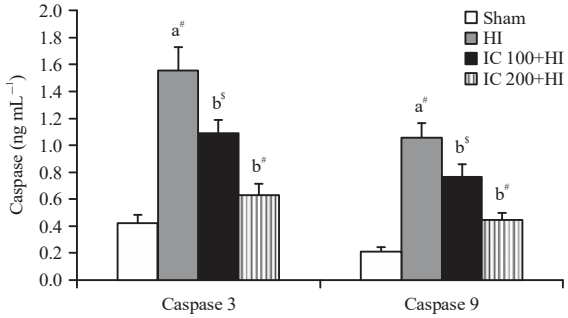

Fig. 2: Efficacy of IC on cerebral apoptotic markers (Caspase-3 and 9) in sham control and experimental pubs

Values are expressed as the mean±standard error of the mean (SEM) for 8 rats in each experimental group, Probability (P) value: $p<0.01, #p<0.001, where 'a' represents the comparison between control and HI model (Sham vs. HI), while 'b' represents the comparison between icariin (IC) and HI model (IC 100/200 vs. HI), HI: Hypoxic-ischemia model, IC: Icariin

neonatal rats as compared to sham control neonatal rats (Fig. 1). However, pre-treatment with 100 ($p<0.05$) or 200 ($p<0.001$) mg kg^{-1} of icariin considerably decreased cerebral infarct size (volume) or damage as well as edema (water content) on comparison with HI insulted neonatal rats.

Table 1: Efficacy of icariin on cerebral antioxidant and lipid peroxidation in sham control and experimental pubs

Groups	Gpx (μg mg^{-1} prot)	SOD (U mg^{-1} prot)	CAT (U mg^{-1} prot)	MDA-LPO (nmol mg^{-1} prot)
Sham	9.02±1.06	5.89±0.65	14.26±1.55	0.55±0.07
HI	6.86±0.81$^{a\#}$	3.95±0.50$^{a\#}$	8.34±0.97$^{a\#}$	1.20±0.13$^{a\#}$
IC 100+HI	7.63±0.90^{b*}	4.73±0.43^{b*}	11.87±1.20$^{b\$}$	0.82±0.09^{b*}
IC 200+HI	8.40±1.10$^{b\$}$	5.58±0.60$^{b\#}$	13.80±1.41$^{b\#}$	0.64±0.08$^{b\$}$

Values are expressed as the mean±standard error of the mean (SEM) for 8 rats in each experimental group, Probability (P) value: *p<0.05, $p<0.01,#p<0.001, where 'a' represent the comparison between control and HI model (Sham vs. HI), while 'b' represent the comparison between icariin (IC) and HI model (IC 100/200 vs. HI), Prot: Protein, HI: hypoxic-ischemia model, IC: Icariin, GPx: Glutathione peroxidase, SOD: Superoxide dismutase, CAT: Catalase, MDA: Malondialdehyde (Lipid peroxidation product). Unit (U for SOD): It corresponds to the amount required to inhibit 50% of pyrogallol autoxidation. Unit (U for CAT): It corresponds to the amount required to decompose hydrogen peroxide (H_2O_2)

Table 2: Efficacy of icariin on cerebral inflammatory markers in sham control and experimental pubs

Groups	NF-κB p65 (pg mg^{-1} prot)	IL-1β (ng mg^{-1} prot)	IL-6 (pg mg^{-1} prot)	TNF-α (ng mg^{-1} prot)
Sham	79.08±8.10	59.05±7.93	71.46±7.50	112.90±12.07
HI	156.43±14.00$^{a\#}$	133.95±16.50$^{a\#}$	140.34±15.70$^{a\#}$	194.72±20.65$^{a\#}$
IC 100+HI	119.94±10.50$^{b\$}$	96.07±10.22$^{b\$}$	103.82±12.91$^{b\$}$	153.37±17.00$^{b\$}$
IC 200+HI	87.74±9.05$^{b\#}$	67.50±8.60$^{b\#}$	82.00±9.11$^{b\#}$	124.22±13.76$^{b\#}$

Values are expressed as the mean±standard error of the mean (SEM) for 8 rats in each experimental group, Probability (P) value: *p<0.05, $p<0.01, #p<0.001, where 'a' represent the comparison between control and HI model (Sham vs. HI), while 'b' represent the comparison between icariin (IC) and HI model (IC 100/200 vs. HI)

Efficacy of icariin on cerebral antioxidant and lipid peroxidation: The results in Table 1 shows the efficacy of icariin on cerebral antioxidant and lipid peroxidation in sham control and experimental pubs. The activities of various cerebral antioxidant enzymes like SOD, Gpx and CAT were concomitantly decreased ($p<0.001$) with a considerable increase in the levels of lipid peroxidation products like MDA in HI-induced neonatal rats. Whereas, pups administered orally with IC (100 or 200) prior to HI induction showed notable improvement ($p<0.05$ or $p<0.001$) in the antioxidant activities (SOD, CAT and GPx) with decreased levels of lipid peroxidation products like MDA than HI insulted neonatal rats.

Efficacy of IC on cerebral apoptotic markers: To cross the anti-apoptotic activity of IC, the cerebral apoptotic markers in sham control and experimental pubs were evaluated (Fig. 2). An exponential increase ($p<0.001$) in the activities of caspase-3 (1.55 ng mL^{-1}) and 9 (1.05 ng mL^{-1}) were noted in HIBD model group on an equivalent with the sham control group. Nevertheless, oral intubation with IC (100/200 mg kg^{-1}) for 7 days before HI induction would significantly rarefy ($p<0.01$/$p<0.001$) the activities of caspase-3 and 9 as compared with the HI-induced neonatal rats.

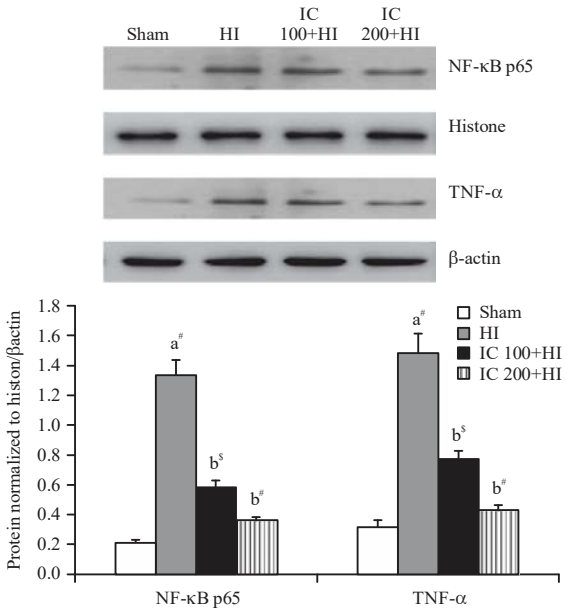

Fig. 3: Efficacy of IC on the protein expression of cerebral inflammatory markers (NF-κB p65 and TNF-α) in sham control and experimental pubs

Values are expressed as the mean±standard error of the mean (SEM) for 8 rats in each experimental group, Probability (P) value: *p<0.05, $^{\$}$p<0.01, $^{\#}$p<0.001, where 'a' represents the comparison between control and HI model (Sham vs. HI), while 'b' represents the comparison between icariin (IC) and HI model (IC 100/200 vs. HI), HI: Hypoxic-ischemia model, IC: Icariin, NF-κB p65: Nuclear factor kappa b p65 subunit, TNF-α: Tumor necrosis factor alpha

Efficacy of icariin on cerebral inflammatory markers: The data in Table 2 shows the efficacy of icariin on cerebral inflammatory markers in sham control and experimental pubs. On comparison with sham control group, the concentration of various cerebral inflammatory markers like NF-κB p65, IL-1β, IL-6 and TNF-α are significantly increased (p<0.001) in hypoxic-ischemia subjected neonatal rats. While, neonatal rats subjected to HI insults but were pre-treated with IC (100; p<0.01 and 200; p<0.001) exhibit better anti-inflammatory activity by considerably lowering the concentration of cerebral inflammatory markers like NF-κB p65, IL-1β, IL-6 and TNF-α as compared to HI insulted neonatal rats.

Efficacy of IC on the protein expression of cerebral inflammatory markers like NF-κB p65 and TNF-α: A marked upregulation (p<0.001) in the protein levels of NF-κB p65 and TNF-α were observed in HI subjected pups. However, neonatal rats supplemented with IC (100 and 200 mg) for 7 days before HI insult showcase significant down-regulation (p<0.001) in the protein expression levels of NF-κB p65 and TNF-α as compared to HI insulted group (Fig. 3).

DISCUSSION

Icariin (IC) is a flavonoid (glycoside) which is the major active phytocomponent of genus *Epimedium*. The IC exhibit a wide range of pharmacological properties including antioxidant, anti-inflammatory, immune-modulatory, aphrodisiac and cardioprotective properties[13,14]. The IC is reported as a phytoestrogen which could pass through BBB and can effectively act as a neuroprotective agent against cerebral ischemia model[10,11,16]. But this is the very first attempt to examine the neuroprotective activity in neonatal rats against hypoxic-ischemic brain damage (HIBD).

Current study results showed that pre-treatment with IC (100 and 200 mg kg^{-1}) for 7 days prior HI induction would significantly abolish the increased cerebral infarct size, edema, apoptosis, lipid peroxidation and inflammatory markers as well as improved the antioxidant status. Moreover, the expression of proteins involved in inflammatory cascade including NF-κB p65 subunit and TNF-α are markedly down-regulated on administration with 100 or 200 mg of IC. Cerebral infarct size/area was evaluated to check the degree of neuronal or brain damage (neuronal deficit) in cerebral hypoxic-ischemic condition[1]. Also, the cerebral edema was calculated based on water content, which also acts as an indicator of BBB rigidity[19,20]. Hence, for the present study, both cerebral infarct area and edema were assessed to check the effect of IC on the protection of brain damage and BBB integrity. The cerebral infarct size and edema were significantly elevated after HI insult due to damage in neural cells attributing to altered BBB and increased oxidative stress. But on pre-treatment with 100 or 200 mg kg^{-1} of icariin dramatically protected the neonatal rat brain owing to neuroprotective and antioxidant activity and thus significantly reduced the cerebral infarct size and edema. Similarly, Liu *et al.*[5] demonstrated that treatment with icariin could considerably lower the neuronal deficit score (infarct size) as well as edema (brain water content) in a focal cerebral ischemia-reperfusion rat model. In addition, icariin is reported to protect brain injury by significantly abolishing the infarct size and edema in middle cerebral artery occlusion mouse model[10].

Increasing number of evidence showed that neonatal brains are highly susceptible to oxidative stress (increased free radical production) induced brain damage/injury owing to its high polyunsaturated fatty acid (PUFA) content increased oxygen demand, low myelinization[3]. Previously, it has been clearly indicated that oxidative stress is one of the major contributors for hypoxic-ischemia induced HIBD and hence to

cross-check the antioxidant efficacy of IC the various cerebral antioxidant enzymes and lipid peroxidation products (MDA) were measured. In HI insulted group the activities of various cerebral antioxidant enzymes like SOD, Gpx and CAT were concomitantly decreased with increase in the levels of lipid peroxidation products like MDA were observed due to increased free radical generation. While neonatal rats exposed to HI and pre-treated with IC showed significant improvement in the activities those cerebral antioxidants with decreased MDA levels due to potent free radical scavenging activity (mainly due to a free hydroxyl group in IC) and antioxidant property[13]. Moreover, Li *et al.*[21] have demonstrated that treatment with an increased concentration of icariin could considerably improve the antioxidant activity with a marked decrease in LPO levels in mice cerebral ischemia/reperfusion model.

Apoptosis is a programmed cell death, which is regulated by a family of protease enzyme (protein) including cysteine-aspartic proteases cysteine aspartases (Caspases). Ample amount of studies has indicated that apoptosis is one the major contributor for cerebral brain damage or injury in hypoxic-ischemic brain damage (HIBD) in a neonatal rat model[22,23]. Wang *et al.*[24] reported that caspase-3 and 9 play a crucial role in the early phase of HIBD, where caspase-9 will activate the caspase-3 to execute apoptosis. Therefore, to cross-check the anti-apoptotic activity of IC, the cerebral apoptotic markers in experimental pubs were evaluated. A pronounced increase in the activities of caspase-3 and 9 was noted in HIBD model group. In contrary, treatment with IC would significantly inhibit the activities of caspase-3 and 9 and thus IC display its potent anti-apoptotic activity in HIBD neonatal rat model. Previously, Liu *et al.*[25] indicated that treatment with icariin could protect against apoptosis in hippocampal neuronal cells by regulating the MAPK pathway.

Oxidative stress and inflammation play a crucial role in HIBD. Since, oxidative stress triggers an inflammatory response by stimulating microglial cells, which in turn release various pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α[26,27]. Current results showed that the levels of cerebral inflammatory markers like NF-κB p65, IL-1β, IL-6 and TNF-α were significantly increased in HI insulted rats as it triggers the oxidative stress and subsequently results in an inflammatory response in the form of release of various pro-inflammatory cytokines. But neonatal rats subjected to HI insults and pre-treated with IC exhibit better anti-inflammatory activity by considerably lowering the concentration of cerebral inflammatory markers like NF-κB p65, IL-1β, IL-6 and TNF-α attributing to its anti-inflammatory activity[13].

To confirm the above results, the protein expression of cerebral inflammatory markers like NF-κB p65 and TNF-α were quantified by western blot technique to confirm its exact mechanism. The protein levels of NF-κB p65 and TNF-α were significantly upregulated in HI subjected pups. However, neonatal rats supplemented with IC (100 and 200 mg) for 7 days before HI insult showcase significant down-regulation in the protein expression levels of NF-κB p65 and TNF-α. Xiong *et al.*[16] highlighted the icariin treatment could effectively attenuate cerebral ischemia-reperfusion injury by suppressing the inflammatory cascade through inhibiting or blocking the activation of NF-κB (release of active nuclear NF-κB p65 subunit). Moreover, icariin is also reported to exhibit better neuroprotective activity in LPS induced rat model by decreasing the protein expression of TNF-α and Il-1β [28]. The above result also endorses that IC protect the brain damage by suppressing inflammatory response by downregulating NF-κB p65 (NF-κB signaling pathway) and thus proving its potent anti-inflammatory activity. The major limitation of this study was the lack of parameters related to mitochondrial dysfunction and TUNEL assay to confirm the anti-apoptotic property of IC.

CONCLUSION

It is concluded that both doses of IC (100 and 200 mg kg^{-1}) can effectively protect the brain damage by suppressing neuronal deficit (infarct area), apoptosis, oxidative stress and neuro-inflammatory response owing to its free radical scavenging/antioxidant, anti-apoptotic and anti-inflammatory activity. However, IC 200 g showed highest neuroprotective activity than IC 100 mg. However, further experiments are required to confirm the exact mechanism (signaling pathway) by which IC exert its neuroprotective property in hypoxic-ischemic brain damage in a neonatal rat model.

SIGNIFICANCE STATEMENT

The outcome of this animal study clearly portrait the neuroprotective activity of icariin (100 and 200 mg) against hypoxic-ischemic brain damage in a neonatal rat model. Result of this animal study would helps the scientist (pharmacist) to develop a novel synergetic neuroprotective agent by combining standard neuroprotective drug (Lamictal) with icariin to enhance the neuroprotection in HIB patients.

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