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NEUROTHERAPEUTIC EFFECT OF MANGIFERIN AGAINST HYPOXIC–ISCHEMIC ENCEPHALOPATHY IN NEONATAL RATS

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Abstract

Background: Hypoxic–ischemic encephalopathy (HIE) in perinatal condition is highly associated with mortality and several neurological disabilities. The present experiment was blueprinted to ascertain the protective efficacy of mangiferin (MF) against hypoxic–ischemic brain injury in neonatal rats.
Materials and Methods: Fourth six neonatal rats (pups) were randomly separated into four groups as a sham group that received only water (control; n=12), pups exposed to Hypoxic–ischemic (HI) insult (HI group-II; n=11), pups receiving 20 (MF 20+HI-III; n=11) or 40 (MF 40+HI-IV; n=12) mg/kg b. wt of MF dissolved in water via i.p. for 7 consecutive days as pretreatment regimen before HI insult as well as one-time post treatment after HI insult.
Results: MF pretreatment for seven days considerably attenuated the cerebral infarct size, edema level, lipid peroxidation (MDA), inflammatory markers (TNF-α, IL-1β, IL-6, and NF-p65 subunit) as well as greatly ameliorated the antioxidant activities of catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GSH) and thereby maintaining the redox balance. The number of viable neuronal count (nissl bodies) was exponentially increased on administration with both the dosages of MF. Both the dosages showed substantial protection against HIE, however, 40 mg of MF exhibit superior neuroprotection in equivalence with 20 mg of MF.
Conclusion: The results of the present study distinctly proving the beneficial effect of MF against HIE by concomitantly improving neuronal count and antioxidant status. Hence, we recommend MF might be used for treating neonatal HIE with some standard drugs.

Key words: Hypoxic–ischemic encephalopathy, Mangiferin, Antioxidant, Inflammation, Neuroprotective

Introduction

Hypoxic-ischemic encephalopathy (HIE) is the condition that highly affects newborn infants after perinatal asphyxia and results in numerous neurological disorders. Statistical data indicated that 55-60% of infants with inborn HIE might suffer from severe disabilities such as epilepsy, mental retardation, cerebral palsy or even die (Kurinczuk et al., 2010; Long & Brandon, 2007). The etiology for HIE are associated with lack of oxygen supply to the brain by interrupting blood flow and finally leads to various metabolic derangements like an energy failure, excitotoxicity, oxidative stress (lipid peroxidation), inflammation, delayed cell death due to alteration in calcium homeostasis (Panickar & Anderson, 2011; Lai & Yang, 2010). Even though some treatment regimen are available for cerebral ischemia (HIE) such as anticonvulsants (antiepileptic), hypothermic treatments, and stem cell transplant, but none of them showed better results against HIE (Sun et al., 2015). Ample evidence inferred that impaired antioxidant with elevated free radicals (oxidative stress), and the neuroinflammatory response is considered to be the major reason for HIE (Sadeghnia et al., 2012; Slemmer at al., 2008). Therefore, a plant drug that might specifically lower the oxidative stress and inflammation with anti-apoptotic effect probably lowers the rate of HIE in the neonatal model. Moreover, several studies had proven that polyphenols might show some beneficial effect against HIE due to their antioxidant and anti-inflammatory properties (West et al., 2007; Rong et al., 2013).

Mangiferin (MF) is a C-glucosyl-xanthone (polyphenol) which are commonly found in the fruits, bark, leaves of *Mangifera indica* L. (Anacardiaceae family) and other plants. MF has unique structural features due to binding of glucose moiety with condensed aromatic ring, which make its higher bioavailability (Mirza et al., 2013). *Mangifera indica* which are often used in traditional medicine for its numerous biological properties (Garrido-Suárez et al., 2014) exhibit a wide array of pharmacological activities like antioxidant, anti-inflammatory, anti-apoptotic, antimicrobial, immunomodulatory, memory enhancer, gastroprotective and neuroprotective properties (Carvalho et al., 2007; Campos-Esparza et al., 2009; Márquez et al., 2010). Moreover, MF possesses the iron-complexing capacity and thus suppresses free radical generation and concomitantly lowers the neuronal lipid peroxidation (Pardo-Andreu et al., 2005). MF has been reported to decrease Ca^{2+} overload and thereby alleviate glutamate excitotoxicity. Furthermore, MF can readily cross the blood-brain barrier (BBB) to reach the brain to exert its pharmacological activities (Amazzal et al., 2007). Moreover, the neuroprotective effect of MF in rats pups against hypoxic–ischemic brain injury (HIBI) or HIE were yet to prove. Owing to its diverse biological activities of MF, it would be worth to elucidate whether MF exerts neuroprotection against HIE by assessing cerebral infarct area, edema as well as various oxidative stress and inflammatory markers in neonatal rats.

Materials and Methods

Chemicals

MF (1, 3, 6, 7-Tetrahydroxyxanthone C_2-β-D-glucoside), 2, 3, 5-tritriphenyl-tetrazolium chloride, glutathione were purchased

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from Sigma-Aldrich (St. Louis, MO, USA). Pyrogallol, sodium citrate, potassium dichromate, heparin, Tris HCL, formaldehyde, isopropanol, chloroform, pentobarbital sodium was supplied by Lingjin Co. Ltd, Shanghai, China. All the other chemicals used in the current study were of analytical grade.

Experimental Animals

Seven days old (P7) Sprague-Dawley (SD) rat pups are weighing 14-18 g of either sex, were procured from animal house of Central Hospital Zhumadian City, China. Pups were maintained at constant room temperature (35-37 ℃) and had free access to food and water (*ad libitum)*. The ethical committee of Zhumadian Central Hospital (CPCSEA24/2015), approved the experiment, which were according to guidelines from the National Institutes of Health Guide for the Care and Use of Laboratory Animals to minimize the animal suffering.

Procedure for HI Insult and Treatment Regimen

Induction of hypoxia-ischemia was carried out as proposed previously by Vannucci et al. (1998) with mild changes. Fourteen days old neonatal SD (P14) rats were anesthetized with 3% isoflurane and maintained by 1.5%. The incision was made in the midline near the neck to isolate the unilateral common carotid artery were dissected and ligated by silk surgical suture and returned to respective dams. After 2 h of the recovery period, pups were transferred from dams to hypoxia chamber that were maintained at 37 ℃ with the assistance of water bath and exposed to 8% O_2 & 92% N_2 by an oximeter (Pro OX Controller, Biospherix, NY, USA) for 2 h. After the hypoxic induction, all pups were taken back to their respective dams and immediately water or MF dissolved in water was administered intraperitoneally (i.p.) in HI or MF group as one-time post treatment. Based on a preliminary dose-dependent study in HI model (data not shown), the doses of 20 and 40 mg/kg of MF were chosen for the present study and administered i.p. for 7 days before induction of HI. In sham control pups, the carotid artery was not ligated and was exposed to the normoxic condition (21% O_2).

Fifty-two rats were utilized for the present study out of which six rats were either dead during HI induction or not active and thus, only forty-six pups were completely involved. Those neonatal pups were divided into four groups as a sham group (control; n=12), which received only water, pups subjected to HI insult (HI group-II; n=11), pups receiving 20 mg/kg b. wt of MF dissolved in water via i.p. (MF 20+HI-III; n=11), pups receiving 40 mg/kg b. wt of MF dissolved in water via i.p. (MF 40+HI-IV; n=12) for 7 days before HI insult (pretreatment). During induction, the time interval was properly maintained to nullify the statistical errors. All the pups were euthanized on the 16th day (P16) by injecting (i.p.) with pentobarbital sodium and brains were removed immediately. Each group was again subdivided into two groups, of which one sub-group brains were fixed in 4% paraformaldehyde for morphological studies (nissl staining) and other sub-group brains for separating cerebral cortex and then homogenized (pooled) by using lysis buffer and used for biochemical analysis.

Morphological Analysis
Infarct Area

The 2, 3, 5-triphenyl-tetrazolium chloride (TTC) staining method was employed to measure the cerebral infarct area as reported elsewhere (Li et al., 2012). In brief, brain was sectioned into four pieces (1 mm) and stained with 0.1% solution of TTC at 37°C for 7 min and rinsed with phosphate buffered saline (PBS) and fixed with 10% formaldehyde. The infarct area was determined using an image analyzing system (ImageJ, Bethesda, MD, USA) and the values are expressed in percentage. Infarct area was determined using the formula,

Infarct area = (total infarct area/whole brain section area) X 100%.

Cerebral Edema

The cerebral edema was analyzed using previously reported method with a few changes (Mdzinarishvili et al., 2007). To evaluate the cerebral edema initially, the wet and dry weight of brain samples was weighed. Cerebral edema was calculated using the formula:

The cerebral edema = (wet weight – dry weight)/wet weight X 100%.

Cresyl Violet Staining (Nissl)

The fixed brain samples were sectioned (4 µm) and those sections were hydrated with 1% toluidine blue at 40 ℃ for 15 min, followed by hydration using double distilled water and dehydrated. The sections were embedded in paraffin and finally staining with cresyl violet. The images of nissl stained cortex are picturized by the computerized imaging system (Imaging-Pro-Plus software; Media Cybernetics, Inc, MD, USA) to analyze nissl bodies (viable neuron).

Biochemical Analysis
Oxidative Indexes

The activities of various antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) as well as the levels of lipid peroxidation product - malondialdehyde (MDA) were assayed and measured by using commercial kit method based on the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively. The Bradford method was employed to assess the protein concentration.

Assay of Inflammatory Markers

The proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in cerebral homogenate were assessed by commercially available ELISA kits (sandwich enzyme immunoassay) based on the manufacturer's instructions (Thermo Fisher Scientific Inc, MA, USA). Similarly, the concentration of the NF-κb free p65 subunit in the nuclear fraction (Cell Biolabs Nuclear/Cytosolic Fractionation Kit) of the cerebral cortex were determined by ActivELISA (Imgenex, San Diego, USA) kit.

Statistical Analysis

The values were explicated as mean ± standard deviation (SD). The changes between control and experimental rats were determined by one-way analysis of variance (ANOVA) followed by Tukeys test using the SPSS 21 version (SPSS Inc., Chicago, USA). A p-value less than 0.05 was denoted as statistically significant.

Results

Impact of MF on Cerebral Infarct Area

The cerebral infarct area in the rat brain was detected by staining with TTC (Fig. 1). To manifest the neuroprotective effect of MF against HIE, we measured the infarct size in experimental and control neonatal pups. The sham stained section displayed a uniform stained region, thus indicating that blood flow was not interrupted. The cerebral infarct area of rats in the HI group was 29.45 ± 2.64%, which is highly significant as compared with the sham control group. Thus, indicating the deleterious events of HI by showing the unstained area as white patches. The cerebral infarct area of both treated MF group (20 and 40 mg/kg) were 19.32 ± 2.05 and 14.79 ± 2.10 respectively, thus effectively ameliorated hypoxia-induced brain damage by displaying less unstained area with the predominantly stained area.

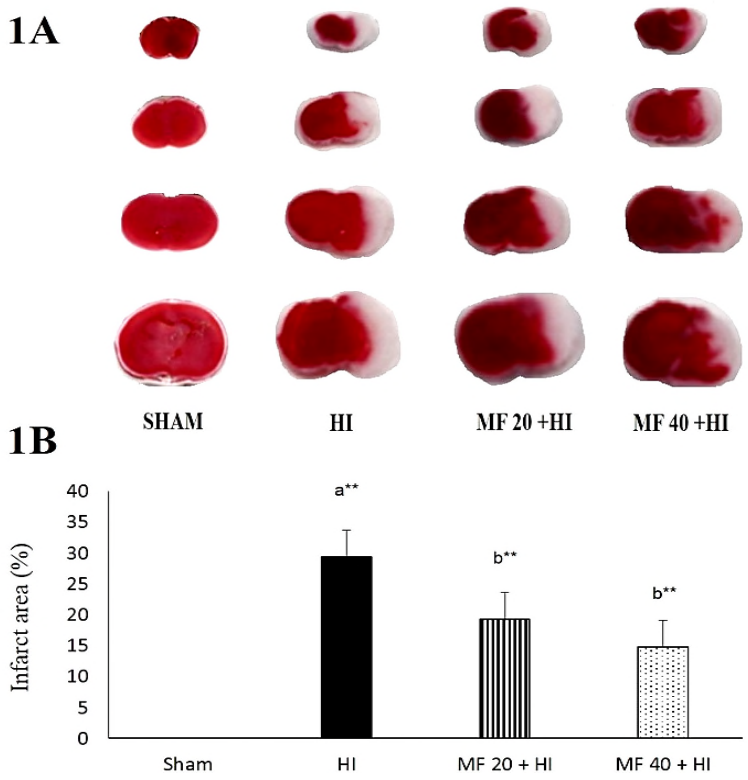

Figure 1: Effects of MF on the cerebral infarct area in sham control and experimental neonatal rats. The pictorial representation of coronal section stained with TTC (1A), showed normal sham control (n=12) with a uniform stained region, whereas HI group (n=11) coronal section portrait few unstained area (white patches) which indicates the ischemic area. However, on treatment with both dosages of MF (n=11; 12) showed less unstained area with the predominantly stained area. The area of cerebral infarct was expressed in percentage (1B). Statistical significance (p value): **p<0.01, *p<0.05 (a) compared with the control group, (b) compared with the HI-induced group.

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Impact of MF on Cerebral Edema

The cerebral edema was evaluated based on the wet and dry weight of sham control and experimental pups, which are illustrated in Fig 2. There were no edematic changes in sham control rats. In the case of HI insult group (85.01%) a substantial escalation was noted on equivalence with the sham group (control). MF (20 and 40 mg/kg) injected groups exhibit the cerebral edema as 71.22% and 63.87%, respectively, thus proving its neuroprotective potential by lowering the edema (water) levels significantly vs. HI group.

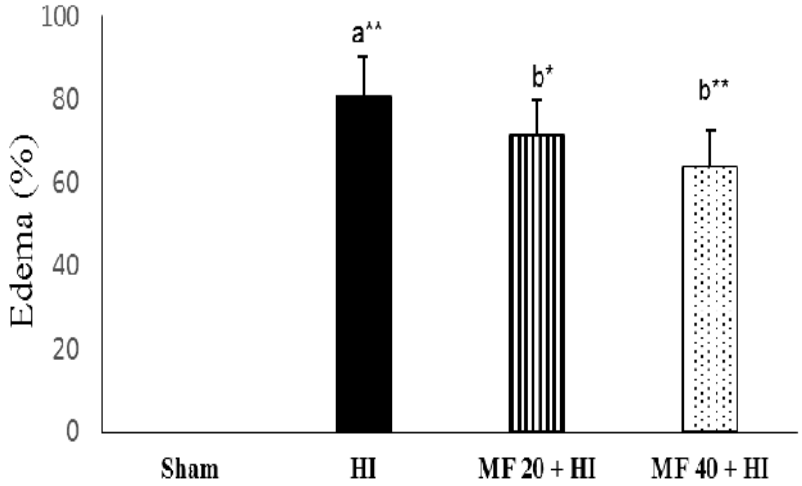

Figure 2: Effects of MF on cerebral edema in sham control (n=12) and experimental neonatal rats (n=12).
Statistical significance (p value): **p<0.01, *p<0.05 (a) compared with the control group, (b) compared with the HI-induced group.

Impact of MF on the Cerebral Section with Nissl Staining

Figure 3 portraits the histopathological changes in a cerebral section of pups in sham control, and experimental neonatal rats. Transection of sham control (n=12) depicts the normal structure with several prominent nissl bodies (3A). Pronounced alterations in neuronal morphology were observed in HI-induced rats (n=11) with pyknotic neurons, interstitial edema and less visible nissl bodies (3B). However, on pretreatment with both doses of MF (n=11; 12) concomitantly improved the number of nissl bodies (3C and D) on equivalence with HI group (data not shown), which indicates its neuroprotective potential.

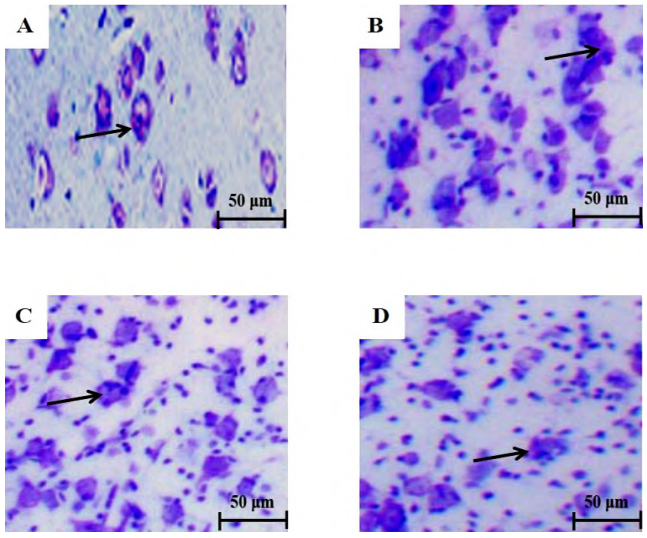

Figure 3: Effect of MF on the cerebral section with nissl staining in sham control and experimental neonatal rats. Transection (400 x) of the sham control portrait normal structure with several prominent nissl bodies (3A). Enormous changes in neuronal morphology were noticed in HI-induced rats with pyknotic neurons and few visible nissl bodies (3B). However, upon supplementation with both doses of MF concomitantly improved the number of nissl bodies (3C and D). Scale bar- 50 µm

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Impact of MF on Oxidative Indexes like LPO and Antioxidants

Table 1 represents the effect of MF pretreatment on the levels of lipid peroxidative products like Malondialdehyde (MDA) and antioxidants in control and experimental neonatal rats. To assess whether MF pretreatment can lower the brain oxidative stress, by elevating the antioxidant levels as well as lower the lipid peroxidative products (MDA) in HI-induced neonatal pups. The cerebral MDA level was considerably raised ($p < 0.01$) in hypoxia group. In the case of antioxidant activities of SOD, CAT, and GSH were substantially suppressed in the HI group ($p < 0.01$) on equivalence with the control group. Compared with HI group, MF injected (20 and 40 mg/kg) pups exhibit a marked decline ($p < 0.05$) in the levels of LPO as well as showed an exponential increase in the activities of SOD, CAT, and GSH.

Table 1: Effect of MF on the activities of brain antioxidants and lipid peroxidation in sham control and experimental neonatal rats

Group	**LPO** (nmols/mg protein)	**SOD** (U/mg protein)	**CAT** (U/mg protein)	**GSH** (µg/mg protein)
Sham control	0.32±0.04	3.52±0.42	67.67±7.25	7.98±0.89
HI	0.85±0.09 a**	2.63±0.26 a**	51.20±4.74 a**	6.05±0.51 a**
MF 20 + HI	0.67±0.08 b**	3.06±0.18 b*	57.56±6.03 b*	6.81±0.40 b*
MF 40 + HI	0.44±0.06 b**	3.36±0.26 b**	62.23±5.34 b**	7.59±0.90 b**

Values were expressed as mean ± S.D for 11 or 12 rats in each group.
Statistical significance (p value): **p<0.01, *p<0.05 (a) compared with the sham control group, (b) compared with the HI injured group.
One unit (U) of SOD activity was defined as the amount of enzyme required for 1 mg tissue proteins in 1 ml of a reaction mixture SOD inhibition rates to 50% as monitored at 550 nm.
One unit (U) of CAT activity was defined as 1 mg tissue proteins consumed 1 µmol H_2O_2 at 405 nm for 1 second.

Impact of MF on the Concentration of Inflammatory Markers

The anti-inflammatory effect of MF evaluated against HI induction were addressed by determining the concentrations of various pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and nuclear factor NF-p65 subunit of NF-κB (Table 2). A considerable elevation ($p<0.01$) in the concentration of those inflammatory markers in brain tissue of HI-induced rats was observed on equating with sham control rats. HI-induced pups treated with MF (20 and 40) dramatically ($p < 0.01$) diminished the concentrations of these pro-inflammatory cytokines, thus proving its anti-inflammatory efficacy.

Discussion

Although, the number of studies indicated the neuroprotective potential of MF in various models (Lemus-Molina et al., 2009; Kasbe et al., 2015), till date, none had proved the protective effect of MF against HIE condition in neonatal rats. Hence, we sought to assess the protective efficacy of MF against HIE by evaluating the cerebral infarct size, edema, lipid peroxidation (MDA), antioxidant status and inflammatory markers in neonatal pups. Typically, the neuronal damage during ischemic condition is evaluated by assessing the area/size of brain infarction by TTC staining method (Yousuf et al., 2009). The fundamental principle of the TTC staining technique is based on the activation of mitochondrial cytochrome oxidase enzyme in the viable (neuronal) cells to produce a red color. In the case of dead cells, this TTC stain will not react and hence the cell (tissue) appears white. The outcome of TTC staining of sham control cerebral section showed uniform stained region, whereas HI group coronal section portrait few unstained areas (white patches) which indicate the ischemic area. However, on treatment with both, dosages of MF showed less unstained areas with a predominantly stained area that clearly depict the neuroprotective nature of MF by elevating the presence of viable that are affiliated with rich red color stain than HI insulted pups. Similarly, Prabhu and his colleagues (2012) inferred that treatment with MF significantly reduced the infarct area in a rat model.

Table 2: Effect of MF on the activities of inflammatory markers in sham control and experimental neonatal rats

Group	TNF-α (ng/mg protein)	IL-1β (ng/mg protein)	IL-6 (pg/mg protein)	NF-p65 (pg/mg protein)
Sham control	110.34±11.93	62.73±4.08	75.23±9.01	81.27±4.51
HI	298.28±21.37 a**	172.90±14.46 a**	255.38±22.82 a**	197.72±17.34 a**
MF 20 + HI	214.34±15.82 b**	121.64±16.06 b*	174.19±16.92 b*	134.92±12.48 b**
MF 40 + HI	152.45±19.94 b**	88.11±10.03 b**	101.46±11.53 b**	99.42±11.66 b**

Values were expressed as mean ± S.D for 11 or 12 rats in each group.
Statistical significance (p value): **p<0.01, *p<0.05 (a) compared with the sham control group, (b) compared with the HI injured group.

Cerebral edema is a major complication related to hypoxic-ischemia, as edema might elevate intracranial pressure with enlargement of brain tissue, which finally leads to cell death (Panickar & Anderson, 2011). It has been hypothesized that during HI condition, the blood flow was interrupted and contribute to an electrolytic imbalance that leads to increased movement of water (increases Aquaporin 4 expression) by altering BBB integrity and thus leads to edema (Wang et al., 2013). To validate the above statement, we found a marked increase in edema level (water content) in HI insult group on equivalence with the sham control group. The cerebral edema levels in MF injected group were substantially reduced, thus proving its neuroprotective potential owing to attenuating oxidative stress and thus ameliorated BBB integrity. Our results are well supported by Tan and his coworkers (2011), also pointed out that MF can effectively reduce the cerebral infarct size in a cerebral ischemic rat model by improving the antioxidant status and thus preserving the integrity of BBB.

The degree of ischemic neuronal injury was determined by checking the number of normal motor neurons using cresyl violet staining procedure based on the presence of nissl bodies (Telles et al., 2014). Enormous changes in neuronal morphology were noticed in a hypoxic-ischemic group with pyknotic neurons as well as few visible nissl bodies, thus indicating the neurotoxicity in HI insult condition with reduced viable neuronal cells. Nevertheless, upon administration with MF concomitantly ameliorated the number of visible nissl bodies (data not included), thus confirm its neuroprotective nature of MF. Previously, Mangifera extract is reported to attenuate ischemia-induced neuronal loss by improving neuronal count, which were evidenced by cresyl violet stained brain section (Sánchez et al., 2001).

Epidemiological studies showed the direct relationship between oxidative stress and HIE. Therefore, we checked the antioxidant status by assessing the activities of enzymic antioxidants like SOD, CAT, GPx as well as oxidative stress marker-Lipid peroxidation products like MDA. A growing body of evidence has demonstrated that neonatal brain tissues are highly susceptible to free radical damage (oxidative stress) because of increased oxygen consumption, lower endogenous antioxidant levels as well as increased lipid content which eventually results in neuronal and glial cells damage (Sadeghnia et al., 2012; Fukui et al., 2002). The enzymic antioxidant activities like SOD, CAT, and GSH were substantially suppressed in HI insulted pups due to elevated oxidative stress. MF (20 and 40 mg/kg) pretreatment for 7 days exhibits an exponential increase in the activities of SOD, CAT, and GSH by activating transcription factor NF-E2-related factor-2 (Nrf2) a key regulator of endogenous anti-oxidant and thus preserving BBB function. We also checked the mRNA expression of Nrf2, where the expression is markedly upregulated upon treatment with MF (data not presented). Several experimental models had shown that MF would act as a protective agent by alleviating oxidative stress by upregulating Nrf2 related genes and thus improved the overall antioxidant status (Mahmoud-Awny et al., 2015; Sethiya, & Mishra, 2014). Also, MF was shown to increase endogenous antioxidants such as SOD, CAT and glutathione in rat and cell line models (Rajendran et al., 2008; Amazzal et al., 2007).

Meanwhile, the cerebral lipid peroxidation product like MDA level was significantly increased in hypoxia group, owing to increased free radical generation that damaged the fatty acids and phospholipids present in the cell membrane and results in increased neuronal damage. Lipid peroxidation is the major contributor for decreased neuronal count, which we found during morphological analysis (nissl stain). Seven days pretreatment successfully protected the neuronal cells by increased endogenous antioxidant system and thus the levels of MDA were significantly reduced. A review from Mirza et al., 2013, also indicated that the antioxidant activity of MF is majorly contributed by catechol moiety (iron complexing capacity), which inhibit the production of free radicals by inhibiting the conversion of Fe^{2+} to Fe^{3+} complex and that would be the reason for maintaining the redox balance by elevating antioxidant status in MF treated pups. Supplementation of MF for 7 days probably eases oxidative stress and lipid peroxidation as well as protects BBB in schizophrenia mice model (Rao et al., 2012).

Existing evidence suggest that impaired antioxidant with elevated free radicals (oxidative stress) and the neuroinflammatory response is considered to be the major reason for HIE (Sadeghnia et al., 2012; Slemmer at al., 2008). Both the events (oxidative stress and inflammation) are interconnected with one another. It is well documented that TNF-α triggered by oxidative stress during HIE is the critical process, which initiate NF-κB transcription factor (specifically nuclear factor NF-p65), which upregulate the upstream genes related to pro-inflammatory cytokines like IL-1β, IL-6, TNF-α (Li et al., 2013; Block et al., 2007). Even our ELISA test also ensured that the levels of all pro-inflammatory markers and nuclear factor NF-p65 were significantly elevated. Administration of MF for 7 days concomitantly lower those inflammatory markers by inactivating NF-κB transcription factor as well as mitigating oxidative stress. Our results are corroborated by the results of Kasbe and his coworkers (2015), they also pointed out that MF 20 and 40 mg/kg showed anti-inflammatory activity by lowering the production of various pro-inflammatory cytokines in brain tissue of aluminum-induced neurotoxicity. Few animal studies had pointed out that MF probably inhibits TNF-α induced IκB degradation and thus subdue the translocation of NF-kB to DNA (activation), thereby preventing the transcription of genes involved in the production of several pro-inflammatory cytokines (Garrido-Suárez et al., 2010). Moreover, Márquez and his coworkers, (2012) also proved that MF can effectively suppress inflammatory response by inhibiting the activation of microglial cells and neutrophil infiltration and thus negatively regulate the expression if NF-κB and subsequent pro-inflammatory cytokines.

We had some limitation in our studies as we checked the neuroprotective effect in neonatal rats for only 7 days (chronic study) but long term effect is yet to be explored. Also, we do not include some apoptotic parameters to check the anti-apoptotic effect pertained to neuroprotective effect of MF. However, in future, we are planning to continue our work by checking the impact of MF by assessing staining, BBB integrity, expression of HIF-1α, endothelial factors as well as a behavioral test to explore the detailed mechanism behind the neurotherapeutic effect of MF in neonatal rats.

Conclusion

Taking together, MF showed a neuroprotective effect against HIE induced neonatal rats by decreasing cerebral infarct size, edema as well as various oxidative stress and inflammatory markers. Also, it improved the viable neuronal count by increasing the activities of antioxidant enzymes. Both the dosages (20 and 40 mg/kg b. wt) showed substantial protection against HIE, however, 40 mg of MF exhibit superior neuroprotection in equivalence with 20 mg of MF. Therefore, MF might be used for treating neonatal HIE with some standard drugs or standard treatment procedure. Also, in future, the precise mechanism underlying the neuroprotective effect of MF has to be explored.

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