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ORIGINAL ARTICLE

Erythropoietin protects the subventricular zone and inhibits reactive astrogliosis in kaolin-induced hydrocephalic rats

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Abstract

Purpose To elucidate the potential role of erythropoietin (EPO) as a neuroprotective agent against reactive astrogliosis and reducing the thinning rate of subventricular zone (SVZ) in kaolin-induced hydrocephalic rats.

Method Thirty-six ten-week-old Sprague-Dawley rats were used in this study. Hydrocephalus was induced with 20% kaolin suspension injected into the cistern of thirty rats and leaving the six rats as normal group. The hydrocephalic rats were randomly divided into hydrocephalic and treatment group. The treatment group received daily dose of recombinant human erythropoietin (rhEPO) from day 7 to day 21 after induction. The animals were sacrificed at 7 (only for hydrocephalic group) and 14 or 21 (for both groups) days after induction. Brain was removed and was prepared for histological analysis by hematoxylin and eosin staining as well as immunohistochemistry for 4-HNE, GFAP, Iba-1, and Ki-67.

Results Histopathological analysis showed that animals treated with rhEPO had a reduced astrocyte reactivity displayed by lower GFAP expression. Hydrocephalic rats received rhEPO also displayed reduced microglial activation shown by lower Iba-1 protein expression. Exogenous rhEPO exerted its protective action in reducing astrogliosis by inhibiting lipid peroxidation that was documented in this study as lower expression of 4-HNE than non-treated group. The SVZ thickness was progressively declining in hydrocephalus group, while the progression rate could be reduced by rhEPO.

Conclusion Erythropoietin has a potential use for inhibiting lipid peroxidation, and reactive astrogliosis in hydrocephalic animal model. The reduced thinning rate of SVZ demonstrated that EPO also had effect in reducing the hydrocephalus progressivity. Further research is warranted to explore its efficacy and safety to use in clinical setting.

Keywords Erythropoietin · Hydrocephalus · Reactive astrocyte · Microgliosis · Subventricular zone · Lipid peroxidation

Introduction

Established treatment for hydrocephalus remains surgical diversion of CSF and elimination of its cause. No other measure can eliminate the need for CSF diversion for hydrocephalic patients. When the CSF diversion cannot be performed in the time being, an effort has to be made to reduce the negative effects of mechanical and biochemical changes to the brain. Despite the failure of system, there are pre-surgical conditions that play role in the successful rate of shunt surgery. Classic factors include pre-surgical condition of cerebral mantle thickness, the severity, and the length of the hydrocephalus. Cellular factors involved in pathophysiology of hydrocephalus are astrocyte reaction and microglial activation that impede the functional recovery. Periventricular astrogliosis in a form of hypertrophy and proliferation of astrocytes and microglia has been documented in several studies [1, 2]. Reactive

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astrocytosis and microglial activation are considered as normal immediate response of the brain to injury in acute phase. Chronic reaction may lead to the scar development that impairs axonal regeneration and remyelination [3]. Once the reactive astrocytosis is established, surgical measure will only slow it down temporarily and it will rise again afterward suggesting that the shunt placement fails to completely ameliorates or reverse this reaction in the long run [4].

Changes of the SVZ had also been observed but it had not been implied as one of the factors in surgical success rate. Hydrocephalus altered the organization and cytoarchitecture of SVZ and its neural stem cell activity. There was a reduction in proliferating neural stem cell (NSC), oligodendroglial precursor cell, and neurogenesis activity. It will be substantially diminished over time as hydrocephalus progresses [5–7]. Efforts had been made to address the pathological hallmark of hydrocephalus with limited success. Non-surgical therapy showed only partial or inconsistent results [8]. A more comprehensive therapy is endorsed.

Erythropoietin (EPO) is an endogenous glycoprotein hormone produced by multiple organs as a response to specific stimulus especially hypoxia. It exerts its neuroprotective activity by binding to EPO receptor (EPOR) that is expressed in neuron, astrocyte, microglia, and oligodendrocyte [9, 10]. EPO-EPOR complex activates the downstream signaling pathway to produce anti-inflammatory, antiapoptotic, growth factors, and antioxidant enzyme. Studies have suggested that exogenous EPO showed these neuroprotective properties in various non-hydrocephalic brain injury models and in vitro experiments.

To elucidate the neuroprotective property of EPO in hydrocephalus, we investigated whether exogenous EPO could reduce the reactive astrocytosis, activated microglia and maintain SVZ thickness.

Methods

Animal

The Institutional Animal Care and Use Committee of Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, approved the experiment protocol. Sprague-Dawley rats of 10 weeks age ($n = 36$) were obtained from the Experimental Laboratory of Universitas Gajah Mada, Yogyakarta, and randomly divided into three groups: normal control, hydrocephalus, and EPO-treated groups. They were housed in standard cages and provided with a normal 12-h dark/light schedule. Access to food and water was given with ad libitum. The rats in hydrocephalus group were sacrificed at 7th, 14th, and 21st days and the rats in EPO-treated group were sacrificed at 14th and 21st day after kaolin induction.

Hydrocephalus induction and EPO administration

Hydrocephalus was induced in 30 rats using percutaneous kaolin suspension injection into the cisterna magna as previously described [11]. In brief, anesthesia was accomplished by injecting the cocktail intravenously. The suboccipital area was shaved and cleaned with 70% ethanol and 10% povidone iodine. Rat was placed on a sponge support so that the neck could be flexed to open the foramen magnum. Slow injection of 0.05 ml of 20% kaolin suspension through foramen magnum was employed. All rats were observed until recovered from anesthesia and housed in a standard environment. Communicating hydrocephalus was expected to establish within 7 days after injection. Recombinant human EPO alpha (rhEPO) (Daewoong Pharmaceutical Company, Indonesia) was administered intraperitoneally at 5000/kg dose daily from day 7 after kaolin induction until the animal was sacrificed while the non-treated hydrocephalus rats received saline injection.

Hematoxylin-eosin and immunohistochemistry staining

The harvested brain was fixed for 48 h in 4% paraformaldehyde at room temperature. Then the brain was embedded in paraffin block and sliced in 5-μm sections. The paraffin sections were dewaxed, rehydrated, and stained with HE. For IHC staining, the paraffin sections were stained with mouse monoclonal antibody (Santa Cruz Biotechnology, Texas) for detecting the expression of 4-HNE (1:300 dilution), Ki-67 (1:500 dilution), GFAP (1:500 dilution), and Iba-1 (1:500 dilution) according to the manufacturer instruction. Images from slides were viewed at × 20 and × 40 magnifications under a Nikon H600L microscope, camera DS Fi2, and analyzed using the NIS Elements Basic Research imaging software (Nikon Corp, Japan). The results were scored using Immuno Reactive Score (IRS) as suggested by Remmele and Stegner [12].

Statistical analysis

Data was presented as mean±S.D. Statistical analysis for comparison among groups was performed using ANOVA. Statistical significance was considered for a P value of 0.05 or less. The test was performed using statistic software SPSS version 24 (IBM, Armonk, NY, USA).

Results

Effect of EPO on 4-hydroxynonenal expressions

To determine the involvement of lipid peroxidation in hydrocephalus pathophysiology, we examined its aldehyde

product among normal control, hydrocephalus, and EPO-treated groups. Immunohistochemical appearance of 4-HNE showed that lipid peroxidation process was involved in hydrocephalus (Fig. 1). The hydrocephalus group revealed a significantly higher 4-HNE expressions ($p < 0{,}001$) on day 7 (8.95 ± 2.98) and day 21 (11.53 ± 0.42) than normal control group (3.60 ± 0.99). In time series, 4-HNE expressions displayed a slight reduction in day 14 and bounced up in day 21 after kaolin induction (Fig. 2A). The expressions were most abundant in periventricular area, hippocampus, external capsule, and striatum. 4-HNE in EPO-treated group (2.80 ± 1.06) was significantly lower than hydrocephalus group ($p < 0.001$). It showed the ability of EPO to decrease lipid peroxidation process comparable to normal state after 7 and 14 days of daily dose administration.

Effect of EPO on reactive astrocytosis

Reactive astrocyte has been considered as the cellular pathological hallmark in hydrocephalus. To reveal the effect of EPO in astrocyte reactivity, we performed an immunohistochemical study for GFAP protein expression (Fig. 1). Hydrocephalus group showed a significantly higher GFAP expression ($p < 0.001$) than in normal group (2.97 ± 0.27). In hydrocephalus group, GFAP expressions were increasing from day 7 (7.38 ± 0.76) to day 21 (10.10 ± 0.10). In EPO-treated group, the GFAP expression was still significantly higher than normal group after 7 days of daily dose administration (5.50 ± 0.10). After 14 days of consecutive dose, the level (4.13 ± 1.33) was decreased approaching the level at normal control group (Fig. 2B). It showed that EPO had the ability to reverse the reactivity of astrocyte.

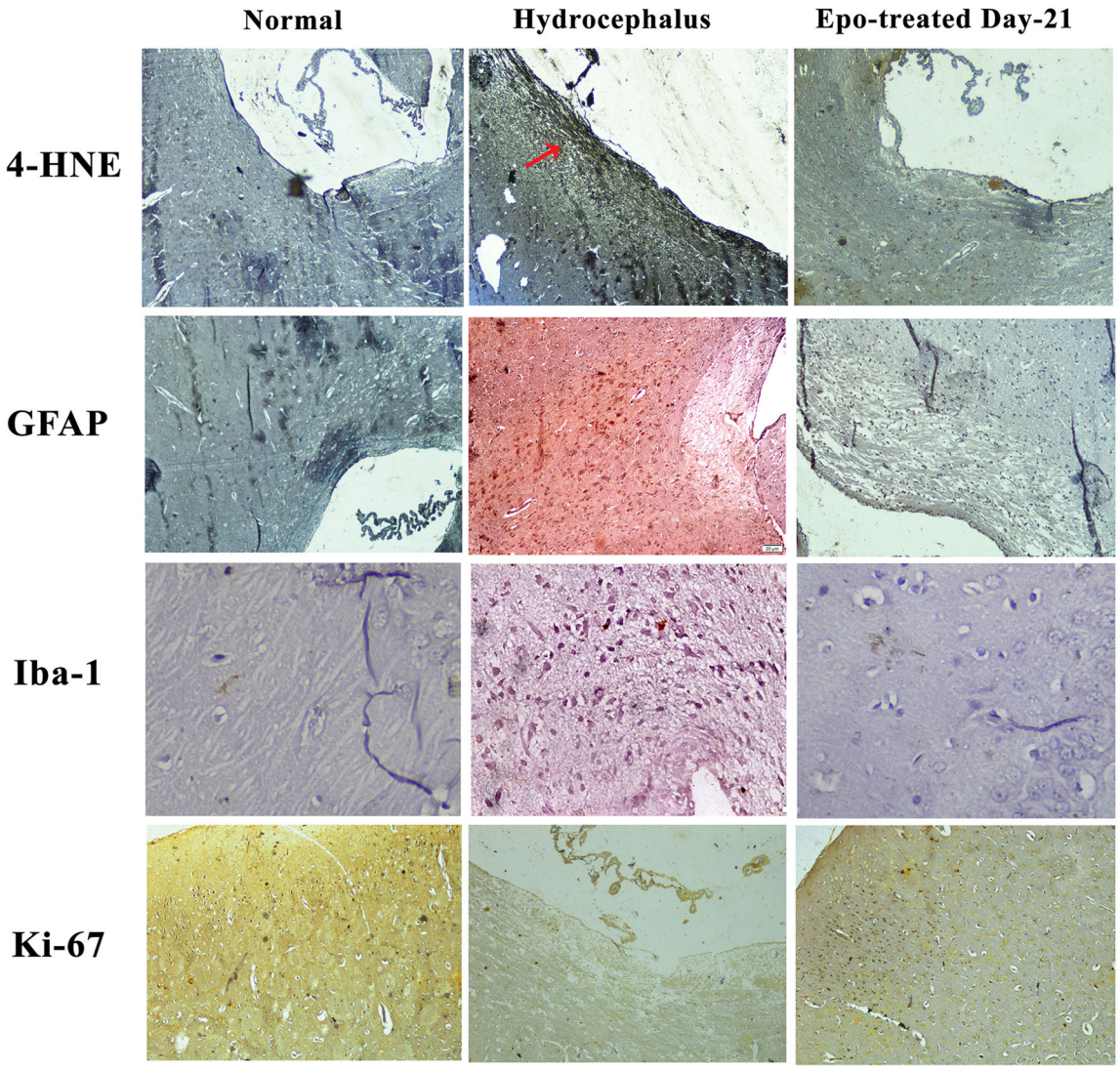

Fig. 1 The representative images of 4-HNE, GFAP, Iba-1, and Ki-67 in periventricular white matter

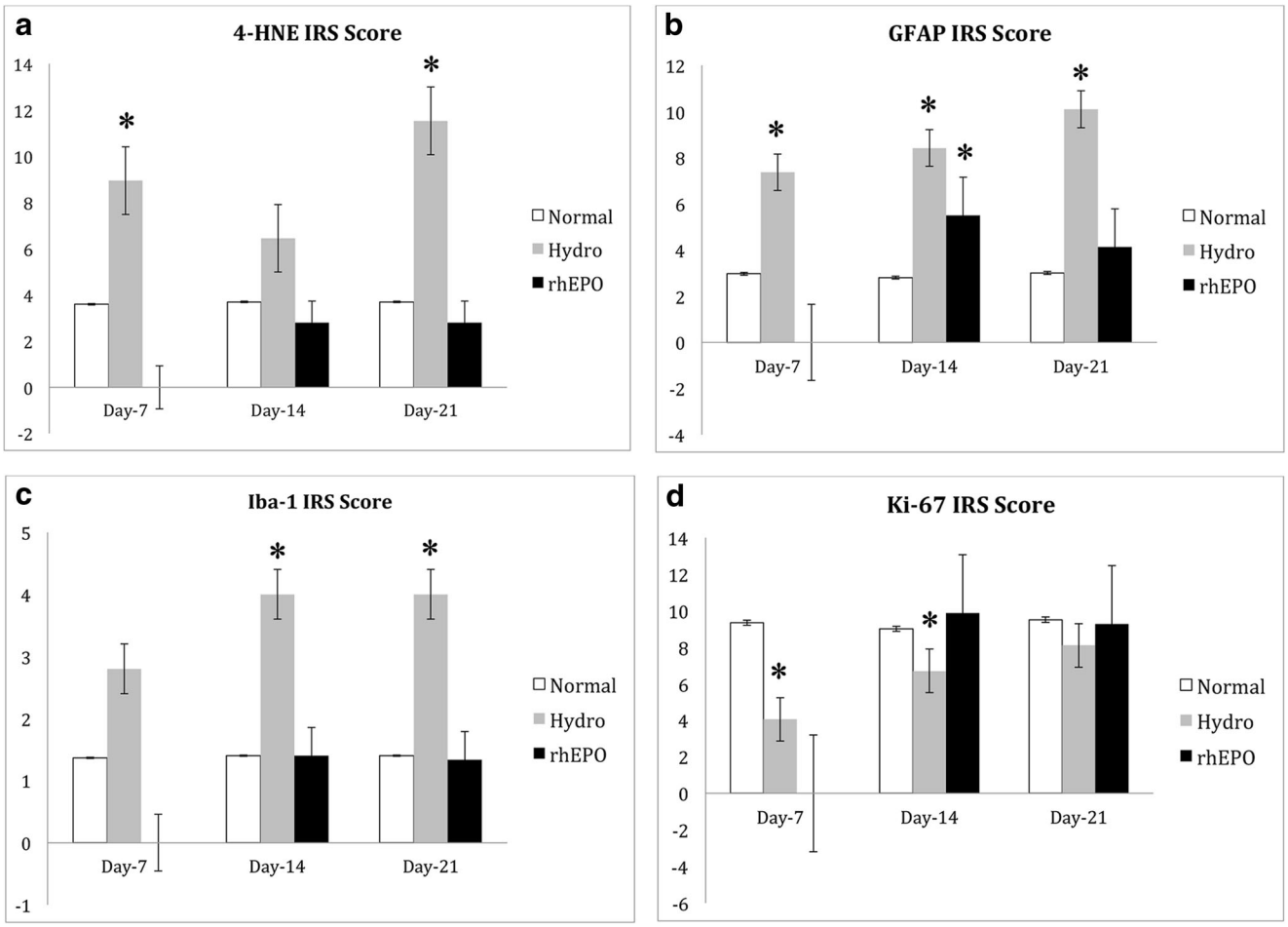

Fig. 2 The expressions of (A) 4-HNE, (B) GFAP, (C) Iba-1, and (D) Ki-67. All protein expressions were documented as IRS score. The difference between hydrocephalic or EPO-treated with normal group is considered significant when $p < 0.05$ (asterisk)

EPO inhibited microglial activation

Immunoreactive score for Iba-1 expression was counted to determine the activation of microglia. Increasing number of microglial activation appeared in the periventricular area, striatum, and white matter including external capsule after induction of hydrocephalus (Fig. 1). Expression of Iba-1 in hydrocephalus group day 14 (4.00 ± 0.91) and day 21 (4.00 ± 0.41) was significantly higher compared to normal group (1.37 ± 0.60) ($p < 0.001$). Administering daily dose of EPO dampened the microglial activation. The EPO-treated groups showed significantly lower score of Iba-1 in day 14 (1.40 ± 0.40) and day 21 (1.33 ± 0.42) after kaolin induction than hydrocephalus group ($p < 0.001$) (Fig. 2C). There was no difference of Iba-1 expressions between normal control group and EPO-treated group.

EPO reduced progressive thinning of SVZ and proliferation rate

SVZ was measured using calibrated micrometer from its border with ependymal line to its border with white matter at dorsolateral part of the lateral ventricle (Fig. 3A). This study documented that SVZ thickness decreased with the progression of the hydrocephalus. The rats in hydrocephalus group day 21 suffered from 41% reduction from normal the thickness. The proliferation activity as shown by Ki-67 expression (Fig. 1) significantly reduced to 50% at day 7 hydrocephalus group (4.05 ± 0.85) ($p < 0.001$). This activity was increasing at day 14 (5.20 ± 1.33) and -21 (7.07 ± 0.31) at hydrocephalus group but never reached the normal activity state (9.87 ± 0.88) (Fig. 2D).

The thickness of SVZ in rats that received EPO injection for 7 days was similar with the normal group. The ability of EPO to maintain the SVZ thickness was decreasing at day 21 post kaolin induction or day 14 after EPO injection. However, EPO demonstrated the ability to maintain proliferative activity. The EPO-treated group showed significantly higher score for Ki-67 at 7 (9.87 ± 0.31) and 14 days (9.27 ± 1.42) after EPO injection than the hydrocephalus group at day 7 and day 14 ($p < 0.001$) (Fig. 3B).

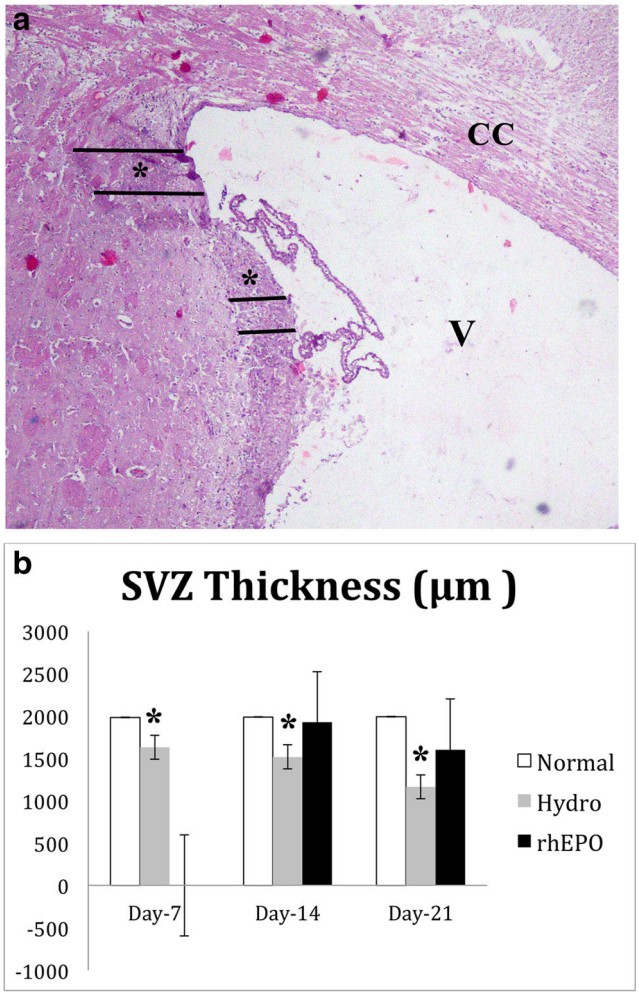

Fig. 3 The representative images of subventricular zone (asterisk) of lateral ventricle (V). CC represents corpus callosum. The thickness of subventricular zone is shown in black line. The results are presented as mean±S.D.

Discussion

There have been several methods to develop animal model of hydrocephalus that aimed to study their pathogenesis, pathophysiology, neurological alteration, and to test various surgical and non-surgical therapies. Obstructing CSF pathway using an irritating agent such as injecting kaolin to cisterna magna had been applied with high successful rate. In terms of technical and budget, the kaolin-induced hydrocephalus model in rat is inexpensive and simple. The mortality rate varied from 20% to 80% even in bigger animal such as cat [6, 13, 14]. In our study, the mortality rate was 40%. It is expected as we used 20% kaolin suspension. A lower mortality rate was reported in animal model that received 3% kaolin suspension. It assumed that higher kaolin concentration led to higher mortality rate [15].

To elucidate the effect of exogenous EPO in hydrocephalic rat, we used rhEPO as it has been used without species boundary in various pathologies. It exerts biological activity similar to endogenous EPO without any immunological issue. The dose applied in this study followed the proven effective dose from other studies [16]. In this study, we employed EPO at day 7 after the kaolin induction in which hydrocephalus had already developed in animal model. The daily dose was given as rhEPO had a short half-life of 8.5 h. The dose was proven effective in several CNS pathologies [17].

Multiple factors involved in pathophysiology of hydrocephalus-induced brain injury including mechanical and biochemical cellular alterations. First structure affected by mechanical injury is ependymal cells that appear as denudation, detachment, or loss of cellularity. In adult brain, ependymal cells play the role in proliferation and migration of precursor cells within SVZ through branched fractal structure that is known as fractone. It is an extracellular matrix that regulates the proliferation of neural stem cells by facilitating the infiltration of cytokines into the SVZ. Increasing ramification of fractones in hydrocephalus allows more inflammatory cytokines to enter the SVZ and reducing the proliferation [5]. In hydrocephalic fetuses, detachment of the lateral ventricles ependyma causes abnormal neurogenesis that is associated with the loss of germinal zone, disorganization of SVZ, and abnormal migration of neuroblasts into the ventricle [18]. Mechanical injury due to ventricle enlargement is also responsible for cerebral blood flow alterations in periventricular white matter at some point [19–22]. Decreasing cerebral blood flow triggers cellular alteration and hypoxic-ischemic cascade. Oxidative stress occurred in cellular level damaged the protein and lipid as the oxygen reactive species rising without sufficient “antioxydative” enzyme. Lipid peroxidation was documented in hydrocephalic [23, 24] and non-hydrocephalic ischemic studies [25, 26]. Lipid peroxidation process produced malondialdehyde (MDA), aldehyde by-product, which was found mostly in periventricular area. Del Bigio et al (2012) also reported the evidence of 4-HNE on the similar area but in a lesser degree. Similar result was observed in the present study but in higher degree. The expression of 4-HNE was increased from 300 to 400% in our hydrocephalus model. 4-HNE is the most toxic product of lipid peroxidation that may cause axonal damage and demyelination. It also involves in modulating downstream signaling [27]. Significant reduction of 4-HNE expressions was found following EPO treatment in our study. EPO protected the cell from oxidative stress by increasing the antioxydative enzyme such as superoxide dismutase (SOD), glutathione peroxidase, and catalase [28, 29]. Inhibition of nitric oxide formation was the other mechanism of EPO to protect the cell from oxidative damage [25].

Astrocyte and microglia reacts to several stimuli such as inflammation, stroke, hypoxic-ischemic, and hydrocephalus. Reactive astrocyte may exert protective or neurotoxic action. Protective actions of astrocyte and microglia are expected at

the early phase of injury via its ability to eliminate extracellular glutamate, to store and to supply adjacent cells with energetic compounds. Protective phenotype will slope down after 7 days after activation and the neurotoxic phenotype will take place [30]. The neurotoxic phenotype loses the protective effects, a loss of ability for synaptogenesis, produces inflammatory chemokine-cytokine and detrimental extracellular matrix which build up glial scar [31, 32]. Glial scar will inhibit remyelination process [33], axonal regeneration, and axonal sprouting [34]. It also produces chondroitin sulfate proteoglycan (CSPG), an extracellular matrix that plays role in demyelination process [35]. Several studies addressed these detrimental effects on neural regeneration and plasticity. Efforts were made to reduce or inhibit the astrogliosis process using surgical [4, 36] or non-surgical measures [8, 37] with different successful rates. Shunting only partially reduced the reactive astrocyte that continues to express an inflammatory phenotype in chronically shunted animal [4]. Suitable agent should be able not only to reduce the reactivity in early days but also able to modulate the inflammatory properties of the astrocyte and microglia. EPO has been proposed in some non-hydrocephalic studies [16, 38] and few hydrocephalic animal studies [39, 40]. Its ability to reduce reactive astrocyte and microglial activation in hydrocephalus has not been reported. This study reports the ability of exogenous EPO to reduce the GFAP protein, reactive astrocyte marker, and Iba-1 expressions, activated microglia indicator. The microglial response was significantly reduced in periventricular area after EPO treatment. The score for astrocyte and microglia in EPO-treated group were similar to normal group. Astrocyte and microglia modulate each other through chemokine and cytokine release. In the first 3 days, activated microglia (M1 phenotype) secretes inflammatory cytokine (TNF-α and IL-1β), which in turn activates astrocyte [31, 41]. Removing this cytokine cannot reverse the reactive astrocyte to the resting state. These findings showed that EPO might rather reduce its further activation than reversing the reactive state to resting or normal state.

The brain keeps the ability to repair itself throughout their life by maintaining their pluripotent neural stem cells in two specific regions known as subgranular zone (SGZ) and subventricular zone (SVZ). This self-repair process occurs in normal condition and as a response to pathological insults. Neurogenesis and oligodendrogenesis are two main responses that are promoted by the SVZ neural stem cells [42, 43]. During embryonic development of the brain, there are two source of neural precursor cells namely primary class precursor cells or radial glia (RG) that reside in ventricular zone (VZ) and secondary class or intermediate progenitor (IP) that reside in subventricular zone (SVZ). In the embryonic period, cells of VZ are developed directly from neural plate, which resembles primitive neuroephitelium cells. Progeny of the VZ become neuroblasts and leaves the VZ to migrate towards pial surface guided by RG. The proliferative activity of the VZ is rapidly declining following the second proliferation phase in the SVZ. The SVZ is exponentially prominent during the late prenatal development as the VZ disappears. This layer is conserved throughout adulthood to maintain brain cellular regeneration [44, 45]. Hydrocephalus altered its cytoarchitecture and cellular organization. Disorganized and diminishing SVZ were observed in animal model of hydrocephalus [5, 18]. The brain loses its self-repair capacity when the SVZ is diminished. Shunt placement may not help to regain the structural and functional recovery once the SVZ is effacing. The present study demonstrated that the SVZ suffered from progressive hydrocephalus. The thickness decreased over time similar to the previous studies. Factors affecting the SVZ came from mechanical stress due to ventricular enlargement and biochemical-cellular changes. Decrease proliferation may contribute to the thinning SVZ. The proliferation rate in present study was acutely decreased in 7 days after kaolin induction and started to show incremental increased in day-14 until day-21 but never reached the normal state. This pattern was similar to other study that showed an initial decrease of SVZ proliferation in 24 h followed by an increase in 14 day and started to decrease again by day 28 after insult [46]. Chronic hydrocephalus resulted in ~ 50% decreased proliferation rate [5]. EPO exhibited a capacity to prevent thinning of the SVZ and maintaining the proliferation rate. We hypothesized that EPO exerted its protective effect on SVZ through vasodilatation of the compressed periventricular capillary vessels, activating Akt signaling that promoted proliferation and differentiation, and suppressing the apoptosis [47]. Inflammatory cytokines, TNF-α, and Il-1, produced by microglia significantly reduced proliferation and survival of NSC in SVZ and hippocampal area [48]. The anti-inflammatory action of EPO on microglia was also suspected as the mechanism to prevent the detrimental effect of hydrocephalus to SVZ by reducing the cytokines production.

Careful note should be taken as EPO could only slow down the progression but not stop the effacement of the SVZ. Surgical measure should be performed as soon as possible. There is no time restriction for surgery after rhEPO administration. Our study suggested that continuing rhEPO administration might be beneficial to reduce the on-going astrogliosis process after CSF diversion. The proposed continuation time is 7–14 days after the surgery. When available, a longer half-life EPO can be used to reduce the daily dosing.

Conclusion

EPO showed the capacity to protect brain from hydrocephalus detrimental effect. It inhibited the lipid peroxidation, regulated astrocyte reactivity, and prevented microglial activation. The reduced thinning rate of SVZ demonstrated that EPO also had

effect in reducing the hydrocephalus progressivity. Further research is warranted to explore its efficacy and safety to use in clinical setting.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest.

Ethical approval All procedures involving animals performed in this study were in accordance with the ethical standards of The Institutional Animal Care and Use Committee of Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, where the study was conducted.

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