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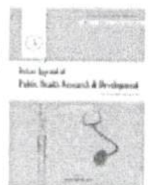

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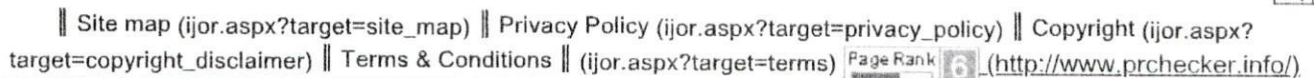
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Erythropoietin Potential as an Antiapoptotic Agent in Ischemic Stroke Using Unilateral Right Unilateral Common Carotid Artery Occlusion (RUCCAO) Model

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Ischemic stroke is insufficient or interrupted of blood flow to an area of the brain, typically caused by blockage of an artery and can result in brain damaged. This study was aimed to investigate the efficacy of Recombinant Human Erythropoietin (rHuEPO) as a neuroprotection (antiapoptotic agent) on rat with ischemic stroke induced by right common carotid artery occlusion. Animals were divided into five groups included sham group, ischemic stroke group and treatment group of rHuEPO. rHuEPO was administered intravenously once a day for 7 days at dose 1000, 5000, 10000 IU/Kg a week after induced by right common carotid artery occlusion surgery. The repair of brain damage was evaluated by Y maze for cognitive repair, ladder rung walking and forelimb asymmetry test for motor repair, measured on day 0, 1, 3 and 7. The result showed that treatment with rHuEPO significantly enhanced spatial memory at 5000 and 10000 IU/Kg in day 7 compare to ischemic group ($p = 0.0260$, $p = 0.0286$ respectively) and improved motoric function in day 7 compare to ischemic group ($p = 0.0064$, $p = 0.0102$ respectively). rHuEPO significantly reduce area infarct at 1000 IU 5000 IU and 10000 IU/Kg compare to ischemic group ($p = 0.0265$, $p = 0.0016$ $p = 0.0024$ respectively). Furthermore, there were significant differences on caspase 3 expression in each group.

Keyword: Ischemic Stroke; rHu Erythropoietin; Anti Apoptotic; Caspase 3

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1. Introduction

Ischemic stroke is greater than 70% of all acute strokes types and is a leading cause of death and disability in worldwide [1]. Ischemic stroke results in inhibition of the oxygen and nutrients supply to the brain resulting neuron death. The damage and neuronal death cause decrease in neurological function. This happens few minutes after the onset of ischemia, resulting tissue damage occurs at the center of the ischemic injury [2].

In ischemic stroke, there are two-pathway mechanism of cell death in neuron there are necrosis and apoptosis. In ischemia, necrosis is the major cause of cell death in the intensely

ischemic core. The core is surrounded by ischemic penumbra, where neurons primarily die by apoptosis, and a highly regulated mechanism of cell death. Penumbra is the term used for the reversibly injured brain tissue around ischemic core. This area is reported an area with progressive apoptosis process. Thus, penumbra is the target for acute ischemic stroke treatment [3, 4].

Erythropoietin is reported as neuroprotective agent working by increasing cell resistance through activation of phosphatidylinositol 3-kinase / Akt and NFkB pathways. These result in the regulation of anti-apoptotic proteins and the inhibition of specific cell of death protease activities, which lead to apoptosis. EPO and its receptor (EPOR) are expressed throughout the brain in glial cells, neurons and endothelial cells, whereas microglia does not produce EPO and is only capable of constitutive EPO-R mRNA expression [5]. In the brain EPO mRNA level have been shown to remain elevated for more than 24 hours during duration of the hypoxic stimuli. Moreover, an

Ischemic/hypoxic dramatic changes have been reported in the expression of EPO and EPOR within and around infarcts in human brain regions [6].

Thus, we conducted the study by animal model of rUCCAO. This model demonstrated a chronic mild reduction in cerebral blood flow, white matter lesions and delayed memory impairment. Furthermore, the levels of pro-inflammatory cytokines increase and those of anti-inflammatory cytokines decrease in the brain [7]. Therefore, we used this model to investigate the potential erythropoietin to repair brain damage as well as cognitive function. Furthermore, we examined the anti-apoptotic effect of EPO using caspase 3 as the marker of apoptosis.

2. RESULTS

2.1 rHuEPO repairs cognitive function in rat with ischemic stroke

Figure 1 shows the potency of rHuEPO on improving the number of alternation as compared to total alternation for 5 minutes in Y maze paradigm. The result showed that rHuEPO significantly increased the percentage alternation as compared to ischemic stroke group. Furthermore the stroke group showed that there was no improvement in cognitive function until the end of the experiment, moreover there was significant increases in cognitive function after rHuEPO treatment with dose of 5,000 and 10,000 IU ($F_{(4, 20)} = 3.583$; Figure 1)

2.2 Potency of rHuEPO to Infarct area

Figure 2 shows the effect of rHuEPO on area infarct in thalamus area of the brain. One way ANOVA test result that showed there was difference among groups with p value < 0.0001. Furthermore ischemic stroke group demonstrated a significant increase in infarct area as compared to sham group. Moreover rHuEPO treatment in all doses demonstrated a significant decrease in infarct area as compared to ischemic stroke group ($F_{(4, 10)} = 13.86$; Figure 2)

2.3 Expression of Caspase 3 in brain after stroke ischemic

Figure 3 shows the immunoreactivity cells were clearly illustrated as cells that absorb the brown color with moderate to strong intensity in the stroke model group (Figure 3B), the stroke caused drastic change in cell shape shown by irregular shape and diminished size. rHuEPO decreased caspase 3 expression in thalamus area as compared to ischemic strokes group (Figure 3 C-E). Semi quantitative result of caspase 3 expression after rHuEPO treatment showed that stroke model group increase the expression of caspase 3 and rHuEPO ameliorated the apoptosis processes by significantly decreasing the expression of caspase

3 ($F_{(4,10)} = 44$; Figure 4)

3. DISCUSSIONS

Our study aimed to determine the effect of rHuEPO ameliorating the brain function after ischemic stroke. Pervious study has been shown that erythropoietin treatment protects brain from neuronal damage when initiated soon after stroke induction, mostly through the decrease in neuronal apoptosis [8]. EPO is a glycoprotein that stimulates differentiation and proliferation of erythroid precursor cells, and hypoxic induction of EPO production increases numbers of red blood cells, leading to better oxygen supply to the tissue [9]. In response to the systemic oxygen caused by decreased oxygen concentration after cerebral ischemia, EPO production is stimulated [10]. It has recently been reported that both erythropoietin and its receptor (EPOR) are found in the human cerebral cortex and hippocampus and that in vitro, the cytokine is synthesized by astrocytes and neurons [11].

The result showed that treatment with rHuEPO for 7 days significantly improved recovery spatial memory, the present study showed that the treatment of stroke with rHuEPO at dose 1,000; 5,000 and 10,000 IU reducing infarct area. Infarct area in the present study was found in thalamus area, one of structures in the limbic system besides the amygdala, hippocampus, para

hippocampal gyrus, cingula gyrus, fornix, hypothalamus, dentate gyrus and entorhinal cortex, where in this area affects cognitive function [12, 13].

Previous studies suggest that EPO may have more general tissue protective effect by targeting different neurodegenerative pathway such as antioxidant, glutamate inhibitory, anti-inflammatory, neurotrophic, angiogenic mechanism, anti-apoptotic and neuroprotective effect in many organs including brain [14, 15, 16].

In the present experiments, rHuEPO at dose 1,000; 5,000 and 10,000 IU of immunohistochemistry prove that EPO reduce expression of caspase 3 at dose 1,000; 5,000 and 10,000 IU. It is suggested that EPO inhibits the expression of caspase 3 by activating to Akt/GSK-3β caspase-3 mediated signaling mechanism. rHuEPO activates Akt, leading to GSK-3β phosphorylation led to inhibition of caspase 3 activation and thus attenuation of apoptosis, in the previous study suggest that conditions of decreased PI3K/Akt signaling caused increased GSK3β activity facilitated caspase-3 activation [17, 18], subsequently this process may phosphorylate STAT5 and activates NF-KB which promote the expression of the anti-apoptotic proteins such as X-linked inhibitor of apoptosis (XIAP) and c-inhibitor of apoptosis-2 [4]. XIAP has been reported to bind and suppress caspase 3 and caspase 9 thus the inhibit activation of procaspase 9 within the apoptosome [3]

The only currently approved medical stroke therapy, tissue plasminogen activator (tPA), is a thrombolytic that targets the thrombus within the blood vessel. Neuroprotective agents is one of alternative approach to stroke treatment [19]. Further clinical evidence rHuEPO show that gives beneficial effects in other brain pathologies such as aneurismal subarachnoid hemorrhage and severe traumatic brain injury. A reduction in delayed ischemic deficits as well as an increase in hospital survival is observed after rHuEPO treatment [20]. In the present study, rHuEPO showed a capability to improve cognitive function and reduce infarct area after ischemic stroke in rodent. We believe that rHuEPO use in clinical setting could be a potential therapy to protect the brain after ischemic stroke. It suggested that further research in rHuEPO as neuroprotective agent may solve the problem in the treatment of stroke.

4. CONCLUSION

Taken all together we suggest that erythropoietin may exert a neuroprotective effect showed by improvement in cognitive function and the reduction of infarct are through the inhibit expression of caspase 3.

5. MATERIAL AND METHODS

5.1 Materials

The materials used in the experiment were rHu Erythropoietin alfa (PT Daewoong Infion), normal saline, TTC 0.5% (2,3,5 triphenyltetrazoliun Chloride) (Sigma Aldrich), distilled water, buffered-formalin 10%, hematoxylin-eosin (Sigma Aldrich), xylazine, midazolam, Antibody caspase 3 (Sigma Aldrich).

5.2 Animal Preparation

Healthy adult Wistar male rats aged 8-10 month weights ranging between 200-240 grams. All animals had free access to food and water *ad libitum*. They maintained under standard laboratory condition that is a well aerated-room with alternating light and dark cycle of 12 h each and at room temperature of 25°C. The experimental protocol was approved by the Animal Ethics Committee, Faculty of Veterinary, Airlangga University, Indonesia.

5.3 Experimental Protocol

Rats were allocated randomly and divided into five groups i.e sham (n=6), ischemic strokes group (n=6), rHuEPO treated (n=18), xylazine (10 mg/kg) and ketamine (80 mg/kg) were administered for anesthesia. Through a small incision in the neck, the right common carotid artery was isolated from the vagal nerve and connective tissue by blunt dissection. The right common carotid artery (CCA) was blocked by bulldog clamp for 90 min. The bulldog clamp was removed (n=24). As controls (n=6), the rats were subjected to the same surgical procedure without carotid blocked. For pain relief and postoperative discomfort, applied lidocaine gel was applied to the wound and 0.5 ml of saline was given intraperitoneally. The drug (rHu Erythropoietin alfa) was administered intravenously at time 24 hours after surgery once a day for 7 days at dose 1,000 IU (n=6), 5,000 IU (n=6), 10,000 IU (n=6), and for control group (n=12) was given by saline.

5.4 Behavioral assessment Y-maze

Y maze protocol was accordance to pervious study [21, 22]. Briefly, Y maze test was performed on day 0 (before surgery) 1, 3 and 7 (after surgery). This behavior, spontaneous alteration was used to evaluate the working memory of rats placed in a new environment. Each rats was placed in one of the arm compartments and was allowed to move freely until it tail completely enters another arm. The sequence of arm entries is manually recorded, the arms being labeled A, B or C. Three possibilities were offered the rats for its first choice: staying in arm A

moving into arm B or moving into arm C, an alternation was considered as correct if the rat visited a new arm and did not return to the two previously visited arm. For each animal the Y maze testing was carried out for 5 minutes. For instance, if the animal makes the following arm entries; ACB-CAB-ABC-BAC-ACB-CAB-ABC, in this example, the animal made 20 arm entries 8 of which are correct alternations. The percentage alternation is calculated as [(actual alternations /maximum alternations) x 100]. The apparatus was cleaned with acetate acid 0.3% and allowed to dry between sessions.

5.5 Infarct area assessment

Infarct area assessment protocol was accordance to pervious study [23]. All animals were sacrificed and brain was removed, placed in brain blocker and sliced in 2 mm thick coronal section in bregma -2.00. The section was stained in TTC 0.5% (2.3.5 triphenyltetrazoliun Chloride) (Sigma Aldrich) saline solution for 20 minute at 37°C. The section was scanned and analyzed using ImageJ software (version 1.6). Brain infarction was visualized as areas of unstained (white) tissue, which contrasted from brick red stained areas of viable tissue.

5.6 Immunohistochemistry

Immunohistochemistry protocol was accordance to pervious study [24]. Immunohistochemistry of caspase-3 was performed using primary rabbit anti-rat caspase-3 antibody. The deparaffinized sections were incubated in 1 % H_2O_2 in PBS containing 0.1 % sodium azide for 10 min. The endogenous peroxidase activity is quenched by incubation with H_2O_2. The sections were incubated with blocking buffer at room temperature for 30 min. The sections were incubated with caspase-3 antibody at appropriate dilution (at least 1:100) in antibody dilution buffer in a humidified chamber for 1 h at room temperature or overnight at 4 °C. To assess nonspecific staining or verify the binding specificities of primary antibodies, the sections were incubated with antibody dilution buffer or equal amounts of nonspecific mouse, rabbit, or goat immunoglobulins without the primary antibody for negative controls. The section were washed the sections with PBS-T for 5 min twice. The section were incubated with HRP-conjugated secondary antibody at appropriate dilution in antibody dilution buffer at room temperature in the dark for 1 h. Wash the sections with PBS-T for 5 min, repeat two times. Incubate the sections in dark with HRP substrate solution. The slides were washed with PBS-T for 5 min twice to remove excess chromogen. The sections were dehydrated with ascending

graded alcohols (for 20 s each in 35, 70 and 95 % ethanol, and 2 min in 100 % ethanol) and clear in xylene. The slides were mounted with mounting medium.

5.7 Statistical analysis

Statistical analysis was performed using Graph-Pad Prism software version 6.0. Data were presented as mean values ± SEM. All behavioral data were analysis using two way analysis of variance (ANOVA) followed by post hoc Tukey's HSD test. Infarction size data were compared among treatment groups by analysis of variance (ANOVA). For Immunohistochemistry data were analysis using one way ANOVA. The difference was considered significant if $p < 0.05$.

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Conflict of interest statement

The authors have declared that no conflict of interest exists

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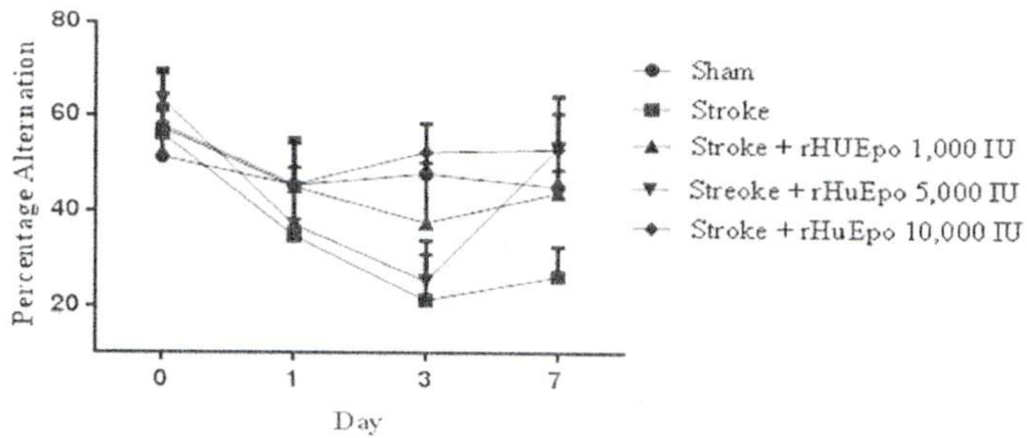

Figure 1. Percentage of behavioral alteration following 5 minute in Y maze exploration. Data represent the mean ± S.E.M $p < 0.05$. EPO administered intravenously once a day for 7 days (n = 6)

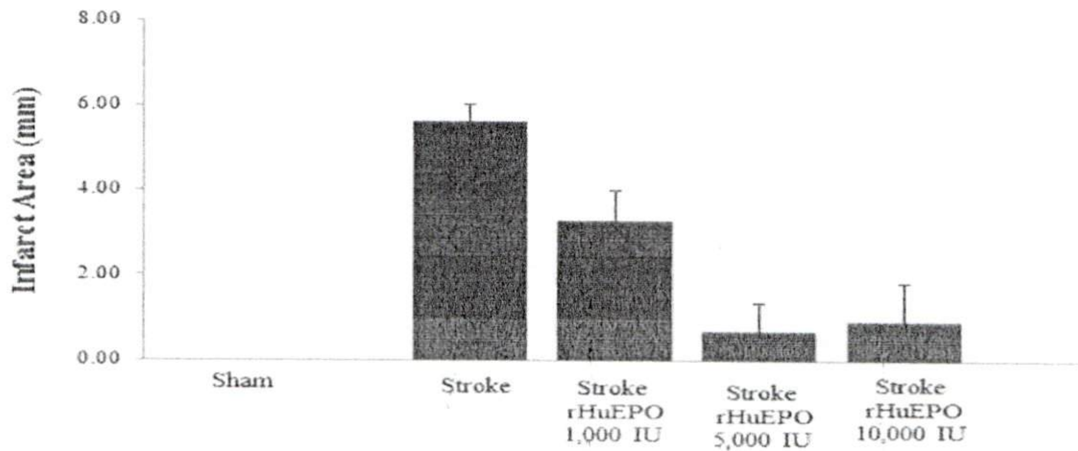

Figure 2. Potency of rHuEPO for reduction the infarct area in brain. Data are presented as mean ± S.E.M (n = 3)

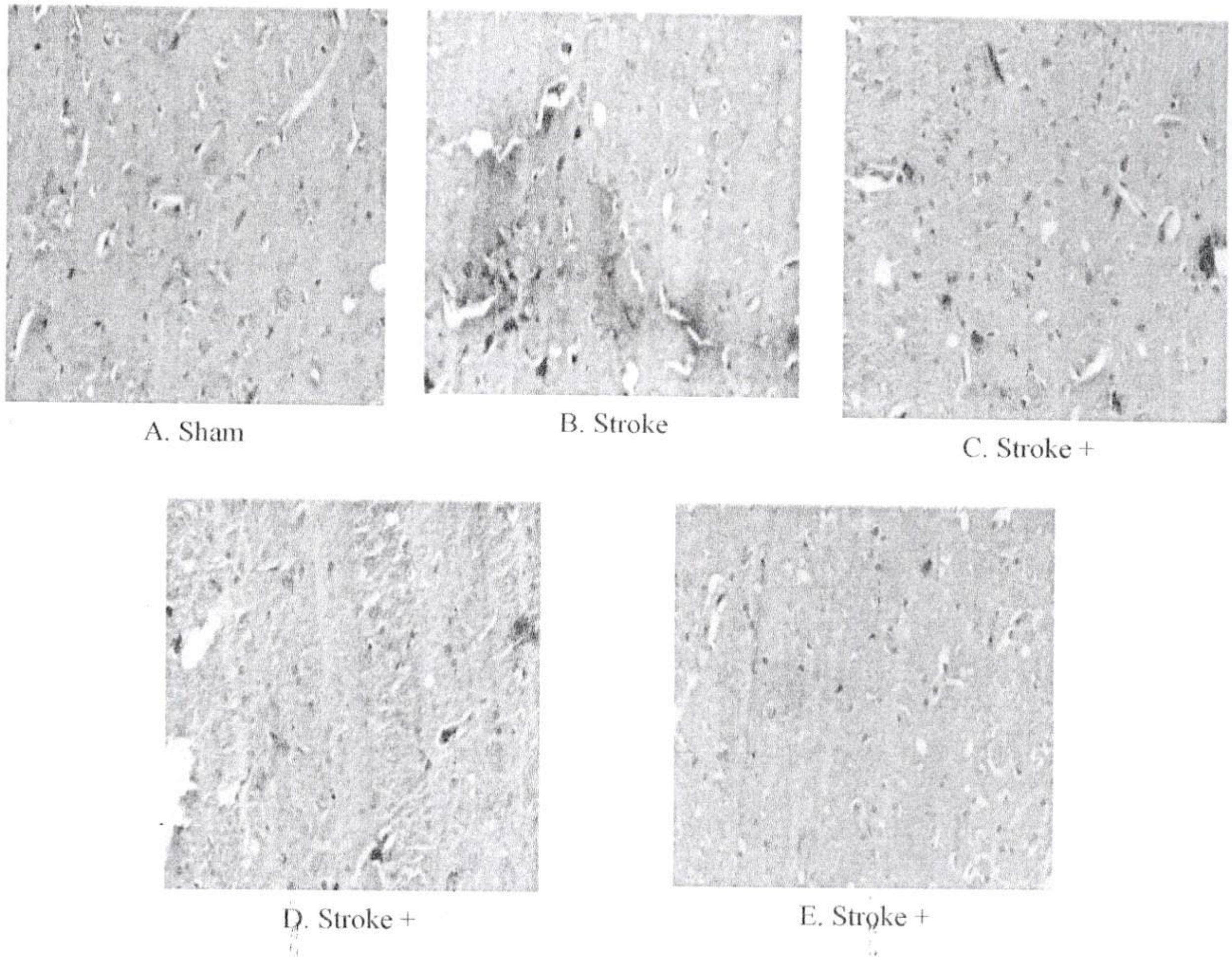

Figure 3. Expression of caspase 3 in thalamus area after ischemic stroke. A Sham; B. Stroke; C. Stroke + rHuEpo 1,000 IU; D Stroke + rHuEpo 5,000 IU; E. Stroke + rHuEpo 10,000 IU

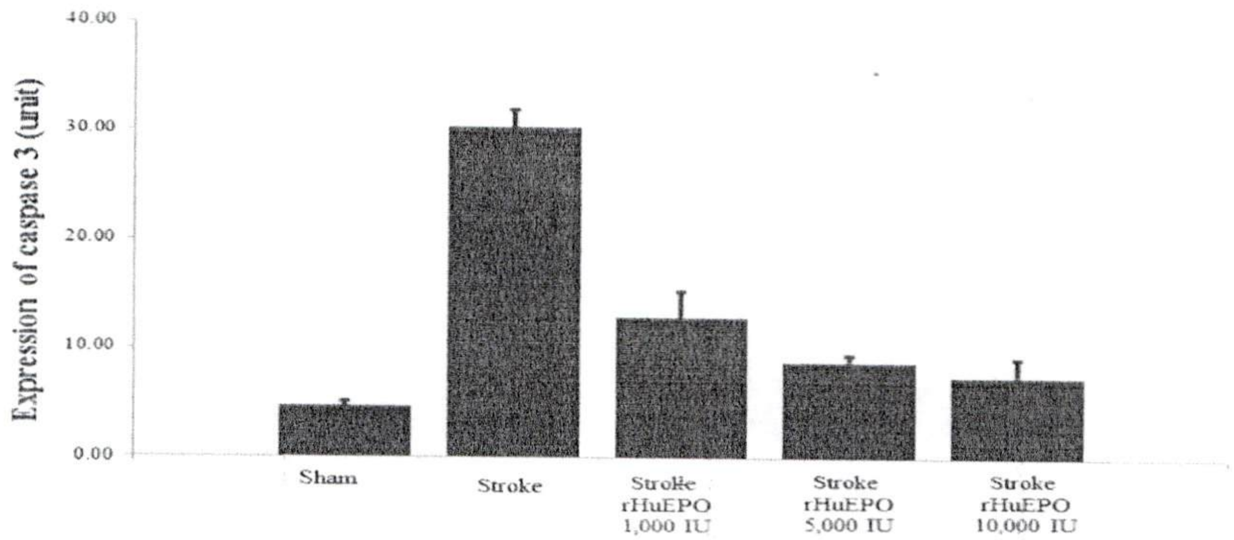

Figure 4. The mean number of caspase 3 expression on the thalamus area. Data are presented as mean ± S.E.M (n = 3)

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