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POTENSI ERITROPOETIN SEBAGAI INDUKSI *CELL SURVIVAL* PADA NEURON SEBAGAI STRATEGI BARU PENGOBATAN STROKE

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RINGKASAN

POTENSI ERITROPOIETIN SEBAGAI INDUKSI CELL SURVIVAL PADA NEURON SEBAGAI STRATEGI BARU PENGOBATAN STROKE

Latar Belakang: Stroke iskemik menyebabkan kekurangan pasokan oksigen dan glukosa, deplesi ATP, kerusakan neuoronal dari akumulasi glutamate yang berlebihan hingga kematian sel secara apoptosis. Erythropoietin (EPO) merupakan sitokin hematopoietik yang juga berperan dalam proteksi otak. Mekanisme induksi *cell survival* yang digunakan dalam konsep kanker dan metastase digunakan untuk melihat efektifitas EPO dalam *neuronal survival.*

Masalah: Potensi induksi cell survival pada mekanisme molekuler EPO untuk memperbaiki kondisi otak paska stroke belum banyak diteliti, khususnya peran jalur MC3/4 receptor yang dikenal menghambat metastasis.

Tujuan: menguji keterlibatan jalur MC3/4 dan alpha MSH dalam mekanisme cell survival pada stroke khususnya hubungannya dengan terapi EPO. Tujuan khusus yakni membuktikan efek pemberian EPO terhadap pengurangan volume infark, perbaikan fungsi motorik dan kognitif, induksi *neuronal survival* pada tikus model stroke iskemik dengan marker apoptosis dan proliferasi, terutama yang tidak berkaitan langsung dengan inflamasi.

Metodologi: Injeksi EPO dilakukan secara intravena dengan menggunakan microsyringe. Model stroke ditentukan dengan metode *unilateral common carotid artery occlusion*, keberhasilan metode model stroke ditentukan dengan pewarnaan Triphenyltetrazolium chloride (TTC), penentuan volume stroke dilakukan secara makro- dan mikroskopik, penentuan kadar melanocortin-3/4 receptor pada area area spesifik di otak dilakukan dengan metode PCR.

Output : Hasil penelitian ini menunjukkan bahwa EPO memiliki efek penekanan terhadap penurunan fungsi kognitif dan motorik yang terjadi pasca stroke. Keterlibatan peptide alpha-MSH dan reseptornya, MC-3/4 juga dieksplorasi kemudian. Dengan pendekatan ini, diharapkan didapat jalur alternatif yang *curative* atau *early preventive* terhadap stroke.

Keywords: Erythropoeitin, Stroke, Cell survival.

PRAKATA

Dengan mengucap syukur alhamdulillah, penulis telah merampungkan beberapa tahapan dari rangkaian tahapan yang harus dilakukan dalam menjalankan penelitian bertemakan Potensi Eritropoietin sebagai Induksi Cell Survival Pada Neuron Sebagai Strategi Baru Pengobatan Stroke dengan dana PDUPT Kemenristek Dikti tahun 2018. Peneliti menghaturkan terima kasih sebesar-besarnya atas dukungan yang diberikan oleh Rektor dalam hal ini melalui Lembaga Penelitian dan Inovasi Universitas Airlangga sehingga penelitian ini dapat memperoleh pembiayaan dana penelitian desentralisasi dari Kemenristek Dikti dan mendapat pembimbingan untuk mencapai luaran yang bermanfaat tidak hanya dalam bidang kesehatan, namun juga berkontribusi dalam peningkatan publikasi dan reputasi akademik Universitas Airlangga. Diharapkan penelitian yang telah diselesaikan tahun ini dan mencapai target luaran, dapat dilanjutkan pada tahun pendanaan berikutnya untuk memberikan luaran dan manfaat lebih bagi pengembangan produk kefarmasian pada khususnya dan pengembangan bidang ilmu kesehatan pada umumnya.

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BAB I
PENDAHULUAN

1.1. Latar Belakang

Stroke merupakan penyakit neurologis dan merupakan penyebab utama kematian dan kecacatan di seluruh dunia (Deb, *et al*, 2009). Stroke dengan defisit neurologik yang terjadi tiba-tiba dapat disebabkan oleh iskemia atau perdarahan otak (Hacke, 2003). Stroke iskemik adalah penyakit heterogen yang disebabkan oleh oklusi fokal pembuluh darah otak yang menyebabkan turunnya suplai oksigen dan glukosa ke bagian otak yang mengalami oklusi yang menyebabkan kematian sel (Park, *et al*, 2014; Hacke, 2003). Prevalensi stroke di dunia adalah 30,7 juta. Setiap 4 menit, 1 orang meninggal dunia karena stroke. Di Indonesia penyakit stroke merupakan penyebab kematian nomor 1 yaitu sebesar 15,4 % dari seluruh kematian (Lukito dan Indra, 2016). Pengobatan stroke iskemik terbatas pada trombolisis menggunakan TPA. Terapi stroke di masa mendatang difokuskan dengan mencegah kematian sel dan meningkatkan mekanisme pemulihan endogen (Hoke, 2006).

Pada keadaan stroke iskemik terjadi kekurangan pasokan oksigen dan glukosa yang akan menyebabkan deplesi ATP, hal inilah yang menyebabkan kerusakan neuoronal dari akumulasi glutamate yang berlebihan serta kematian sel oleh program apoptosis sel. Akibat deplesi ATP, gradien ion tidak dapat dipertahankan (ion Na^+, K^+) dan neuron menjadi didepolariasi, peristiwa tersebut menyebabkan hilangnya "*neuronal excitability*" dan pembebasan (*release*) *glutamate* secara besar-besaran (masif). Kekurangan energi juga mengurangi *uptake* glutamate yang dilakukan oleh astrosit. Timbunan (*build-up*) *glutamate* yang berlebihan di sinaps, mempercepat kematian nekrotik dari berbagai neuron yang merupakan target sinaps. Ketika glutamate berikatan dengan reseptor glutamate yaitu *N-methyl-D-* aspartate (NMDA) menyebabkan konsentrasi Ca^{2+} dalam intraneuronal meningkat, peningkatan konsentrasi kalsium intraseluler ini mengakibatkan *excitotoxicity* yang pada akhirnya menyebabkan nekrosis sel. (Graham *et al* 2002; Anugro *et al* 2014). Terjadinya akumulasi Ca^{2+} dan nitric oxide akibat adanya kelebihan glutamate, menyebabkan produksi ROS (*reactive oxygen species*). ROS membantu memindahkan *cytochrome C* dan *apoptosis inducing factor* (AIF) dari dalam membran mitokondria ke ruang intermembran (Hagberg *et al,2016)*. Pelepasan *cytochrome C* akan membentuk kompleks dengan APAF-1 dan procaspase 9. Procaspase 9 ini akan terpecah menjadi bentuk yang aktif yaitu caspase 9. Caspase 9 akan terpecah kembali dan menjadi

bentuk caspase lain yang lebih aktif yaitu caspase 3 (efektor). Caspase 3 ini nantinya akan bekerja untuk menginaktifkan protein *DNA repair*, pemecahan dari protein cytoskletal dan pemecahan ICAD suatu inhibitor dari CAD yang berhubungan dengan program kematian sel.

(EPO) merupakan sitokin hematopoietik yang memberi efek proliferasi dan diferensiasi progenitor erythroid dan kelangsungan hidup pada sel erythroid yang matang (Hoke, 2006). EPO tidak hanya berperan dalam eritropoiesis tetapi juga memiliki efek proteksi otak dengan merangsang *protein of repair*, mengurangi eksitotoksisitas neuron, mengurangi inflamasi, menghambat apoptosis neuron dan merangsang neurogenesis dan angiogenesis. Pada penelitian eksperimental cedera iskemia, hipoksia dan cedera toksik, EPO juga memperbaiki *outcome* neurologik dan fungsi mental (Fuadi dan Bisri, 2015).

Berdasarkan uraian di atas maka diperlukan adanya penelitian mengenai potensi induksi *cell survival* dari erythropoeitin sebagai terapi baru dalam pengobatan stroke. Pembuktikan hal tersebut dilakukan dengan pengujian pada tikus yang dikondisikan menderita stroke iskemik dengan menggunakan model eksperimental oklusi carotid arteri. oklusi carotid arteri adalah metode operasi yang dilakukan untuk memblok aliran darah di otak (iskemik) dengan atau tanpa memasukkan benang monofilament ke dalam *eksternal carotid artery* sampai pada *middle carotid artery* (Rupadevi *et al*, 2011). Setelah 24 jam pasca oklusi carotid arteri, total presentase terjadinya infark sebesar 40% dari total hemisphere (Chiang *et al*,2011). Dengan menggunakan model ini maka akan menciptakan lesi iskemik yang lebih besar yang dimana melebihi daerah *middle carotid artery* untuk mencapai bagian thalamus, hippocampus dan substantia nigra (Canazza *et al*,2014). Untuk melihat perkembangan perbaikan dari stroke iskemik dengan menggunakan eritropoetin maka akan dilihat volume infrak pada otak, perbaikan motorik dan kognitif, poliferasi sel pada otak serta melihat peningkatan level VEGF dengan stimulasi angiogenesis pada hewan coba.

BAB 2
TINJAUAN PUSTAKA

2.1 Stroke

Istilah stroke atau penyakit serebrovaskular menngacu pada setiap gangguan neurologik mendadak yang terjadi akibat pembatasan atau terhentinya aliran darah melalui sistem suplai arteri otak (Price *et al*, 2006). Stroke adalah suatu tanda klinis yang berkembang cepat akibat gangguan otak fokal (atau global) dengan gejala yang berlangsung selama 24 jam atau lebih serta dapat menyebabkan kematian tanpa adanya penyebab lain yang jelas selain vaskuler (Caplan, 2009; WHO, 2006)

2.1.1 Epidemiologi

Data epidemiologis menunjukan bahwa stroke merupakan penyebab kematian nomor dua di dunia setelah penyakit jantung. WHO memperkirakan bahwa ada 6,7 juta kematian terkait stroke pada tahun 2012, dan akan mengalami kenaikan pada tahun 2030 dengan 7,8 juta kematian (Bannet, 2011; Kim *et* al, 2012; Kulshreshtha *et al*, 2012). Jumlah penderita penyakit stroke di Indonesia tahun 2013 berdasarkan diagnosis tenaga kesehatan (Nakes) diperkirakan sebanyak 1.236.825 orang (7,0‰), sedangkan berdasarkan diagnosis Nakes/gejala diperkirakan sebanyak 2.137.941 orang (12,1‰) (Kementerian Kesehatan, 2013).

2.1.2 Klasifikasi Stroke

Menurut Davenport dan Dennis (2000), secara garis besar stroke dapat dibagi menjadi dua bagian yaitu stroke iskemik dan stroke hemoragik. Sedangkan klasifikasi stroke menurut Caplan (2011) stroke dibagi menjadi dua kelompok yaitu:

a. Stroke iskemik dikarenakan trombosis, embolism atau hipoperfusi sistemik:

1) Trombosis

Umumnya mengacu pada lokasi obstruksi di arteri. Obstruksi terjadi dikarenakan adanya penyakit dinding arteri, seperti arteriosklerosis, diseksi, atau fibromuskular displasia (Caplan, 2011; Crowin, 2009).

2) Embolism

Stroke embolik berkembang setelah oklusi arteri oleh embolus yang terbentuk di luar otak. Sumber umum embolus adalah jantung setelah infark miokardium atau fibralasi atrium, dan embolus yang merusak arteri karotis komunis atau aorta (Caplan, 2011; Crowin, 2009).

b. Stroke hemoragikdengan 2 penyebab, antara lain:

1) Intracerebral hemorrhage

Perdarahan pada intraserebral hemorrhage (ICH) biasanya berasal dari arteriol atau arteri kecil.Perdarahan langsung ke otak, membentuk hematoma lokal yang menyebar di sepanjang jalur *White* matter (Caplan,2011)

2) subarachnoid hemorrhage

Disebabkan oleh ruptur suatu aneurisma vaskular dan trauma kepala. Pecahnya aneurisma melepaskan darah secara langsung ke dalam cairan cerebrospinal (CSF) di bawah tekanan arteri, darah menyebar cepat ke dalam CSF, sehingga meningkatkan tekanan intrakranial (Caplan, 2011; Price, 2006).

2.2 Patofisiologi Stroke

2.2.1 Patofisilogi Stroke Iskemik

Sel-sel otak tergantung pada oksigen dan glukosa untuk bertahan hidup. Metabolisme glukosa mengarah ke konversi adenosindifosfat (ADP) menjadi adenosin trifosfat (ATP). Pasokan konstan ATP diperlukan untuk menjaga integritas neuronal (Caplan *et al*,2009).

Stroke iskemik terjadi karena kurangnya pasokan aliran darah dan energi ke otak, yang memicu setidaknya lima dasar mekanisme yang menyebabkan kematian sel yaitu excitotoxicity dan ketidakseimbangan ion, oksidatif / stres nitrosative, inflamasi, apoptosis, dan peri-infract depolarisasi. (Amantea *et al*, 2008; Gonzalez *et al*, 2011).

Penurunan ATP dan deplesi energi menginduksi serangkaian peristiwa biokimia meliputi: ketidakseimbangan ion, rilis dari *excess glutamate* di ruang ekstraselular yang mengarah ke *excitotoxicity*, peningkatkan kalsium intraseluler yang pada gilirannya mengaktifkan beberapa kematian jalur intraseluler seperti disfungsi mitokondria, disfungsi sawar darah otak, oksidatif dan nitrosative stres serta produksi reaktif spesies oksigen (ROS), spesies nitrogen reaktif (RNS) yang mengarah pada kematian sel neuron, glia dan sel endotel. (Gonzales *et al*, 2011; Kanyal, 2015)

2.2.2 Gangguan keseimbangan ion dan eksitoksisitas

Deplesi akut ATP memicu kerusakan neuronal dari akumulasiL-glutamate yang berlebihan. Proses ini dinamakan *excitotoxicity*,melibatkan aktivasi reseptor-reseptor glutamate, akumulasisitosol Ca^{2+} aktivasi kaskade yang dipicu oleh Ca^{2+} generasi radikal bebas oksigen dan disfungsi mitokondria.

2.2.3 Mekanisme Kematian Sel Neuron Pada Stroke Iskemik

Ada dua mekanisme kematian pada stroke iskemik yaitu nekrosis dan apoptosis dimana kedua proses ini tergantung pada lama durasi dan intensitas iskemik yang dialami. Terdapat area yang mengelilingi *core* yang disebut sebagai area *penumbra*, pada area ini

pengurangan aliran darah tidak separah yang terjadi pada area *core*. Kematian sel pada daerah *penumbra* di perantarai oleh proses apoptosis (Nguyen *et al*, 2014).

a. Mekanisme Kematian Sel Secara Apoptosis Pada Stroke Iskemik

Sebuah lesi stroke ditandai dengan kematian sel nekrotik di area *core* yang terbentuk dengan cepat setelah cedera dandapat mewakili jaringan yang ireversibel, dan area*penumbra*adalah area yang mengelilingi *core*. Peran jalur molekuler yang mendasari terjadinya neuronal apoptosis adalah mitokondria, caspases, dan keluarga protein Bcl-2 (Yuan,2009 ; Zeng *et al* 2003). Mekanisme caspase-dependent untuk kematian sel membutuhkan energi dalam bentuk ATP, apoptosis terutama terjadi di penumbra iskemik dibanding pada inti iskemik. Pori-pori mitokondria merupakan jalur melintasnya sitokrom c dan protein pro-apoptosis dari ruang intramembran mitokondria ke sitoplasma. Pelepasan sitokrom c mengaktifkan jalur caspases melalui pembentukan apoptosome. Caspases mengaktifkan caspase-activated deoksiribonuklease (CAD). Caspase independent melibatkan aktivasi poly-ADP (ribose) polymerase (PARP) yang mendorong pelepasan faktor apoptosis-inducing (AIF), yang bertranslokasi ke dalam inti, mengikat DNA, dan mengakibatkan kematian sel (Gonzales *et al*, 2011; Zeng *et al*, 2003).

2.3 Erythropoeitin (EPO)

Erythropoietin (EPO) merupakan sitokin hematopoietik yang memberi efek proliferasi dan diferensiasi progenitor erythroid dan kelangsungan hidup pada sel erythroid yang matang. Pengobatan pada stem cell saraf otak depan dengan EPO menjelaskan adanya produksi pada progenitor saraf secara in vitro. Selanjutnya, EPO meningkatkan angiogenesis baik pada in vitro dan in vivo (Hoke, 2006).

2.3.1 Struktur Kimia

EPO alfa merupakan glikoprotein 34.000 Dalton, yang terdiri dari 60% protein dan 40% karbohidrat yang mempengaruhi eritropoisis sel darah merah. Human gen EPO merupakan single- Copy-gen, yang terletak pada kromosom 7 yang terdiri dari 5 ekson dan 4 intron, 165 asam amino peptida. EPO memiliki berat molekul glikoprotein 30.000, deglikosilat EPO 18.000 terdiri dari 2 buah rantai disulfida, 4 a-helical bundle, dengan proporsi karbohidrat berupa fruktosa, galaktosa, manosa, N-asetilgalaktosamin, asam N-asetilneuraminik, 3 N linked, 1 O-linked glykosilasi (Fuadi dan Bisri, 2015).

2.3.2 Mekanisme Kerja EPO di Sistem Saraf Pusat

Ekspresi EPO di otak meningkat pada hipoksia dan stres metabolik akut. Mekanisme kerja EPO sebagai neuroprotektor diduga multifaktorial baik secara

langsung maupun tidak langsung pada neuron. EPO dapat melawan efek sitotoksik dari glutamat, meningkatkan ekspresi enzim-enzim antioksidan, mengurangi pembentukan radikal bebas, memperbaiki aliran darah serebral, mempengaruhi pelepasan neurotransmiter, dan meningkatkan angiogenesis. Pada neuron daerah cortex pada tikus EPO berefek proteksi dengan cara EPO–R mengaktifasi JAK2 kemudian mengaktifasi kaskade nuclear factor (NF)–kB dan meningkatkan ekspresi gen inhibitor apoptosis yaitu XIAP dan c-IAP2. EPO juga mempunyai efek proteksi terhadap cedera akibat iskemia pada neuron melalui regulasi gen anti apoptosis yaitu gen Bcl–x. Efek neuroprotektor juga dapat dilihat dari efek penurunan inflamasi dari daerah cedera otak. NF–kB yang diaktifasi oleh EPO adalah regulator dari gen inflamasi.

EPO berefek maturasi oligodendrosit dan melindungi dari interferon-gamma (IFN-γ), toksisitas lipopolisakaraida, ekspresi *inducible nitric-oxide synthase* (iNOS) dan produksi nitrit. EPO mempunyai efek antioksidan yakni secara langsung sebagai *free radical scavenging* atau tidak langsung dengan mengaktifasi enzim-enzim antioksidan (Fuadi dan Bisri, 2015)

2.3.3 Peran EPO pada Sistem Saraf

Gen EPO ditemukan pada jaringan otak manusia daerah korteks temporal, *hippocampus*, dan *amygdala*. EPO dapat dideteksi dalam cairan serebrospinal manusia dewasa dan neonatus. Pada tingkat sel, EPO diproduksi oleh astrosit dan neuron. Reseptor EPO secara luas diekspresikan pada kebanyakan sel otak, termasuk neuron, sel endotel, sel *microglial*, dan astrosit. EPO berperan sebagai faktor neurotropik pada neuron.

2.3.4 Jalur Sinyal yang Berperan dalam Proses Neuroproteksi dari EPO

Berbagai penelitian telah mengeksplorasi proteksi otak dari EPO melalui jalur sinyal EPO–EPOR kompleks. EPO endogen dan eksogen dapat berikatan dengan EPOR menyebabkan homodimerization dan fosforilasi JAK–2 mengakibatkan proses aktivasi *downstream signaling* yang rumit. Fosforilasi JAK–2 mengaktifkan phosphatidylinositol 3– kinase (P13K) dan menginduksi aktifasi NF–kβ (nuclear factor) dan menstimulasi homodimerisasi STAT-5. Selain itu fosforilasi JAK–2 mengaktifasi *Ras-Mitogen activated protein kinase* (MAPK) *signaling pathways*, dan modulasi konsentrasi kalsium pada sel yang tereksitasi, aktifitas elektrik dan pelepasan neurotransmiter dengan mengaktifkan phospholipase C. Penelitian in vivo menunjukkan inhibisi JAK–2 atau PI3K menghilangkan efek neuroproteksi dari EPO. Aktifasi Akt yang dimediasi melalui

PI3K, memodulasi beberapa sinyal intrasel yang berperan pada apoptosis, *synaptic signaling*, dan sintesa glikogen. Molekul target dari Akt adalah p53, GSK–3 dan cytochrome c yang berperan pada siklus sel dan kematian sel. Jalur Ras/Raf/MEK/extracellular *signal-regulated* kinase (ERK)–1/2 berperan pada respon neuroproteksi dari EPO melalui efek antiapoptosis dengan meningkatkan transkripsi dari gen-gen antiapoptosis (Bcl–2, Bcl–xL). STAT–5 yang sudah berfosforilasi berhomodimerisasi dan memasuki inti sel dimana gen antiapoptosis Bcl-2 and Bcl-xL ditranskripsi. Bcl–2 dan Bcl–xL mencegah pelepasan cytochrome c dari mitokondria. EPO meningkatkan STAT–5 dan konsentrasi gen-gen antiapoptosis. Beberapa jalur baru yang yang mungkin berperan dalam kemampuan EPO untuk mencegah apoptosis seluler berkaitan erat dengan Akt-1. EPO memodulasi *pro-apoptotic* FOXO3a yakni faktor transkripsi gen-gen apoptosis. Selain itu, EPO mengaktivasi NFkβ untuk mencegah apoptosis setelah pemaparan β-amyloid peptide di sel saraf (Ponce, *et al.*, 2012).

2.4 Model Stroke Iskemik pada Hewan

Stroke yang disebabkan oleh oklusi pembuluh serebral akut bisa direproduksi dengan beberapa teknik yang berbeda, yaitu dengan Oklusi mekanik baik arteri serebri proksimal (PMCAo) (oklusi pembuluh besar) atau distal MCA (dMCAo) (Oklusi pembuluh kecil), atau dengan oklusi trombotik baik melalui injeksi bekuan darah atau trombin ke MCA atau dengan foto-trombosis setelah injeksi intravena Rose Bengal. Beberapa model hewan yang bisa digunakan dalam iskemik serebral yaitu *Mechanical occlusion of the MCA, Thromboembolic Models, The endothelin and the photothrombosis model* dan *Cerebral venous thrombosis models* (Bacigaluppi, *et al.*, 2010).

MCAO adalah metode operasi yang dilakukan untuk memblok aliran darah di otak (iskemik) dengan cara memasukkan benang monofilament kedalam *eksternal carotid artery* sampai pada *middle carotid artery*. Setelah 24 jam pasca MCAO total presentase terjadinya infark sebesar 40% dari total hemisphere (Chiang *et al,* 2011). Model ini menciptakan lesi iskemik yang lebih besar yang dimana melebihi daerah *middle carotid artery* untuk mencapai bagian thalamus, hippocampus dan substantia nigra (Canazza *et al*,2014).

BAB 3
TUJUAN DAN MANFAAT PENELITIAN

1.2. Tujuan Penelitian

Penelitian ini mempunyai beberapa tujuan antara lain:

a. Membuktikan efek pemberian Eritropoetin terhadap pengurangan volume infark pada otak setelah mengalami stroke
b. Membuktikan efek pemberian EPO terhadap perbaikan fungsi motorik dan kognitif pada tikus model stroke
c. Membuktikan efek pemberian Eritropoetin pada cell survival baik melalui induksi proliferasi maupun penghambatan proses kematian sel secara apoptosis pada tikus model stroke iskemik dengan marker MC4 receptor ataupun alpha-MSH

1.3. Pentingnya atau Keutamaan Rencana Penelitian Ini

Penelitian ini mempunyai kemanfaatan antara lain :

a. Kefarmasian dan Kedokteran

Hasil penelitian ini akan memberikan dampak perkembangan pengobatan bagi dunia farmasi dan kedokteran berkaitan dengan penanganan Stroke yang lebih spesifik, efisien dan bersifat kausatif. Dengan pendekatan ini, pengobatan dapat bersifat menyembuhkan dan tidak diperlukan penggobatan jangka panjang atau selama pasien hidup.

b. Pasien Stroke

Dengan adanya temuan ini maka penderita Sroke yang disebabkan oleh iskemik kembali sehat. Sehingga kualitas hidup pasien dapat mengalami peningkatan.

c. Asuransi dan Pemerintah

Akan menurunkan biaya pengobatan sehingga beban asuransi dan pemerintah dapat mengalami penurunan. Penurunan terjadi karena tidak diperlukan pengobatan jangka lama dan perawatan di rumah sakit.

BAB 4
METODE PENELITIAN

3.1 Metode

3.1 Jenis Penelitian

Penelitian yang dilakukan adalah eksperimental laboratorium (*true experimental*) dengan tipe *pre-test post-test control group test*. Melalui penelitian ini dapat dikaji pengendalian apoptosis dan angiogenesis sel neuron tikus (*Rattus novergicus*) pada model stroke iskemik

3.2 Bahan

Bahan-bahan yang digunakan antara lain ketamine, midazolam, NaCl 0,9%, rHu Eritropoetin 10000 IU/kg, RNAase free water, RNA purification system, Quantus RNA quantification system, Water for Injection (WFI), Benang nylon monofilament, suture, TTC 0,5% (2,3,5 triphenyltetrazoliun Chloride), Primer untuk beta actin, primer untuk MC4 receptor, antibody HSP70

3.3 Alat

Alat-alat yang digunakan antara lain alat-alat gelas, Jarum suntik 26G dan *Syringe* 1 mL, kandang Tikus dan perlengkapannya, alat bedah, Neraca analitik Ohaus *Adventurer*, *Curved Forceps*, Mikrotom dan Mikroskop cahaya dengan kamera digital dan *graticule*.

3.4 Subyek Penelitian

Hewan coba yang digunakan adalah mencit (*Rattus Novergicus*) yang berasal dari Laboratorium Hewan Universitas Airlangga dengan kriteria berjenis kelamin jantan, berasal dari satu galur (galur wistar), berumur 8-12 minggu dengan berat badan 200-250 gram dan dalam keadaan sehat dan normal.

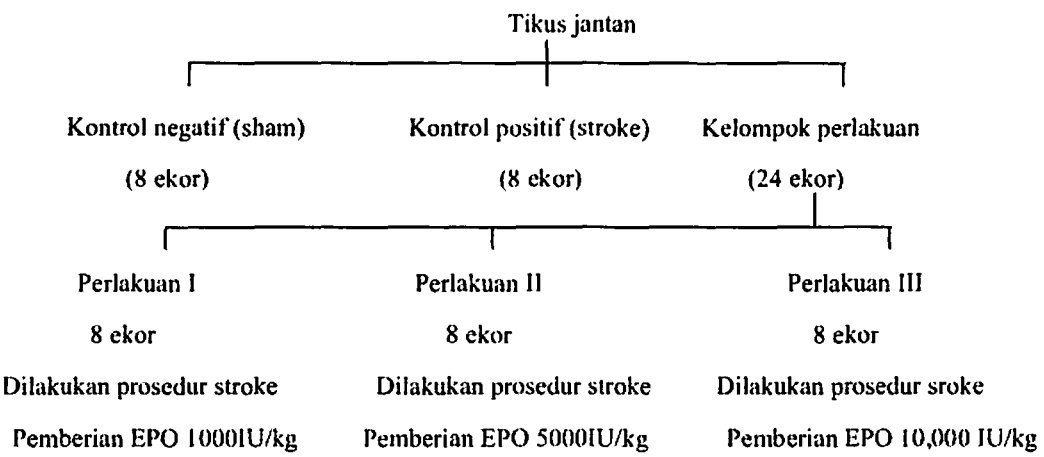
3.5 Protokol Penelitian

Tikus (*Rattus Novergicus*) jantan berumur 8-12 minggu ditempatkan secara berkelompok dalam kandang dengan temperatur 30 ± 1°C dan siklus penerangan 12 jam terang/gelap. Selama penelitian, makanan dan minuman tersedia *ad libitum*. Untuk menginduksi terjadinya Stroke iskemik, hewan coba di anastesi dengan menggunakan ketamin 80 mg/KgBB dan midazolam 5 mg/KgBB. Cukur bulu tikus yang pada area bagian leher, Buat irisan di area permukaan leher dengan menggunakan pisau bedah. Buat panjang irisan sekitar 3 cm. isolasi *Common carotid artery* dari nervus vagus dan ikat sementara. Ikat percabangan ECA di dua bagian dan pasang clamp vascular di antara percabangan CCA ke ECA dan ICA, buat irisan pada ujung ECA, masukan benang melalui ECA sampai pada batas MCA, ikat kuat (ikatan yang terdapat pada ECA). Dan lepas clamp,

Setelah 2 jam pasca operasi, benang di ambil kembali. Pemberian eritropoetin dilakukan 15 menit pasca operasi, hari pertama sampai hari ke tujuh, dengan dosis 100,1000 dan 5000 IU/KgBB. Untuk kelompok kontrol positif dan negatif diberi saline. Dilakukan uji motorik dan kognitif.

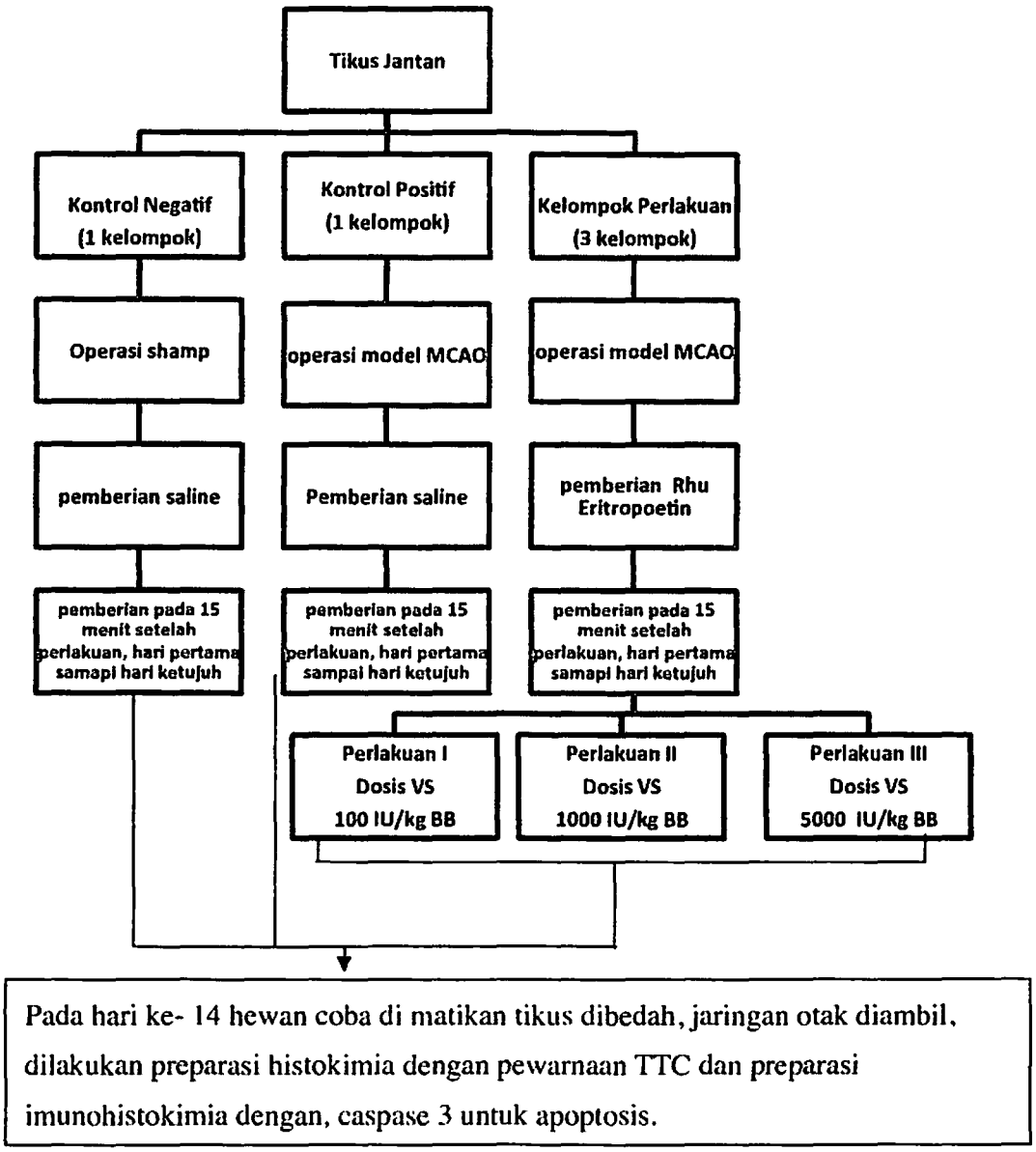
Setelah hari ke-14 tikus dimatikan, jaringan otak diambil dan dibekukan -8° C selama 15 menit, potong pada titik bregma 0 mm hingga bregma -2 mm. Jaringan direndam pada 2,3,5 triphenyltetrazoliun (TTC) selama 15-20 menit pada suhu 37° C Jaringan otak difiksasi dengan formaldehid 10% v/v dan dibilas dengan normal saline. Setelah itu dilakukan analisis marker-marker angiogenesis, proliferasi maupun apoptosis. Pemotongan dan pewarnaan jaringan baik secara histokimia maupun imunohistokimia dilakukan dengan bantuan ahli histologi dari Fakultas Kedokteran Hewan Universitas Airlangga.

3.6 Pengelompokan Hewan Coba dan *Timeline* Kelompok Perlakuan



Gambar 3.1 Pengelompokan Hewan Coba

3.7 Kerangka Operasional



Gambar 3.2 Diagram Pengelompokan Hewan Coba dan Perlakuan yang Diberikan pada Tiap Kelompok

3.7 Uji Fungsi Motorik

Pada test ini, tikus diuji dengan ditempatkan pada silinder akrilik dan diamati penggunaan forelimb kanan dan kirinya sebagai tumpuan untuk mengeksplor dinding silinder. Jumlah penggunaan forelimb kiri dibandingkan total penggunaan semua forelimb merupakan proporsi/prosentase penggunaan contralateral forelimb. Semakin tinggi penggunaan forelimb kiri maka prosentase akan semakin besar, menunjukkan forelimb kiri mengalami normalisasi penggunaan setelah stroke, demikian sebaliknya.

3.8 Uji Fungsi Kognitif

Pengujian kognitif pada model hewan stroke iskemik menggunakan uji Y maze (*evaluation using spontaneous alternation*) tujuan menggunakan uji adalah untuk mengevaluasi memori kerja hewan coba. Tikus ditempatkan di suatu lengan (no 1.) tiga kemungkinan ditawarkan kepada tikus,yaitu tetap berada pada lengan 1 atau pindah ke lengan 2 atau pindah ke lengan 3. Sebuah pergantian dianggap benar jika tikus mengunjungi lengan baru dan tidak kembali lengan sebelumnya. Periode observasi dihitung untuk memberikan frekuensi alternasi spontan

3.9 Preparasi dan Pengamatan Jaringan Otak

3.9.1 Preparasi dan pengamatan histokimia dan imunohistokimia

Organ otak dipotong kemudian difiksasi dengan buffer formalin, selanjutnya dekalsifikasi dengan EDTA 7,4. Setelah itu dilakukan deparafinisasi dengan xilol 1 selama 1 menit, xilol 2 selama 1 menit dan xilol 3 selama 1 menit. Proses berlanjut yaitu hidrasi dengan alkohol 96% selama 2 menit, alkohol 96% selama 2 menit, alkohol 80% selama 2 menit dan alkohol 70% selama 2 menit. Kemudian masukkan kedalam air mengalir lalu celupkan pada cat utama Meyer's Hematoksilin selama 15 menit. Inti sel akan berwarna biru terang dan sitoplasma jernih. Celupkan cat pembanding eosin 1% selama 30 detik. Selanjutnya proses dehidrasi dengan konsentrasi alkohol 80% selama 2 menit, alkohol 90% selama 2 menit, alkohol 96% selama 2 menit lalu alkohol 96% selama 2 menit. Proses *clearing* dengan xilol 1 selama 5 menit, xilol 2 selama 5 menit kemudian mounting medium lalu tutup dengan *cover glass*. Hasil pewarnaan jaringan otak dengan *hemtoxyline-eosin* diamati di bawah mikroskop dan diambil gambarnya. Diamati perubahan morfologi jaringan otak. Preparasi jaringan untuk pewarnaan imunohistokimia dilakukan dengan merujuk pada protokol masing-masing Kit pada HSP70.

3.8.2 Preparasi perhitungan volume infark

Volume infark merupakan volume area otak yang mengalami kerusakan oleh karena stroke.Area infark ditandai dengan area yang lebih pucat daripada daerah sekitarnya. Volume infark dihitung dengan rumus: m. L merupakan daerahμ(L1+L21+L41+L61)*200 dengan area infark dan angka di belakang L merupakan nomer potongan slide, sebagai contoh L21 berarti area permukaan infark potongan slide ®ke 21. L dihitung dengan menggunakan CellSens Digital Imaging Software. Irisan pertama dimulai dari kiasma optikus, dan jarak di antara potongan mμslide ialah 10

3.9 Analisis Hasil

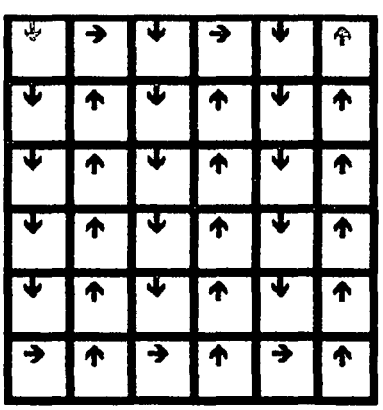
3.9.1 Analisis Statistik

Hasil pengukuran kadar glukosa darah merupakan data yang bersifat *continuous* sehingga dapat dianalisis secara statistik dengan ANOVA. Akan dilakukan pembandingan kadar glukosa darah antar kelompok percobaan sehingga dilakukan analisis ANOVA satu arah. Jika dari analisis statistika ANOVA dua arah ini ada perbedaan bermakna ($p<0,05$) maka analisis statistika dilanjutkan dengan analisis *Tukey*.

3.9.2 Analisis preparat histokimia dan imunohistokimia

Kuantifikasi dari sel nucleus dilakukan sebagai berikut:

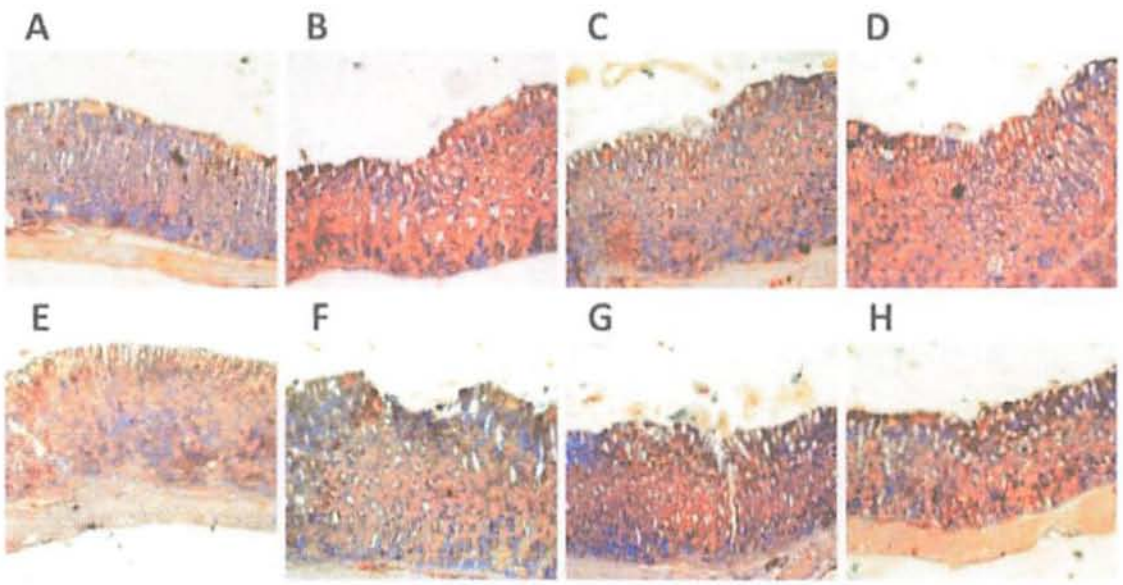
1. Masing-,masing slide ditempatkan pada optical photomicroscope dan diamati pada lensa objektif dengan perbesaran 40 atau lebih kemudian gambar diambil. Area yang diperbesar sebanding dengan slide asli yang berukuran 0.34 mm2.
2. Jumlah sel positif-negatif dihitung secara manual pada tiap gambar dan tiap frame.
3. Data hasil pengamatan (misalnya jumlah sel nucleus positif dan negatif dan jumlah sel dihitung sebagai berikut: % sel positif= sel nucleus positif/total nucleus sel x 100). Indeks dipresentasikan sebagai persen positif sel per millimeter persegi jaringan.



Gambar skematik dari grid yang digunakan. Grid dibagi menjadi 36 persegi dengan dimensi yang sama. Panah hijau (kiri atas) menunjukkan mula perhitungan, perhitungan berlanjut mengikuti panah hitam sampai berakhir pada panah merah.

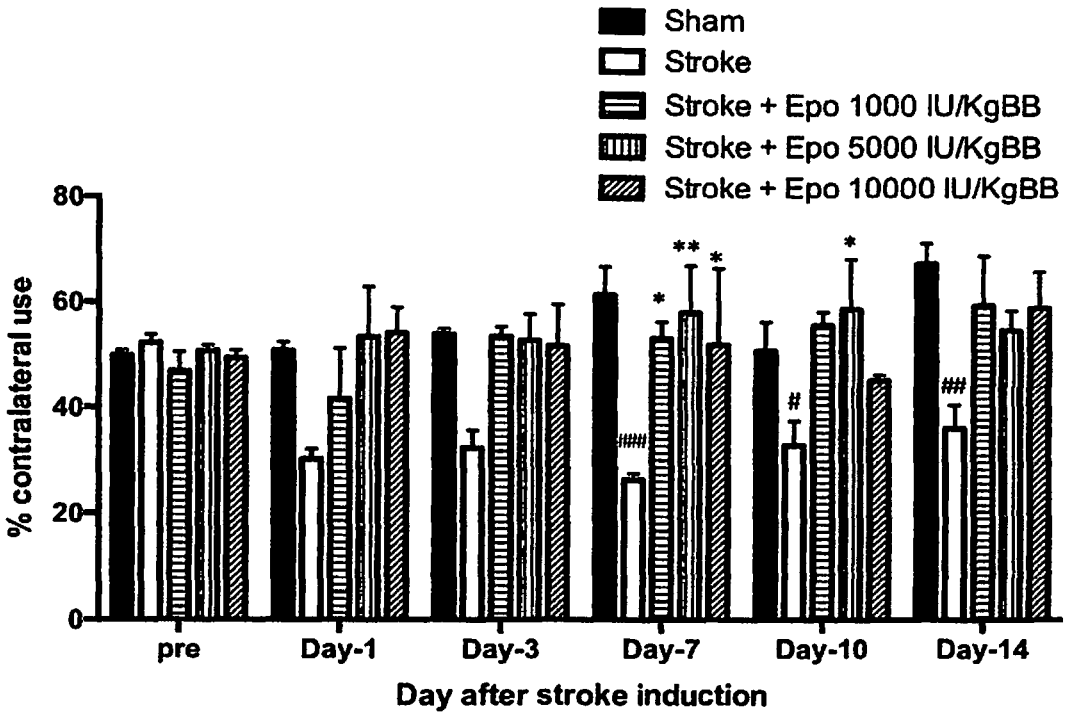
BAB 5
HASIL DAN LUARAN PENELITIAN

Hasil optimasi antibodi HSP70 pada perbaikan jaringan menunjukkan bahwa HSP meningkat dengan baik pada kondisi jaringan ulcer yang membaik. Diharapkan pada penggunaan pada stroke dengan perlakuan EPO, HSP70 dapat menjadi marker perbaikan jaringan yang sesuai (gambar 1).



Gambar 1. Performa antibodi HSP70 yang dicoba pada model perbaikan jaringan menggunakan perbaikan jaringan lambung yang diinduksi fluvoxamine pada stress ulcer.

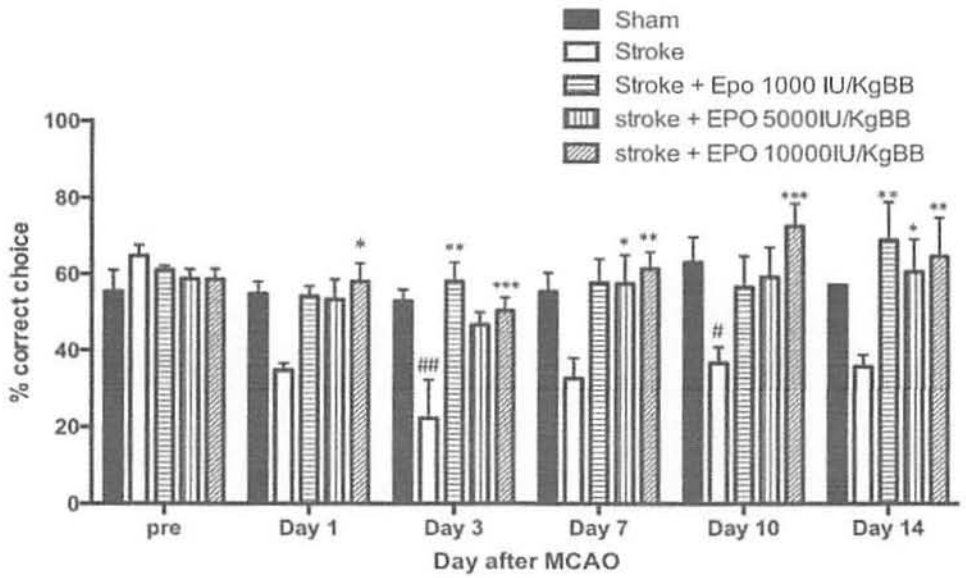
Hasil penelitian ini menunjukkan bahwa tikus yang mengalami stroke dengan metode oklusi arteri karotid menunjukkan perubahan pada fungsi motorik ditunjukkan dengan gangguan penggunaan tungkai kontralateral dan gangguan kognitif ditunjukkan dengan kemampuan memori dan eksplorasi.



Gambar 2. Efek EPO terhadap fungsi motorik diamati dengan menggunakan metode FUAT. Pengamatan dilakukan pada hari sebelum induksi stroke (pre), hari ke 1, 3, 7, 10, dan 14. Penentuan skor dilakukan dengan menghitung prosentase penggunaan tungkai depan kiri (kontralateral terhadap arteri karotid kanan) terhadap total penggunaan tungkai depan pada tes FUAT. Nilai ditampilkan sebagai rerata dan S.E.M dari 5-6 ekor tikus. #p<0.05, ##p<0.01 vs kelompok Sham. *p<0.05, **p<0.01 vs kelompok Stroke.

Hasil penelitian ini menunjukkan bahwa pada kondisi stroke penggunaan tungkai depan kiri sebagai tungkai yang kontralateral terhadap oklusi di arteri karotid kanan mengalami penurunan. Hal ini ditunjukkan dengan menurunnya proporsi penggunaan tungkai depan kiri dibandingkan total penggunaan tungkai depan kanan dan kiri sebagai tumpuan berdiri (standing) pada metode FUAT. Penurunan proporsi penggunaan tungkai depan kiri terjadi secara persisten dari hari ke 1 hingga hari ke 14 jika dibandingkan dengan kelompok Sham.

Pemberian Epo segera setelah induksi stroke menunjukkan penekanan terhadap penurunan penggunaan tungkai depan kiri yang diinduksi stroke. Secara konsisten penghambatan kerusakan fungsi motorik ditunjukkan sejak hari ke 1 pasca stroke hingga hari ke 14. Perbaikan pada fungsi motorik oleh Epo menunjukkan tingkat perbaikan yang sama dari dosis 1,000; 5,000 maupun 10,000 IU/kgBB.



Gambar 3. Efek EPO terhadap fungsi kognitif diamati dengan menggunakan metode Y-maze. Pengamatan dilakukan pada hari sebelum induksi stroke (pre), hari ke 1, 3, 7, 10, dan 14. Penentuan skor dilakukan dengan menghitung prosentase tikus memasuki *arm* yang berbeda dari sebelumnya (*arm* yang benar) terhadap total eksplorasi arm pada Y-maze test. Nilai ditampilkan sebagai rerata dan S.E.M dari 5-6 ekor tikus. #p<0.05, ##p<0.01 vs kelompok Sham. *p<0.05, **p<0.01, ***p<0.001 vs kelompok Stroke.

Pengukuran fungsi kognitif dengan menggunakan metode Y-maze menunjukkan bahwa pada kondisi stroke proporsi pemilihan *arm* yang benar atau tidak mengulang terhadap total jumlah eksplorasi terhadap *arms* mengalami penurunan proporsi jika dibandingkan dengan kelompok Sham. Penurunan proporsi pemilihan *arm* yang benar terjadi secara persisten dari hari ke 1 hingga hari ke 14 jika dibandingkan dengan kelompok Sham. Pemberian Epo segera setelah induksi stroke menunjukkan peningkatan terhadap proporsi pemilihan *arm* yang benar jika dibandingkan dengan kelompok stroke. Secara konsisten penghambatan terhadap penurunan proporsi pemilihan *arm* yang benar ditunjukkan sejak hari ke 1 pasca stroke hingga hari ke 14. Perbaikan pada fungsi motorik oleh Epo menunjukkan tingkat perbaikan yang *dose-dependent* pada hari ke 10.

Dari hasil di atas dapat disimpulkan bahwa Epo melakukan penghambatan kerusakan fungsi motorik dan fungsi kognitif secara persisten mulai hari pertama pasca stroke. Terlebih, Epo pada beberapa tingkat dosis yang berbeda memberikan efek yang baik terhadap fungsi motorik dan kognitif pasca stroke.

Uji coba kinerja antibodi HSP70 pada jaringan lambung yang telah dirusak untuk dapat dijadikan acuan peneliti dalam menggunakan marker perbaikan jaringan dilakukan sebagai

data awal. Hasil menunjukkan jaringan lambung yang mengalami perbaikan menunjukkan peningkatan HSP70. Sehingga pada tahap selanjutnya antibodi HSP70 dapat dijadikan alat ukur yang valid untuk induksi EPO pada perbaikan jaringan otak pada daerah penumbra yang diinduksi stroke.

Adapun luaran yang dicapai penelitian ini adalah berupa 2 manuskrip pada jurnal internasional berreputasi antara lain:

1. Laporan mengenai antibodi HSP70 yang efektif untuk mengukur perbaikan jaringan sehingga tahun depan dapat digunakan pada jaringan otak yang stroke, dengan judul "Selective serotonin reuptake inhibitor fluvoxamine increases HSP-70 level to ameliorate stress- and NSAID induced peptic ulcer" dengan status **ACCEPTED** pada *Journal of Basic and Clinical Physiology and Pharmacology* (Q3, non predatory).
2. Laporan mengenai efek perbaikan motorik dan kognitif yang dihasilkan EPO dengan metode Y maze dan FUAT dengan judul "Erythropoietin Ameliorates Stroke-Induced Motoric And Cognitif Deficits Evaluated By Forelimb Use Assymetry Test And Y-Maze" status **SUBMITTED** 2 November 2018 pada Jurnal *VASCULAR* (Q2, non predatory) dan hingga saat laporan akhir ini dilaporkan masih dalam proses review. Diperkirakan proses review dan acceptance akan selesai pada bulan Maret 2019.

BAB 6
RENCANA TAHAP BERIKUTNYA

Penelitian telah mendapatkan data pengaruh fisiologis EPO terhadap motorik dan kognitif model hewan coba tikus yang mengalami stroke. Pasca pengamatan behavior tikus, telah dilakukan pengambilan dan fiksasi jaringan otak untuk dilakukan uji biologi molekular.

Perencanaan tahap selanjutnya adalah meliputi screening perubahan ekspresi protein atau ekspresi mRNA dari protein fungsional yang mungkin berperan dalam *neuronal survival*. Pengukuran mRNA dari *melanocortin receptor*-3/4 (MC-3/4) akan dilakukan dengan teknik PCR untuk mengetahui adanya perubahan regulasi MC-3/4 receptor di bagian otak yang mengatur kognisi seperti hippocampus atau yang mengatur motorik seperti cortical area, stria dan thalamus. Rencana selanjutnya juga meliputi pengukuran ekspresi protein *alpha-melanocyte stimulating hormone* (alpha-MSH) yang diduga berperan dalam proses *healing* dari *brain injury*, namun belum ada evidence yang pasti mengenai keterlibatannya dalam mekanisme perbaikan yang diinduksi oleh EPO.

Ditargetkan pada tahap tahun ke dua luaran yang didapatkan akan bertambah 1 artikel pada jurnal internasional berreputasi dan 1 artikel pada jurnal nasional terakreditasi, sehingga total capaian 3 tahun adalah 3 artikel jurnal internasional dan 1 artikel jurnal nasional.

BAB 7
KESIMPULAN DAN SARAN

7.1 Kesimpulan

Dari hasil penelitian hingga tahun pertama ini, dapat disimpulkan bahwa:

1. EPO memberikan efek perbaikan terhadap fungsi motorik tikus yang mengalami stroke iskemia
2. EPO memberikan efek perbaikan terhadap fungsi kognitif dari tikus yang mengalami stroke iskemia

7.2. Saran

Adapun saran yang dihasilkan pada tahun pertama ini adalah:

1. Perlu dilakukan screening molekular terkait protein-protein fungsional yang mungkin berperan dalam mekanisme neuroproteksi oleh EPO pada stroke, baik berupa receptor, transporter, neuropeptida, transmitter lainnya dan signaling intraseluler.
2. Perlu dilakukan eksplorasi peran bagian otak tertentu yang selama ini belum pernah mendapat perhatian peneliti namun berpotensi untuk terlibat dalam efek neuroproteksi EPO pada stroke seperti hippocampus, cortex, amygdala, hypothalamus dan brain stem.

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Selective serotonin reuptake inhibitor fluvoxamine increases HSP-70 level to ameliorate stress- and NSAID-induced peptic ulcer

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Section/Category:	• Oxidative Stress
Keywords:	Gastric ulcer, stress, fluvoxamine, SSRI, Hsp70
Abstract:	Background: SSRIs has recently become a potential candidate for new therapeutic approach for ulcer and gastric bleeding. Hsp70 plays an important role in cellular resistance to NSAIDs. However, there is lack of evidence that fluvoxamine recruits Hsp-70 to affect stress-induced gastric ulcer. Thus, we investigated the effect of fluvoxamine on NSAID- and stress-induced gastric ulcer and the possible involvement of Hsp70. Method: ICR mice were used. Stress induction was made by water-immersion plus restraint method. NSAID-induced gastric ulcer was produced by oral administration of indomethacin. Fluvoxamine was given 30 minutes orally before the stress induction and indomethacin treatment. Results: Stress and indomethacin treatment significantly increased the ulcer index and intraluminal bleeding score. Stress and indomethacin treatment significantly increased the expression of Hsp70. Fluvoxamine significantly decreased the ulcer index and intraluminal bleeding in both

	ulcer models. Moreover, fluvoxamine further increased the expression of Hsp70 in gastric tissue of stress- and indomethacin-treated mice. Conclusion: These results indicate that fluvoxamine may exhibit a protective effect against stress-induced as well as NSAID-induced gastric ulcer. In addition, it is suggested fluvoxamine inhibits ulcer formation through the up regulation of gastric Hsp70 expression. ABSTRACT Rahmadi et al 2018.doc

1. INTRODUCTION

Stress affects many people in the world and induces numerous psychiatric disorders such as depression, PTSD, anxiety and schizophrenia.[1] Stress has been widely reported as one of the cause of gastric ulcer.[2] Gastric ulcer affects 4 million people around the world per year. The life-threatening perforation occurs about 2%-14% of the ulcers. [3,4] In critically ill patients, the prevalence of stress-related gastric ulcer followed with bleeding ranges between 15-50%. The stress-induced mucosal bleeding is considered as a severe complication with high mortality. [5,6] It is challenging to provide an ideal management to completely treat gastric ulcer, particularly stress-induced gastric ulcer. The use of antacids and H2 blocker has become inadequate in the management of gastric ulcer. Long course of proton pump inhibition may lead to several serious adverse events.[7,8] Thus, the development of new approach in treating stress-induced ulcer is still needed.

The extensive reports on the protective effect of several antidepressants on gastric ulcer have raised a new challenge to explore new therapeutic approach for ulcer and the underlying mechanism. Selective-serotonin reuptake inhibitor (SSRI) drug is one of the drug classes effectively used for the treatment of depression, a psychiatric disorder induced mainly by stressful life event. Studies show that SSRIs may become a potential candidate for new therapeutic approach for ulcer and gastric bleeding. Fluoxetine dose-dependently decrease the intraluminal bleeding in rat with indomethacin-induced gastric ulcer.[9] In stress model, fluoxetine suppresses acute cold restrain stress-induced gastric ulcer possibly by decreasing malondialdehyde and increasing gastric catalase and nitric oxide.[10] Repeated administration of paroxetine suppresses water immersion stress-induced gastric ulcer. Similarly, paroxetine treatment attenuates the corticosterone level increase during stress and demonstrates the anxiolytic and antidepressive effects.[11]

Even though studies demonstrate a detrimental effect of SSRIs on gastric mucosal tissue, studies also suggest that fluvoxamine effectively reduce the ulceration. Fluvoxamine suppresses NSAID-induced gastric ulcer by increasing total glutathione and nitric oxide levels. Moreover, fluvoxamine reduces the increase of oxidant parameters level in indomethacin-induced gastric ulcer.[12] However, to date, there is no evidence regarding the effect of fluvoxamine on stress-induced gastric ulcer. Moreover, there is lack of evidence in the gastroprotective mechanism of fluvoxamine. Thus, further examination on the exact mechanism how fluvoxamine affects NSAID- and stress-induced ulcer is still needed.

Heat shock proteins (HSPs) are family of molecular chaperones that play a vital role in protein folding and protein transport to sub-cellular compartment. Hsp70 is one of the HSPs widely known to have protective effect against gastric lesion and inflammation. It is known that various stressors, including NSAIDs, induce HSPs expressions.[13–16] Induction of Hsp70 expression provides cellular resistance to NSAIDs. Study using transgenic mice shows that the absence of HSF-1, a transcription factor of HSP genes, increases indomethacin-induced gastric lesion. Moreover, *in vitro* study demonstrates that Hsp70 silencing promotes a higher indomethacin-induced apoptosis.[17] These evidences suggest the important protective role of Hsp70 in gastric ulcer. However, the role of Hsp70 in the effect of fluvoxamine on stress- and NSAIDs-induced ulcer is still remained to be determined. Thus, in the present study, we investigated the effect of fluvoxamine on NSAIDs- and stress-induced gastric ulcer, and the possible involvement of Hsp70 in the fluvoxamine action.

2. MATERIALS AND METHODS

Drugs

Materials used were fluvoxamine (Wako Pure Chemical Industries, Kyoto, Japan), Tween 80 (WakoPure Chemical Industries), normal saline (PT. Otsuka Indonesia, Malang, Indonesia)

and antibodyfor HSP-70 (Santa CruzBiotech, CA, USA). All true solution and drug suspension in 1% tween were freshly prepared.

Experimental animals and treatments

Male 6-8 week-old ICR mice were used. All mice were cared with equal treatment, standard chow diet *ad libitum*, and 12 hours light-dark cycle. All experiment was performed in accordance with The Guiding Principles for the Care and Use of Animal Research of Universitas Airlangga No. 683-KE. All efforts were made to minimize animal suffering and to reduce the number of animal used. Animals were used only once.

After an acclimatization period of 14 days, all mice were randomly divided into control group, stress group, stress group treated with vehicle or fluvoxamine, indomethacingroup, and indomethacin group treated with vehicle or fluvoxamine.Fluvoxamine was administered in dose of 50 and 100 mg/kg.

Gastric ulcer induction

Stress-induced gastric ulcer was done in accordance to Ji et. al.,[9] Animals were food-deprived overnight. Thirty minutes after fluvoxamine, or saline injection, stress induction was started. Animal was restrained in polypropylene tube with sufficient holes for air circulation. The tube was vertically immersed in water bath at 25°C for 6 h.

NSAID-induced gastric ulcer was produced by treating animal with indomethacin. Animals were food-deprived overnight. Thirty minutes after administration of fluvoxamine or vehicle, indomethacin 25 mg/kg were orally administered.[18]

Assessment of gastric mucosal injury

After water immersions for 6 h, or 6 h after ulcer induction with indomethacin, the animals were then sacrificed. The stomach tissue was rapidly removed, opened along the greater curvature and gently washed by normal saline. Scoring of ulcer was performed toward macroscopic mucosal lesions to calculate ulcer index and intraluminal bleeding.[9,18]

Briefly, stomach was pinned out flat and observed for the presence of blood or mucus. Severity of intraluminal bleeding was evaluated according to the arbitrary scale,[19] as follows; 0, no blood detectable; 1, thin blood follows the rugae; 2, thick blood follows the rugae; 3, thick blood follows the rugae with blood clots in certain areas; 4, extensive covering of the whole of mucosal surface with thick blood. Then the stomachs were gently rinsed with saline to remove the gastric contents, the wiped blood off, spread and pinned in a flat altitude for subsequent examination of the ulcer index. Color photographs of the mucosal surface were taken. Each lesion area was measured in square millimeters, and the cumulative area of all lesions served as the measure of erosion damage.[9] The tissue was fixated and stained with hematoxylin-eosin and HSP 70 antibody.

Statistical Analysis

All data are presented as means ± S.E.M. One-way analysis of variance (ANOVA) followed by a post hoc Bonferroni test was used to compare groups. Student's t-test was used to compare two groups, where appropriate. Differences were considered statistically significant when $p < 0.05$.

3. RESULTS

Effect of fluvoxamine on NSAID-induced gastric ulcer

The photograph of gastric lumen showed that oral administration of indomethacin 25 mg/kg increased the gastric lesion (Figure 1A-B). Dark spot in the gastric lumen represented the lesion. Fluvoxamine 50 mg/kg decreased the dark spots appearance in the lumen. Fluvoxamine 100 mg/kg decreased the dark spots appearance as compared to indomethacin-treated group and fluvoxamine 50 mg/kg group (Figure 1C-D).

The calculation of ulcer index showed that indomethacin treatment significantly increased the ulcer index. Fluvoxamine 50 mg/kg markedly decreased the ulcer index. Furthermore,

fluvoxamine 100 mg/kg notably reduced the ulcer index (Figure 2A). Indomethacin treatment significantly increased the intraluminal bleeding score. Fluvoxamine 100 mg/kg, but not 50 mg/kg, suppressed the indomethacin-induced rise in the intraluminal bleeding score (Figure 2B).

The histological measurement using hematoxylin-eosin staining showed the damage of epithelial tissue after treatment with indomethacin. It is demonstrated that fluvoxamine decreased the damage induced by indomethacin (Figure 3A-D).

Effect of fluvoxamine on stress-induced gastric ulcer

The photograph of gastric lumen showed that stress using water immersion and restraining method increases the gastric lesion (Figure 4A-B). Fluvoxamine 50 mg/kg decreased the gastric lesion in the lumen. Fluvoxamine 100 mg/kg decreased the gastric lesion in the lumen as compared to stress group and fluvoxamine 50 mg/kg group (Figure 4C-D).

Pretreatment with stress significantly increased the ulcer index as compared to non-stress group. However, fluvoxamine 50 mg/kg markedly decreased the ulcer index as compared to saline-treated stress group. Furthermore, fluvoxamine 100 mg/kg notably reduced the ulcer index (Figure 5A). Accordingly, stress induction significantly increased the intraluminal bleeding score. Fluvoxamine 50 and 100 mg/kg suppressed the stress-induced increase in the intraluminal bleeding score (Figure 5B).

The histological measurement using hematoxylin-eosin staining showed the damage of epithelial tissue after 6 hours stress induction. The result showed that fluvoxamine decreased the gastric ulcer induced by stress (Figure 6A-D).

Effect of fluvoxamine on Hsp70 expression in gastric ulcer

Immunohistochemistry was conducted using anti-Hsp70. Indomethacin treatment increased the expression of Hsp70 (Figure 7A-B). Injection of fluvoxamine 50 mg/kg and 100 mg/kg prior to indomethacin treatment further increased the expression of Hsp70 as compared with

indomethacin-treated group (Figure 7B-D). Accordingly, water immersion restraint stress increased the expression of Hsp70 (Figure 7E-F). Injection of fluvoxamine 50 mg/kg and 100 mg/kg prior to stress induction further increased the expression of Hsp70 as compared with stress group (Figure 7F-H).

4. DISCUSSION

The gastric ulcer models induced by NSAIDs administration and stress induction have been commonly used to evaluate the efficacy of gastroprotective agents. In the present study, we clarified that NSAIDs-induced gastric ulcer is successfully produced by oral administration of indomethacin 25 mg/kg. This result is in agreement with the previous study byJi[9]showing that single administration of indomethacin successfully induces ulceration.Furthermore, our result showed that fluvoxamine dose-dependently decreased the indcx ulcer induced by indomethacin. Moreover fluvoxamine 100 mg/kg suppressed the intraluminal bleeding induced by indomethacin. This is in agreement with the previous study by Dursun et. al.,[20] showing that fluvoxamine affects antioxidant and oxidant parameters in gastric tissue to inhibit ulcer formation.[12]

The inhibitory effect of fluvoxamine on indomethacin-induced gastric ulcer has raised the potential gastroprotective effect of fluvoxamine on stress-induced ulcer. Reports show that some SSRIs have shown a protective effect against stress-induced ulcer. For example, fluoxetine suppresses acute cold restrain stress-induced gastric ulcer.[10] Moreover, repeated administration of paroxetine decreases water immersion stress-induced ulcer possibly by modulating corticosterone level increase during stress.[11] The present study demonstrated that 6 h stress induction produced by water immersion and restrain procedure in mice generates gastric ulcer and intraluminal bleeding. This result is in agreement with previous

study showing that 6 h water immersion in restraining cage effectively induced ulcer formation and intraluminal bleeding.[9]

Furthermore, the present study demonstrated that fluvoxamine dose-dependently and significantly decreased the rise in ulcer index induced by stress. Furthermore, fluvoxamine dose-dependently and significantly attenuated the stress-inducedincrease in intraluminal bleeding score. The similar result with other SSRIs, fluoxetine, is also previously reported. Fluoxetine attenuates the ulcer index and gastric intraluminal bleeding in either NSAIDs- or stress-induced gastric ulcer.[9,10] Since there is no evidence showing the mechanism underlying the protective effect of fluvoxamine on stress-induced gastric ulcer, we further investigated the possible molecule related to cell damage in gastric tissue.

Hsp70 is known as a gastroprotective molecule in ulceration event. In the present study, we found that the expressions of Hsp70 increase in gastric ulcer induced by indomethacin. This is in agreement with the study by Suemasu et. al.,[17] showing that down regulation of Hsp70 expression increases cell damage induced by indomethacin. Moreover, deleting the transcription factor of Hsp70, HSF, increases the lesion index induced by indomethacin. Subsequently, our results showed that fluvoxamine administration before ulcer induction by indomethacin further increases the expression of Hsp70 in gastric tissue. Together with the result showing an ameliorative effect of fluvoxamine on gastric ulcer, this result suggests that fluvoxamine may decrease indomethacin-induced gastric ulcer by up regulating Hsp70 expression in gastric tissue.

Previous study shows that the high expression of Hsp70 is associated with the protective effect of moxibustion on stress-induced ulcer.[21] Similarly, our result showed that the expression of Hsp70 increases in stress-induced gastric ulcer condition. These suggest that Hsp70 may play as a protective response to stress-induced ulcer. Furthermore, our present study showed that the treatment with fluvoxamine further increased the Hsp70 expression in

gastric tissue. This suggests that fluvoxamine may ameliorate gastric ulcer by upregulates the expression of Hsp70 in gastric tissue.

Mechanism on how fluvoxamine upregulates Hsp70 expression is remained unknown. Sigma-1 receptor, the receptor for fluvoxamine, has been reported to bind Hsp70 in endoplasmic reticulum. Binding by fluvoxamine on sigma-1 receptor may brake down the sigma-1 receptor-Hsp70 binding, and activates the chaperoning activity each component.[22] Further research is needed to clarify this issue.

5. CONCLUSION

The present study demonstrates that fluvoxamine ameliorates the gastric ulcer induced by stress as well as NSAID in mice. Moreover, fluvoxamine further increases the upregulation of Hsp70 expression in gastric epithelial tissue. The further study on the detail mechanism of fluvoxamine's antiulcer activity may lead to the prospectivedevelopment of effectivetreatment to stress-induced gastric ulcer.

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AUTHORS CONTRIBUTION

Study conception and design by MR, CA, KN, DWS, TA, S; Acquisition of data by MR, KN, RO, AR, YD; Analysis and interpretation of data by MR, CA, KN; Drafting of manuscript by MR, CA, KN, RO, AR, YD; Critical revision by MR, CA, RO, AR, YD, DWS, TA, S.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in the conduct of this study.

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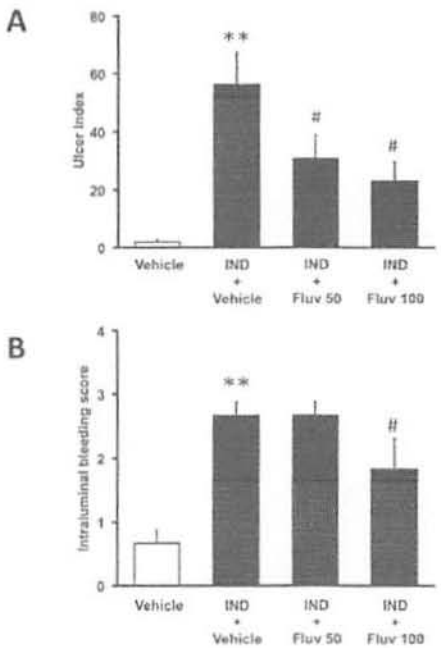

Rahmadi et al., Figure 2

Figure 2. Effects of fluvoxamine on indomethacin-induced increase in ulcer index (A) and intraluminal bleeding score (B). Each column represents the mean ± S.E.M. of 6 mice. ***p < 0.001 vs vehicle group. #p < 0.05, ##p < @0.01 vs IND + vehicle group.

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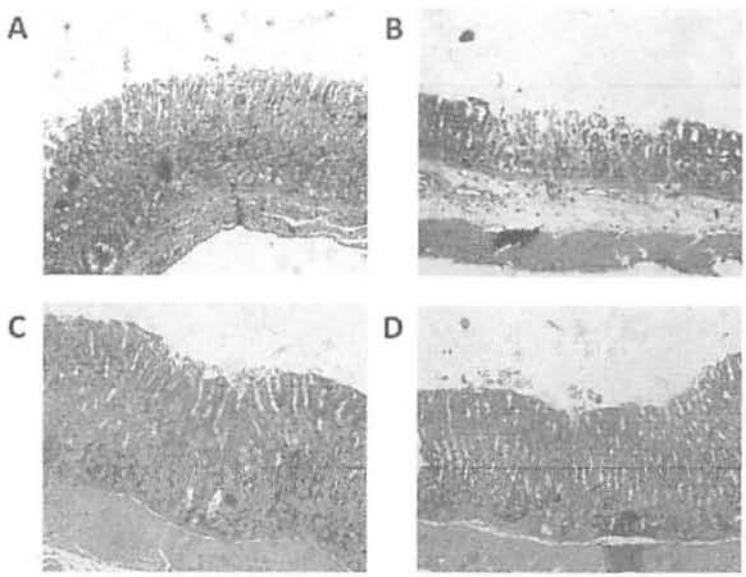

Rahmadi et al., Figure 3

Figure 3. Representative microphotograph of gastric epithelial tissues of mice treated with vehicle (A), indomethacin-tween-80 (B), indomethacin-fluvoxamine 50 mg/kg (C), and indomethacin-fluvoxamine 100 mg/kg (D). The transverse sections were stained with hematoxylin eosin to identify epithelial tissue damage.

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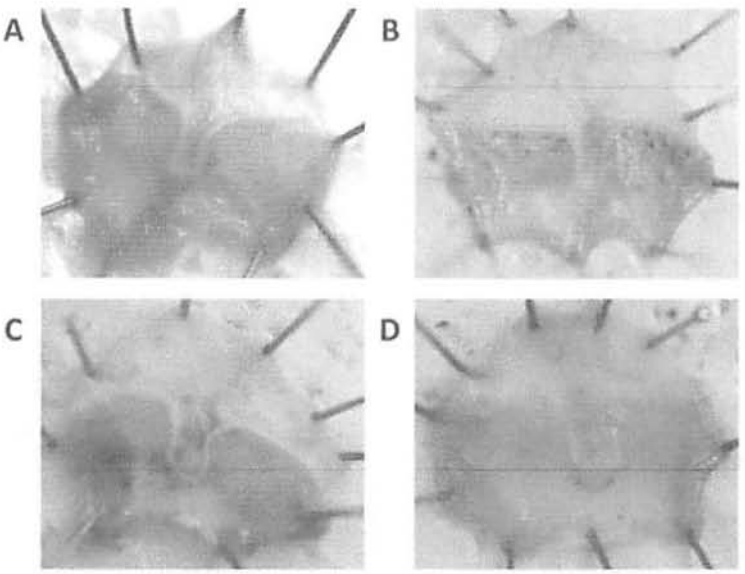

Rahmadi et al., Figure 4

Figure 4. Representative photograph of gastric lumen of mice treated with no stress (A), stress followed with tween-80 (B), stress followed with fluvoxamine 50 mg/kg (C), and stress followed with fluvoxamine 100 mg/kg (D). Tween-80 and fluvoxamine were injected 30 min prior to stress induction. Tissues were sampled soon after the termination of 6 h stress induction.

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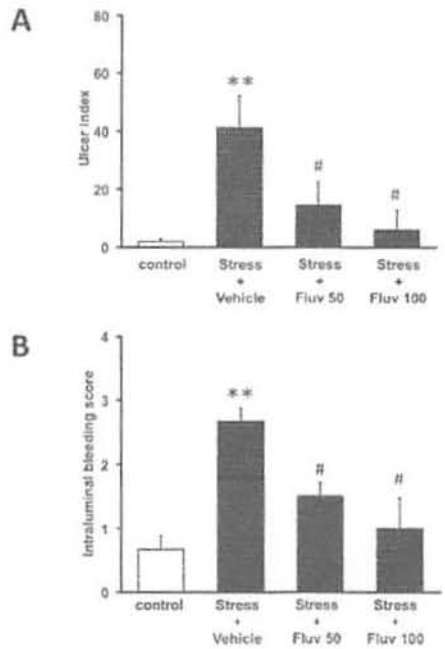

Rahmadi et al., Figure 5

Figure 5. Effects of fluvoxamine on stress-induced increase in ulcer index (A) and intraluminal bleeding score (B). Each column represents the mean ± S.E.M. of 6 mice. **p < 0.01, ***p < 0.001 vs control group. #p < 0.05, ##p < @0.01 vs stress + vehicle group.

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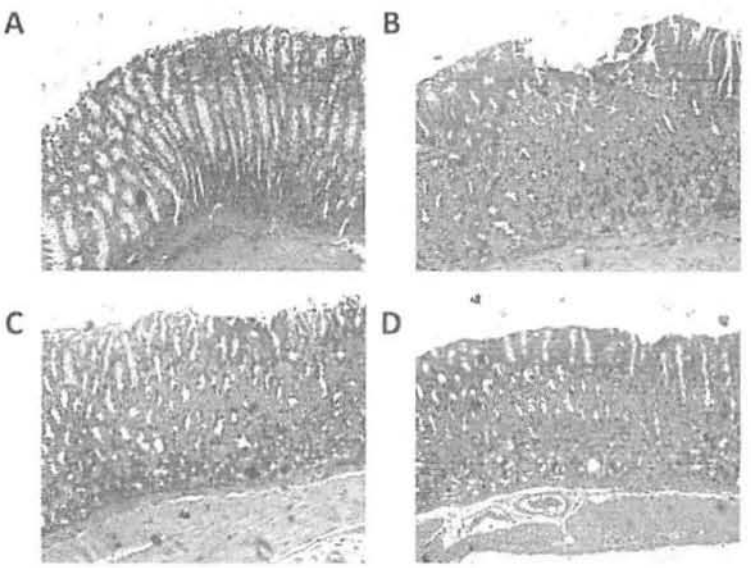

Rahmadi et al., Figure 6

Figure 6. Representative microphotograph of gastric epithelial tissues of mice treated with no stress (A), stress followed with tween-80 (B), stress followed with fluvoxamine 50 mg/kg (C), and stress followed with fluvoxamine 100 mg/kg (D). The transverse sections were stained with hematoxylin eosin to identify epithelial tissue damage.

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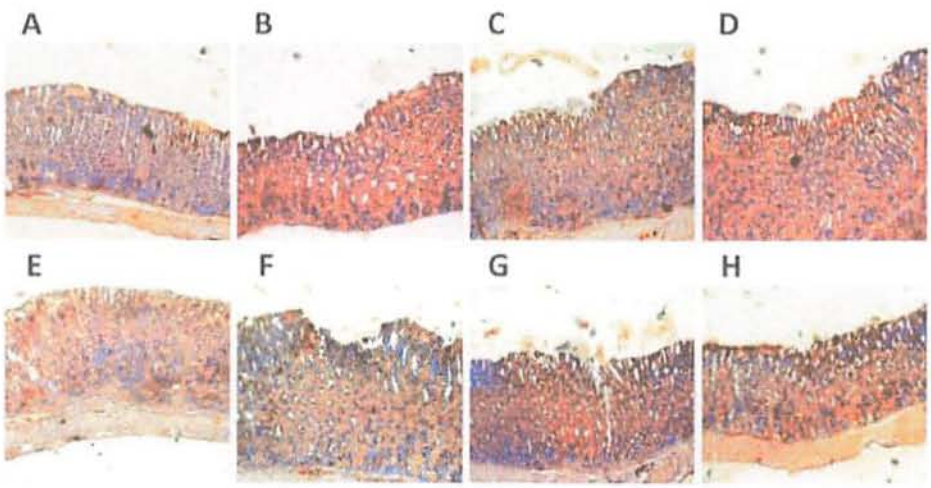

Rahmadi et al., Figure 7

Figure 7. Representative microphotograph showing Hsp70 expression in gastric tissues after treatment with oral administration of normal saline (A), indomethacin-tween-80 (B), indomethacin-fluvoxamine 50 mg/kg (C), and indomethacin-fluvoxamine 100 mg/kg (D), stress followed with tween-80 (E), stress followed with fluvoxamine 50 mg/kg (F), and stress followed with fluvoxamine 100 mg/kg (G). The transverse sections were stained with anti-Hsp70.

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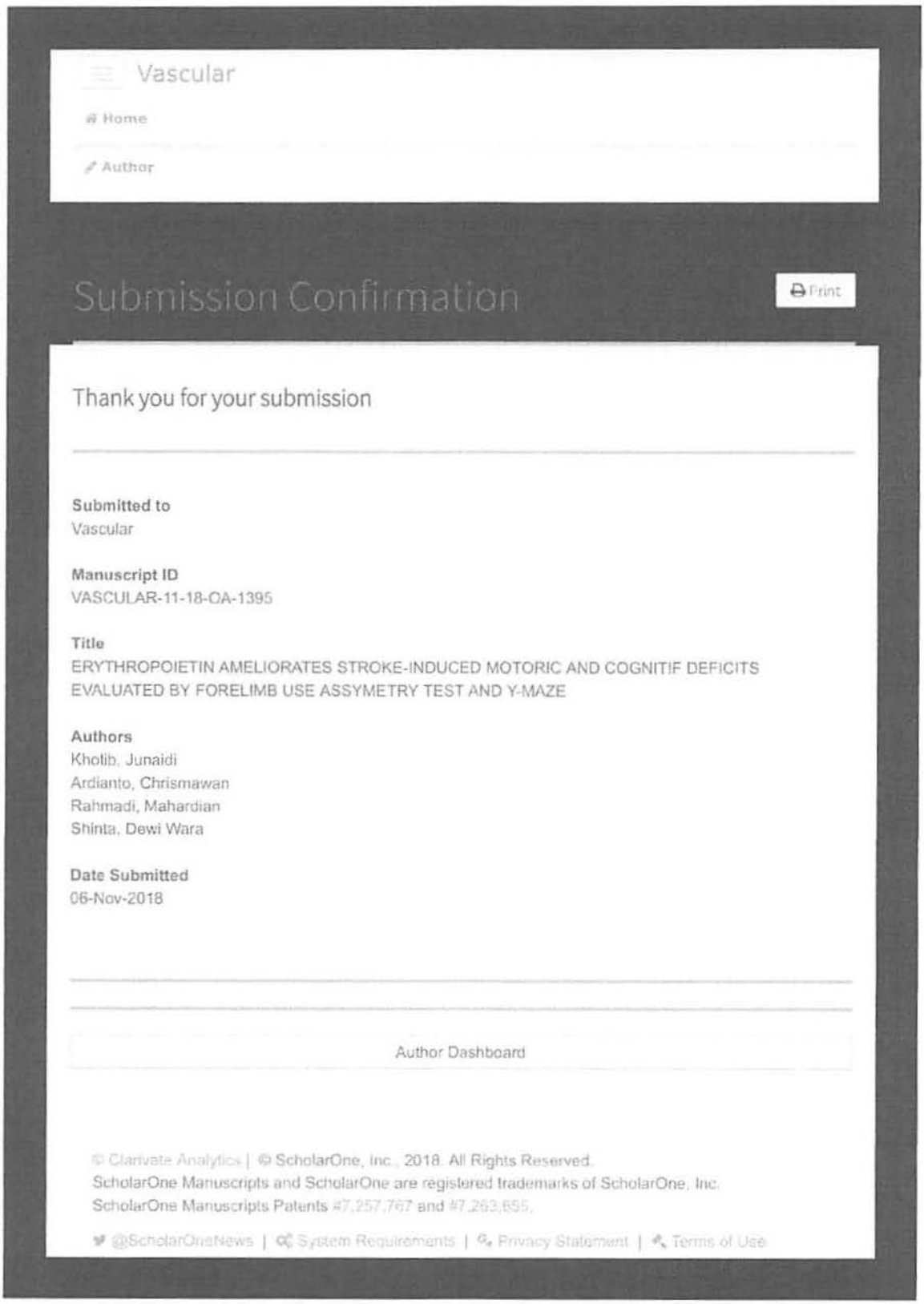

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-Title page-

ERYTHROPOIETIN AMELIORATES STROKE-INDUCED MOTORIC AND COGNITIF DEFICITS EVALUATED BY FORELIMB USE ASSYMETRY TEST AND Y-MAZE

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Abstract

Background Stroke has been known as the leading cause of disability and death. The neuronal death caused by ischemic stroke leads to loss in motoric and cognitive function, which lowered the quality of life. Erythropoietin (Epo) has been proposed to protect brain from injury through the inhibition of apoptosis in penumbra area its interaction with vascular endothelial growth factor (VEGF) and insulin growth factor (IGF-1) to provide an angiogenic effects on injured brain area. However, robust evidence on the protective effect of Epo on motoric and cognitive function, and the dose dependent character of Epo treatment remains to be determined. **Method** Male 6-8 week old Wistar rats were used. The stroke was induced by right unilateral carotid artery occlusion. Examination on motoric and cognitive function were conducted using FUAT and Y-maze test, respectively, before stroke induction and 1; 3; 7; 10; and 14 days after stroke. **Results** The results showed that the induction of stroke decreased the contralateral forelimb use in FUAT test and % correct arm entry in Y-maze test. Epo ameliorated the suppressive effect of Epo on contralateral forelimb use and % correct arm entry. Epo at dose 1,000; 5,000; and 10,000 IU/kg showed a rather equal protective effect on behavior deficits. The onset of protective effect of Epo on motoric and cognitive functions was shown from 1 day after stroke. **Conclusion** To conclude, the present study demonstrates an early and robust neuroprotective effect of Epo treatment on motoric and cognitive deficits induced by ischemic stroke.

(248 words)

Keywords: Erythropoietin, ischemic stroke, FUAT, Y-maze

Short/running title: Epo improves cognitive and motor function in stroke

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Introduction

Stroke is one of life threatening diseases, which commonly triggered by chronic courses of metabolic disorder. Stroke causes disability and lowering the quality of life by affecting the brain function. Ischemic stroke is the common form of stroke that made by focal occlusion of blood vessels in the brain, which lead to the suppression of oxygen supply to the certain part of the brain and causes neuronal death. More than 80% of stroke causes by ischemic infarctions, and the remaining are caused by hemorrhages and subarachnoid hemorrhage 4. In a year, there are more than 20 million people in the world experiencing stroke. More than 500,000 stroke attacks per year, mostly primary strokes, occur in the U.S [1,2]. There is an emergence need of therapeutic approach to reduce the disability by preventing further neuronal deficits in the infraction area.

It is reported that hypoxia promotes multiple gene activation to produce functional protein and peptides such as Epo and VEGF that will improve oxygen delivery to the tissues. The hypoxia-induced upregulation of Epo and Epo receptor suggests that exogenous Epo administration exhibits a potential therapeutic role in preventing further tissue damage from ischemia in the central nervous system [3,4,5]. There is an increasing evidences shows that angiogenesis after Epo treatment results in improved collateral circulation and may impact to the recovery of motoric and cognitive functions. Epo has been proven to inhibit the apoptosis in penumbra area and increase the proliferative factors [5,6,7]. However, there is still lack of evidence in the robust protective effect of Epo on motoric and cognitive function and the onset of immediate Epo administration post stroke event. More over, there is evidence showing the use of different level of Epo dose, yet without pointing out the dose size effect of Epo on stroke-induced deficits in motoric and cognitive functions. Thus,

present study aimed to examine the effect of Epo on the stroke-induced deficits in motoric and cognitive functions after immediate administration upon stroke attack and its dose dependent effect. The stroke induction was made by unilateral carotid artery occlusion and Epo was administered intravenously in the same day. The motoric and cognitive tests were made by using FUAT and Y-maze, respectively.

Material and Method

Materials

The materials used in the experiment were rHu Erythropoietin alfa (PT Daewoong Infion, Pandaan, Indonesia), normal saline (PT. Widatra Bhakti, Pandaan, Indonesia), purified water made by water purifier, buffered-formalin 10%, xylazine, midazolam.

Animals model and treatments

Male 6-8 week old Wistar rats were used. The conduct of the research has been approved by ethical committee on animal use of faculty of veterinary medicine, Universitas Airlangga. All animals were used only once. All efforts were made to reduce the animal number and their suffering. The stroke was induced by unilateral carotid artery occlusion in the right side. Briefly, rats were randomly divided into sham, stroke with Epo 1,000 IU, stroke with Epo 5,000 IU, and stroke with Epo 10,000 IU. Intraperitoneal injection of xylazine (10 mg/kg) and ketamine (80 mg/kg) were used as anesthesia. The animal was fixed to the surgical table using adhesive tape. A small incision (2 - 3 cm) in the midline neck and the right common carotid artery was isolated from the connective tissue and vagal nerve. in groups with stroke, the right common carotid artery (CCA) was blocked by

bulldog clamp for 90 min under unconscious condition. Sham group was subjected to the same procedure without clamping the artery. The incision was closed with sutures. 0.5 ml of saline was given intraperitoneally to substitute the water loss during surgery. Epo was administered intravenously not more than 2 hours after the stroke-induction or the surgery finished. Examination on motoric and cognitive function were conducted using FUAT and Y-maze test, respectively, before stroke induction and 1; 3; 7; 10; and 14 days after stroke.

Forelimb Use Asymmetry Test (FUAT)

Forelimb Use Asymmetry Test was conducted before stroke induction, and at 1, 3, 7, 10 and 14 days after stroke induction. Forelimb use during exploratory activity was analyzed by videotaping rats in a transparent cylinder (20 in cm diameter and 30 cm in height) for 3 to 10 minutes depending on the degree of activity during the trial. A mirror was placed to the side of the cylinder at an angle to enable the recording of forelimb movements even when the animal was turned away from the camera. Scoring was done by blinded, trained-examiner. Independent use of the left or right forelimb for contacting the wall during a full rear to initiate a weight-shifting movement or to regain center of gravity while moving laterally in a vertical posture is scored as 1 point for the respective forelimb used. Simultaneous use of both the left and right forelimbs for contacting the cylinder wall during a full rear and for alternating lateral stepping movements along the wall is scored as 1 point for each forelimb. The total score of impaired (contralateral) forelimb and unimpaired (ipsilateral) forelimb was used as a percentage of total number of limb use. The disability score was measured by comparing the use of impaired contralateral (left) forelimb to the total use of forelimb.

Y-maze assay

Y maze test was conducted in accordance to Onalopo et al (2012) and Wahl et al (1992). Briefly, Y maze test was performed before stroke induction, and at 1, 3, 7, 10 and 14 days after stroke induction. This behavior, spontaneous alternation 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 entries a new arm and did not return to the two previously visited arm. The percentage of correct choices was calculated as a percentage of corrects alternations to the total alternations. The apparatus was cleaned by swapping tap water, followed by 0.3% acetic acid, and allowed to dry between sessions.

Results & Discussion

The present study showed that the percentage of contralateral forelimb use was not significantly affected by sham surgery from day 1 to 14 observations. The results showed that the induction of stroke significantly decreased the contralateral forelimb use in FUAT test at day 7, 10 and 14. Epo ameliorated the deteriorating effect of stroke on contralateral forelimb use. Epo at dose 1,000; 5,000; and 10,000 IU/kg showed a rather equal protective effect on behavior deficits. Epo significantly ameliorated the motoric functions deficits induced by stroke at day 7 and 10 (fig. 1).

The result of the present study showed that the percentage of correct alternation choices was not significantly affected by sham surgery from day 1 to 14 observations. The

induction of stroke significantly decreased the correct alternation choices in Y-maze test at day 3 and 10. Moreover, The induction of stroke steeply but not significantly decreased the correct alternation choices in Y-maze test at day 1, 7 and 14. Epo significantly ameliorated the suppressive effect of stroke on the correct alternation choices at day 1 to day 14. Epo at dose 1,000; 5,000; and 10,000 IU/kg showed a rather equal protective effect on cognitive deficits (fig. 2).

The activation of EpoR by EpoR during hypoxia suggests that Epo has a pivotal therapeutic role for tissue damage during brain ischemia or neuronal hypoxia in the central nervous system. Epo has shown an efficacy to improve cognitive and sensorimotor deficits in rats model of chronic contrictive injury and cryogenic lesion injuries. Thus, it is possible that the reduction of cell death in the motor area in brain improves motor function in stroke.

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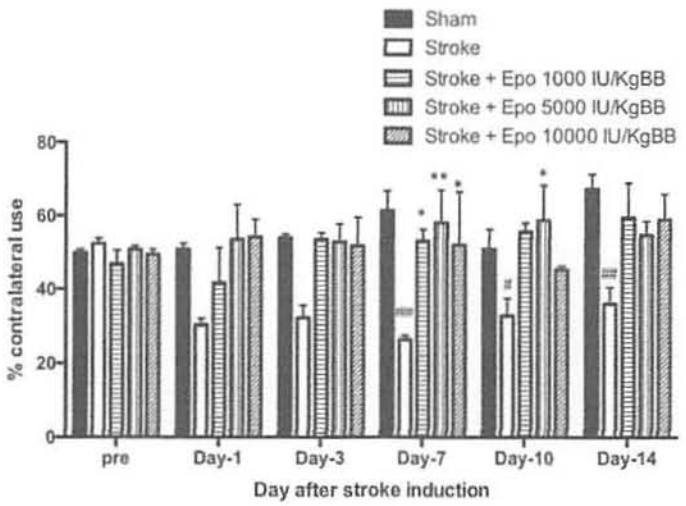
Figure captions

Figure 1. The effect of Epo on motoric function is evaluated by FUAT method. The forelimb use was examined before stroke induction, and 1, 3, 7, 10, and 14 days after stroke induction. The score are performed as the percentage of contralateral (left) forelimb use to the total forelimb use. Scores are performed as the mean ± S.E.M of 5-6 rats. #p<0.05, ##p<0.01 vs Sham group. *p<0.05, **p<0.01 vs Stroke group.

Figure 2. The effect of Epo on cognitive function is evaluated by Y-maze method. The forelimb use was examined before stroke induction, and 1, 3, 7, 10, and 14 days after stroke induction. The score are performed as the percentage of correct alternation choices to the total alternation done during the test. Scores are performed as the mean ± S.E.M of 5-6 rats. #p<0.05, ##p<0.01 vs Sham group. *p<0.05, **p<0.01, ***p<0.001 vs Stroke group.



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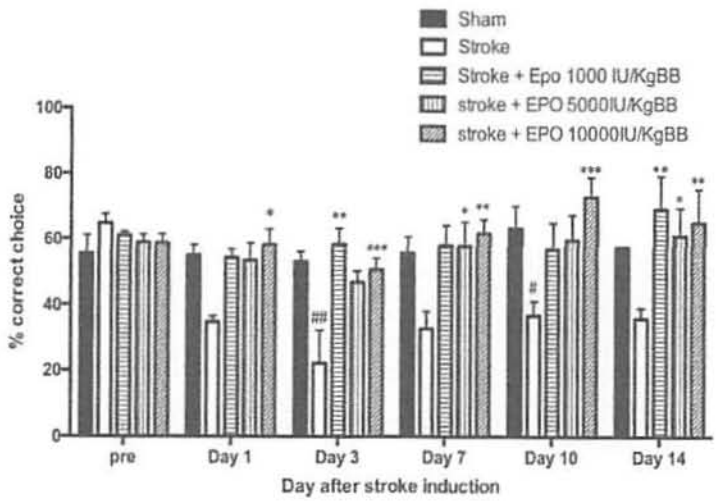

The effect of Epo on cognitive function is evaluated by Y-maze method. The forelimb use was examined before stroke induction, and 1, 3, 7, 10, and 14 days after stroke induction. The score are performed as the percentage of correct alternation choices to the total alternation done during the test. Scores are performed as the mean ± S.E.M of 5-6 rats. #p<0.05, ##p<0.01 vs Sham group. *p<0.05, **p<0.01, ***p<0.001 vs Stroke group.

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