ABSTRACT

ADIPOSE DERIVED NEURAL STEM CELL PREVENTS BRAIN DAMAGE PROGRESSION IN BRAIN ISCHEMIA

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Introduction
Until now there has been no neuroprotective therapy that has proven to be effective in improving brain hypoxic ischemia (HI) and apoptosis. Stem cell therapy has the potential role to repair neurological damage caused by HI. Adipose cells are a widely available source of stem cells, without invasive action. The mechanism of Adipose-derived Neural Stem Cell (ADNSC) on HI is not yet explained

Objective
This study aimed to investigate the role of intracerebrally tranplanted ADNSC in prevention of brain damage progressivity in brain ischemia.

Methods
A post test only control group design was used in 20 weeks-old male Wistar rats that randomized into control, ligation of carotid communis artery (CCA) and treatment (Ligation of CCA and ADNSC intracerebral transplantation) groups. Brain ischemia was induced by ligation of CCA for 2 hours and hypoxia procedure for 60 minute. ADNSC was intracerebrally tranplantated after hypoxia procedure to the treatment group. After 48 hours all rats were sacrified and their brain were immunohistochemically analyzed for expression of IL-10, NGF, MDA, Caspase-3 and TUNEL assay procedure for apoptotic neuron. ANOVA and Kruskal Wallis test were used for statistical analysis.

Results
There were 24 rats divided into 8 groups each, participating in the study. There were no decrease in TNF-α expression ($P > 0.05$), an increase in IL-10 expression ($P < 0.05$), an increase in NGF expression ($P < 0.05$), a decrease in MDA exppression ($P < 0.05$), a decrease in Caspase-3 expression ($P < 0.05$) and a decrease in the number of apoptotic neurons ($P < 0.05$) in brain ischemia induced CCA ligation after ADNSC transplant intracerebrally. Path analysis showed ADNSC decreased apoptotic neuron through Caspase-3 pathway.

Conclusion
Prevention of the progression of brain damage in brain ischemia by administering ADNSC intracerebraly can reduce MDA expression and apoptotic neurons through Caspase-3 pathway.

RINGKASAN

Mekanisme pencegahan progresivitas kerusakan otak melalui transplantasi intraserebral *Adipose Derived Neural Stem Cell* pada iskemia otak (Analisis TNF-α, IL-10, NGF, MDA, Caspase 3 dan Neuron yang mengalami Apoptosis pada Hipokampus Tikus Wistar)

Hipoksia-iskemia (HI) adalah salah satu penyebab utama kematian dan kecacatan di seluruh dunia. Masih belum ada terapi neuroprotektif farmakologis yang terbukti efektif secara klinis yang mampu mengurangi tingkat keparahan HI. Inflamasi merupakan kontributor utama untuk cedera sekunder dan melibatkan peningkatan produksi kemokin dan sitokin. TNF-α sebagai sitokin inflamasi dilepaskan pada tahap awal HI dan mendorong respon inflamasi dengan meningkatkan ekspresi faktor kemotaksis, yang menginduksi perekrutan makrofag ke dalam area yang cedera. Ekspresi IL-10 meningkatkan kelangsungan hidup sel neuronal dan glial, dan mengurangi respon peradangan melalui sejumlah jalur sinyal. HI juga akan menginduksi stres oksidatif dengan akibat keluarnya *malondialdehyde* (MDA) sebagai produk sekunder peroksidase lipid. HI mengaktifkan jalur apoptosis mitokondria dengan aktivasi Caspase-3. Caspase-3 telah diidentifikasi sebagai mediator utama apoptosis pada hewan model HI. Terapi sel punca mempunyai potensi untuk memperbaiki kerusakan neurologis yang disebabkan oleh cedera otak HI. Secara in vitro, *Neural Stem Cell* merangsang pertumbuhan akson dan menunjukkan efek protektif melalui sekresi *Nerve Growth Factor* (NGF). MSC juga menunjukkan adanya supresi yang signifikan pada pelepasan radikal bebas yang dibuktikan tingkat MDA yang lebih rendah pada HI. Mekanisme efek potensial *Adipose-derived Neural Stem Cell* (ADNSC) pada kerusakan otak HI masih belum dapat dijelaskan

Tujuan dari penelitian ini adalah menjelaskan mekanisme pencegahan progresivitas kerusakan otak pasca transplantasi intraserebral ADNSC pada iskemia otak yang diinduksi ligasi AKK dan hipoksia.

Jenis penelitian yang digunakan adalah eksperimental *post test only control group design*. Unit eksperimen menggunakan tikus putih jantan Rattus norvegicus strain Wistar berusia 2 bulan. Hewan coba dibagi menjadi 3 kelompok secara random yakni kelompok kontrol, kelompok ligasi AKK dan kelompok ADNSC. Variabel yang diteliti Ekspresi TNF-α, Ekspresi IL-10, Ekspresi NGF, ekspresi MDA, Ekspresi Caspase-3 dan neuron yang mengalami Apoptosis. Penelitian dilakukan bulan Maret 2018 – Juli 2019. Pemeliharaan hewan coba, proses isolasi dan kultur ADNSC serta pemeriksaan imnositokimia dilakukan pada Laboratorium Pusat Penelitian dan Pengembangan Stem Cell Universitas Airlangga Surabaya. Pemeriksaan imunohistokimia dan TUNEL *Assay* di Lab Histologi Fakultas Kedokteran Universitas Airlangga Surabaya. Alur penelitian adalah sebagai berikut : Hewan coba dilakukan adaptasi terhadap lingkungan barunya selama 1 minggu. Setiap kandang berisi 2 ekor tikus yang telah diberi tanda sesuai kelompok perlakuannya. Tikus pada kelompok kontrol tidak dilakukan intervensi apapun. Pada kelompok ligasi dilakukan ligasi AKK (selama 2 jam) dan dimasukkan ke dalam

chamber hipoksia (selama 1 jam), sedangkan pada kelompok ADNSC dilakukan ligasi AKK (selama 2 jam) dan dimasukkan ke dalam *chamber* hipoksia (selama 1 jam) diikuti pemberian transplantasi *ADNSC* intraserebral. Dilakukan skor neurologi untuk mengevaluasi fungsi neurologi secara klinis setelah 24 jam dan 48 jam.Setelah 48 jam semua tikus dikorbankan dengan menggunakan anestesi xylazine/ketamin. Jaringan otak dilakukan analisa secara imunohistokimia untuk mengetahui jumlah sel yang mengekspresikan TNF-α , IL-10, NGF, MDA serta Caspase-3 dan TUNEL *Assay* untuk apoptosis.

Hasil penelitian menunjukkan tidak terdapat penurunan ekspresi TNF-α , terjadi peningkatan ekspresi IL-10, terjadi peningkatan ekspresi NGF, terjadi penurunan ekspresi Caspase-3 dan terjadi penurunan jumlah neuron yang mengalami Apoptosis pada iskemia otak yang diinduksi ligasi AKK dan hipoksia. ADNSC menurunkan ekspresi MDA, dan menurunkan apoptosis melalui penurunan jalur Caspase-3.

Dalam sistem saraf pusat, sinyal TNF memainkan peran ganda, yaitu yang meningkatkan inflamasi melalui TNFR1 pada sel-sel imun sambil memberikan sitoproteksi melalui TNFR2 pada sel-sel saraf. Dengan adanya fungsi ganda itu, maka akan jadi sulit di prediksi fungsi mana yang dominan dari TNF-α setelah di terapi dengan ADNSC. Kadarnya bisa tetap, naik atau turun. *Upregulation* dari IL-10 memainkan peran neuroprotektif potensial terhadap iskemia otak dan menyediakan lingkungan mikro yang menguntungkan untuk neurogenesis setelah iskemik otak. Secara spesifik,NGF meregulasi plastitas sinaptik dan memproteksi neuron dari stres oksidatif dan apoptosis yang dapat menstimulasi neurogenesis. Pada HI yang diberikan MSC memiliki tingkat MDA yang lebih rendah, menunjukkan adanya supresi yang signifikan pada pelepasan radikal bebas dan memberikan perlindungan antioksidan pada sel dan membran organel. Caspase-3 memainkan peran penting dalam kematian sel apoptosis, dan penghambatan caspase-3 telah terbukti memperbaiki cedera iskemik serebral. Transplantasi NSC dapat secara signifikan mengurangi jumlah sel apoptosis dalam penumbra pada 7 hari dengan meningkatkan pengaturan ekspresi Bcl-2.

Kesimpulan dari penelitian ini adalah tranplantasi intraserebral ADNSC tidak dapat menurunkan ekspresi TNF-α , meningkatkan ekspresi IL-10, meningkatkan ekspresi NGF, menurunkan ekspresi MDA, menurunkan ekspresi Caspase-3 dan menurunkan neuron yang mengalami apoptosis pada iskemia otak yang diinduksi ligasi AKK dan hipoksia. Pencegahan progresivitas kerusakan otak pada iskemia otak dengan pemberian ADNSC yakni dengan penurunan MDA dan penurunan neuron yang mengalami apoptosis melalui jalur Caspase-3.

SUMMARY

Mechanisms for preventing the progression of brain damage through intracerebral Adipose Derived Neural Stem Cell transplantation in brain ischemia
(Analysis of TNF-α, IL-10, NGF, MDA, Caspase 3 and Apoptotic neurons in the hippocampus of the Wistar rat)

Hypoxia-ischemia (HI) is one of the leading causes of death and disability throughout the world. There is still no pharmacological neuroprotective therapy that has been proven clinically effective to reduce the severity of HI. Inflammation is a major contributor to secondary injury and involves increased production of chemokines and cytokines. TNF-α as an inflammatory cytokine is released in the early stages of HI and promotes the inflammatory response. IL-10 expression increases neuronal and glial cell survival, and reduces inflammatory responses through a number of signaling pathways. Cerebral ischemia will also induce oxidative stress due to the release of malondialdehyde (MDA) as a secondary product of lipid peroxidase. HI activates the mitochondrial apoptotic pathway by caspase-3 activation. Caspase-3 has been identified as the main mediator of apoptosis in HI animal models. Stem cell therapy has the potential to repair neurological damage caused by HI brain injury. In vitro, Neural Stem Cells stimulate axon growth and show protective effects through the secretion of Nerve Growth Factor (NGF). MSC also shows a significant suppression of free radical release and provides antioxidant protection as evidenced by lower MDA levels in HI. The mechanism of the potential effects of Adipose-derived Neural Stem Cells (ADNSC) on HI brain damage remains unclear.

The purpose of this study is to explain the mechanism of preventing the progression of brain damage after ADNSC intracerebral transplantation in brain ischemia induced by carotid communis artery (CCA) ligation and hypoxia.

This type of research is an experimental post test only control group design. The experimental unit used male Wistar strain Rattus norvegicus strain 2 months old. The experimental animals were divided into 3 groups randomly namely the control group, CCA ligation group and ADNSC group. The variables studied were TNF-α expression, IL-10 expression, NGF expression, MDA expression, Caspase-3 expression and cells undergoing apoptosis. The study was conducted in March 2018 - July 2019. Maintenance of experimental animals, isolation and ADNSC culture and imnocytochemical examinations were carried out at the Stem Cell Research and Development Laboratory of Airlangga University Surabaya. Immunohistochemical examination and TUNEL Assay in the Histology Lab, Faculty of Medicine, Airlangga University, Surabaya. The research procedure were as follows; animals were adapted to their new environment, for 1 week. Mice in the control group did not do any intervention. In the ligation group; CCA ligation was performed for 2 hours and then they was put into the hypoxia chamber for 1 hour, whereas in the ADNSC group, CCA ligation was performed for 2 hours and then they was inserted into the

hypoxia chamber for 1 hour followed by ADNSC intracerebrallly transplantation. Neurological scores were performed to evaluate neurological function clinically after 24 hours and 48 hours. After 48 hours all mice were sacrificed using xylazine / ketamine anesthesia. Brain tissue was analyzed by immunohistochemistry to determine the number of cells expressing TNF-α, IL-10, NGF, MDA and Caspase-3 and TUNEL Assay for apoptosis.

The results showed no reduction in TNF-α expression, an increase in IL-10 expression, an increase in NGF expression, a decrease in MDA expression, a decrease in Caspase-3 expression and a decrease in the number of neurons undergoing Apoptosis in cerebral ischemia induced by AKK ligation and hypoxia. APNSC decreases apoptosis by decreasing Caspase-3 to the MDA and NGF pathways.

In the central nervous system, TNF signaling plays a dual role, which is to increase inflammation through TNFR1 in immune cells while providing cytoprotection via TNFR2 on nerve cells. With this dual function, it will be difficult to predict which function is dominant in TNF-α after being treated with ADNSC. Levels can be fixed, up or down. Upregulation of IL-10 plays a potential neuroprotective role against brain ischemia and provides a favorable microenvironment for neurogenesis after brain ischemia. Specifically, NGF regulates synaptic plasticity and protects neurons from oxidative stress and apoptosis which can stimulate neurogenesis. The HI given by MSC has a lower MDA level, showing a significant suppression of the release of free radicals and providing antioxidant protection in cell and membrane organelles. Caspase-3 plays an important role in apoptotic cell death, and inhibition of caspase-3 has been shown to improve cerebral ischemic injury. NSC transplantation can significantly reduce the number of apoptotic cells in the penumbra at 7 days by increasing the regulation of Bcl-2 expression.

The conclusion of this study is that ADNSC intracerebral transplantation cannot reduce TNF-α expression, increase IL-10 expression, increase NGF expression, decrease MDA expression, decrease Caspase-3 expression and decrease neurons that undergo apoptosis in brain ischemia induced by CCA ligation and hypoxia. Prevention of the progression of brain damage in brain ischemia by administering ADNSC can reduce MDA expression and apoptosis through the Caspase-3 pathway.