How to Cite:

**TNF-α and IL-10 levels in hipoxic ischemic rat brain receiving stem cell**

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**Abstract**---To investigate the effect of intracerebral adipose neural stem cell transplantation in the inflammatory process after brain HI. The study used 16 male Wistar rats aged 2 months which were randomly divided into the following group: Ischemia-Reperfusion group (IR, n=8) and ANSC-treated group (IR+ANSC, n=8). IR was performed by right common carotid artery occlusion for 2 hours, hypoxia procedure for 1 hour and reperfusion. ANSC was prepared from rats adipose tissue and processed in Laboratory. ANSC was inserted intracerebally after hypoxia procedure and after 48 hours the blood sample were analyzed by using ELISA. T-test for independent samples was used for statistical analysis. There were no significant differences in the TNF-α level between the IR group and IR+ANSC group. There were statistically significant difference in IL-10 level between groups (P< 0.05). Intracerebral ANSC transplantation cannot reduce TNF-α expression and increase IL-10 expression in brain ischemia induced by CCA ligation and hypoxia.

**Keywords**---Adipose derived neural stem cell, Hypoxic ischemic encephalopathy, IL-10, Mortality, TNF-α.

**Introduction**

Acute brain injury after cerebral ischemia due to stroke or ischemic hypoxic ischemic encephalopathy (HIE) together is one of the leading causes of death and disability worldwide[1]. Stroke in children more than 1 month occurs at 13 per
100,000 children per year, whereas in neonates it is higher in 25-40 cases per 100,000 babies\textsuperscript{(2)}. Similarly, HIE produces 2-9 per 1000 babies born in developed and developing countries\textsuperscript{(3)}. Most affected children are physical and / or neurological disabilities in a sustainable life \textsuperscript{(4,5)}.

Inflammation is a major contributor to secondary injury and involves increased production of chemokines and cytokines. TNF-α is released in the early stages of HIE and promotes an inflammatory response by increasing the expression of chemotaxis factors, which induce the recruitment of neutrophils and monocytes / macrophages into the injured area \textsuperscript{(6)}. Interleukin-10 (IL-10) as an anti-inflammatory cytokine uses a large number of immunomodulatory functions during the inflammatory response and is very important during the resolution phase. IL-10 expression in the brain increases with central nervous system (CNS) pathology, increases neuronal and glial cell survival, and reduces inflammatory responses through a number of signaling pathways \textsuperscript{(7)}.

Stem cell therapy has the potential to repair neurological damage caused by HIE \textsuperscript{(8)}. The immunoregulatory and anti-inflammatory effects of neural stem cells have been confirmed by in vitro studies. Various types of stem cells, doses, and effective administration methods, are still being investigated. Adipose cells is thought to be the source of stem cells is widely available and easily obtained without invasive action \textsuperscript{(9)}. The biomolecular mechanism of neurological improvement associated brain inflammation process in HIE after adipose derived neural stem cells (ANSC) administration still cannot be explained. The purpose of this research is to investigate the effect of intracerebral adipose neural stem cell transplantation in the inflammatory process after brain HI.

**Methods**

Unit experiment

The research was experimental randomized post test only control group design. All animal protocols were approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Airlangga University No: 2.KE.019.02.2018. The study used 16 male Rattus norvegicus strain Wistar rats aged 2 months, mean weight 204.20 gram, which were randomly divided into the following group: Ischemia-Reperfusion group (IR, n=8), ANSC-treated group (IR+ANSC, n=8). The unit of analysis examined in this study is the hippocampus.

Cerebral ischemia model

The cerebral ischemia model was established according to Vanucci Methods. Anesthesia for Wistar mice was induced with xylazine 10% intramuscularly. An incision in the midventral cervical area in the middle of the neck on the upper edge of the sternum was made. This will be accessible to the sternocleidomastoid muscle and the sternohyoid muscle. Slow pull of the sternocleidomastoid muscle deep inside the sternohyoid muscle (until the sternohyoid muscle appears), there will be a common carotid artery (CCA) that is wrapped by fibrous connective tissue, which contain vagus nerve in it. Carefully separate the CCA from the attached tissue using ophthalmic forceps. Unilateral CCA are bound twice with 4-0 silk, the second stitch is next to each other, then the surgical wound is stitched
back with suture. The mice were then placed for 60 minutes in an 8% O₂ hypoxic atmosphere chamber and 92% N₂\textsuperscript{10,11}. After hypoxia, reperfusion is performed by opening the 2 hours CCA ligation.

Adipose neural stem cell procedure in vitro development and characterization

This protocol includes the following steps:

\textbf{a)} Taking and isolating adipose tissue; Adipose tissue was obtained from abdominal surgery from wistar rats. Rat adipose tissue was commonly found in the area around the kidneys. Adipose tissue was then inserted into the transport medium for processing at the Stem Cell Research and Development Laboratory of Airlangga University, Surabaya.

\textbf{b)} MSC development in vitro; The adipose tissue is chopped manually until soft. Then, it is transferred to the erlenmeyer tube plus with digest enzyme (0.01% Colagenase type I). Stopper is given by adding a medium containing Fetal Bovine Serum (FBS); then it was collected for centrifuge. The resulting pellet was accumulated and resuspended with a new alpha-MEM culture medium. Then it was planted in petri dish. When the cell has grown and reached 80% confluence, passage preparation is carried out. Cells were grown until they reach passage 4 and when the confluence was seen. MSC characterization was carried out by immunocytochemical methods at passage 4 for MSC validation of adipose cells. MSC showed positive expression on CD29, CD44, CD73, CD90, and CD105 and negative expression on CD45.

\textbf{c)} ANPSC in vitro differentiation and characterization. MSC was then differentiated towards ANSC. Neurobasal supplement, B27 2%, FBS 20% and FGF 10ng/mL, penicillin streptomycin 1%, amphotericin 1% were added for neuronal culture, expansion and differentiation. Culture was carried out for approximately 3 weeks followed by immunocytochemistry characterization. ANPSC characterization was performed using Nestin and SOX2.

\textbf{ANSC transplantation procedure}

ANSC was inserted intracerebrally through the right temporal burrhole process of rat calvaria using a 1 mL needle. Intracerebral transplantation is performed before reperfusion. The dose was 2x10⁵ cells.

\textbf{Specimen preparations}

After 48 hours of CCA ligation all rats were anesthetized with xylazine. The blood specimen were taken for ELISA procedure to measure the level of TNF-α and IL-10.

\textbf{Statistical analysis}

To ensure that the sample came from a population whose weight and age were homogeneous with the Levene test. To test whether the data comes from a normal distribution, the Normality test is used using Saphiro Wilks and to test the
differences in the effects of each exposure group, a statistical test with T test for independent sample was conducted. Statistical significance was set at P<0.05.

Results

TNF-α data normality test results in each group showed normal data distribution. Homogeneity test using Levene test showed data with homogeneous variations. There was no statistically significant difference in TNF-α levels among groups (P=0.418) (Table 1).

Table 1. TNF-α expression comparison among groups

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean ± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR</td>
<td>8</td>
<td>0.779 ± 0.101</td>
<td>0.418*</td>
</tr>
<tr>
<td>IR+ANSC</td>
<td>8</td>
<td>0.742 ± 0.079</td>
<td></td>
</tr>
</tbody>
</table>

Note: * Not Significant α=0.05 (T test for independent sample)

IL-10 data normality test results in each group was not in normal distribution. Homogeneity test using Levene test shows data with homogeneous variations. There were statistically significant difference in IL-10 expression among groups (P<0.05). The median IL-10 expression in the IR+ANSC group was higher than other groups (Table 2).

Table 2. IL-10 expression comparison among groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean ± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR</td>
<td>8</td>
<td>0.730 ± 0.033</td>
<td>0.000*</td>
</tr>
<tr>
<td>IR+ANSC</td>
<td>8</td>
<td>0.793 ± 0.094</td>
<td></td>
</tr>
</tbody>
</table>

Note : * Significant α=0.05 (T test for independent sample)

Discussion

This study used The Rice-Vannucci rat HI model. This model allows the study of the mechanisms underlying HI-related brain damage during development. In brief, this model is carried out as follows: ACC is bound unilaterally and the animals rest briefly before being exposed to hypoxic air (8% oxygen) for 3.5 hours at 37°C \(^{12}\). Hypoxia alone (without carotid occlusion) does not cause brain damage, nor does carotid unilateral occlusion (without hypoxia) will produce the same thing \(^{13}\).

Clinical proof of the occurrence of HI injury in the rat brain in this study was evaluated by the Neurology score according to Longa. The stroke model in mice is very important for understanding the pathophysiology of stroke development and recovery. There is no consensus for neurological assessment for stroke models in mice. There are studies aimed at determining and comparing the sensitivity of three commonly used neurological assessments - the Garcia, Longa, and Modo tests. All three tests correlate well with each other and can be used for neurological evaluation in mice after stroke \(^{14}\). This study showed neurological deficits in all mice that had CCA ligation with different degrees.
The inflammatory response that occurred after brain ischemia occurred in this study. When compared with control group, there was a significant increase in TNF-α level in the IR group. However there was no significant change in TNF-α expression in the IR+ANSC group compared to the IR group. TNF-α performs its function by binding to two receptors (TNFR1 and TNFR2) to regulate the survival, proliferation, migration, and differentiation of target cells, especially immune cells. 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. These molecules also interact with MSC to modify or mediate their therapeutic effects (15). With this dual function, it will be difficult to predict which function is dominant in TNF-α after being treated with ANPSC. Levels can be fixed, up or down. The ups and downs of TNF-α levels depend also on various factors including the sex, the course of the disease, the concentration of cytokines, and the length of time of treatment (15). Ex-vivo stimulation test with lipopolysaccharide or concanavalin A, proved that stroke-mediated immune cell dysfunction was not modified by MSC. In addition, the level of systemic inflammatory cytokines (IL-6, TNF, interferon-γ, chemoattractant protein-1, monocytes) remained unchanged in rat serum after cerebral ischemia and treated with cell transplants (16).

In this study IL-10 levels increased significantly in experimental animals given ANSC compared to controls. Immunological analysis shows that MSC/IL-10 transplantation significantly inhibits microglial activation and expression of proinflammatory cytokines compared to MSC alone. In addition, IL-10 suppresses neuronal degeneration and increases the survival of MSC in the ischemic area. These results suggest that IL-10 overexpressed enhances the neuroprotective effect of MSC transplants with anti-inflammatory modulation and thus supports the survival of neurons during the acute ischemic phase (17). Upregulation of endogenous IL-10 plays a potential neuroprotective role against brain ischemia and provides a favorable microenvironment for neurogenesis after ischemic stroke (18). Increased IL-10 can support hBMSC survival and support the proliferation of endogenous cells in the subependimal layer of the ventricular zone (19). MSC has the ability to modulate the immune system related to inflammation and cytokines as a cerebral inflammatory response induced by TBI. It offers new insights into the mechanisms responsible for the immunomodulatory effects of MSC transplants, with implications for neurological functional recovery after TBI (20). The injection of hUCB-MSCs during the early stage of cerebral ischemia reduced the IL-1β, IL-6 expression levels in the serum and enhanced IL-10 expression levels. Western blot analysis for IL-1β, IL-6 and IL-10 three days after hUCB-MSC transplantation strengthened these findings. The results suggest that hUCB-MSC transplantation could modulate the balance of pro- and anti-inflammatory cytokines (21).

The limitation of the study was the setting to analyze effects of ANSC on biomolecular reactions in brain ischemia is only in a the short-term. Long-term biomolecular effects are expected to be able to see the mechanism more completely with clinical monitoring of the effects of therapy.
Conclusion

Intracerebral ANSC transplantation cannot reduce TNF-α level and increase IL-10 level in brain ischemia induced by CCA ligation and hypoxia. It is need for further research to determine the effects of ANSC in the long term period by monitoring the clinical condition and biomolecular mechanism.

Acknowledgments

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References


