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# Characteristic of neurogenic secretome under hypoxia: Organoleptic, IL-6 and IL-10 profile

Bagus Wibowo Soetojo[1,2], Teddy Heri Wardhana[1,2], Heri Suroto[1,2,3*]
[1]Department of Orthopedics and Traumatology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia; baguswibowosoetojo@gmail.com (B.W.S.).
[2]Department of Orthopedics and Traumatology, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia; teddy-heri-w@fk.unair.ac.id (T.H.W.).
[3]Cell & Tissue Bank-Regenerative Medicine, Faculty of Medicine, Universitas Airlangga, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia; heri-suroto@fk.unair.ac.id (H.S.).

**Abstract:** Understanding the physical characteristics and IL-6/IL-10 cytokine levels of neurogenic secretome is crucial for evaluating the effectiveness of stem cell therapy in nerve injury treatment. This study utilized an in vitro experimental design to analyze the organoleptic properties, pH levels, moisture content, and structural features of neurogenic secretome under hypoxia. Electron microscopy was used to examine structural details, while ELISA tests were performed to measure IL-6 and IL-10 cytokine concentrations. The control group exhibited a pale pink color in both liquid and freeze-dried forms, while the neurogenic secretome appeared light orange in liquid form and yellow when freeze-dried. Both samples had a neutral pH of 7.00. The control group showed a higher moisture content (5.510%) compared to the neurogenic secretome (5.099%). Electron microscopy revealed more pores in the neurogenic secretome than in the control ($p=0.237$). Additionally, cytokine analysis showed significantly lower levels of IL-6 and IL-10 in the neurogenic secretome compared to the control group ($p<0.0001$). The neurogenic secretome exhibited distinct physical characteristics, including more pores and lower cytokine levels, which may enhance cell proliferation and adhesion, potentially improving its effectiveness in stem cell therapy for nerve injuries. The findings suggest that neurogenic secretome, with its unique properties, could offer advantages in therapeutic applications involving nerve regeneration.

***Keywords:*** *IL-6, IL-10, Neurogenic secretome, Organoleptic, Stem cell therapy.*

## 1. Introduction

Peripheral nerve injuries, affecting both sensory and motor nerves, are prevalent in the community. These injuries are often caused by trauma, which can occur anywhere on the body, with traffic accidents being the most common cause. In the United States, there are approximately 200,000 cases of peripheral nerve injuries annually due to traffic accidents, while Europe sees about 300,000 cases each year. A study on brachial plexus injuries in Surabaya reported 423 cases over 12 years, predominantly among adults aged 21 to 30, mainly due to motorcycle accidents.[1]

Therapeutic options for peripheral nerve injuries include both operative and non-operative treatments. Operative treatments involve microsurgery, nerve grafting, and nerve tubulization. Non-operative treatments include laser therapy, electrical stimulation, physical exercise, and medication. Additionally, stem cell therapy has emerged as an alternative treatment. Mesenchymal stem cells (MSC), grown in a conditioned medium known as MSC-conditioned media (MSC-CM), are beneficial due to their growth factors, cytokines, and growth factors (GF) that influence cellular activities at the molecular level.[2]


* Correspondence: heri-suroto@fk.unair.ac.id

The pro-inflammatory cytokines that govern the early immune response consist of interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α. TNF-α is a subject of substantial research in sepsis because of its involvement in the release of cytokines. The release of these pro-inflammatory cytokines also impacts the release of anti-inflammatory cytokines in order to sustain immunological homeostasis. IL-10 is secreted in order to counteract the effects of pro-inflammatory cytokines such as TNF-α. Nevertheless, TNF-α has the ability to stimulate NF-κB, resulting in the establishment of a positive feedback loop. The simultaneous elevation of pro-inflammatory cytokines and IL-10 signifies an unbalanced inflammatory reaction, referred to as a cytokine storm, which can lead to harm to organs and subsequent infections, finally resulting in higher mortality rates. An investigation demonstrated that the administration of secretions from human embryonic mesenchymal stem cells (MSCs) had a substantial positive effect on the survival and histopathological scores of mice with sepsis. This treatment increased the levels of IL-10, a beneficial cytokine, without altering the levels of IL-1β and TNF-α, which are other cytokines associated with sepsis.[3]

IL-6 and IL-10 are crucial cytokines; IL-6 plays a role in cell differentiation, proliferation, regeneration, and degeneration, while IL-10 prevents neuronal degeneration in pathological conditions or brain injuries. Growth factors related to peripheral nerve regeneration include nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), neurotrophin 3 (NT-3), neurotrophin 4 (NT-4), neurotrophin 5 (NT-5), and fibroblast growth factor 2 (FGF-2).[4]

This research aims to compare the physical, chemical, and pro-neurogenic cytokine (IL-6 and IL-10) content of the neurogenic secretome under hypoxic conditions (5% oxygen), which enhance MSC regeneration and have cytoprotective effects. Physical characteristics evaluated include color, odor, and moisture content, while chemical properties assessed include physiological pH. Both sample groups are analyzed microscopically using scanning electron microscopy (SEM) to evaluate inter-particle relationships, surface conditions, and the structure of IL-6 and IL-10 particles. The samples are freeze-dried for stability, longevity, and ease of application, potentially useful for peripheral nerve injury treatment. This research aims to provide an alternative therapy for peripheral nerve lesions using secretomes.

## 2. Methods

### *2.1. Study Design*

This study is in vitro experimental laboratory research comparing the characteristics of the neurogenic secretome under hypoxic conditions. The research was conducted at the Tissue Bank of Dr. Soetomo General Academic Hospital from January to May 2024.

### *2.2. Preparation of Adipose Derived Mesenchymal Stem Cell (ADMSC)*

In order to collect fat tissue samples measuring 1 $cm^3$, it is necessary to prepare alpha MEM transport medium. These samples should be free from any connective tissue or blood clots. The collected samples should be promptly loaded into the transport medium and expeditiously transported to the stem cell laboratory for isolation of MSCs.

The adipose tissue is extracted from the transport medium and rinsed with phosphate-buffered saline (PBS) until all remaining red blood cells are eliminated. The adipose tissue is subsequently chopped into small pieces and combined with collagenase enzyme prior to being transferred into a container equipped with a magnetic stirrer. The bottle is placed on a hot plate and kept at a temperature of 37°C for a duration of 30 minutes until the fat tissue is completely dissolved. Following dissolution, a stopper medium is introduced and incubated for a further 10 minutes to ensure a uniform solution. The solution is strained through a sterile gauze filter into a 50 ml conical tube to extract any fat tissue that has not disintegrated. The liquid that has passed through the filter is then spun at a speed of 3000 revolutions per minute for a duration of 5 minutes to create a solid mass. The pellet is reconstituted in

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alpha MEM medium to provide a uniform solution. Ultimately, the suspension is placed on a 10 cm Petri dish and kept in a CO2 incubator for 24 hours until the cells attach to the dish. The culture medium is replaced every 2 days until the cells aggregate and achieve 80% confluence.

The MSCs that have formed colonies successfully are further cultured until the desired dosage for clinical use is attained. Cells that have grown to 80% confluence in a single layer need to be refreshed by transferring to a new culture vessel. Passaging entails the retrieval of the medium from the Petri dish, followed by rinsing the monolayer with phosphate-buffered saline (PBS). Subsequently, a solution containing three enzymes is introduced, and the dish is then incubated for a duration of 5 minutes to separate the monolayer from the surface of the dish. After the monolayer is separated, a stopper medium is introduced, and the cells are mixed until they form a suspension consisting of individual cells. Subsequently, the individual cells are moved to a conical tube and subjected to centrifugation in order to create a compact mass. The pellet is reconstituted in alpha MEM medium to form a uniform solution, which is subsequently placed on a new Petri dish. This technique is iterated till the fifth iteration in the study.

In this study, stem cell cultures are subjected to hypoxic conditions with 5% oxygen for several days of cultivation to reach passages 4 and 8. MSCs obtained from the first stage of the research are grown until passage 4 and passage 8. The cells are aseptically collected and plated in culture dishes with a density of 2x10^7 cells/cm² in 10 cm dishes with IMDM medium containing 15% FBS, MSC simulator supplements, and antibiotics (100 U penicillin/100 µg/mL streptomycin) at 37°C, 5% CO2, and 95% air. The maintenance of cell quiescence is achieved by placing the culture flasks in a specialized hypoxia incubator (modular incubator chamber) with 5% oxygen concentration.

Characterization of MSCs isolated from adipose tissue is performed using specific markers: CD-34 (cluster differentiation 34, indicating stemness), CD-45 (hematopoietic stem cells), and CD-105 (mesenchymal stem cells), which are markers specific for crude secretome. Flow cytometry and immunocytochemistry are used for analysis. A positive result for CD-105 is indicated by the presence of staining on the surface of the MSC membrane, while CD-45, a specific marker for hematopoietic stem cells, is used to confirm the isolation from adipose tissue MSCs. A negative result for CD-45 is shown by the absence of staining on the MSC membrane.

*2.3. Secretome Collection*

The obtained conditioned medium is transferred into a 50 ml dialysis tubing, which is securely sealed at both ends. Subsequently, the tube is immersed in a 500 ml beaker filled with chilled PBS solution. A magnetic rod is introduced, and the beaker is placed on a magnetic stirrer hot plate. The combination is agitated at a speed of 500 revolutions per minute for the duration of the night until the color of the conditioning medium inside the dialysis tubing has become less intense. After the color has diminished, the tube is extracted from the PBS solution, and then it is severed using sterile scissors. The substance is placed into a 250 ml beaker, and the resulting product is filtered using a 0.22-micron filter and then moved into a 50 ml conical tube. Subsequently, the substance is introduced into a Sterile Medipack, securely sealed, and the resultant liquid is gathered as crude secretome.

*2.4. Preparation of the Neurogenic Secretome*

The ADMSC culture, which has reached 80% confluence, is seeded by introducing a fresh frozen nerve measuring 1 cm x 0.5 cm x 0.5 cm into a 10 cm petri dish already containing mesenchymal stem cell culture. The culture is then incubated for 2x24 hours at a temperature of 30 degrees Celsius with a 5% CO2 concentration until the medium changes color to yellowish. Harvesting is then performed by aspirating the culture medium.

The production of freeze-dried preparations from liquid preparations involves two processes: deep freezing at -80°C for 24 hours, followed by lyophilization (cooling and vacuuming to 0.001 torr) at -50°C in an AMSCO apparatus for 48 hours.

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*2.5. Characterization and Analysis*

The organoleptic examination of the secretome involves assessing its color and odor to evaluate its basic sensory attributes. Surface morphology is analyzed using scanning electron microscopy (SEM), where samples are freeze-dried to observe their surface features at various magnifications. Moisture content is determined by weighing the samples before and after drying in an oven, which helps in quantifying the water content. Additionally, the pH of neurogenic secretomes is measured to ensure it is close to physiological pH, which is crucial for maintaining biological compatibility. The levels of IL-6 and IL-10 in the secretomes are quantified using enzyme-linked immunosorbent (ELISA). Reagents and samples are prepared, and the ELISA process involves multiple incubation and washing steps, followed by optical density (OD) measurement at 450 nm.

## 3. Results

*3.1. Organoleptic*

Neurogenic secretomes are odorless, as is the case with the controlled group, indicating comfortable use. Even after freeze-drying, the secretome remains odorless. Researchers observed a color difference between the two groups: the controlled group is light pink, whereas the neurogenic secretome is light orange. The controlled group retains its light pink color, while the neurogenic secretome turns yellow Figure 1.

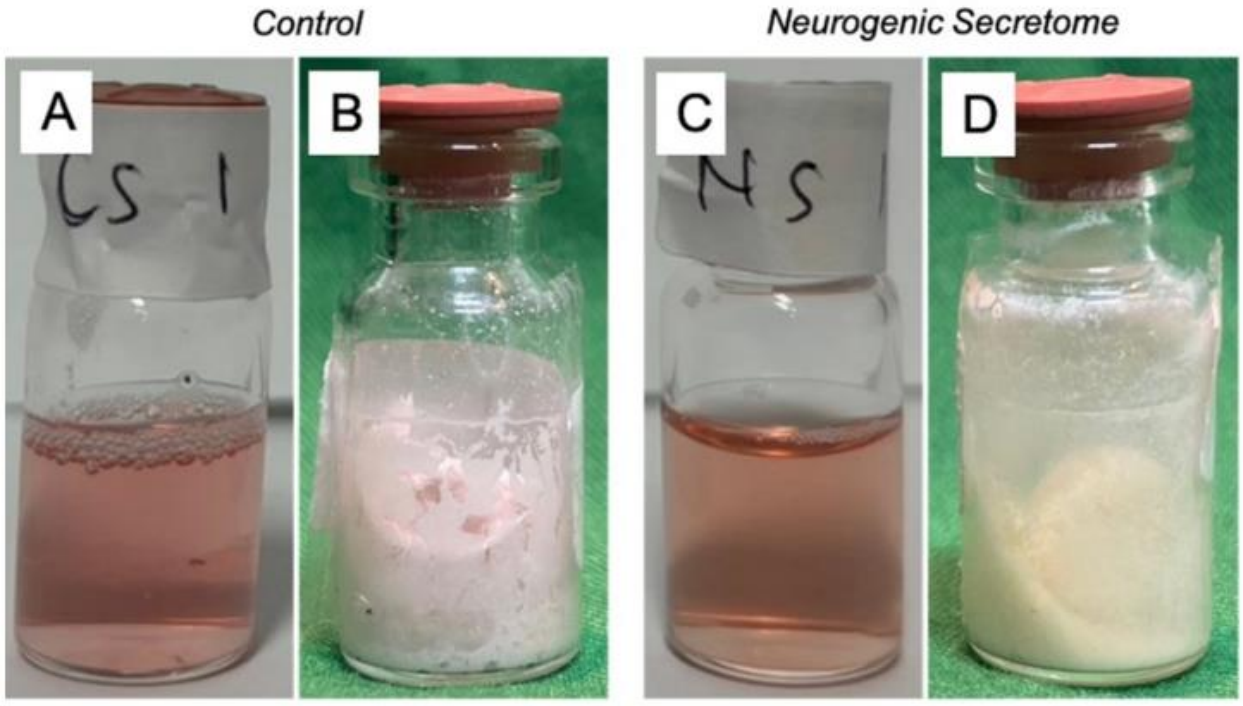


**Figure 1.**
A, B.) The control secretome shows a light pink color in the liquid and freeze-dried secretome. C, D) Neurogenic Secretome show light orange in liquid and yellow.

*3.2. Physical Characterization with Microscopic Evaluation using SEM*

The deposits of the neurogenic secretome were examined at 150x, 500x, 1000x, and 5000x magnifications. At 150x and 500x magnifications, the surface of the neurogenic secretome shows pore formations. At 1000x and 5000x magnifications, the surface appears to be a patch with white spots. At

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5000x magnification, the neurogenic secretome exhibits branched dot-like structures resembling a Christmas tree, while the controlled group shows round dot-like structures resembling wheels or flowers Figure 2. From Table 1, At 150x magnification, the neurogenic secretome has a higher number of pores, with 30 pores and pore sizes ranging from 53.8 to 323.6 nm, with an average size of 139.56 ± 71.76 nm. Meanwhile, the controlled group has 25 pores with pore sizes ranging from 51.4 to 252.9 nm, with an average size of 116.61 ± 68.00 nm, as shown in Table 1. This indicates that neurogenic secretome pores have more and larger pores compared to the controlled group, but is not significant (P = 0,237).

**Table 1.**
Pores analysis in control and neurogenic secretome.

| | **Control group** | **Neurogenic secretome** | ***P value*** |
|---|---|---|---|
| Total pores /Field | 25 | 30 | P = 0,237 |
| Mean pore sizes | 116.61 ± 68.00 | 139.56 ± 71.76 | |
| Size range (Min. – Max.) | 51.4 – 252.9 | 53.8 – 323.6 | |

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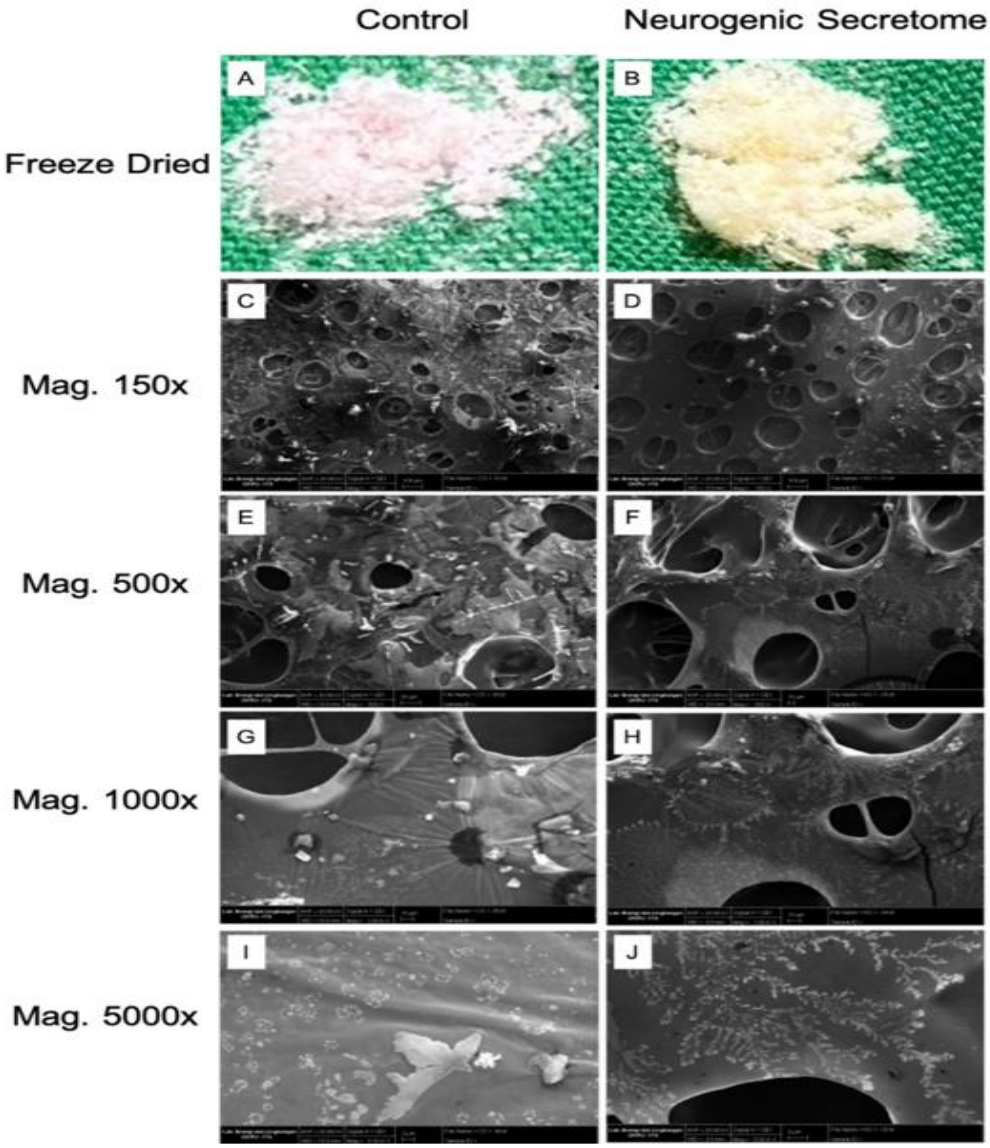


**Figure 2.**
Shows the microscopic surface images of the neurogenic secretome. A, B) Control and neurogenic freeze dried secretome in macroscopic; C, D) Control and neurogenic secretome showed pores in 150x Magnification; E, F) Control and neurogenic secretome showed pores in 500x Magnification; G, H) Control and neurogenic secretome showed pores and some white spots in 1000x Magnification; I) Control secretome in 5000x magnification showed white spot with rossete or wheel formation; J) Neurogenic secretome in 5000x magnification.

*3.3. Moisture Content*

The study on the moisture content of freeze-dried secretome showed that the moisture content of neurogenic secretome was 5.099%, while that of the of the controlled group was 5.510%. This analysis involved a single replication, with 16 samples included in each neurogenic secretome and controlled group. These findings indicate slightly lower moisture content in the neurogenic secretome than in the controlled group. The single replication with 16 samples per group helps provide an initial understanding of the moisture content characteristics of each secretome type (Table 2).

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**Table 2.**
Moisture content in freeze dried control group and neurogenic secretome

| Sample | % moisture content |
|---|---|
| Freeze dried neurogenic secretome | 5.099 % |
| Controlled group | 5.510 % |

*3.4. pH Testing*

Researchers analyzed the pH levels in both sample groups under hypoxic conditions. The results of the pH measurements for both samples are shown in Table 3 below. The pH level of the neurogenic secretome is 7.0. pH measurements were conducted 16 times for each sample group to ensure that the pH values obtained adequately represent each sample. The pH measurement for the controlled group also yielded a result of 7.0.

**Table 3.**
The result of pH testing in control group and neurogenic secretome

| Sample | pH testing with lakmus paper |
|---|---|
| Neurogenic secretome | 7.0 |
| Controlled group | 7.0 |

*3.5. ELISA for IL-6 and IL-10*

The researchers evaluated the levels of IL-10 and IL-6 in the neurogenic secretome using ELISA. The study included a total of 32 IL-6 and IL-10 samples for each neurogenic secretome and controlled group, with each sample being assessed in duplicate for greater accuracy. The IL-10 level in the neurogenic secretome group was 0,643 ± 0,078, whereas in the controlled group it was 0,913 ± 0,083 ($p < 0,05$). For IL-6, the neurogenic secretome had a level of 0,628 ± 0,100, while the controlled group had a level of 0,938 ± 0,068 ($p < 0.05$). These findings suggest that IL-10 levels are lower in the neurogenic secretome compared to the controlled group. IL-6 levels appear to be lower in the neurogenic secretome than in the controlled group. These differences indicate distinct cytokine profiles between the two types of secretome studied Table 4.

**Table 4.**
Concentration of IL-10 and IL6 in control and neurogenic secretome group.

| Variable | IL-10 | IL-6 | p value |
|---|---|---|---|
| Controlled group | 304.610 ± 33.85 | 131.3824 ± 15.01 | < 0.005 |
| Neurogenic secretome | 201.4873 ± 28.51 | 74.5746 ± 16.51 | < 0.005 |
| Conc: *Concentration* | | | |

## 4. Discussion

This study shows that neurogenic secretions are odorless. The freeze-dried secretome from both sample groups also lacks any odor. Neurogenic secretomes exhibit different colors: the neurogenic secretome appears light orange, while the controlled group is light pink. After freeze-drying, neurogenic secretome turns yellow, and controlled group retains its light pink color. These findings align with a study by Perwira et al. on freeze-dried chondrogenic secretomes.[5]

This study showed that the water content in freeze-dried neurogenic secretome was 5.099% compared to 5.510% in the controlled group. According to Merivara et al., optimal residual moisture content for good freeze-dried material ranges from 0.5% to 3%[6]. This range ensures prolonged stability of proteins and product stability. Susa et al. similarly suggest that residual moisture content between 1% and 3% supports the long-term stability of active pharmaceutical ingredients.[7] Franks et al. warn against conditions of overdrying, where moisture content below 0.5% can lead to irreversible damage due to unstable culture conditions possibly caused by excessive temperatures surpassing

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thresholds.[8] Laggner et al. found that freeze-dried peripheral blood mononuclear cell-derived (PBMC) secretome ideally maintains a moisture content of 2.6% to 3%.[9]

The study utilized SEM to examine deposits of neurogenic secretome under hypoxic conditions at magnifications of 150x, 500x, 1000x, and 5000x. At 150x magnification, neurogenic secretome exhibited a higher number of pores, specifically 30 pores, with pore sizes ranging from 53.8 to 323.6 nm and an average size of 139.56 ± 71.76 nm. In comparison, controlled group had 25 pores with pore sizes ranging from 51.4 to 252.9 nm and an average size of 116.61 ± 68.00 nm. This indicates that neurogenic secretome has more and larger pores compared to controlled group, although this difference was not statistically significant ($P = 0.237$). At 5000x magnification, neurogenic secretome showed branched dot-like structures resembling a Christmas tree, whereas controlled group displayed rosette or wheel.

Han et al. demonstrated that pore sizes of 100 μm and 200 μm are most suitable for chondrogenic differentiation of cells and gene expression. Both of these groups showed significant advantages compared to other pore size groups in immunofluorescence analysis and qRT-PCR. Conversely, the group with 50 μm pores exhibited much lower results, indicating that smaller pores are not suitable for chondrogenic differentiation of stem cells.[10] Furthermore, deeper investigations into BMSC chondrogenic differentiation suggest that microscopic pores do not optimally support cell growth and differentiation. While larger pores (>300 μm) provide sufficient space for cell proliferation, excessive growth can lead to decreased differentiation efficiency. Therefore, to achieve efficient tissue repair using tissue engineering frameworks, it is crucial to provide adequate cellular support without disrupting cellular. Although Han et al.'s study did not find significant differences between the 100 μm and 200 μm pore size groups, they highlighted that these pore sizes offer sufficient adhesion support and appropriate growth space for cells, resulting in better performance.[10]

Teixeira et al. show that the secretome has similar effects in supporting the differentiation of human neural progenitor cells in vitro. Additionally, it has been demonstrated that the production of proangiogenic factors (e.g., IL-6 and VEGF) by MSCs increases under hypoxic preconditioning, with the assumption that these beneficial effects may also be regulated by various complex signaling pathways such as the Akt and ERK pathways.[11]

Semita et al. demonstrated a significant difference in the number of cells expressing IL-10 between the spinal cord injury (SCI) + secretome group and the SCI + non-secretome group ($p < 0.05$). The SCI + secretome group had a greater number of IL-10 expressing cells. Administering IL-10 into the bloodstream has a notable effect on protecting the nervous system and improving the ability to function after a spinal cord injury (SCI). IL-10 exerts trophic effects on neurons by reducing the levels of pro-apoptotic molecules such as caspase 3, cytochrome c, and Bax, while simultaneously boosting the levels of anti-apoptotic molecules such as B-cell lymphoma 2 (Bcl-2) and X-linked Bcl-2 (Bcl-xl). The beneficial outcomes encompass decreased size of lesions and improved motor recovery.[12]

The stem cell secretome acts through various pathways to protect degenerating nervous tissue. The anti-inflammatory effects of the stem cell secretome are partially mediated by soluble immunoregulatory molecules. Anti-inflammatory cytokines found in the stem cell secretome include tumor necrosis factor 1 (TNF-1), interleukin (IL) 10, IL13, IL27, IL18-binding protein, IL1 receptor antagonist, IL17E, IL12p70, ciliary neurotrophic factor, and neurotrophin 2. On the other hand, pro-inflammatory cytokines in the stem cell secretome include IL1b, IL6, IL8, and IL9.[12]

Severe acute inflammation has been shown to occur immediately after spinal cord transection injury. Levels of interleukin-1, interleukin-6, and tumor necrosis factor-α are highly expressive initially but then sharply decline within 24 hours.[1,13]

Our research results present a different finding in the neurogenic secretome, the concentration of IL-10 in the neurogenic secretome is lower than in the controlled group. This discrepancy may be attributed to the influence of sample transport over long distances due to the different study locations, potentially altering the microenvironment of the secretome and reducing IL-10 levels.

IL-6 is a significant cytokine that promotes inflammation and has diverse effects on different types of cells. It is largely secreted by macrophages/monocytes and TH2 cells, together with IL-1β and

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tumor necrosis factor-α. IL-6 and other cytokines are produced and secreted by mast cells and Schwann cells in the peripheral region, as well as microglial cells in the central nervous system (CNS). IL-6 plays a role in the generation of stem cells, the increase in neural stem cell populations, the control of established neuronal markers, and the growth of neural tissue in cases of nerve injury. IL-6, via its primary agent STAT3, facilitates the fast generation of induced pluripotent stem cells and sustains the immature condition of pluripotent stem cells.[4]

IL-6 acts as a neurotrophic factor during neurogenesis, promoting neuron differentiation. Together with neurotrophic growth factors (NGF), IL-6 leads to increased activation of extracellular regulated kinase-1 (ERK1). Deficiency in IL-6 disrupts neurogenesis by reducing the number of neural progenitor cells.[14] Other research by Semita et al. indicates that neurogenic secretome reduces MMP-9. MMP-9 protein is an endopeptidase that contributes to spinal cord injury repair by breaking down ECM molecules. MMP-9 reduction can be promoted by reactive oxygen species (ROS) and pro-inflammatory mediators like TNF-α and IL-1β through the NFκB pathway.[12,15]

Semita et al.'s study also demonstrates a rise in TGF-β. TGF-β, a cytokine with anti-inflammatory properties, stimulates the development of regulatory T cells and facilitates the transformation of macrophages into the M2 phenotype. TGF-β inhibits the production of M1 cells that are triggered by TNF-α, IL-1β, IL-6, inducible nitric oxide synthase, ROS, glutamate, and pro-inflammatory proteases. TGF-β plays a role in the process of nerve repair and regeneration. It is commonly reported to reverse nerve damage and promote the survival, growth, proliferation, differentiation, and invasion of neurons and glial cells.[12]

The current study remains in vitro in nature, which limits the applicability of the findings to real world conditions. To enhance the accuracy and reliability of the pH measurements, it is recommended to use a pH meter for this purpose, as it provides more precise and consistent pH readings compared to other methods. Furthermore, it is crucial to have a sufficiently large sample size for water content analysis to ensure that the results are statistically significant and can be reliably compared. Another significant limitation of the study was the constant change of research locations, which could have introduced variability in the experimental conditions and affected the consistency of the results. Additionally, the study did not include a comparison of samples under normoxic conditions, which would have been valuable for understanding the differences between normoxic and experimental conditions. Addressing these issues in future research will help improve the study's robustness and the generalizability of the findings.

## 5. Conclusion

In this study, notable differences were observed between the neurogenic secretome and the controlled group under hypoxic conditions. One of the differences was in color, where the neurogenic secretome exhibited a more yellow hue compared to the controlled group. However, no differences in odor were detected between the neurogenic and controlled groups under hypoxic conditions. Additionally, the neurogenic secretome demonstrated a higher number and larger size of pores compared to the controlled group in hypoxia, which facilitated better cell proliferation and adhesion. Although the water content in both the freeze-dried neurogenic secretome and the controlled group under hypoxia was similar, it was found to be outside the expected range. No significant differences in pH levels were observed between the neurogenic and controlled groups in hypoxia. However, significant differences were noted in the concentrations of IL-6 and IL-10, with the neurogenic secretome showing lower levels of both cytokines compared to the controlled group under hypoxic conditions. Despite the differences in individual cytokine levels, the ratio of IL-6 to IL-10 remained consistent at 2.5:1 for both secretomes.

## Ethical Statement:

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The Health and Research Ethics Committee of Dr. Soetomo General Academic Hospital has granted ethical approval with the Ethics Number 0872/KEPK/I/2024.

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