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Physical, chemical, and microbiological stability test of liquid neurogenic Secretome at temperature 0-4°C and 20-25°C

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Abstract: Nerve tissue is vital for motor, sensory, and autonomic activities, and peripheral neuropathy is a common disorder. Trauma causes 2-3% of peripheral nerve damage, with radial and plexus injuries being the most prevalent. Sciatic, peroneal, and tibial nerves are most impacted in 18-35-year-olds and 8% of 55-year-olds have peripheral neuropathy. Tissue engineering, including secretome studies, may be useful. This study aims to assess the stability of liquid neurogenic secretome at different temperatures by evaluating its physical, chemical, and microbiological properties. This in vitro experimental laboratory study employed a post-test only group design to compare the stability of liquid neurogenic secretome stored at temperatures of 0-40°C and 20-25°C, both on the first day and after 30 days. Stability was assessed through physical, chemical, and microbiological tests. Brain-derived neurotrophic factor (BDNF) concentration was similar in the room and refrigerator groups, indicating neurogenic secretome stability. Additionally, no bacterial growth was observed from day 1 to day 30, suggesting the secretome's resistance to contamination under both storage conditions. The secretome from purified mesenchymal stem cell (MSCs) along with antibiotics was effective in clearing clearing methicillin-resistant staphylococcus aureus (MRSA) biofilms in a mouse wound model and showed promise in treating multidrug-resistant chronic wound infections in a clinical trial involving dogs. This study provides insights into the physical, chemical, and microbiological stability of liquid neurogenic secretome under different storage conditions, highlighting its potential for therapeutic use.

Keywords: *Brain-derived neurotrophic factor, Mesenchymal stem cell, Neurogenic secretome.*

1. Introduction

Management in therapy and regeneration in the field of Orthopaedics remains a challenge in the modern health era. In the human body, nervous tissue is one of the elements of the musculoskeletal system that has motor, sensory, and autonomic functions. One of the most frequently encountered nerve diseases is peripheral neuropathy. The term peripheral neuropathy is usually used to describe symmetrical and universal damage to adjacent nerves. The damage and clinical manifestations are usually located distally and progressively move to the proximal part.[1] Treating the symptoms and managing the underlying illness process are the two main objectives of peripheral neuropathy treatment. Treating the underlying illness or making up for dietary inadequacies will help with the first. These actions are essential to stop neuropathy from getting worse and might even make symptoms better. Intravenous immunoglobulin therapy is more vigorous and urgently needed for acute inflammatory neuropathy. It is crucial to assist patients in managing the pain and numbness associated with peripheral neuropathy illnesses as well as to lessen the handicap brought on by weakening.

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Pharmacological alternatives for treating neuropathic pain include antidepressants (like amitriptyline) and certain antiseizure drugs (including gabapentin, topiramate, carbamazepine, and pregabalin). For certain people, topical creams and sprays containing lidocaine or capsaicin can also be used to ease discomfort.[2,3]

Secretome is defined as a collection of factors or molecules secreted into the extracellular space. These factors include, among others, proteins, nucleic acids, lipids, and extracellular vesicles. The latter can be further divided into microparticles and exosomes. The secretions of individual cells and tissues are specific and change in response to fluctuations in physiological states or pathological conditions.[4] The secretome is preferred because it has several advantages, including being non-immunogenic, and to date, there have been no reported cases of the secretome causing tumor.[5] Currently, there is an increase in research to study the characterization and potential of the secretome from various types of cells. The main challenge in this research is the difficulty in collecting samples due to the very small amount of secretome, measured in nanograms.[6–8]

2. Materials and Methods

This research was conducted at the Cell and Tissue Bank-Regenerative Medicine, Dr. Soetomo General Academic Hospital, Surabaya, the Microbiology Laboratory, Diagnostic Center, Dr. Soetomo General Academic Hospital, Surabaya, and the Tropical Disease Center, Universitas Airlangga, Surabaya, from November 2023 to September 2024. The research material is liquid neurogenic secretome. In this study, a comparative test will be conducted between 2 independent groups. Thus, the number of replications per group is 6. The implementation of this research is divided into 5 stages.

2.1. Isolation and Culture of Mesenchymal Stem Cells (MSCs)

Isolated and cultured MSCs from adipose tissue are used in various clinical and research applications, including regenerative therapies for tissue repair, inflammation modulation, and studies on cell behavior and differentiation. Prior to collecting samples, it is essential to first create the MEM alpha transport medium. This medium is used to transport fat tissue samples that are 1 cm3 in size and free from any connective tissue or blood clots. The collected samples are promptly deposited in a transport medium and instantly sent to the stem cell laboratory for the isolation process of mesenchymal stem cells.

Once the fat tissue is extracted from the transport medium, it is thoroughly washed with a PBS solution until all the red blood cells adhered to the fat tissue are eliminated. The adipose tissue is finely minced and combined with the enzyme colagenase, before being transferred into a container equipped with a magnetic stirrer. The tissue in the bottle is thereafter placed on a hot plate and incubated at a temperature of 37° degrees Celsius for a duration of 30 minutes, until the fat tissue is fully dissolved. After the substance has completely dissolved, introduce the stopper medium and allow it to incubate for an additional 10 minutes until it achieves a uniform and consistent solution. The solution is subsequently transferred into a 50 ml container after being filtered using sterile gauze to remove any leftover insoluble fat tissue. The filtered findings were subsequently subjected to centrifugation at a speed of 3000 revolutions per minute for a duration of 5 minutes, resulting in the formation of pellets. The pellets were subsequently reconstituted in MEM alpha medium until achieving a uniform solution. Subsequently, the specimens were placed in a 10 cm petri dish and subjected to incubation in a CO2 incubator for a duration of 24 hours, allowing the cells to adhere to the bottom of the dish. The cells that were attached were subsequently substituted with fresh media every 2 days until colonies formed and reached 80% confluence. Once mesenchymal stem cells have effectively developed into colonies, they can be expanded in number to meet the necessary dosage for therapeutic use.

2.2. Administration of Human Nerve Scaffold

The cells derived from MSC collected during the first phase of the study were cultivated until the second phase and then collected on the sixth day. The cells were aseptically collected and then placed on a culture dish with a density of 2x106 cells/cm2. They were distributed into 10 5 cm dishes containing IMDM media with 15% FBS, MSC simulator supplement, and antibiotics (100 U penicillin/100µg/mL

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Streptomycin). The dishes were kept at a temperature of 37° C, with 5% CO2 and 95% air. It is necessary to analyze mesenchymal stem cells obtained from adipose tissue by employing CD-105, a particular marker for identifying mesenchymal stem cells. Subsequently, the mesenchymal stem cells that had been marked with labels were analyzed using flow cytometry and immunocytochemical methods. The presence of color luminescence on the surface of the mesenchymal stem cell membrane indicates a positive test for CD-105. Additionally, the utilization of CD 45, a distinct indicator for hemopoitic stem cells, must be conducted to verify the purity of mesenchymal stem cells obtained from adipose tissue isolation. This is demonstrated by a negative test result where there is no presence of color luminescence on the surface of the MSC membrane.

2.3. Secretome Collection

The accumulated conditioned medium is introduced into the tubing dialysis membrane, with a volume of 30 ml. Securely fasten the dialysis tubing at both ends to ensure it is taut, and thereafter immerse it in a 30 ml beaker filled with chilled PBS solution. Subsequently, carefully put the magnetic bar into the designated location and position the beaker onto the hot plate of the magnetic stirrer. Subsequently, rotate the dialysis tube at a velocity of 500 revolutions per minute and allow it to remain undisturbed overnight until the hue of the conditioning media within the tube has diminished. Once the color disappears, extract the substance from the PBS solution and carefully sever the dialysis tubing using sterile scissors. Transfer the substance into a 15 ml beaker and proceed to filter the metabolite product to a size of 0.22 microns. Finally, package the filtered product in 30 ml conical containers.

2.4. Microbiological Stability Testing

The microbiological stability test involves replacing a liquid preparation derived from the neurogenic secretome with a liquid form and examining it under a microscope to determine if there is any microbial growth present. Data was collected on both day 1 and day 30.

2.5. Data And Statistical Analysis

The results of the expression analysis utilizing the ELISA method in this study were observed both descriptively and quantitatively. The data were subjected to descriptive analysis, and hypothesis testing was conducted using the ANOVA test.

3. Results

Statistical analysis showed that there was no significant difference between the secretome groups stored at room temperature and in the refrigerator in terms of brain-derived neurotrophic factor (BDNF) concentration. The mean, median, standard deviation, minimum value, and maximum value for both variables, BDNF concentration and Optical Density (OD), did not show significant differences between these groups. The analysis results also indicated that there was no significant difference in BDNF concentration between the Room and Refrigerator groups. The p-values obtained from the statistical analysis confirmed that the differences between these groups were not statistically significant.

The conclusion from the room and refrigerator samples is that on day 1, no bacterial growth was observed in either condition. Statistical analysis showed that there was no significant difference in optical density between the secretome groups stored at room temperature and in the refrigerator. However, the results indicated a significant difference in BDNF concentration between the Room and Refrigerator groups on day 30. On day 30, the average BDNF concentration in the Room group was 80.045 with a standard deviation of 0.000, while in the Refrigerator group, it was 66.670 with a standard deviation of 44.015. The obtained p-values were 0.000, indicating that the difference between these groups was highly significant. Thus, it can be concluded that the storage condition, specifically refrigeration, has a significant impact on BDNF concentration on day 30. The conclusion from the room and refrigerator samples is that on day 30, no bacterial growth was observed in either condition.

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4. Discussion

Stem cell transplantation is a popular approach in regenerative medicine, where healthy stem cells are introduced into damaged tissues in patients to promote regeneration.[9] Interestingly, researchers have found that stem cell transplantation often has regenerative effects even if the stem cells fail to differentiate and integrate into the tissue.[10] This has led to the formulation of the "paracrine hypothesis": the beneficial effects of stem cell transplantation may not be due to cell differentiation and replacement, but rather to the release of beneficial factors that promote regeneration.[10] This hypothesis is particularly intriguing because stem cell transplantation faces many issues. Immune incompatibility and infection transmission are always sources of concern when transplanting foreign cells into a host, and uncontrolled stem cell proliferation can lead to tumorigenicity.[11]

Isolating therapeutic factors from the stem cell secretome and using them to treat patients would avoid these complications.[12] Additionally, using the secretome instead of stem cell transplantation offers many logistical advantages, including lower costs, increased scalability, availability, and longer shelf life.[12] In this study, we will conduct a stability test on the neurogenic secretome by assessing the concentration of BDNF at room temperature and refrigerator temperature. The analysis results also showed that there was no significant difference in BDNF concentration between the room and refrigerator groups, indicating that the neurogenic secretome has good stability as it can maintain BDNF concentration.[13]

In our study, we also found no bacterial growth from the first day to the 30th day, indicating that the neurogenic secretome is not easily contaminated, even when stored at room temperature and in the refrigerator. This finding is supported by the study by Marx et al., which first demonstrated that the secretome secretes proteases that inhibit biofilm formation and dissolve biofilms of various bacteria. Although the inhibition of planktonic bacterial growth by secretome-released factors is not as strong as conventional antibiotics, the secretome is superior in reducing bacterial growth within biofilms compared to antibiotics.[14]

The study by Marx et al. showed that methicillin-resistant staphylococcus aureus (MRSA) biofilms can be dissolved by proteases released by the secretome. Additionally, they found that antibiotics unable to inhibit MRSA growth in biofilms become effective after the biofilms are treated with the secretome, likely due to its protein degradation effects. Overall, the results of Marx et al. suggest that the secretome could be an effective adjunct treatment for various bacterial infections in wounds, especially those caused by antibiotic-resistant bacteria such as MRSA, not only by killing bacteria through antimicrobial peptides (AMPs) but also by enhancing the efficacy of antibiotics through biofilm degradation by secretome-released proteases.[14]

5. Conclusion

From this study there is no difference in the physical stability of the liquid neurogenic secretome at storage temperatures of 0-40C and 20-250C on the first day and after 30 days of storage, there is no difference in the chemical stability of the liquid neurogenic secretome at storage temperatures of 0-40C and 20-250C on the first day and after 30 days of storage, also there is no difference in the microbiological stability of the liquid neurogenic secretome at storage temperatures of 0-40C and 20-250C on the first day and after 30 days of storage.

Ethical Clearance:
Ethical clearance for this research was granted by the Ethical Committee of Dr. Soetomo General Academic Hospital (No. 0858/KEPK/XII/2023).

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