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Analysis of MSC and EVS-MSC administration to the liver of biliary atresia model rats using liquid chromatography-mass spectrometry examination

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Abstract: This study aims to investigate the therapeutic potential of mesenchymal stem cells (MSCs) and extracellular vesicles derived from MSCs (EVs-MSCs) in reducing toxic compound accumulation in a biliary atresia model. Biliary atresia is a progressive fibro-inflammatory disease of the bile ducts that often requires surgical intervention and liver transplantation, highlighting the need for alternative therapies. Experimental rat livers subjected to bile duct ligation (BDL) were divided into untreated and treated groups. The treated group received MSCs and EVs-MSCs. Liquid chromatography–mass spectrometry (LC-MS) was employed to identify and compare compounds present in both groups. LC-MS analysis revealed 27 compounds in untreated BDL livers, including toxic metabolites such as dimethyl sulfoxide and pyrogallol. In contrast, treated livers exhibited only 18 compounds, notably safer agents such as nicotinamide, butylparaben, caffeine, and indole-3-carbinol. Two compounds, nicotinamide (a vitamin) and butylparaben (an antifungal), were consistently detected in both groups. Importantly, compounds in treated samples were less toxic and potentially protective. Administration of MSCs and EVs-MSCs reduced the presence of harmful compounds, suggesting a protective biochemical effect against biliary atresia progression. These findings provide preclinical evidence that MSC-based therapies may serve as safer, non-toxic alternatives or adjuncts to surgery and transplantation in biliary atresia management.

Keywords: *BDL, Butylparaben dan nicotinamide, EVS-MSC, LCMS, MSC.*

1. Introduction

Biliary atresia is a severe neonatal disease caused by inflammatory and fibrotic obliteration of the extrahepatic bile ducts resulting in cholestasis and progressive liver failure. Babies with biliary atresia have increased fibrosis of the bile ducts, which obstructs the bile ducts and is a severe inflammatory condition [1]. If left untreated, biliary atresia will inevitably lead to death from end-stage liver disease within the first two years of life [2]. The female to male ratio for biliary atresia is 1.4:1. Approximately 25–30% of cases of extrahepatic cholestasis are caused by biliary atresia. This disease is very common in East Asian countries, especially in Taiwan, its prevalence has been observed to be around 1:5000 [3]. Although the cause of biliary atresia is unknown, it is thought to be caused by an organ disorder; chromosomal abnormalities trisomy 17, 18, and 21; and infection. Biliary atresia progresses to liver fibrosis and can become liver cirrhosis caused by persistent fibrosis via paracrine and autocrine pathways [4]. Liver transplantation is the only effective treatment for patients with liver cirrhosis and biliary atresia [5].

Liver transplantation is the most successful treatment for end-stage liver failure, but it is challenging due to immunological rejection, lack of donors, and accessibility of transplant centers [6]. To prevent patients from developing cirrhosis or slow the course of the disease if they have reached the

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cirrhosis stage, many efforts have been made to stop the process of liver fibrosis, including the use of MSCs and EVs-MSCs, which have been studied extensively for their potential in preventing liver damage [7]. MSCs are considered preferable among other types of stem cells because they pose lower ethical risks, exhibit minimal immunogenicity, are widely available, have a lower risk of viral transmission, and are more primitive. Due to their potential to differentiate into hepatocytes, paracrine effects, and immunomodulatory functions, MSCs have been shown to ameliorate liver fibrosis [8]. According to Heo and Kim, in 2022, the therapeutic potential of stem cells has been linked to exosome-based paracrine factors [9]. MSC-derived exosomes have a similar molecular load to host MSCs, which also significantly reduces HSC activation [10].

Mice are the most widely used animal model for liver fibrosis research. The two most commonly used techniques to create mouse animal models of liver fibrosis are bile duct ligation (BDL) and carbon tetrachloride (CCl4) injection. The most popular technique is CCl4 injection, but it is very toxic, while the BDL model has a lighter risk. After BDL, obstructive jaundice appears within two weeks and progresses to cirrhosis or fibrosis within four to six weeks [11]. Bile duct obstruction causes various pathophysiological consequences, including hepatic dysfunction and portal hypertension, by inhibiting hepatic blood flow and disrupting the metabolic and synthetic capacities of the liver [12]. Biliary duct ligation causes the development of various stages of liver disease caused by cholestasis, which can be classified as induced cholestasis, then accompanied by liver inflammation and finally liver fibrosis and liver cirrhosis [13]. The aim of this study was to analyze the administration of MSCs and EVs-MSCs to a rat model of biliary atresia compared to bile duct ligation rat samples using LC-MS examination.

2. Materials and Methods

2.1. Method

This research is an in vitro experimental study of MSCs, EVs-MSCs and frozen liver organs stored in a -80 freezer^{0}C in the Stem Cell and Tissue Engineering cluster of the Indonesia Medical Education and Research Institute (SCTE-IMERI) FKUI Jakarta.

2.2. Material

Biological material from rat liver used in research by Rico et al. used in this research. Mouse organs were stored at -80^{0}C in the freezer. These organs originate from the right medial lobe and left lateral lobe of the rat liver. The first sample of rat liver used was from a rat whose bile duct was ligated as a negative control. The second sample was from rat organs that had bile duct ligation and were given 1 x 10 MSCs^6 injected intrasplenically within one day, and administration of 150 µL EVs-MSC injected intravenously within 7 days.

Characterization of EVs-MSCs

EVs-MSC characterization was carried out using various techniques, including MSC culture, secretome collection, EVs-MSC isolation and evaluation of EVs-MSC results using a respiratory flow chip kit, flow cytometry, particle size analysis (PSA), and zeta potential.

2.2.1. Culture MSC

Cryotubes containing MSCs from Wharton's jelly umbilical cord were removed from a nitrogen tank with code 1, 2, 2, P5, 1 million on April 8, 2022, and thowed in a beaker filled with water at 37^{0}C. Complete medium was prepared for MSCs in the form of 10% PRP, 1% heparin (1000U/mL), 1% amphotericin B (250ug/mL), 1% penicillin-streptomycin (10,000U/mL penicillin and 1000 g/mL streptomycin), and α-MEM. 9 mL of complete media was put into a centrifuge tube, and 1 mL of cells from the cryotube, centrifuged at 1200 rpm for 10 minutes, the supernatant was discarded, and the pellet formed at the bottom of the tube was taken, and 1-2 mL of complete media was added, then the number of cells was counted using the tryptophan blue method.

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Cells were cultured at a density of $5x10^3$ cells per cm2 in a 75 cm flask, and 9 mL of complete medium was added. The flask containing the cells was stored in an incubator at 37°C^{0}C and humidity with 5% CO2. The culture medium was changed every 2-3 days. Cell harvesting is carried out if cell confluence reaches 80%. Harvesting begins by removing the pumpkins from the incubator and placing them in the BSC. Complete medium was discarded and cells were washed with PBS 2 times. Three milliliters of triple-select was added and incubated for 3-5 minutes in the incubator. The cells are observed under a microscope to determine whether they have detached. If cells had detached, they were transferred to a centrifuge cube, and 3–6 ml of complete medium was added. Cells and complete medium were transferred to a centrifuge tube and centrifuged at 1200 rpm for 10 min. The supernatant was discarded, the pellet was removed and 1-2 mL of complete medium was added. The number of cells was counted using the tryptophan blue method. The research was conducted at SCTE IMERI-FKUI Jakarta.

2.2.2. Ultracentrifuge

The thowing condition medium MSC (CM-MSC) was carried out in warm water at a temperature of 37^{the}C. The CM solution was centrifuged at a speed of 750 g and a temperature of 20°C for 15 minutes. The supernatant was collected and centrifuged at 2000 g for 15 min. The supernatant was collected and centrifuged at 10,000 g and temperature 4^{the}C for 45 minutes. The supernatant was collected and filtered using a 22 um filter syringe. The filtrate was then transferred to an ultracentrifuge tube. Ultracentrifugation was performed at a rate of 100,000 × g on 4^{the}C for 90 minutes. The supernatant was discarded, and the pellet containing the EVs was transferred from the ultracentrifuge tube to a 15 ml falcon tube. DPBS was added to a volume of 5 ml and the solution was resuscitated. EVs were put into 1 ml cryovial tubes and stored in a refrigerator cryobox -20^{the}C or refrigerator -80^{the}C. The examination was carried out at MBVCF IMERI-FKUI Jakarta.

2.2.2.1. Respiratory Flow Chip Kit

This examination is an in vitro diagnostic tool used to identify the main pathogens that can cause acute respiratory tract infections. Can detect 20 types of viruses and 3 types of pathogenic bacteria that cause acute respiratory infections. The examination was carried out at the Microbiology Laboratory, Faculty of Medicine, University of Indonesia, Jakarta.

2.2.2.2. Flow Cytometry examination

The sample observation process using flow cytometry is based on the protocol issued by BD Sciences. The flow tube containing the EVs-MSC suspension was then centrifuged using a Fresco 17 microcentrifuge at 500 g for 5 minutes. The centrifuged supernatant was discarded and the resulting pellet was labeled with a solution of CD63 and CD81 antibodies by pipetting 2.5 µL of each antibody into a flow tube containing the EVs pellet. Antibodies were homogenized with pellets using a 10 µL pipette with up and down pipetting, then the tubes were incubated for 20 minutes in a room without light. Next, the tube containing the mixture of EVs-MSCs and antibodies was inserted into the flow cytometry device for a few seconds. Data were analyzed using BD FACS ARIA III with an incidence of 50,000—100,000 cells/second and FACSDiva 8.0, software for determination of gates obtained from EVs-MSC populations that were not incubated with antibodies. This research was conducted at SCTE IMERI-FKUI. Jakarta.

2.2.2.3. Particle size analyzer (PSA) examination

PSA examination is used to measure the size, shape and distribution of particles in a particular sample. Held at the Integrated Laboratory and Research Center of the University of Indonesia (ILRC UI), Depok, West Java.

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2.2.2.4. Zeta Potential Analyzer Examination

Zeta potential examination to measure the size of nano and micro particles. The research was conducted at the Integrated Laboratory and Research Center of the University of Indonesia (ILRC UI), Depok, West Java.

2.2.3. Uji Liquid Chromatography-Mass Spectrometry (LC-MS)

The LCMS test was carried out at the Forensic Laboratory Center of the Indonesian Police Criminal Investigation Agency (Puslabfor Bareskrim Polri) Sentul, Bogor. High resolution spectrometry tests were carried out using an ultra -performance liquid chromatography (ULPC) unit (LC: ACQUITY UPLC® H - Class System, Waters, USA) and a mass spectrometer (Xevo G2—S QTof, Waters, USA). The test involved a C18 column (1.8 µm 2.1x100 nm, ACQUITY UPLC® HSS, Waters, USA) at temperatures of 50°C (column) and 25°C (room). Liquid chromatography analysis uses a mobile phase in the form of water mixed with 5 mM ammonium formate and acetonitrile mixed with 0.05% formic acid. Mass spectrometry analysis was carried out using electrospray ionization (ESI) in positive mode with a mass range of 50-1200 m/z, source temperature of 100°C, and desolvation temperature of 350°C. A cone flow velocity of 0 L/hour and a desolvation gas flow velocity of 793 L/hour were also used, with the collision energy varied at 4-60 eV. Masslynx software version 4.1 was used for data acquisition and analysis.

2.3. Data Analysis

Analysis of data from the LCMS test to determine the metabolite profile of extracellular vesicle samples was carried out qualitatively using Masslynx 4.1 software. From the results of the analysis in Masslynx 4.1 software, a chromatogram will be produced from liquid chromatography (LC) and a mass spectrum from mass spectrometry (MS). In the chromatogram, the abscissa axis shows the retention time, and the ordinate axis shows the relative abundance of the compounds detected at each retention time. The percentage area of each peak in the chromatogram represents the percentage of compound content in the sample being analyzed. Each compound in the chromatogram peaks has a specific mass spectrum. The mass spectrum is a visualization of the partial fragmentation experienced by each compound due to ion collisions in a mass spectrometry machine. In a mass spectrum, the abscissa axis shows the molecular mass in m/z units, and the ordinate axis shows the relative abundance. The m/z value shows the ratio of the mass of the ion (in Dalton units) to the total amount of charge on the ion. Relative abundance is obtained from the ion intensity detected from each m/z value and compared to the ion that has the highest intensity [14]. The mass spectra obtained from MassLynx software were then compared with the mass spectra database. There are five database sources that can be used for interpretation of masslynx analysis, namely PubChem, Chemspider, MassBank of North America (MoNA), Human Metabolome Database (HMDB), and mzCloud.

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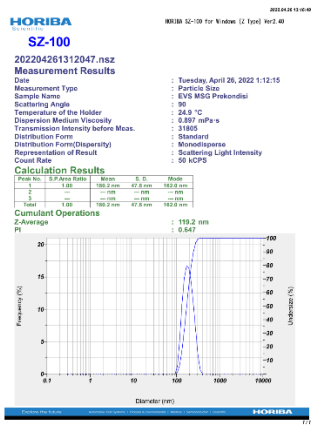
3. Results

3.1. EVs-MSC Characterization Results

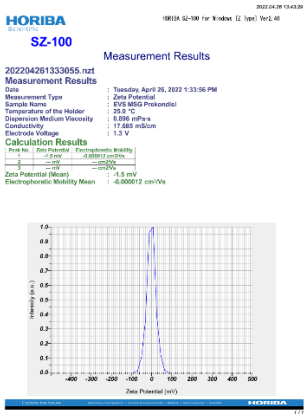
A. respiratory flow chip kit

Molecular examination	Result
Package ISPA III	
Virus Influenza A H1N1 2009	Negatif
Human Metapneumovirus	Negatif
Human Parainfluenza Virus Type 3	Negatif
Rhinovirus	Negatif
PCR Mycoplasma pneumonia	Negatif
Virus Influenza Type A	Negatif
Virus Influenza Type B	Negatif
Bordetella pertusis	Negatif
Human Coronavirus 229E	Negatif
Virus Influenza A H3	Negatif
Respiratory Syncytial Virus Type B	Negatif
Human Parainfluenza Virus Type 1	Negatif
Human Parainfluenza Virus Type 2	Negatif
Human Parainfluenza Virus Type 4	Negatif
Adenovirus	Negatif
Bocavirus	Negatif
Human Coronavirus HKU1	Negatif
Human Coronavirus NL63	Negatif
Human Coronavirus OC43	Negatif
Bordetella Parapertusis	Negatif
Enterovirus	Negatif
Human Coronavirus SARS COV-2	Negatif
Respiratory Syncytial Virus Type A	Negatif

B. Particle size analizer



C. Zeta potentiated analyzer



D. Flow cytometry

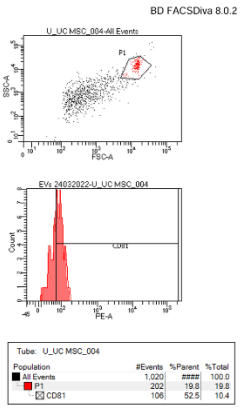


Figure 1.
EVs-MSC characterization was carried out using various techniques, including MSC culture, EVs-MSC isolation, and EVs-MSC evaluation using: A. Respiratory flow chip kit examination results; B. Results of particle size analyzer inspection; C. Zeta potential analyzer results; D. Results of flow cytometry examination.

EVs-MSCs were characterized using a respiratory flow chip kit, particle size analyzer, zeta potential analyzer and flow cytometry. In the respiratory flow chip kit examination, all examination results obtained negative test values. When examining the particle size analyzer, the Z average value was 119.2 nm and P1 was 0.647. In examining the zeta potential analyzer, the zeta potential (mean) results were -1.5 mV and the electroporetic mobility mean was -0.000012 cm^2/Vs and flow cytometry examination obtained a parent percentage value of 52.5% (Figure 1).

3.2. LCMS Examination Results

When examining the BDL sample, LCMS data was obtained as follows:

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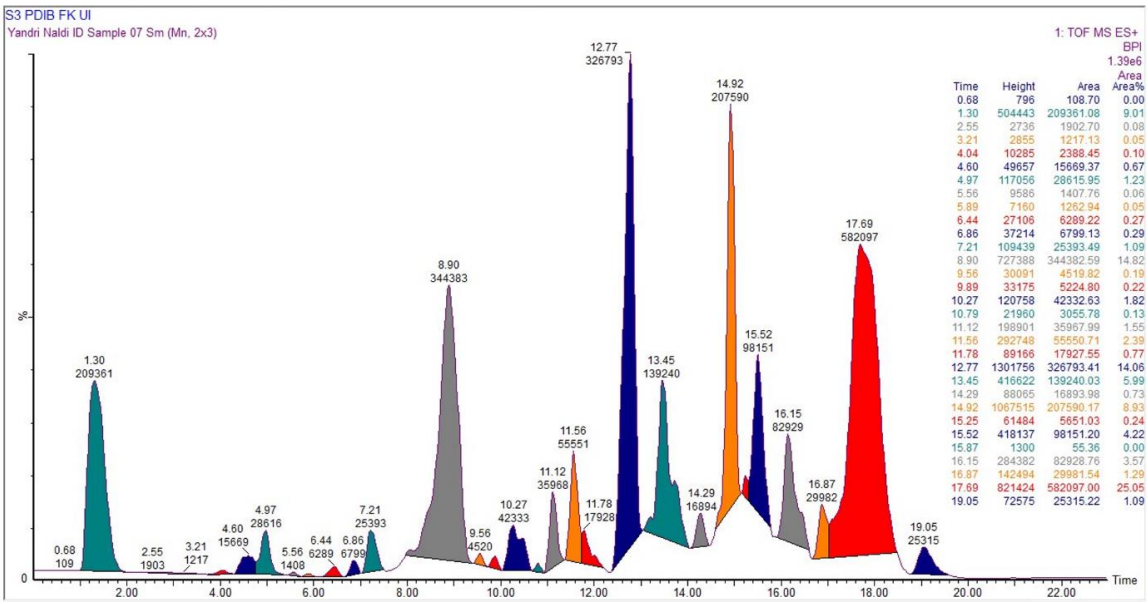

Figure 2.
Results of examining BDL samples using LCMS examination, data obtained from 31 real time examinations and 16 types of compounds detected.

When examining the BDL sample, the LCMS examination results were obtained as follows: 31 real time peak data were obtained, and 16 compounds were obtained, namely: *Dimethyl sulfoxide*, *Nicotinamide*, *butylparaben*, *Pyrogallol*, *Amidrine*, *Dibenzylamine*, *Ciclopirox*, *Chrysin*, *Octylamine*, *Valyl*, *Daidzein*, *N,N-dimethyl-4-nitroaniline*, *N-Acetyl-L-glutamic acid - L-lysine (1:1)*, *3,4-Ethylenedioxy-N-methylamphetamine*, *2-[5-(Ethylsulfanyl)-1H-tetrazol-1-yl]-N-(2-methoxyethyl) ethanamine* And *Methyl(2ξ)-6-deoxy-3,4-O-[ethoxy(phenyl)methylene] -2-O(sulfanyl carbonothioyl)-β-L- lyxo-hexopyranoside.*

Meanwhile, when examining the BDL + MSC + EVs-MSC samples, the following data were obtained:

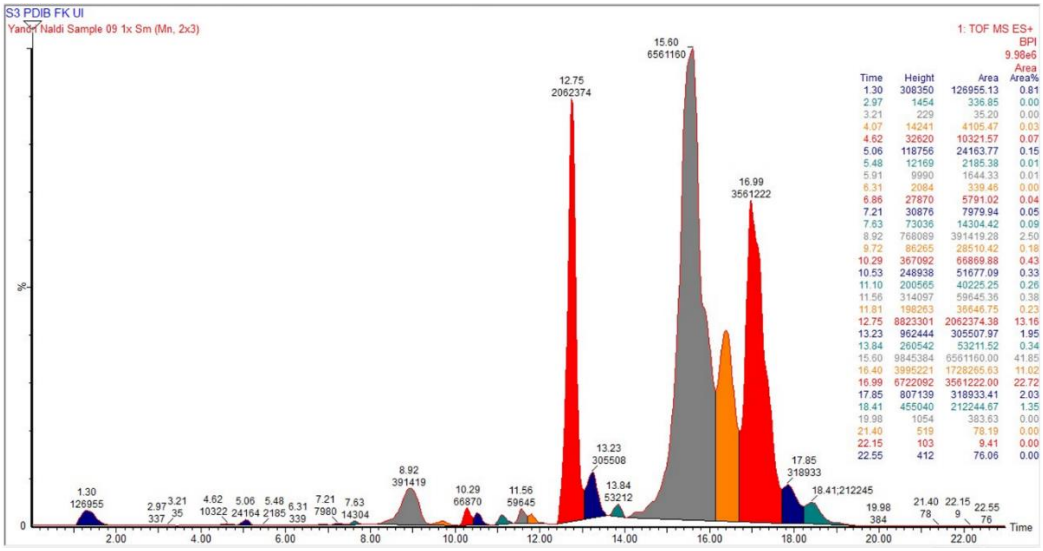

Figure 3.
Results of examination of treatment samples during LCMS examination, data obtained from 31 real time examinations and 15 types of compounds detected.

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Examination of the treatment samples obtained the following examination results: 31 real time peak data, and among them there were 15 compounds, namely: *Butylparaben*, nicotinamide, *Caffeine*, *Indole-3-carbinol*, *5-amino-4-cyano-1-methyl-1,2-pyrazole*, *3,3-Dinitro-1,5-pentanediaminium*, *4-Methoxy-N,N-diphenyl benzamide*, *N-Benzyl-N-isopropyl-2-naphthalenesulfonamide*, *5,5'-[(E)-1,2-Diazenediyl]bis(1-methyl-1H-tetrazole)*, *1-Isothiocyanato-9-(methylthio) nonane*, *2-(3-Methyl-1-benzofuran-2-yl)-2-oxoethyl 3-[(methylsulfonyl)methyl] benzoate*, *5,8-Quinolinedione*, *6,7-bis(benzylthio)*, *1-[(4aS,5S,6S,8aS)-6-Hydroxy-5,8a-dimethyloctahydro-2(1H)-isoquinolinyl]-1-dodecanone* And *2-{(3S,6R,9E,14S, 15S) -3-Isopropyl-6-methyl-11-methylene-15-[(2R)-2-octanyl]-2,5,8,13-tetraoxo-1-oxa-4,7,12-triazacyclopentadec-9-en-14-yl}ethyl stearate*.

4. Discussion

4.1. Characterization of EVs-MSCs

Test results using a sterility test using a respiratory flow chip kit (fig. 1A). All test results were negative. This shows that the EVs-MSC samples examined were sterile or free from contaminants in the form of bacteria and fungi. This analysis is important to obtain samples that are free from contaminants, which ensures that research using EVs-MSC samples is only based on pure EVs-MSC and not other substances such as the presence of bacteria or fungi. Because deposits or small particles of salt, proteins, or other elements can hinder nanometric sample analysis, Vitro [15] states that maintaining sterile conditions during this procedure will help lower the background and maintain sample integrity [15]. Based on the MISEV 2018 criteria, namely small EVs (MVB-derived exosomes) measuring 40-150 nm, large EVs (microvesicles) measuring 100-1000 nm, extracellular autophagic vesicles measuring 40-1000 nm, and apoptotic vesicles measuring 100-1000 nm. When examining the particle size analyzer (figure 1B), the sample value was 119.2 nm. This shows that small EVs or MSC-derived exosomes are the material examined by researchers. According to information from Théry, et al. [16] which states that extracellular vesicles (EVs) are particles that are naturally released from cells, are surrounded by a lipid layer, and are unable to replicate. Ectosomes and exosomes are the two main groups that can be divided into. Ectosomes are vesicles originating from the plasma membrane and their diameter varies from 50 nm to 1 mm. In contrast, exosomes originate from endosomes and their diameter ranges from approximately 40 to 160 nm, with an average diameter of approximately 100 nm [16]. The zeta potential value tested by researchers was -1.5 mV, and the electrophoretic mobility was -0.000012 cm^2/Vs (figure 1C). A negative zeta potential reading indicates that the sample membrane is negatively charged, indicating that the sample has good penetration into the cell membrane. Samples can pass through cell membranes more easily due to their very low average electrophoretic mobility. Compared with stem cells, their membranes are richer in microdomains enriched in tetraspanins, lectins, and highly mannosylated epitopes, as well as cholesterol, sphingomyelin, and hexanamides, according to de Gassart, et al. [17]. Figure 1D Flow cytometry revealed that the percentage of CD 63+ and CD 81+ parents was 52.5%. Good results are indicated by a high parent percentage, namely > 50%. This shows that the samples examined by flow cytometry had CD values of 63+ and 81+. MSCs are one of the easiest primary stem cells to extract from the human body and secrete many EVs, which makes them one of the best sources of EVs, according to Ramos, et al. [18]. In addition to having markers for MSCs including CD29, CD73, CD90, and CD44, EVs-MSCs also include markers for CD107, CD63, and CD81 Ramos, et al. [18]. Andronico, et al. [19] claims that this research explains how flow cytometry can be used to examine EVs-MSCs [19].

4.2. LCMS Check

From the results of liquid chromatography/LC spectroscopy and mass spectroscopy/MS (LC-MS) examinations on BDL samples consisting of 31 real time examinations and 16 types of compounds were detected. In the BDL samples, compounds were found, including: Dimethyl sulfoxide, nicotinamide, butylparaben, pyrogallol, amidrine, ciclopirox, chrysin, anandamide and others. Meanwhile, the treatment sample consisted of 31 real time examinations and 15 types of compounds were detected.

Edelweiss Applied Science and Technology
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Meanwhile, in the treatment samples the following compounds were found: butylparaben, nicotinamide, caffeine and others.

In both BDL and treatment groups there are two types of the same compound, namely a compound with a molecular weight of 123.0589 with the molecular formula C6H7N2O, namely the Nicotinamide compound, and a molecular weight of 195.0916 with the molecular formula C11H15O3, namely the Butylparaben compound. On average, the LCMS results in the treatment group had a greater west mass than the BDL group so that many compounds in the treatment group were not detected.

Nicotinamide is an amine that functions as a vitamin: An organic substance that is distributed in food, and is necessary for the normal nutrition of the organum concerned. Nicotinamide is generally considered safe when taken in recommended doses. However, high doses may cause side effects such as nausea, vomiting, and hepatotoxicity [20]. Butylparaben is an antifungal agent, an antimicrobial agent that destroys fungi by suppressing their ability to grow or reproduce. Another function is as an antimicrobial agent, namely a substance that kills or slows the growth of microorganisms, including bacteria, viruses, fungi and protozoa. Some studies suggest that parabens can mimic estrogen (estrogenic effects) and may be linked to certain health problems, including endocrine disruption and potential cancer risk. However, regulatory bodies such as the FDA (Food and Drug Administration) and EFSA (European Food Safety Authority) still consider butylparaben safe in the concentrations used today [21].

In rat liver samples with BDL there were compounds dimethyl sulfoxide, nicotinamide, butylparaben, pyrogallol, amidrine, ciclopirox, chrysin, and anandamide. Dimethyl sulfoxide is an organosulfur compound with the chemical formula $(CH_3)_2SO$. It is a colorless, odorless and hygroscopic liquid that has the unique ability to dissolve various chemical compounds, both polar and nonpolar. DMSO is used in the preservation of cells, tissues, and organs for transplantation or research because of its ability to protect cells from damage caused by freezing. DMSO also has antioxidant properties and can stabilize cell membranes. Side effects of DMSO include allergic reactions, high doses can cause toxicity, including liver or kidney damage. DMSO can also cause eye and respiratory tract irritation and is flammable [22]. Pyrogallol has antioxidant properties and can scavenge free radicals, although in high concentrations it can be pro-oxidant. Pyrogallol is used in the preservation of biological specimens due to its anti-microbial properties. Possible side effects include skin, eye and respiratory tract irritation. Long-term exposure can cause liver and kidney damage. Pyrogallol is also carcinogenic in some animal studies, so handling it requires special precautions [23]. Dibenzylamine is used in the synthesis of pharmaceutical compounds, including drugs that have biological activity such as antimicrobials, anticancer and antidepressants, and is also used as an intermediate in the synthesis of more complex organic compounds, including drugs, dyes and special chemicals. Side effects of this compound include skin and eye irritation, respiratory toxicity, dangerous for aquatic organisms and the environment if not disposed of properly [24].

The Ciclopirox compound has benefits for treating fungal infections of the skin (Tinea), Onychomycosis, dandruff and seborrheic dermatitis and Candidiasis. Has antibacterial activity against some gram-positive and gram-negative bacteria and anti-inflammatory effect. While the side effects are local irritation such as redness, itching, or a burning sensation in the treated area, allergic reactions, there is a potential risk to the fetus or baby [25]. The Chrysin compound has benefits as a strong anti-oxidant, reduces inflammation by inhibiting the COX-2 enzyme, has anti-cancer potential, reduces anxiety and increases relaxation. Meanwhile, the side effects of this compound include having low bioavailability when consumed orally, which limits its effectiveness in the body. disrupts hormonal balance, can interact with certain drugs, especially those metabolized by cytochrome P450 enzymes. digestive disorders such as nausea or diarrhea when taking high doses of chrysin [26]. Anandamide compounds have the benefits of increasing feelings of happiness and reducing anxiety and depression, analgesic effects, neuroprotective properties that can help protect nerve cells from damage due to oxidative stress or inflammation, reducing inflammation by modulating the immune response through CB2 receptors. The side effect of this compound is that in high concentrations it can cause psychoactive

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effects similar to THC (tetrahydrocannabinol), the main psychoactive compound in cannabis. Too high levels can interfere with cognitive functions, such as memory and concentration. Excessive amounts can cause psychological dependence [27].

Meanwhile, in DBL liver samples given MSC and EVs-MSC there were caffeine, nicotinamide and butylparaben compounds. Caffeine is a natural alkaloid compound found in various plants, such as coffee beans, tea leaves, cocoa beans, and kola. Chemically, caffeine is known as 1,3,7-trimethylxanthine, with the molecular formula $C_8H_{10}N_4O_2$. Caffeine works by inhibiting adenosine receptors in the brain, which reduces drowsiness and improves alertness, physical performance, and cognitive function [28].

From the statements above, it can be seen that the compounds in the BDL samples have many side effects and can be toxic when used in high doses, while the compounds in the samples given MSC and EVs-MSC are safer and less toxic than the compounds found in the BDL samples alone.

5. Conclusion

EVs-MSC characterization stated that the sample examined by researchers was pure EVs-MSC and was not contaminated with bacteria or viruses. There are two compounds that are the same in the bile duct ligation samples and the treatment samples, namely the compounds butylparaben and nicotinamide. BDL samples administered with MSC and EVs-MSC have compounds that are safer and non-toxic.

Transparency:

The authors confirm that the manuscript is an honest, accurate, and transparent account of the study; that no vital features of the study have been omitted; and that any discrepancies from the study as planned have been explained. This study followed all ethical practices during writing.

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