DETECTION OF SYNOVIAL FLUID-DERIVED EXOSOMES INTERNALIZATION BY THP-1 CELLS

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DETECTION OF SYNOVIAL FLUID-DERIVED EXOSOMES INTERNALIZATION BY THP-1 CELLS

by

HANINA SOFEA BINTI FAIZAL

Dissertation submitted in partial fulfilment
of the requirements for the degree
of Bachelor of Health Science (Honours)
(Biomedicine)

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CERTIFICATE

This is to certify that the dissertation entitled “Detection of Synovial Fluid-Derived Exosomes Internalization by THP-1 Cells” is the bona fide record of research work done by Ms Hanina Sofea Binti Faizal during the period from September 2024 to January 2025 under my supervision. I have read this dissertation and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfilment for the degree of Bachelor of Health Science (Honours) (Biomedicine).

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

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Date: 25 MARCH 2025 ……..

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LIST OF SYMBOLS

%	-	Percentage
℃	-	Degree celsius
Ml	-	Milliliter
U/Ml	-	Unit per milliliter
Ml	-	Microliter
Mm	-	Micromolar

LIST OF ABBREVIATIONS

AML	-	Acute Monocytic Leukemia
APB	-	Apoptotic Bodies
ATCC	-	American Type Culture Collection
CFDA-SE	-	Carboxyfluorescein Diacetate Succinimidyl Ester
CGT	-	Cell And Gene Therapy
CLP	-	Common Lymphoid Progenitor Cell
CMP	-	Common Myeloid Progenitor Cell
DAPI	-	4',6-Diamidino-2-Phenylindole
DMSO	-	Dimethyl Sulfoxide
DPBS	-	Dulbecco's Phosphate Buffered-Saline
dsDNA	-	Double Stranded Deoxyribonucleic Acid
EDTA	-	Ethylenediamine Tetraacetic Acid
EV	-	Extracellular Vesicle
FBS	-	Fetal Bovine Serum
FSC	-	Forward Scatter
GMP	-	Granulocyte-Monocyte Progenitor
HSC	-	Hematopoietic Stem Cells
HUSM	-	Hospital Universiti Sains Malaysia
ILV	-	Intraluminal Vesicleskl
KL	-	Kellgren-Lawrence
LAS X	-	Leica Application Suite X
MHC	-	Major Histocompatibility Complex
miRNA	-	Micro Ribonucleic Acid
mRNA	-	Messenger Ribonucleic Acid
MVB	-	Multivesicular Bodies
OA	-	Osteoarthritis
PBS	-	Phosphate Buffered Saline
PE	-	Phycoerythrin
PSG	-	Penicillin-Streptomycin-Glutamine
SF	-	Synovial Fluid
SSC	-	Side Scatter
ssDNA	-	Single Stranded Deoxyribonucleic Acid

TEM	-	Transmission Electron Microscopy
TKA	-	Total Knee Arthroplasty
TLR	-	Toll-Like Receptor
WBC	-	White Blood Cells

PENGESANAN PENGAMBILAN EKSOSOM YANG BERASAL DARI CECAIR SINOVIAL OLEH SEL THP-1

ABSTRAK

Eksosom adalah vesikel kecil yang terlibat dalam komunikasi antara sel, membawa molekul bioaktif yang mempengaruhi tindak balas imun dan keradangan. Eksosom yang berasal dari cecair sinovial telah menarik perhatian kerana potensi peranannya dalam penyakit sendi seperti osteoartritis dan artritis reumatoid. Walaupun terdapat minat yang semakin meningkat terhadap eksosom yang berasal dari cecair sinovial dan peranannya dalam modulasi imun serta penyakit sendi, pemahaman tentang bagaimana eksosom ini diserap oleh sel imun, terutamanya monosit, masih terhad. Kajian ini bertujuan untuk menangani jurang pengetahuan ini dengan memeriksa proses penyerapan eksosom oleh satu sel titisan monosit manusia (THP-1). Kajian ini meneroka penyerapan eksosom ini oleh sel THP-1 untuk memahami kesannya terhadap modulasi imun. Sel THP-1 yang dilabelkan DAPI telah dikultur dengan eksosom yang dilabelkan PKH67 dari cecair sinovial, dan interaksi mereka diperhatikan menggunakan mikroskop pendarfluor. Penyerapan dianalisis pada masa inkubasi yang berbeza (24 dan 48 jam) serta nisbah sel-ke-eksosom (1:10, 1:20, dan 1:40) menggunakan perisian ImageJ. Sitometri aliran mengesahkan ekspresi CD14 pada sel THP-1. Keputusan menunjukkan penyerapan eksosom yang signifikan, dengan nisbah sel-ke-eksosom menjadi faktor utama yang mempengaruhi penyerapan, sementara tiada perbezaan yang signifikan diperhatikan antara dua tempoh inkubasi. Kajian ini menekankan peranan nisbah sel-ke-exosom dalam proses penyerapan, sekaligus menyumbang kepada pemahaman bagaimana eksosom ini berinteraksi dengan sel imun.

DETECTION OF SYNOVIAL FLUID-DERIVED EXOSOMES

INTERNALIZATION BY THP-1 CELLS

ABSTRACT

Exosomes are small vesicles involved in intercellular communication, carrying bioactive molecules that influence immune responses and inflammation. Synovial fluid-derived exosomes have garnered attention for their potential roles in joint diseases like osteoarthritis and rheumatoid arthritis. Despite the growing interest in synovial fluid-derived exosomes and their potential role in immune modulation and joint diseases, there is limited understanding of how these exosomes are internalized by immune cells, particularly monocytes, which this study aims to address by examining the internalization process in a human monocytic cell line (THP-1 cells). This study explores the internalization of these exosomes by THP-1 cells to understand their impact on immune modulation. DAPI-labelled THP-1 cells were cultured with PKH67-labelled synovial fluid-derived exosomes, and their interactions were observed using fluorescence microscopy. The uptake was analysed at two incubation times (24 and 48 hours) and cell-to-exosome ratios (1:10, 1:20, and 1:40) using ImageJ software. Flow cytometry confirmed CD14 expression on THP-1 cells. Results showed significant exosome internalization, with the cell-to-exosome ratio being the key factor affecting uptake, while no significant difference was observed between the two incubation periods. This study emphasizes the role of exosome-to-cell ratio in the internalization process, contributing to the understanding of understanding of how these exosomes interact with immune cells.

CHAPTER 1: INTRODUCTION

1.1 Background of the study

Exosomes are tiny vesicles, typically 30 to 150 nm in size, released by various cell types to facilitate communication between cells. These vesicles carry a range of bioactive molecules, including proteins, lipids, and nucleic acids, which can influence the functions of the cells they interact with (Chen et al., 2024). Exosomes can be found in all types of body fluids (Liu et al., 2019) including synovial fluid which is found in joint cavities. These synovial fluid-derived exosomes can provide insight into joint health and diseases such as rheumatoid arthritis and osteoarthritis.

Studying how synovial fluid-derived exosomes are internalized by immune cells like monocytes is crucial to understand their role in immune regulation and inflammation (Swami et al., 2024). THP-1 cells, a widely used human monocytic leukemia cell line, serve as a model to explore monocyte and macrophage biology (Qin, 2011). Investigating the interaction between THP-1 cells and synovial fluid-derived exosomes can offer insights into how these vesicles influence immune cell behaviour and uncover potential therapeutic applications (Swami et al., 2024).

In this study, DAPI-labelled THP-1 cells and PKH67-labelled synovial fluid-derived exosomes were co-cultured, and their interactions were observed using fluorescence microscopy. The uptake of exosomes by THP-1 cells was analysed using ImageJ software to visualise their internalization at different time points and concentration ratios. To ensure the accuracy of the experiments, the CD14 expression of the THP-1 cells was confirmed using anti-CD14-PE antibody markers through flow cytometry before starting the co-culture process.

1.2 Problem statement

Exosomes derived from synovial fluid have garnered significant interests due to their potential roles in intercellular communication and their involvement in joint diseases such as rheumatoid arthritis and osteoarthritis. While substantial research has focused on their molecular composition and secretion, there is limited understanding of how these exosomes interact with immune cells, particularly regarding their internalization by monocytes. Monocytes are likely to internalize exosomes, which could influence inflammatory responses and other immune functions. However, the specific uptake patterns and factors influencing exosome internalization remain unclear.

This research aims to address this gap by investigating whether THP-1 cells internalize synovial fluid-derived exosomes and how factors such as incubation time and the ratio of THP-1 cells to exosomes impact internalization efficiency. By examining the uptake of exosomes over different time points and concentration ratios, this study will provide insights into the dynamics of exosome internalization and contribute to understanding how these interactions may play a role in immune modulation and inflammation in joint diseases.

1.3 Rationale of study

This study aims to deepen the understanding of how synovial fluid-derived exosomes are internalized by THP-1 cells. Gaining insight into this process is important to understand the role exosomes play in immune responses, particularly in joint diseases like osteoarthritis. By focusing on exosome internalization, this research could help shed light on how exosomes influence inflammation and immune modulation in the context of these conditions.

In addition to studying the internalization process, this research will examine how varying exosome-to-cell ratios and incubation times impact exosome uptake. Identifying the optimal conditions for co-culturing THP-1 cells with exosomes will provide useful guidelines for future studies. The findings could contribute to the development of new therapeutic approaches that use exosomes to modulate immune responses, offering potential benefits in treating joint diseases.

1.4 Research objectives

1.4.1 General objective

To assess internalization of synovial fluid-derived exosomes by THP-1 cells.

1.4.2 Specific objectives

I. To characterize THP-1 cells by flow cytometry
II. To assess PKH67-labelled exosomes internalization within the DAPI-labelled THP-1 cells

1.5 Hypothesis

1.5.1 Null hypothesis (H_0)

I. H_{01}: THP-1 cells do not show significantly higher CD14 expression compared to HeLa cells.
II. H_{02}: PKH67-labelled exosomes are not internalized by DAPI-labelled THP-1 cells.

1.5.2 Alternative hypothesis (H_1)

I. H_{11}: THP-1 cells show significantly higher CD14 expression compared to HeLa cells.
II. H_{12}: PKH67-labelled exosomes are internalized by DAPI-labelled THP-1 cells.

CHAPTER 2: LITERATURE REVIEW

2.1 Monocytes

Monocytes are a type of white blood cells (WBC) that play a crucial role in the immune system. They make up for about 2 to 8% of the total white blood cells in our body and are characterized by their large, kidney-shaped nuclei and abundant cytoplasm. These cells lack specific granules in their cytoplasm hence being a part of agranulocytes (Tigner, Ibrahim and Murray, 2022). They are about 12 to 20 µm in diameter, approximately twice the size of RBC, which makes them the largest WBC (Espinoza and Emmady, 2023).

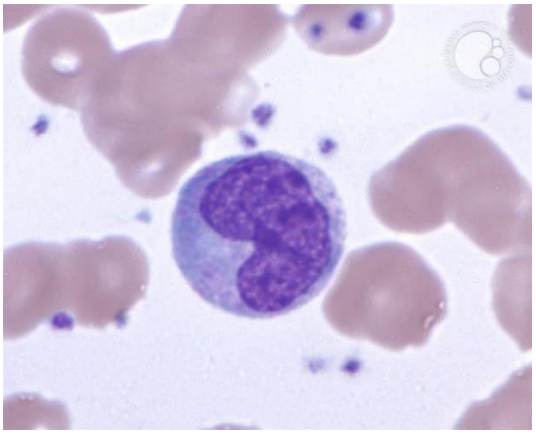

Figure 1: Monocyte in blood. Nucleus of monocyte have a "kidney-bean" shape or a cerebriform appearance and appears blue in color. Their cytoplasm is abundant and appears blueish-grey (Maslak, 2008)

Monocytes are produced in the bone marrow from a shared stem cell of blood cells which are pluripotent hematopoietic stem cells (HSCs). HSCs are divided into two types which are common lymphoid progenitor cells (CLPs) and common myeloid progenitor cells (CMPs). The CMPs differentiate into granulocyte-monocyte progenitor cells (GMPs) which further differentiates into monoblasts and promonocytes. Lastly, promonocytes matures into monocytes and released to circulate in the bloodstream before

migrating to tissues where they differentiate into macrophages or dendritic cells (Omman and Kini, 2020).

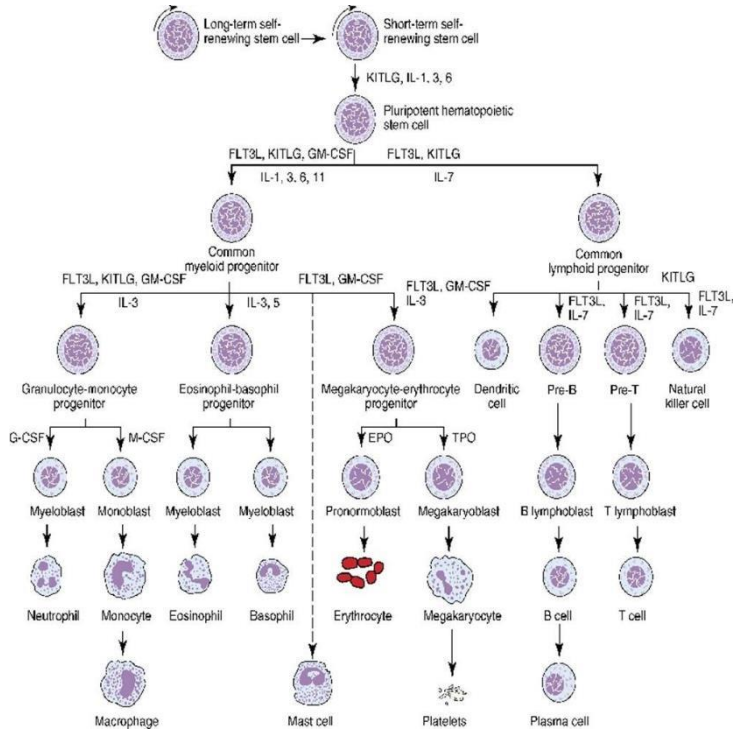

Figure 2: Hematopoiesis process. The hematopoiesis of monocytes begins with pluripotent hematopoietic stem cells, which give rise to all blood cell types. These stem cells differentiate into common myeloid progenitors, which further develop into granulocyte-monocyte progenitors. From this stage, the progenitor cells commit to the monocyte lineage by forming monoblasts, the earliest recognizable precursors of monocytes. As monoblasts mature, they develop into monocytes, which are released into the bloodstream. Once monocytes migrate into tissues, they further differentiate into macrophages (Keohane et al., 2015)

Monocytes are vital to the innate immune response, playing a key role in the body's first line of defence against pathogens through phagocytosis. They also act as messengers, directing other immune cells to sites of infection. Monocytes achieve this by presenting antigens via MHC molecules to T lymphocytes or by secreting pro-inflammatory cytokines that recruit additional phagocytes, such as neutrophils, and activate immune cells like NK cells (Janeway et al., 2001). Given the vital functions of monocytes in immune defence, their study is of significant importance. Their diverse roles, including pathogen recognition, immune regulation, and differentiation into macrophages and dendritic cells, make them an essential focus for research.

Due to the critical roles of monocytes in immune responses, various cell lines have been developed for different research purposes, allowing for in-depth studies of monocytes as well as the cells they differentiate into, such as macrophages and dendritic cells. For example, THP-1 and U937 are commonly used monocytic cell lines (Nascimento et al., 2022), while the RAW 264.7 cell line is frequently utilized to study murine macrophage functions (Taciak et al., 2018). Additionally, the MONO-MAC-6 cell line is often used to study the phenotypic and functional features of in vivo mature monocytes since it expresses mature monocyte markers that cannot be found on the THP-1 and U937 cell lines, such as M42, LeuM3, 63D3, Mo2 and UCHMI (Chanput et al., 2015).

2.1.1 THP-1 cell line

The THP-1 cell line is a human monocytic cell line widely used to model monocyte and macrophage biology. It was originally derived from the peripheral blood of a male patient with acute monocytic leukaemia (AML). The cell line which was established by Tsuchiya et al. in 1980 has distinct monocytic markers such as lysozyme production, and ability to phagocytose latex particles and sensitised sheep erythrocytes. These abilities make it a suitable and one of the most commonly used monocytic cell line for monocyte research.

Since THP-1 cells are a monocytic cell line directly involved in the immune system, they are widely used in immunology and immune disorder research. These suspension cells are particularly useful for 3D cell culture which enable researchers to mimic the natural behaviour of monocytes *in vivo* through *in vitro* studies. Moreover, since THP-1 cells is a cancer cell line, they are also used in immune-oncology studies and cell and gene therapy (CGT) development. Additionally, THP-1 can be easily induced to differentiate into macrophages and dendritic cells (Hölken and Teusch, 2023).

2.1.2 Benefits of using THP-1 cell line and its limitations

A comprehensive literature review by Qin (2011) thoroughly compiles and emphasizes the benefits of using THP-1 cells over primary human monocytes. To summarize, the homogenous genetic makeup of THP-1 cells allows for the variability in cell phenotype to be reduced. Hence, THP-1 cells offer more consistency compared to primary cells, which can be influenced by donor variability that may affect experimental outcomes. Moreover, THP-1 cells can be preserved in liquid nitrogen for extended periods without compromising their monocytic characteristics and viability, whereas primary cells have a limited lifespan even in culture and can be contaminated by other blood components such as platelets (Chanput et al., 2015). These advantages make THP-1 cells a preferred choice over primary monocytes for research.

THP-1 cells are one of the most commonly used monocytic cell line. Other monocytic cell line that are used to replace primary human monocytes are U937, and MONO-MAC-6. Both are mostly similar with THP-1 in terms of its uniformity in shape, exhibition of monocyte function and ability to differentiate into macrophages and dendritic cells. However, since both U937 and MONO-MAC-6 are derived from the blood of older patients, they mostly resemble mature monocytes or monocyte-derived macrophages whereas since THP-1 is derived from the blood of a 1 year-old patient, the cells mostly resembles promonocytes, which are precursors of monocytes (Nascimento et al., 2022). Hence, it is able to replicate the early stages of monocyte development which is important in disease modelling studies such as atherosclerosis, and osteoarthritis.

Although there are many benefits to using THP-1 cells in research, it is important to note its limitations in representing the human monocytes. For instance, THP-1 when compared to its human counterparts, are less responsive to lipopolysaccharide (LPS) due to the its low CD14 expression compared to primary monocytes. Even when THP-1 are

transfected with CD14, the production of IL-8, a cytokine produced by monocytes and macrophages, are lower compared to primary monocytes when stimulated with LPS (Bosshart and Heinzelmann, 2016). Moreover, THP-1 is found to be more responsive towards M1 stimuli and skewed towards M1 phenotype while U937 cells are more responsive towards M2 stimuli and skewed to M2 phenotype (Nascimento et al., 2022). This highlights that although THP-1 is a commonly used model of monocytes, it is also important to acknowledge its limitations compared to primary monocytes and verify the results using primary monocytes or *in vivo* models to be able to generalize the findings to monocytes (Qin, 2011).

2.1.3 Culture of THP-1

Roswell Park Memorial Institute 1640, or more commonly known as RPMI-1640, is developed by Moore, Gerner and Franklin in 1967 was first developed to cultivate human leukemic cells but has now been modified to cater to most type of cells of both suspension and adherence cells. FBS is supplemented to support cell growth by providing nutrients and growth hormones. 2-mercaptoethanol is mostly used to reduce oxidative stress to the cells. However, the addition of 2-mercaptoethanol is particularly useful in serum free medium. In contrast, addition of 2-mercaptoethanol in FBS supplemented media is less crucial since FBS itself has provided an array of growth hormone and nutrients to support cell growth (Click, 2013).

Another commonly used method of cultivating THP-1 is using RPMI-1640 media supplemented with 10% FBS, and 1% penicillin-streptomycin-glutamine (PSG) (Omage et al. 2024). The addition of PSG is crucial to prevent contamination and enhance cell growth. Penicillin is an antibiotic used against gram-positive bacteria whilst streptomycin is an antibiotic that targets gram-negative bacteria. Thus, this prevents both gram-positive

and gram-negative bacteria from growing in the culture media. Addition of L-Glutamine also serves as an energy source for cell growth and proliferation.

It is also noted that a study by Aldo et al. in 2013 has found that the confluency that a culture is maintained also plays a significant role in the phenotype of the cells. Culture maintained at a low confluency (2.5×10^5/ml) maintained its homogenous condition characterized by its small size and low density. In contrast, the cells maintained at high density (2.0×10^6/ml) becomes heterogeneous characterized by some large cells and high density. Hence, it is important to maintain the cells in a low density to avoid heterogenous cells which can affect the reproducibility of the results and also skewing the findings by introducing a variability in size.

2.1.4 Flow cytometry

Due to the advancements of monoclonal antibodies, flow cytometry becomes a widely used technique for analysing cells by measuring their size, complexity, and the expression of specific markers (Abeles et al., 2012). It works by passing single cells through a laser beam in a fluid stream, where they scatter light and emit fluorescence signals that are detected by the system. This provides detailed data on individual cells, allowing researchers to examine characteristics like cell surface markers, size and granularity of the cell.

The technique is especially useful for assessing the expression of cell populations. By staining cells with fluorescently-labelled antibodies that bind to specific markers, flow cytometry can determine the proportion of cells expressing these markers. The resulting data can be analysed to quantify the percentage of the targeted population in the sample (Aprahamian, 2019). This ensures that only the appropriate cell type is used for further

experiments. It can also be used to determine whether a cell line matches the human or animal counterpart that they want to replicate.

CD14 is a glycoprotein found on the surface of monocytes and macrophages, playing a key role in the immune response. It acts as a co-receptor for certain receptors like Toll-like receptors (TLRs), helping the cells detect pathogens and trigger immune reactions. Due to CD14 is mainly found on monocytes and macrophages, it serves as a reliable marker for these cell types (Lambert et al., 2015).

In THP-1 cells, CD14 is commonly used to confirm their monocytic identity (Lambert et al., 2015). Antibodies that specifically bind to CD14 is usually labelled with a fluorescent dye like phycoerythrin (PE) will attach to the CD14 molecules on the surface of THP-1 cells, and the fluorescence emitted by these cells can then be measured by the machine. The results are analysed to get the percentage of CD14-positive cells. This ensures that the culture consists mainly of monocytes and only monocytes are used for subsequent experiments. Hence, flow cytometry provides a reliable way to ensure that the cells remain homogeneous and appropriate for studying monocyte.

2.1.5 Staining of THP-1

THP-1 cells are often stained using DAPI (4',6-diamidino-2-phenylindole), a fluorescent stain that binds and fluoresces 20 times strongly to the AT-rich regions of double-stranded DNA (dsDNA) than in the unbound region. When exposed to ultraviolet light at around 345 nm, DAPI fluoresces blue, peaking at 455 nm. This makes DAPI ideal for visualizing the nuclei of both live and fixed THP-1 cells.

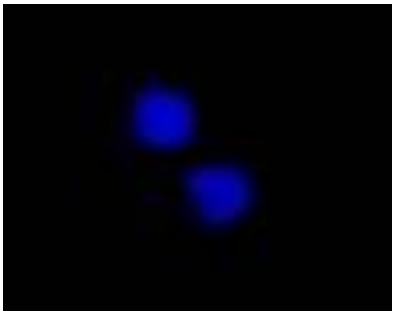

Figure 3: DAPI-stained THP-1 cell. The nucleus of THP-1 cells fluorescent blue when stained with DAPI (Qiu et al., 2022).

DAPI is commonly used in cellular research because it allows for detailed examination of nuclear features, such as size, shape, and overall integrity (Tarnowski et al. 1991). These observations are particularly useful for studying processes like the cell cycle, apoptosis, and differentiation. Its strong binding to dsDNA, along with its high fluorescence intensity, ensures that even small amounts of DNA can be detected with clear imaging under fluorescence microscopy (Button and Robertson, 2001). Moreover, DAPI is frequently combined with other stains or markers for multi-parameter analyses (Jayaprakash et al., 2014). Due to its low toxicity and suitability for live-cell imaging (Huschka et al., 2010), DAPI is a valuable tool for tracking the health and growth of THP-1 cells in various experiments, particularly those focused on immune response, inflammation, and cell signalling.

2.2 Extracellular vesicles

Extracellular vesicles (EVs) are a group of membrane-bound nano-sized particles with varying sizes, contents and surface markers that are released by cells into the extracellular environment through various mechanisms. They usually carry a variety of origin's cell membrane and cytoplasm components including proteins, mRNA, miRNA, metabolites, lipids, and even DNA (Shi et al., 2019). Larger EVs like apoptotic bodies may even contain organelles like mitochondria, ribosomes and endoplasmic reticulum (Ramesh Kakarla et al., 2020).

EVs are released by a wide variety of organisms, ranging from prokaryotes to unicellular eukaryotes such as protozoa, yeasts, and fungi, as well as mammals. They can be extracted from all bodily fluids, including blood, plasma, serum, saliva, urine, sweat, cerebrospinal fluid, breast milk, and semen. In humans, nearly every cell type, including immune cells, endothelial cells, red blood cells, liver cells, and epithelial cells, has been reported to secrete EVs (Ibrahim and Khan, 2023).

EVs are categorized into three main types: apoptotic bodies, microvesicles, and exosomes. Although the sizes may vary, sometimes the EVs might overlap in sizes hence, the EVs are not categorised solely by size and instead accompanied by categorising through their distinct process that they are released from the cells of origin. Firstly, apoptotic bodies (APBs) comes from cells during their apoptosis process. APBs arises either from apoptopodia which emerges during the apoptosis process or from the separation of membrane blebs (Ibrahim and Khan, 2023). They are the largest EVs among the three types ranging from 50 to 250 nm in diameter making it quite distinguishable compared to the other two. Meanwhile, ectosomes, or more commonly known as microvesicles are heterogenous particles that arise by direct budding of the cell membrane to the outside of the cell. Hence, its larger size compared to the third type

which are exosomes where the sizes are 50 to 1000 nm in diameter and 40 to 100 nm in diameter respectively (Creese, 2019).

2.2.1 Function of extracellular vesicles

EVs have many functions in the human body mainly because its function depends on what it carries from the origin cell. Since humans have many types of cells, the contents of the EVs that these cells produce are also different and exhibit different functionalities. Interestingly, some EVs has a direct function and does not need a recipient cell. For example, some exosomes may carry remodelling enzymes like hyaluronidases on their surface matrix that perform directly in the extracellular environment (Ibrahim and Khan, 2023). EVs can also act as decoy by capturing and neutralising the effects of pathogens or foreign molecules (Buzas, 2022).

One of the most important function is that EVs allows for cells that are apart to have an intercellular communication without having to move. These cells communicate by releasing EVs into the body fluids and when the EVs reach their target cell, the surface molecules on the EVs will act as ligands to stimulate the surface receptor on the recipient cells. The way cells communicate affects the physiological processes in the body (Buzas, 2022). For example, platelet-derived EVs that are only released during injuries or endothelial damage acts as activators of the coagulation cascade. In contrast, platelets in healthy circulating blood releases EVs that inhibits platelet aggregation (Eustes and Dayal, 2022). Cell communication through EVs is crucial so that the body can act in harmony and maintain the homeostasis balance.

Unfortunately, since EVs are released by all cells and not only the cells in the human body, it becomes a weapon used by both host and pathogen. Pathogens like bacteria, viruses, fungi, and parasites use EVs to evade the immune system by inhibiting complement activity and degrading antibodies produced by immune cells. Not only that, EVs may also be directly used for disruptive behaviours of pathogens like cytotoxicity, delivery of virulence factors and drug resistance. For instance, bacteria with b-lactamase protein may transfer their enzyme to b-lactamase deficient bacteria leading to antibiotic resistance. On the other hand, the immune system also uses EVs to combat against these invading pathogens. The immune cells depend on the information that they get from the EVs from the pathogen itself, the non-immune cells and other immune cells in order to orchestrate the fight against the pathogen (Peregrino et al., 2024). EVs from microbes, dendritic cells or B lymphocytes has also been found to be able to activate T lymphocytes by directly transferring antigens to the T cells. Not only immune cells, epithelial cells are also able to release exosomes containing an antiviral that can neutralise the virus (Peng et al., 2023). This shows that EVs play an important role in the survival of a host and pathogen.

2.2.2 Exosomes

Exosome is the smallest EV ranging in size from 30 to 150 nm in diameter (Zhang et al., 2019). These exosomes appear as cup-shaped vesicles when viewed with Transmission Electron Microscopy (TEM)s and are homogenous in shape compared to other EVs. Exosomes carry functional proteins, lipids and nucleic acids like dsDNA, ssDNA, mRNAs, and miRNAs from origin cells and are released into the extracellular environment hence their involvement in various biological processes, including immune modulation, cell signalling, and waste removal (Mousavi et al., 2022).

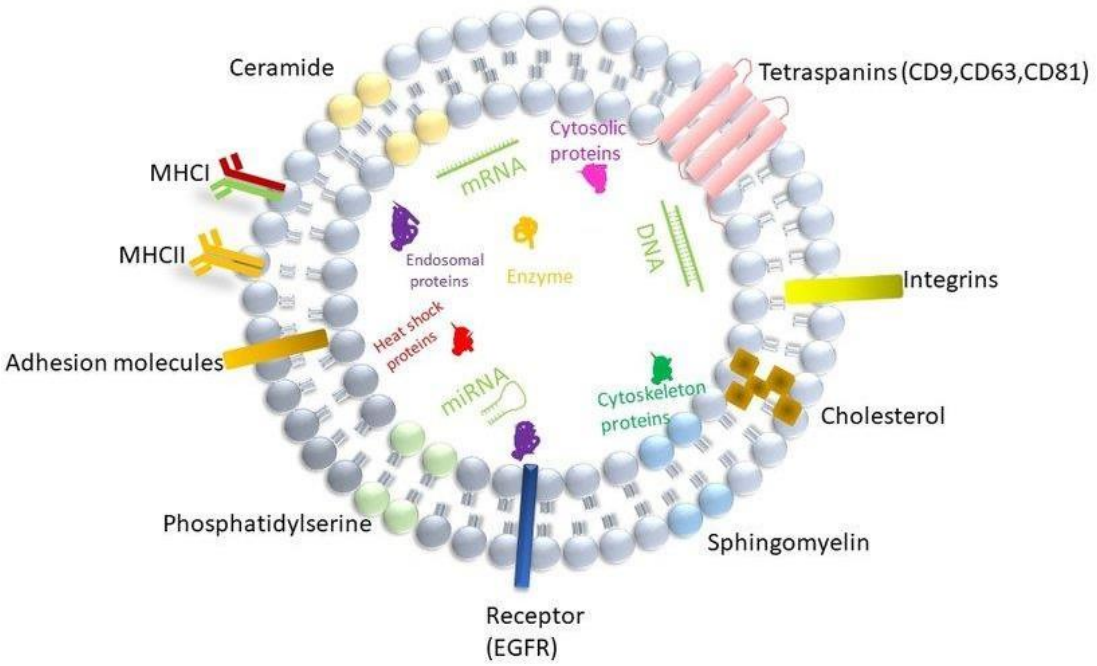

Figure 4: Structure and composition of exosome. Exosome has a lipid bilayer structure that contains lipids, proteins and nucleic acids. Sphingomyelin, phosphatidylserine, cholesterol and ceramides are highly distributed on the membrane. Exosomes also contain a variety of proteins such as major histocompatibility complex I and II (MHCI and MHCII), tetraspanins (CD9, CD63, CD81), endosomal origin proteins (ALIX, Tsg101), heat shock proteins (HSP70, HSP90), enzymes (GAPDH, nitric oxide synthase, catalase), receptor (EGFR), adhesion proteins, integrins, cytoskeleton proteins (actin, gelsolin, myosin, tubulin) and cytosolic proteins, as well as RNA, miRNA and DNA (Xiao et al., 2021).

Exosome structure can be divided into two which is the membrane structure and the composition of the exosome itself. The membrane structure of exosomes is quite similar to cells where they are surrounded by phospholipid bilayer. However, the composition of the membrane is different since exosome membranes are highly distributed with sphingomyelin, phosphatidylserine, cholesterol and ceramides. Another unique feature of the membrane is the high level of the lysoglycerophospholipid, which is absent in the other cellular membranes (Sun et al., 2019). In addition, the membrane also has a multitude of proteins like major histocompatibility complex (MHC I and MHC II), and tetraspanins (CD63 and CD81). Since the tetraspanins are found in most exosomes, it is used as exosomal markers for exosome detection. The composition of

exosome also reflects that of a regular cell where they have cytoskeleton proteins, enzymes, and nucleic acids like DNA, mRNA and miRNA (Xiao et al., 2021).

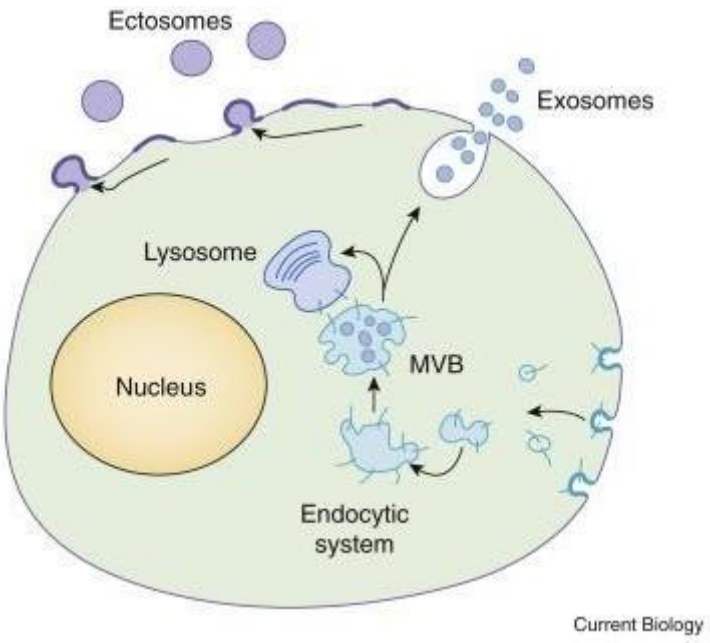

Figure 5: Formation of exosomes. Exosome formation occurs through the endocytic pathway, starting with the invagination of the plasma membrane to form early endosomes. These early endosomes mature into multivesicular bodies (MVBs), which contain intraluminal vesicles formed by the inward budding of the endosomal membrane. MVBs have two potential fates: they can either fuse with lysosomes for degradation or merge with the plasma membrane, releasing their vesicles into the extracellular space as exosomes (Meldolesi, 2018).

Exosome are distinctly different from the other EVs since they are formed from the endosomal pathway. Firstly, the membrane of endocytic cisternae forms intraluminal vesicles (ILVs), which is the precursor of exosomes, by the budding of microdomains. Once ILVs have accumulated in the cytosol, the cisternae forms into multivesicular bodies (MVBs). These MVBs remain in the cytosol for a period of time and undergo either two pathways which are lysosomal degradation, where the MVBs fuse and are degraded by lysosomes, or exocytosis where they fuse with the plasma membrane and are released from the cells as exosomes (Meldolesi, 2018).

2.2.3 Isolation of exosomes

Exosomes are isolated using methods such as differential ultracentrifugation, and size-exclusion chromatography. Differential ultracentrifugation has long been the gold standard for exosome isolation. This technique allows for the gradual separation (and collection) of the EVs since it involves a series of centrifugation steps at different speeds to separate the components. To remove the large components like cells and cell debris, the sample will be subjected to a low-speed spin at 2,000 xg. Enzymatic treatment may follow to remove contaminants, ensuring a purer exosome preparation. The samples were then centrifuged at 10,000 xg to pellet larger sized EVs like apoptotic bodies and microvesicles. Lastly, ultracentrifugation at 100,000 xg was performed to pellet exosomes (Dilsiz 2024).

Another method that are used to isolate exosomes are ultrafiltration where the components in the sample are separated under centrifugal force based on filtration and size. This method requires the least amount of time compared to differential ultracentrifugation and is generally cheaper than differential ultracentrifugation. Ultrafiltration can be used for small amount of sample which means that it is not practical for scalability. Moreover, exosomes isolated by ultrafiltration was found to vary in size and shape compared to other methods. This means that the isolated exosomes may be contaminated by other vesicles (Ansari et al., 2024).

In recent years, the method of exosome isolation has evolved and expanded by using methods like immunomagnetic beads, nanolithography, and negative magneticelectrophoresis. These methods are used to achieve a rapid and high yield exosome isolation. For example, since it is known that CD9, CD63 and ALIX are exosome markers, the use of beads coated with these antibodies allows for the binding of the beads to the exosome and easier isolation of the exosome. Although this method

produces a low yield of exosomes, the exosomes isolated are highly pure and specific, thus reduces the risk of contamination with other particles (Jiawei et al., 2022). It is found that combination of traditional methods with modern ways were able to produce exosome isolation at a higher yield and purity however, it would be time-consuming and costly especially when combined with methods like ultracentrifugation which is already costly and time-consuming on its own.

2.2.4 Staining of exosome

Exosomes can be stained with a variety of fluorescent dyes and markers to visualise its structure and interactions with other components. Exosomes can be stained with PKH67, a green fluorescent dye that labels lipid membranes. The principle of PKH67 staining lies in its ability to integrate into the phospholipid bilayer of vesicles through their two long lipophilic tails, providing stable and long-lasting fluorescence (Bao et al., 2023). This method allows for the visualization and tracking of exosomes in co-culture experiments. Under a fluorescence microscope, PKH67-labeled exosomes exhibit bright green fluorescence. The dye is excited at a wavelength of 490 nm and emits fluorescence at 502 nm. This enables the visualization of exosome uptake by cells and their intracellular localization.

Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) is another type of dye that can be used to label exosomes. These dyes are amine-reactive dyes which means that they bind to the amine groups of proteins which are generally carried by exosomes. These dyes penetrate the cell membrane, bind to the intracellular protein inside the exosome and emits green fluorescence after hydrolysis. CFCA-SE does not affect the size or function of the exosome and certainly avoids aggregation problems like membrane-dyes. However, it has its limitations in acidic conditions where the fluorescence weakens (Bao et al., 2023).

2.3 Role of exosome in inflammation

Exosomes can induce inflammation by releasing their contents and activating receptor cells. Exosomes from immune cells such as macrophages and dendritic cells can carry pro-inflammatory cytokines or microRNAs to recipient cells and enhancing the inflammatory response. They can also activate more macrophages and dendritic cells to release pro-inflammatory cytokines. In contrast, exosomes from regulatory T cells may release anti-inflammatory molecules that can counteract the pro-inflammatory molecules. Moreover, in chronic inflammatory diseases like rheumatoid arthritis and osteoarthritis, exosomes from synovial fibroblasts or even immune cells can worsen the condition by transferring complex molecules and activating macrophages to produce chemokines and proinflammatory cytokines which may worsen the joint condition (Tian et al., 2022).

2.3.1 Osteoarthritis

Osteoarthritis or OA is the most common type of arthritis in the world. that can be characterized with joint pain and loss of function. OA can be divided into two types which is primary and secondary OA. Primary OA has no known cause but is typically due to aging and wear and tear on the joints whereas secondary OA is usually caused by another disease, injury or deformity (Sen and Hurley, 2023).

Primary osteoarthritis is the most common in adults and the elderly where the hands, knees and hips being the most commonly affected joints. Primary osteoarthritis can be divided into four stages. The first stage is where there is a loss of cartilage. The second stage is where the joint space starts to narrow and there are occurrence of osteophytes which are bone spurs that grow on the bones especially around the joints. The third stage is where the gaps in the cartilage starts to expand until the surface of the bone is visible. Lastly, the fourth stage of primary osteoarthritis is characterized by the loss of approximately 60% of the cartilage, significantly increasing bone friction during

movement, leading to severe pain and difficulty in joint mobility. In some cases, this stage may require joint replacement surgery or the insertion of supportive structures, such as metal implants, to mimic the function of the natural joint and alleviate symptoms (Aboulenain & Saber, 2022).

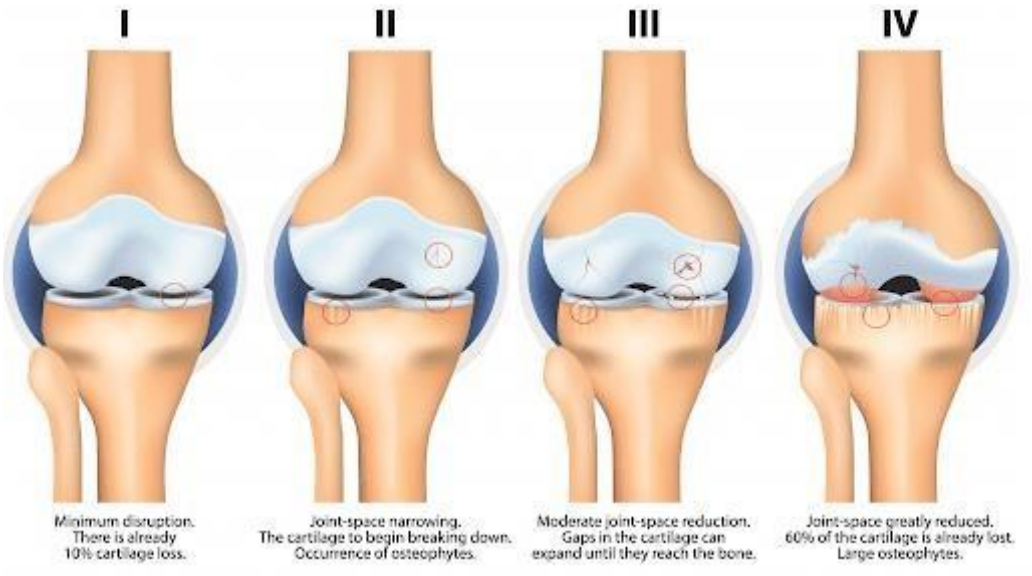

Figure 6: Stages of primary osteoarthritis. Osteoarthritis progresses through four distinct stages. In Stage I (Early Osteoarthritis), there is minimal disruption with approximately 10% cartilage loss, and small osteophytes (bone spurs) begin to form, though symptoms are usually mild or absent. In Stage II (Mild Osteoarthritis), joint-space narrowing becomes noticeable as the cartilage starts breaking down, and osteophytes become more prominent. In Stage III (Moderate Osteoarthritis), significant joint-space reduction occurs, with visible cartilage loss and gaps that extend closer to the bone. Osteophytes enlarge, and inflammation may cause pain and swelling. In Stage IV (Severe Osteoarthritis), the joint space is greatly reduced, with approximately 60% cartilage loss. Large osteophytes form, and bone-on-bone contact occurs, leading to severe pain, stiffness, and limited mobility (TeachMeSurgery 2022).

The treatment of primary osteoarthritis focuses on reducing symptoms, enhancing joint function, and slow the disease's progression. Non-pharmacological approaches include weight control, engaging in regular low-impact exercises, and undergoing physical therapy. Pain and inflammation are often managed with medications like acetaminophen and nonsteroidal anti-inflammatory drugs (NSAIDs), while more severe cases may benefit from corticosteroid injections. For advanced osteoarthritis, surgical procedures such as joint replacement or the use of supportive implants may be necessary to improve mobility and alleviate pain. In cases where both pharmacological and non-

pharmacological treatment fails, the patient will undergo partial or total removal of the joint (Aboulenain & Saber, 2022). A combination of lifestyle changes, medical treatments, and, when needed, surgical options can help effectively manage the condition and enhance the patient's quality of life.

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Overview of the study

The flowchart of the study is shown in Figure 3.1. This study involved the collection of synovial fluid (SF) from primary osteoarthritis (OA) patients that underwent total knee arthroplasty (TKA). The synovial fluid was kept at -80°C until it was processed using enzymatic method which incorporated the use of hyaluronidase and DNase I, followed by ultracentrifugation to isolate the exosomes from the SF. The isolated exosomes were kept at -80°C.

THP-1 cells were acquired from American Type Culture Collection (ATCC) and maintained in complete medium in an incubator at 37°C with 5% CO_2 supply. The cells were maintained by appropriate subculture procedure. THP-1 cells were characterized using flow cytometry. For each passage, 2.4 x 10^6 cells were collected and stained with DAPI. The DAPI-stained cells were divided into 6 wells accounting for three different ratios which were 1:10, 1:20, and 1:40 and incubated at 24 hours and 48 hours.

To detect internalization of exosome by THP-1 cells, exosomes were stained with PKH67 and distributed into 6 wells containing DAPI-stained cells with the ratio of 1:10, 1:20 and 1:40 for 24 hours and 48 hours before being observed under fluorescence microscope. Stained THP-1 cells alone and exosomes alone were also prepared and observed under fluorescence microscope as the negative control. Images obtained from fluorescence microscope were analysed using ImageJ software and the statistical analysis were performed using SPSS software.

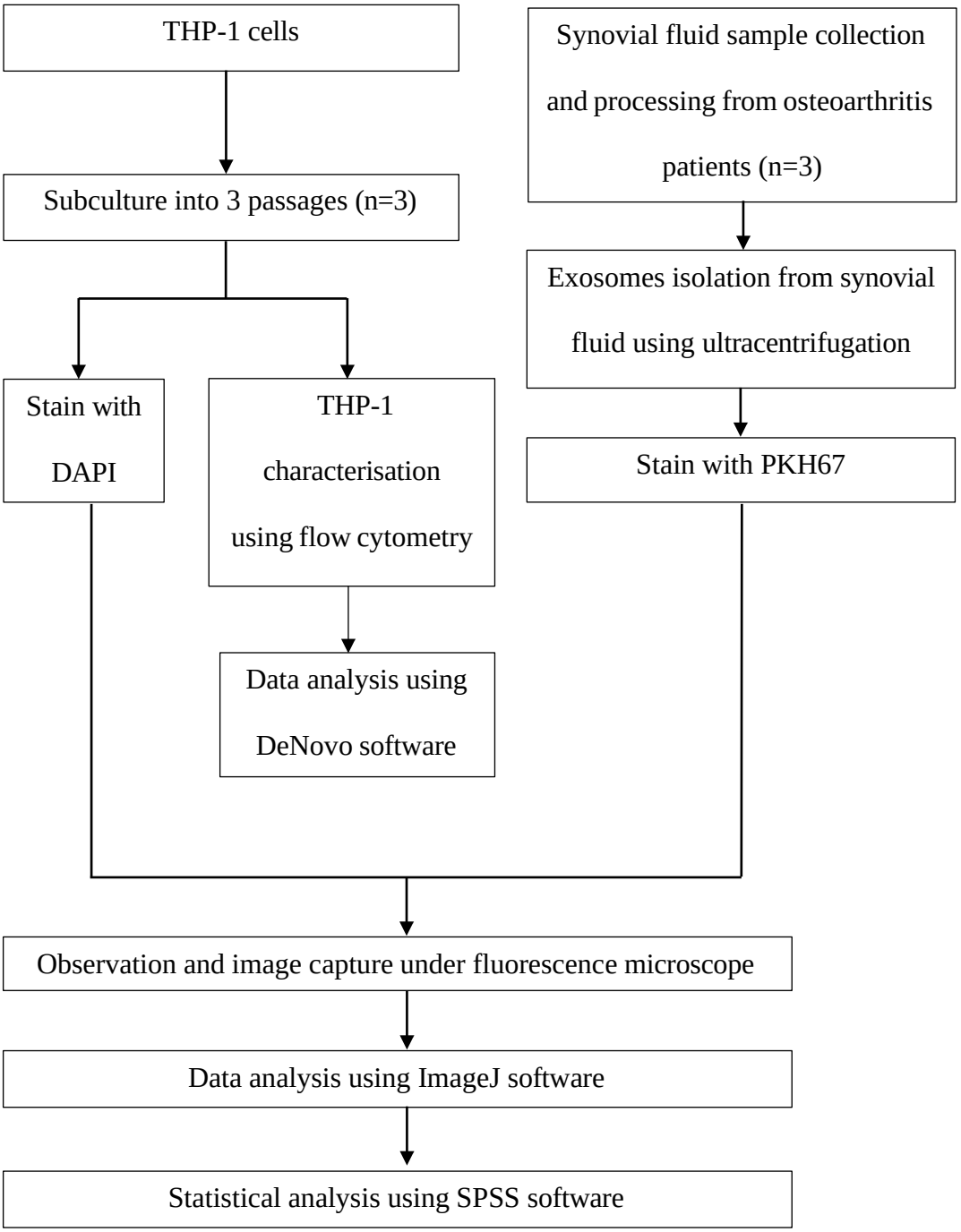

Figure 7: Flowchart experiments carried out in this study

3.2 List of chemicals, reagents, kits, and consumables

All chemicals, reagents, kits and consumables used in this study are as listed in Table 1, Table 2, and Table 3, respectively.

Table 1: List of chemicals and reagents

Chemicals and reagents	Manufacturer
Absolute ethanol	HmbG, Germany
Bleach	Clorox, USA
Anti-CD14 PE	BD Biosciences, USA
DAPI staining solution	Macklin, China
Dimethyl sulfoxide (DMSO)	Thermo Fischer Scientific, UK
DNase 1	Novagen, USA
Dulbecco's phosphate buffered-saline (DPBS)	Corning, USA
Ethylenediamine tetraacetic acid (EDTA)	Sigma-Aldrich, USA
Glycerol	Thermo Fischer Scientific, UK
Hyaluronidase	Sigma-Aldrich, USA
Methanol	Bendosen, USA
Penicillin-Sterptomycin-Glutamine (PSG)	Nacalai Tesque, Japan
Phosphate buffered saline (PBS) tablets	Thermo Fischer Scientific, UK
RPMI-1640 media	Sigma-Aldrich, USA

Table 2: Lits of kits

Kits	Manufacturer
PKH67 Green Fluorescent Cell Linker Mini Kit	Sigma-Aldrich, USA