ASSESMENT OF MONOCYTE PHAGOCYTOSIS IN THE PRESENCE OF SYNOVIAL-FLUID-DERIVED EXOSOMES FROM OSTEOARTHRITIS PATIENTS

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by

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for the degree of
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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.

Signature

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‘Muhammad Akid Bin Ruhani’

Date: 27 / 1 / 2025

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LIST OF ACRONYM, ABBREVIATIONS, AND SYMBOLS

APC-H7	Allophycocyanin H7
BSA	Bovine Serum Albumin
DNase I	Deoxyribonuclease I
DPBS	Diluted Phosphate Buffered Saline
EDTA	Ethylenediaminetetraacetic Acid
EVs	Extracellular Vesicles
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
g	Gravitational force unit
IL-1	Interleukin-1
IL-6	Interleukin-6
kDa	Kilodalton
mL	Milliliter
mg/mL	Milligrams per milliliter
MFI	Mean Fluorescence Intensity
MMPs	Matrix Metalloproteinases
MVs	Microvesicles
nm	Nanometer
NS	Non-Significant
OA	Osteoarthritis
P	Probability
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
RPMI	Roswell Park Memorial Institute
rpm	Revolutions per minute
SF	Synovial Fluid
TNF-α	Tumor Necrosis Factor-alpha
USM	Universiti Sains Malaysia
U/mL	Units per milliliter

µL	Microliter
µM	Micromolar
°C	Degree Celsius
±	Plus/minus
+/-	Standard error or variation
>	Greater than
<	Less than
×	Times
%	Percentage

LIST OF APPENDICES

PENILAIAN FAGOSITOSIS MONOSIT DALAM KEHADIRAN EXOSOME YANG DIPEROLEHI DARIPADA CECAIR SINOVIAL PESAKIT OSTEOARTRITIS

ABSTRAK

Osteoartritis (OA) adalah penyakit sendi degeneratif yang boleh dikenal pasti oleh degradasi rawan, keradangan, dan ketidaknormalan fungsi sendi, dengan monosit memainkan peranan penting dalam patogenesisnya melalui proses fagositosis dan modulasi keradangan. Kajian ini menyelidik kesan exosome yang diperoleh daripada cecair sinovial pesakit OA terhadap aktiviti fagositosis monosit. Cecair sinovial (SF) diperoleh daripada pesakit OA yang menjalani artroplasti lutut, dan exosome diasingkan melalui proses ultrasentrifugasi. Monosit daripada penderma sihat telah dikultur bersama exosome pada kepekatan yang berbeza (1:10, 1:20, dan 1:40) selama 24 dan 48 jam. Fagositosis monosit telah dinilai dengan menggunakan konjugat pHrodo™ Green *E. coli* dan dianalisis melalui mesin sitometri aliran. Keputusan kajian telah menunjukkan peningkatan signifikan dalam aktiviti fagositosis monosit dengan kehadiran exosome, sekaligus membuktikan pengaruh modulasi exosome. Penemuan ini telah memberikan pemahaman tentang interaksi antara exosome dan monosit dalam patofisiologi OA, serta menunjukkan potensi terapi berasaskan exosome untuk menguruskan keadaan degeneratif penyakit ini.

ASSESMENT OF MONOCYTE PHAGOCYTOSIS IN THE PRESENCE OF SYNOVIAL-FLUID-DERIVED EXOSOMES FROM OSTEOARTHRITIS PATIENTS

ABSTRACT

Osteoarthritis (OA) is a degenerative joint disease characterised by cartilage degradation, inflammation, and joint dysfunction, and monocytes play an important role in its pathogenesis via phagocytosis and inflammatory regulation. This study investigated the effect of synovial fluid-derived exosomes from OA patients on monocyte phagocytic activity. Synovial fluid (SF) was harvested from OA patients undergoing total knee arthroplasty, and exosomes were isolated by ultracentrifugation. Monocytes from healthy donor were cultured for 24 and 48 hours at different exosome concentrations (1:10, 1:20, and 1:40). Then, pHrodo™ Green *E. coli* conjugates were used to measure monocyte phagocytosis, which was quantified by flow cytometry. The findings demonstrated a significant increase in monocyte phagocytic activity upon exosome treatment, indicating a modulatory effect. These findings provide insight into the interaction between exosomes and monocyte in OA pathogenesis, underlining the possibility of exosome-based treatments to manage this degenerative illness.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The immune system plays a vital role in maintaining homeostasis and defending the body against pathogens. However, in certain disorders, including osteoarthritis (OA), dysregulation of the immune response can exacerbate disease progression. Soluble and cellular mediators of the immune system significantly influence OA pathogenesis, with monocytes playing a particularly important role (Chow & Chin, 2020). Monocytes are known for their phagocytic capabilities, a critical process in clearing apoptotic cells, debris, and pathogens within the joint environment. This phagocytic activity plays a significant role in maintaining joint homeostasis and mitigating inflammatory responses. The involvement of monocytes in OA pathophysiology has been strongly linked to their role in phagocytosis and inflammatory modulation (Gómez-Aristizábal et al., 2019). Additionally, monocytes are implicated in synovitis, where they attract other immune cells, further amplifying joint inflammation. The involvement of monocytes has been strongly linked to the pathophysiology of OA (Gómez-Aristizábal et al., 2019).

The ability of monocytes to interact with extracellular vesicles (EV) such as exosomes has drawn significant attention in recent research. Exosomes are small membrane-bound vesicles that mediate intercellular communication and regulate various biological processes (Ni et al., 2020). In OA, exosomes have been found in the articular cavity, where they can induce changes that drive disease

progression. Joint cells, including chondrocytes, synovial fibroblasts, osteoblasts, and tenocytes, secrete exosomes that alter the biological activities of target cells.

Osteoarthritis (OA) is a chronic degenerative joint disease characterized by the progressive destruction of cartilage, leading to joint pain, stiffness, and reduced mobility. OA primarily affects weight-bearing joints such as the knees and hips but can also involve the hands and spine. The disease is more common in older individuals, particularly women, and typically manifests in middle age (Sen & Hurley, 2023). Primary OA arises without a known underlying cause, while secondary OA may result from trauma or defective cartilage, as seen in rheumatoid arthritis (Hsu & Siwiec, 2023).

Current treatments for OA include physical therapy, pharmacological interventions, and surgical options. Physical therapies, such as weight loss and exercise, aim to reduce mechanical stress and improve joint function (Chen et al., 2021). Pharmacological treatments, including NSAIDs and acetaminophen, primarily address pain and inflammation but cannot reverse cartilage damage. Surgery, such as total joint replacement, is reserved for severe cases but has limitations (Zhang et al., 2016). Despite these treatment options, no cure exists, emphasizing the need for novel therapeutic strategies.

To investigate monocyte phagocytosis in the presence of synovial fluid-derived exosomes from OA patients, exosomes were isolated from the synovial fluid (SF) of primary OA patients by using ultracentrifugation. Healthy donor-derived monocytes were cultured with SF-derived exosomes at various concentrations and incubation period. Phagocytosis was assessed using pHrodo Green *E. coli* conjugate and quantified by flow cytometry, providing valuable insights into the interactions between monocytes and OA-derived exosomes.

1.2 Problem statement

Monocytes play an important role in osteoarthritis (OA) development, especially during the inflammatory phase. These immune cells play a significant role in disease pathogenesis due to their phagocytic activity and interaction with extracellular signals. Exosomes have received attention because of their potential to influence essential physiological processes such as immune responses, inflammation, and tissue remodelling. Despite the known existence of synovial fluid-derived exosomes in OA patients, their exact role in affecting monocyte phagocytosis is unclear. Exosomes consist of a variety of signalling chemicals which can modify monocyte behaviour, making it critical to investigate their influence on immune cells. A better knowledge of how synovial-derived exosomes influence monocyte phagocytosis might give important insights into their role in OA pathogenesis and discover potential therapies in this degenerative disease.

1.2 Rationale of the study

The purpose of this study is to investigate phagocytosis of monocytes in the presence of synovial fluid-derived exosomes from osteoarthritis patients. Monocytes play an important part in the inflammatory response and tissue breakdown in OA, whereas exosomes are involved in cell communication and immunological control. Understanding how exosomes regulate monocyte activity in OA may provide useful insights into the underlying immunological processes which contribute to disease development. This study will further our understanding of how exosomes contribute to OA pathogenesis and clarify their possible function in immune regulation within affected joints.

1.3 Research Objectives

1.4 General Objective

To investigate monocyte phagocytosis in the presence of synovial-fluid-derived exosomes from OA patients.

1.5 Specific Objective

i) To optimize the concentration of anti-CD14 antibody for monocyte characterization

ii) To analyse monocyte surface markers and phenotypic profiles using flow cytometry.

iii) To assess phagocytosis of monocytes in the presence of synovial fluid-derived exosome.

CHAPTER 2

LITERATURE REVIEW

2.1 The immune system

The immune system makes an important contribution to the development of OA. Both innate and adaptive immune responses contribute to joint inflammation and tissue damage. Macrophages in the innate immune system release pro-inflammatory cytokines such as IL-1β and TNF-α, which cause cartilage destruction and bone remodelling (Thomson & Hilkens, 2021). Synovial fibroblasts enhance inflammation by releasing pro-inflammatory cytokines (Ospelt, 2017). Furthermore, regulation of the complement system could contribute to inflammation and cartilage degradation in OA patients.

While the innate immune system is heavily implicated, the adaptive immune system also plays a role, with T and B lymphocytes found in the synovial fluid of OA patients, indicating their involvement in inflammatory processes (Sengprasert et al., 2023).

2.2 Overview of monocytes

Monocytes are white blood cells that play an important role in the immune system. They are the largest type of white blood cell, accounting for around 2-10% of the overall leukocyte population (Espinoza & Emmady, 2023). Monocytes are phagocytic cells, which consume and digest cellular debris, foreign substances, and pathogens. They also participate in antigen presentation, a key mechanism that initiates the adaptive immune response and connects innate and adaptive immunity (Espinoza & Emmady, 2023).

Monocytes originate from haematopoietic stem cells (HSCs) in the bone marrow, which are the source of all blood cells. Monocytes go through numerous phases of differentiation. HSCs, which are pluripotent and may produce a variety of blood cell

types, first develop into common myeloid progenitors (CMPs) (Höfer & Rodewald, 2018). CMPs can differentiate into granulocytes, monocytes, and macrophages. CMPs then develop into granulocyte-monocyte progenitors (GMPs), which will become either granulocytes or monocytes (Lieu & Reddy, 2012). GMPs grow into monoblasts, the first cell type specialised to the monocyte lineage. These monoblasts differentiate into promonocytes, which are larger and have more developed organelles. Finally, promonocytes mature into monocytes, which are released into the circulation and prepared to conduct immunological activities (Höfer & Rodewald, 2018).

Monocytes are characterized by the expression of CD14, a co-receptor for bacterial lipopolysaccharide (LPS), which is required for pathogen identification and the activation of innate immune responses. CD14 is predominantly associated with monocyte activity and acts as a reliable marker for identifying these cells (Sampath et al., 2018). Beyond CD14, monocytes may express other markers such as CD16, allowing for further classification into subsets with unique inflammatory or regulatory functions. For example, classical monocytes ($CD14^{++}$ $CD16^{-}$) are primarily involved in phagocytosis, whereas non-classical monocytes ($CD14^{+}$ $CD16^{++}$) monitor endothelium surfaces and aid in tissue repair and inflammation resolution (Sampath et al., 2018).

2.2.1 Cell surface expression of monocytes

Monocyte express a glycoprotein CD14. Therefore, by using anti-CD14 antibodies tagged with a fluorochrome allows for precise identification and gating of monocytes within a sample (Ahrazoglu et al., 2024). There are many protocols to isolate monocytes from the peripheral blood, and one of the most common is by using immunomagnetic beads coupled to anti-CD14 antibody. The isolation procedure must be

carried out to ensure that the monocyte population is pure and thus reduces possible contamination with other white blood cells (Sharygin et al., 2023).

2.2.2 Mechanism of monocytes phagocytosis

Phagocytosis is an important mechanism in which monocytes engulf and destroy foreign particles such as bacteria, viruses, and cellular debris (Chiu & Bharat, 2016). The process begins with the recognition of target particles by specialised surface receptors. Pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and cell surface receptor (CLRs), recognise conserved molecular patterns on pathogens (Xuan et al., 2023). Furthermore, recognition via Fc and complement receptors is improved by opsonisation, in which target particles are coated with complement proteins or antibodies (Vandendriessche et al., 2021).

After recognition, monocytes extend pseudopods to engulf the target, enclosing it in a membrane-bound vesicle known as a phagosome. The phagosome matures by fusing with lysosomes to form a phagolysosome (Rosales & Uribe-Querol, 2017). Lysosomal enzymes, which include proteases, nucleases, and lipases, later break down the material that was consumed into basic components such amino acids, lipids, and nucleotides. These products are released into the cytoplasm, where they act as energy sources or structural components for cellular processes (Lee et al., 2020).

The usage of pHrodo Green *E. coli* conjugates offer a useful way for studying phagocytosis in monocytes utilising fluorescence-based, pH-sensitive technology. These conjugates allow for accurate, real-time quantification of phagocytic activity. The pHrodo dye has low fluorescence at neutral pH but becomes highly luminous in acidic settings, making it excellent for monitoring the phagocytic process when ingested particles enter the acidic phagosome environment (Figure 2.1) (Zhang et al., 2016).

The dye is covalently attached to the surface of *E. coli* bacteria, allowing them to function as both phagocytic targets and fluorescent emitters. Monocytes phagocytose the dye, and the acidic environment of the phagosome activates it, dramatically enhancing fluorescence. This signal can be detected and quantified with flow cytometry or fluorescence microscopy, providing an accurate approach to evaluate phagocytic activity (Lenzo et al., 2016).

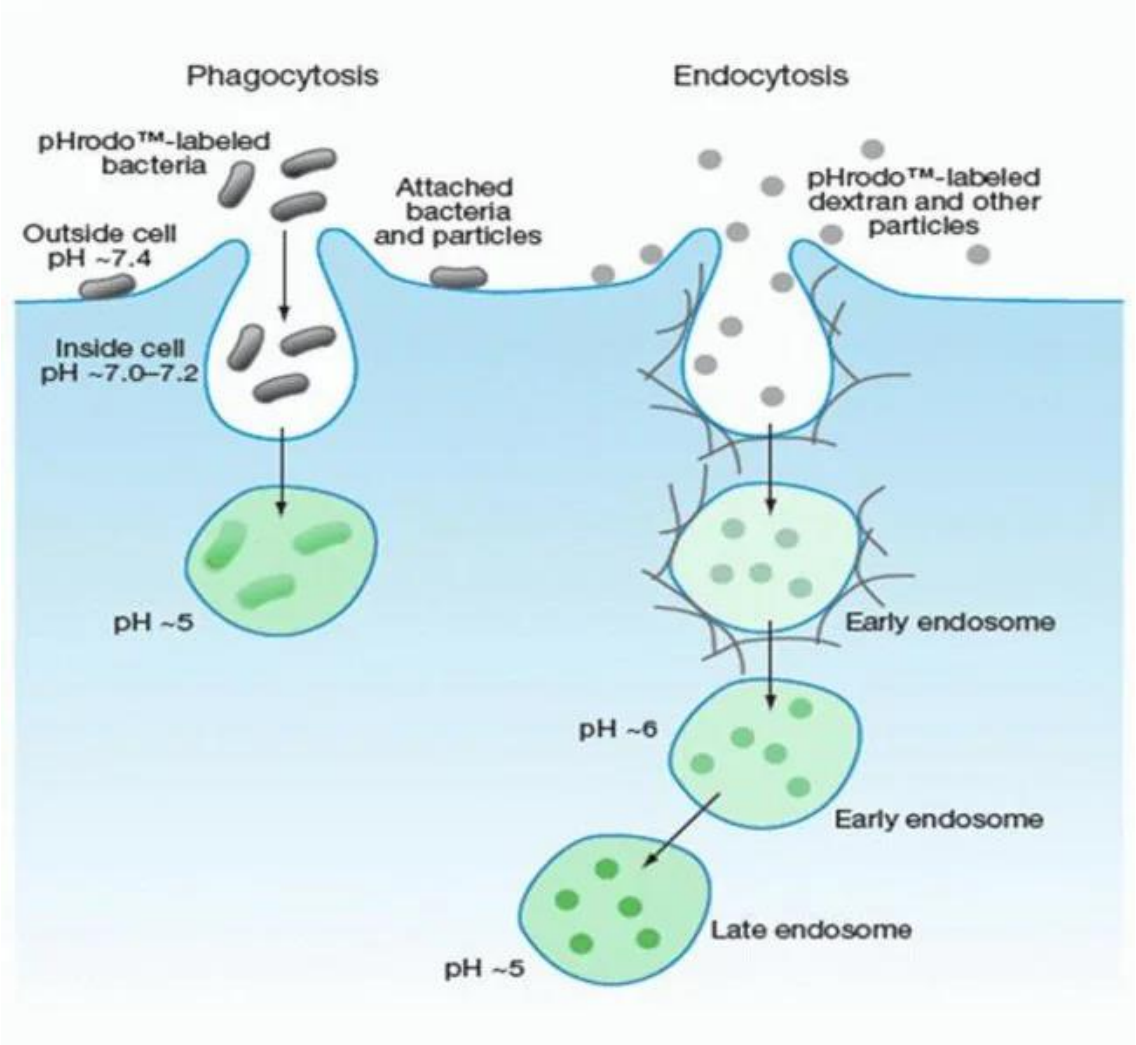

Figure 2.1 The process of phagocytosis using pHrodo Green *E. coli* conjugates.

During phagocytosis, monocytes phagocyte pHrodo Green-labeled *E. coli*, producing phagosome. As the phagosome grows, its internal pH lowers from 7.4 (extracellular) to 5 (intracellular), resulting in increased fluorescence, allowing for fluorescence detection of particle phagocytosed and intracellular processing (Adapted from Dolman et al., 2013).

Flow cytometry is an excellent method to analyse monocyte phagocytosis using pHrodo Green *E. coli* conjugates. Its high throughput capabilities enable quick examination of large cell populations, which produces statistically robust data and increased result reliability (Drescher et al., 2021). Furthermore, its single-cell resolution allows for the identification of individual monocytes with different phagocytic capabilities, providing insights into population heterogeneity (McKinnon, 2018).

A key advantage of flow cytometry is its ability to provide precise quantitative measurements. It measures the fluorescence intensity of pHrodo green-labeled *E. coli* within monocytes, enabling precise comparisons of phagocytic activity across different experimental settings, such as concentrations and time points (Ramesha et al., 2020). The approach also enables the simultaneous evaluation of multiple characteristics, such as cell size, granularity, and surface marker expression, therefore linking phagocytic activity to other cellular characteristics (McKinnon, 2018).

The pH-sensitive pHrodo green dye enhances phagocytosis detection by selectively fluorescing in acidic environments, ensuring that fluorescence corresponds to active phagocytosis (Rubey et al., 2022). Flow cytometry also allows for real-time analysis of living cells, allowing researchers to study phagocytosis kinetics and stimulus responses (Campbell et al., 2024). Furthermore, advanced statistical analysis of flow cytometry data allows for rigorous interpretation and detection of significant differences between experimental groups, making it an important tool for studying monocyte phagocytosis (McKinnon, 2018).

2.2.3 Roles of monocytes in OA

Monocytes play a complex function in the inflammatory processes associated with OA. Although they are necessary for immune defence, their participation in OA

frequently increases the damage to joints. Monocytes contribute to OA development by infiltrating the joint, differentiating into inflammatory macrophages and osteoclasts, and secreting pro-inflammatory cytokines (Wu et al., 2020).

During inflammation or joint injury, monocytes are migrate into the joint cavity through the synovial membrane (Haubruck et al., 2021). Within the joint membrane, monocytes differentiate into macrophages towards pro-inflammatory (M1) or anti-inflammatory (M2). M1 macrophages produce cytokines such as TNF-α, IL-1β, and IL-6, which cause cartilage degradation and bone deterioration. In contrast, M2 macrophages promote tissue repair and inflammation clearance. However, in OA, the balance changes towards the pro-inflammatory M1 phenotype, which accelerates the progression of the disease (Luo et al., 2024).

Monocytes can also differentiate into osteoclasts, which are specialised cells responsible for bone resorption. Excessive osteoclast activity causes bone loss and structural degradation, which are characteristic of OA (Guillem-Llobat et al., 2024). Monocytes and their derivatives promote joint inflammation by releasing cytokines such as IL-1, TNF-α, and IL-6 found that IL-1 promotes inflammatory mediators, TNF-α promotes cartilage and bone disintegration, and IL-6 leads to systemic inflammation (Van Den Eeckhout et al., 2021).

While monocytes are essential for immunological defence and tissue regeneration, their dysregulated activation and differentiation in OA emphasise their dual function as defenders and contributors to disease development. This duality emphasises their potential as therapeutic targets in OA to reduce inflammation and prevent joint deterioration (Haubruck et al., 2021).

2.3 Osteoarthritis (OA)

Osteoarthritis (OA) is a prevalent degenerative joint disease that causes cartilage breakdown, joint inflammation, and discomfort. It typically affects older persons. OA normally impacts other joints only if prior injury, extreme stress, or a cartilage disorder has occurred. Cartilage is an elastic substance that covers bone ends in healthy joints, deforms under pressure to minimise friction and absorb shocks. OA causes cartilage to stiffen and lose suppleness, making it more susceptible to harm. It may eventually wear away, lessening its shock-absorbing effectiveness. This degradation can cause tendons and ligaments to overstretch, resulting in discomfort and, in severe cases, bone-to-bone contact (Seed, 2024). Hence, its clinical symptoms include joint pain, stiffness, swelling, and restricted mobility, which can contribute to substantial disability and impaired quality of life (Collins et al., 2022).

Osteoarthritis is classified into two types: primary and secondary, each with a unique set of characteristics and underlying reasons. Primary OA is also known as 'idiopathic' OA, is an articular degeneration that has no apparent underlying cause. This is commonly thought of as degeneration due to age and wear and tear, which usually affects persons over 65 (Hsu & Siwiec, 2023). Secondary osteoarthritis develops when a pre-existing disorder causes cartilage degradation in a joint. It is frequently the consequence of joint damage or misalignment. These variables may include previous joint injuries (e.g., fractures or ligament rips), obesity, inflammatory illnesses (e.g., rheumatoid arthritis), metabolic disorders (e.g., gout or diabetes), congenital joint abnormalities, or conditions such as avascular necrosis. Secondary OA, unlike primary OA, can develop at a younger age and affect joints that are not usually implicated in primary OA (Sen & Hurley, 2023).

2.3.1 Pathophysiology of OA

OA is a degenerative joint disease characterised by the gradual breakdown of articular cartilage and surrounding tissues. This complex procedure involves a complex interaction of biochemical and biomechanical components (Coaccioli et al., 2022). Articular cartilage, predominantly made of type II collagen and proteoglycans, performs critical biomechanical roles in the joint (Wu et al., 2021). Type II collagen contributes to tensile strength and structural integrity, whereas proteoglycans hydrate the skin and resist compression (Wang et al., 2015).

Cartilage degradation in OA is caused by an imbalance of matrix production and degradation. Chondrocytes, the primary cell type within cartilage, become dysregulated, causing reduced synthesis of extracellular matrix (ECM) components such as type II collagen and aggrecan (Maldonado & Nam, 2013). Concurrently, the synthesis of matrix-degrading enzymes, such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), is dramatically increased (Mixon et al., 2021). This imbalance causes enhancing ECM loss, which compromises cartilage structural integrity and results in softening, fibrillation, and degradation. Chondrocyte apoptosis exacerbates this process since dead chondrocytes cannot maintain or repair the damaged ECM (Mixon et al., 2021).

As the cartilage deteriorates, the underlying subchondral bone undergoes extensive remodelling. Increased bone turnover causes sclerosis, which is characterised by bone stiffening and the production of osteophytes (Yunus et al., 2020). These bony outgrowths contribute to joint abnormalities and reduce mobility. The altered biomechanics caused by these alterations put additional stress on adjacent tissues such as

the synovium, tendons, and ligaments, potentially leading to serious damage (Yunus et al., 2020).

An important factor in the development of OA is inflammation, specifically the activation of the synovium. Synovitis is characterised by the invasion of immune cells such as macrophages and monocytes, which results in the generation of inflammatory mediators (Haouimi & Weerakkody, 2019). Proinflammatory cytokines, such as IL-1β and TNF-α, stimulate chondrocytes to create MMPs, further damaging cartilage and preventing the formation of protective matrix components. This results in a continuous process of tissue damage and inflammation (Liu et al., 2022). The inflammatory synovial membrane develops synovial hyperplasia and increased synovial fluid production, which contributes to joint swelling, pain, and restricted mobility. Changes in synovial fluid composition, such as higher levels of inflammatory cytokines and decreasing levels of lubricating compounds, worsen joint dysfunction (Ashraf et al., 2024).

In OA joint cavities, there are occurrence of synovial thickening, inflammation, activation of inflammatory M1 macrophages, articular cartilage erosion, release of inflammatory factors such as TNF-α, IL-1, IL-12, IL-6, and IL-16, increase in cartilage destruction factors, and destruction of cartilage formation factors as compared to normal joints (Figure 2.2). An imbalance in the homeostasis of the OA joint cavity microenvironment promotes OA development and progression (Yin et al., 2022).

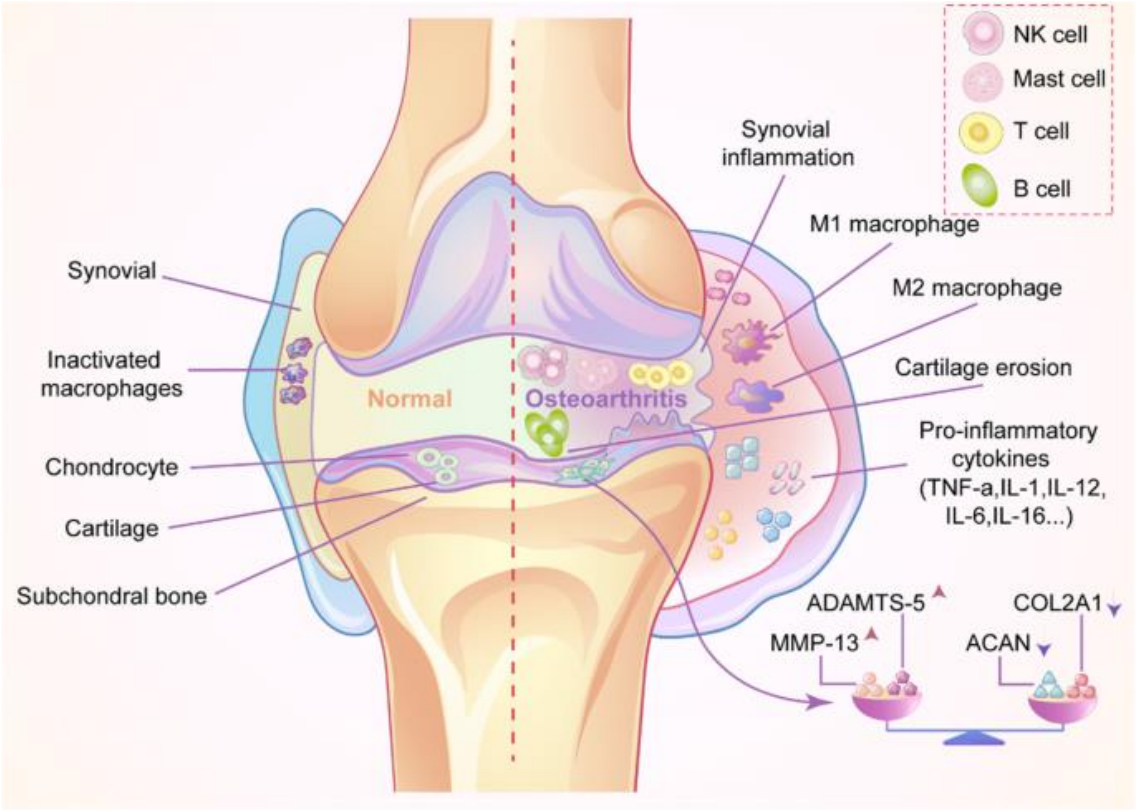

Figure 2-2 Comparison of the microenvironment of normal and OA joint cavities.

The synovium, the lining of the joint, thickens and becomes inflammatory, activating macrophages. Immune cells produce inflammatory chemicals such as TNF-α and IL-1, causing cartilage degradation. This damage, in turn, causes the production of more inflammatory chemicals, resulting in a vicious cycle. Concurrently, immune cells such as B cells, T cells, mast cells, and NK cells penetrate the joint cavity, contributing to the overall inflammatory response. The cumulative effect of these processes disrupts the delicate balance inside the joint, resulting in cartilage degradation and the advancement of OA (Adapted from Yin et al., 2022).

2.3.2 Treatments of OA

In OA, many treatment options are available to help patients manage symptoms, improve joint function, and improve their quality of life. Treatment options include non-pharmacological, pharmacological, and surgical procedures, all of which aim to relieve pain and improve general well-being (Larson, 2021).

Surgical interventions are considered when conservative management strategies fail or when OA significantly restricts mobility and daily function. Arthroscopy, a minimally invasive procedure, allows for joint assessment and removal of loose cartilage fragments that can cause pain or restrict movement (Kalashnikov et al., 2022). Osteotomy, a surgical method that involves realigning bones to redistribute weight away from the afflicted joint region, is especially useful for knee OA and may prevent the need for more invasive operations (Osadchuk et al., 2023).

In severe OA cases when other therapies have failed, joint replacement surgery, such as complete knee or hip arthroplasty, is often recommended. This major surgical treatment replaces damaged joint surfaces with artificial components, restoring function, relieving pain, and significantly enhancing patient quality of life (Usiskin, 2023). While invasive, surgical procedures are highly effective in treating severe OA symptoms and restoring mobility (Usiskin, 2023).

Non-pharmacological approaches to OA care are vital, focusing on patient education, exercise, and weight control. Patient education offers individuals self-management skills such as ergonomics and activity pacing. Visual aids are frequently used in conjunction with structured programs to improve comprehension and physical function. Exercise therapy, which involves strength, aerobic, and range-of-motion exercises, is important. Regular low-impact exercises, such as walking or swimming, increase muscular strength, and flexibility, and reduce stiffness (Geneen et al., 2017).

Pharmacological treatments primarily comprise analgesics and nonsteroidal anti-inflammatory drugs. Acetaminophen is a common analgesic that relieves pain but not inflammation (Richard et al., 2022). NSAIDs, like ibuprofen and naproxen, are more effective in treating pain and inflammation. Topical NSAIDs provide localised relief with fewer adverse effects. While, selective COX-2 inhibitors, such as celecoxib, treat pain

and inflammation while reducing the likelihood of gastrointestinal adverse effects (Richard et al., 2022).

Many molecules within OA synovial joints, including those carried by extracellular vesicles (EVs), contribute to the inflammatory cascade and cartilage degradation. Previous research has shown that OA patients' synovial fluid contains significantly more EVs, particularly those derived from inflamed synovial cells, than healthy individuals (Swami et al., 2024).

2.4 Extracellular vesicles

Extracellular vesicles (EVs) are nanosized, membrane-bound vesicles released by cells into the extracellular space. These vesicles are carriers for various biological substances, including proteins, lipids, and nucleic acids. EVs play an important role in intercellular communication, allowing cells to exchange information and impact the behaviour of nearby cells or distant tissues (Petroni et al., 2023). EVs are typically classified based on their size and biogenesis which are apoptotic bodies, microvesicles (MVs), and exosomes.

The apoptotic bodies are large vesicles (500-2000 nm in diameter) produced during programmed cell death or apoptosis. They consist of cellular organelles and nuclear fragments and are involved in the clearance of apoptotic cells (Fang et al., 2022).

MVs are smaller vesicles (100–1000 nm in diameter) that emerge from the plasma membrane. MVs have cytoplasmic components such as proteins, lipids, and mRNA. They contribute to various biological functions, including cell signalling, coagulation, and inflammation (Ståhl et al., 2017).

Exosomes are tiny vesicles (30-150 nm in diameter) derived from the endosomal pathway (Figure 2.2). They are produced within multivesicular bodies (MVBs) and released into the extracellular space when MVBs fuse with the plasma membrane (Liu, Wu, et al., 2022). Exosomes are rich proteins, lipids, and nucleic acids, and they serve critical roles in a variety of biological processes such as immune response, cell proliferation, and differentiation (Kalluri & LeBleu, 2020).

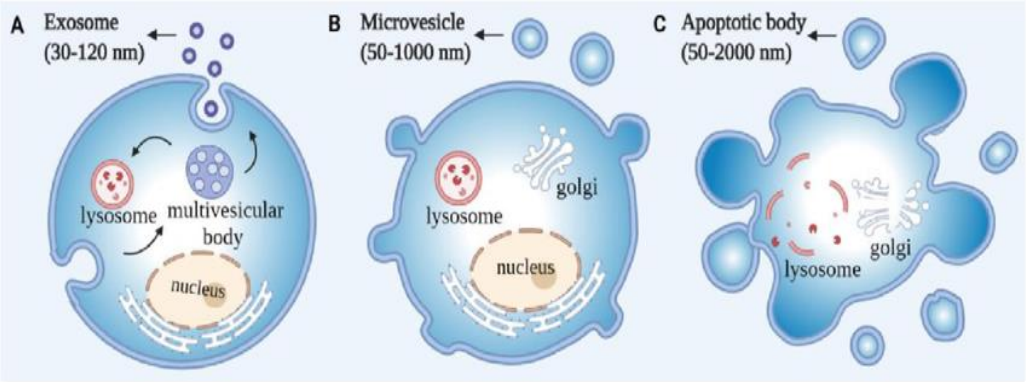

Figure 2.2 The biogenesis and release mechanisms of extracellular vesicles (EVs).

The figure highlights three main types. Exosomes, ranging from 30–120 nm in size, are formed within multivesicular bodies (MVBs) as intraluminal vesicles (ILVs) and are released when MVBs fuse with the plasma membrane. Microvesicles, which are 50–1,000 nm in size, are generated through the direct outward budding and fission of the plasma membrane. Apoptotic bodies, measuring 50–2,000 nm, are produced during apoptosis as membrane-bound fragments containing cellular organelles and components. Each type of EV plays a distinct role in intercellular communication and physiological processes (Adapted from Liu, Wu, et al., 2022).

2.4.1 Biogenesis of exosomes

Exosomes are nano-sized vesicles released by cells that play crucial roles in intercellular communication. Their biogenesis begins within the endosomal pathway, where early endosomes mature into late endosomes, also known as multivesicular bodies (MVBs) (Figure 2.3) (Kaur & Lakkaraju, 2018). During this process, the endosomal

membrane invaginates, forming intraluminal vesicles (ILVs) within the MVB (Fan et al., 2022).

Following the formation, MVBs can either fuse with lysosomes for degradation or undergo exocytosis. In exocytosis, MVBs migrate to the plasma membrane, fuse with it, and release the ILVs as exosomes into the extracellular space (Hessvik & Llorente, 2017). The process of release is tightly controlled, involving cytoskeletal dynamics and specific molecular cues that promote membrane fusion. The precise regulation of exosome release highlights their importance in intercellular communication and their potential roles in various physiological and pathological processes (Fan et al., 2022).

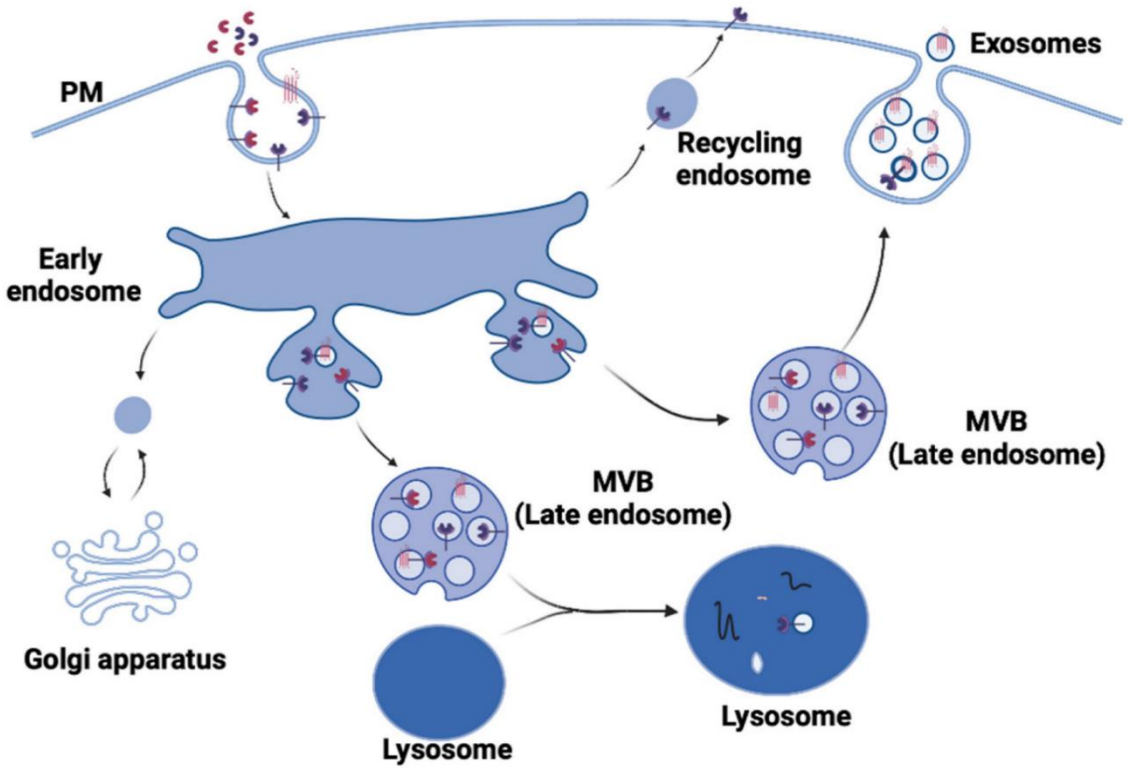

Figure 2.3 The biogenesis of exosomes through the endosomal pathway

Exosome formation begins with the internalization of plasma membrane (PM) components, leading to the generation of early endosomes. These early endosomes can recycle back to the membrane or mature into late endosomes, also known as multivesicular bodies (MVBs). During this process, the endosomal membrane

invaginates, forming intraluminal vesicles (ILVs) within the MVBs. MVBs have two possible fates: they either fuse with lysosomes for degradation or merge with the plasma membrane to release ILVs into the extracellular space as exosomes. This process highlights the intricate mechanisms of intracellular vesicle trafficking and exosome release (Adapted from Yang et al., 2024)

CHAPTER 3

METHODOLOGY

3.1 Overview of the study

The flowchart for this research is presented below (Figure 3.1). Briefly, the main purpose of this study is to assess the monocyte phagocytosis in the presence of synovial-fluid-derived exosome from OA patients. Primary knee OA patients were recruited by collecting their synovial fluid (SF) during total knee arthroplasty (TKA). The SF was stored at -80°C until it was needed. To isolate exosomes, frozen SF was treated enzymatically before being separated using two-step ultracentrifugation. The isolated exosomes were stored at -80°C until being used.

On the other hand, blood samples from healthy individuals were obtained. A phlebotomist collected approximately 30 mL blood from each donor. Blood was immediately processed to isolate peripheral blood mononuclear cells (PBMC) by density gradient centrifugation. Then, human monocytes were further isolated from PBMC using Mojosort Human $CD14^+$ Selection Kit.

The isolated monocytes were stained using PE mouse anti-human anti-CD14 antibody before being characterized by flow cytometry. Isolated $CD14^+$ monocytes were cultured in the presence of exosomes in various ratios which were 1:10, 1:20, and 1:40 for 24 and 48 hours. After 24 and 48 hours, pHrodo green *E. coli* conjugate was added to the cultured cells to determine the phagocytosis of monocytes followed by flow cytometry analysis. Flow cytometry data were analysed using FCS Express version 5.0 and statistical analysis was carried out using GraphPad Prism software version 9.0.

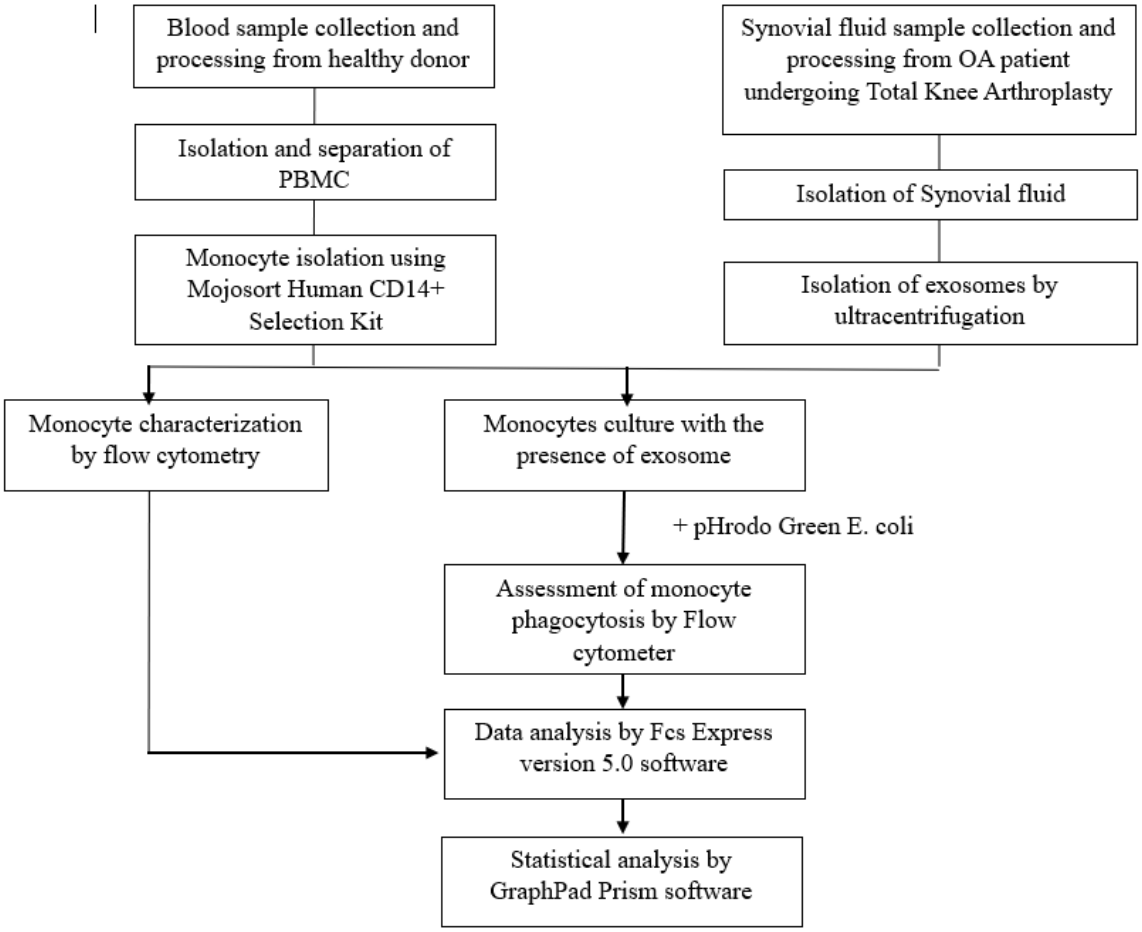

Figure 3.1 Flowchart of the experiments in this study

3.2 Chemicals, reagents, kits and consumables

The chemicals, reagents, kits, and consumables used in this study are listed in Table3.1, 3.2, and 3.3, accordingly.

Table 3.1 List of chemicals, reagents and antibodies

Chemicals and reagents	**Manufacturer**
0.4% Trypan blue	Sigma-Aldrich, USA
100X PSG (Penicillin-Streptomycin-Glutamine)	Thermo Fisher Scientific, USA
10X Dulbecco's Phosphate Buffered Saline (DPBS)	Mediatech, USA
20 U/ml Recombinant DNase 1	Roche Diagnostics, Germany
Absolute ethanol	HmbG, Germany
Accutase Cell Detachment Solution	Capricon Scientific, Germany
5X Mojosort Buffer	BioLegend, USA
Bleach	Clorox, USA
Ethylenediamine tetraacetic acid (EDTA)	Sigma-Aldrich, USA
Hyaluronidase	Sigma-Aldrich, USA
Lymphocyte Separation Medium (LSM)	Corning USA
Phosphate buffered saline (PBS) Tablets	Thermo Fisher Scientific, UK
RPMI 1640 w/o L-glutamine	Sigma-Aldrich, USA
Fetal Bovine Serum (FBS)	Sigma-Aldrich, USA
APC Mouse Anti-human CD3	BD Biosciences, USA
PE Mouse Anti-human CD14	BD Biosciences, USA
pHrodo^{TM} Green BioParticles^{TM} conjugate	Thermo Scientific, USA

Biolegend MojoSort Human $CD14^+$ Monocytes Selection Kit	BioLegend, USA
BD® CompBeads Compensation Particles Set	BD Biosciences, USA

Table 3.2 List of consumables

Consumables	**Manufacturer**
21-G butterfly needle	BD Biosciences, USA
Alcohol swabs	Hospitech, Malaysia
6-well Cell culture plate	SPL Lifesciences, KR (SPL#30006)
Centrifuge tube (15 & 50 mL)	Fisher Scientific, Singapore
10 mL vacutainer EDTA tubes	BD Biosciences, USA
10 mL tips	Eppendorf, USA
Falcon tube (50 mL)	Biologix, USA
Gloves	IronSkin, Malaysia
Microcentrifuge tube (1.5 mL)	Eppendorf, USA
Parafilm	Thomas Scientific, USA
Pasteur pipettes	PorLab Scientific, China
Pipette tips (10 µL, 200 µL,1000 µL)	Axygen Scientific, USA
Sterile filter (0.22 µM)	TPP, Germany
Syringe (10 mL)	Terumo, Japan
Ultracentrifuge tube (4mL)	Thermo Scientific, USA

3.3 List of laboratory equipment and apparatus

All laboratory equipment, apparatus and software used in this study are listed in Table 3.3 and 3.4 respectively.

Table 3.3 List of laboratory equipment and apparatus

Laboratory equipment and apparatus	**Manufacturer**
Autoclave steriliser	Amerex Instruments, USA
pipette 10 mL	JoanLab, China
Biological safety cabinet Class II	Esco Micro, Malaysia
CO_2 incubator	Esco Micro, Malaysia
Deep freezer -80°C	ILSHIN BioBase, South Korea
Fusion Fx7-826 Molecular Imager	Vilber Lourmat, Germany
Glass reagent bottle with cap (500 mL & 1000 mL)	Schott Duran, Germany
Hemocytometer	Thermo Fisher Scientific, USA
Laboratory freezer -20°C	Toshiba, Japan
Light microscope	Leica Microsystems GmbH, Germany
Pipettes (10 µL, 200 µL, 1000 µL)	Sartorius, USA
Rocking platform shaker	Heidolph, Germany
Sorvall WX 100+ Ultracentrifuge	Thermo Fisher Scientific, USA
Universal 320 & Micro 22R centrifuge	Hettich Zentrifugen, Germany
Vortex mixer	ERLA Technologies, Malaysia
Water bath	Memmert, Germany