GOLD NANOPARTICLE-INDUCED CHONDROGENIC DIFFERENTIATION OF HUMAN UMBILICAL CORD BLOOD MESENCHYMAL STEM CELLS (hUCB-MSC) FOR OSTEOARTHRITIS THERAPY

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CONTENTS

LIST OF TABLE

LIST OF FIGURES

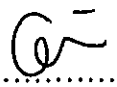
LIST OF ABBREVIATIONS AND SYMBOLS

ACI	Autologous Chondrocyte Implantation
AD-MSCs	Adipose-Tissue-Derived Mesenchymal Stem Cells
AMIC	Autologous Matrix-Induced Chondrogenesis
ANOVA	Analysis of Variance
ASCs	Adipose-Derived Stem Cells
BM-MSCs	Bone Marrow-Derived Mesenchymal Stem Cells
BMP	Bone Morphogenetic Protein
BMP-2	Bone Morphogenetic Protein 2
BMP-7	Bone Morphogenetic Protein 7
BSE	Backscattered Electron
COOH	Carboxyl Group
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FGF-2	Fibroblast Growth Factor 2
hUCB-MSCs	Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells
IGF	Insulin-like Growth Factor
MACI	Matrix-Induced Autologous Chondrocyte Implantation
MSC	Mesenchymal Stem Cell
nM	Nanomolar
nm	Nanometer

NP	Nanoparticle
OA	Osteoarthritis
OH	Hydroxyl Group
PDGF	Platelet-Derived Growth Factor
PEG	Polyethylene Glycol
PLL	Poly(L-lysine)
PVP	Poly(vinylpyrrolidone)
ROS	Reactive Oxygen Species
RT-PCR	Real Time Polymerase Chain Reaction
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
TGF-β	Transforming Growth Factor-beta
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor

DECLARATION

I hereby declare that this thesis is based on my original work except for quotations and citations which have duly acknowledged.



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PENGGUNAAN NANOPARTIKEL EMAS UNTUK PEMBEZAAN KONDROGENIK SEL STEM MESENKIMAL DARI DARAH TALI PUSAT MANUSIA (hUCB-MSC) UNTUK TERAPI OSTEOARTRITIS

ABSTRAK

Kepentingan untuk mendapatkan penyelesaian baru dalam terapi osteoartritis boleh dilihat pada impak negatif globalnya terhadap berjuta-juta pesakit, peruntukkan dalam bidang kesihatan, dan aktiviti harian, di samping kesukaran penyembuhan kartilaj dan had penggunaan sel stem mesenkimal dari sumsum tulang berikutan usia, Kajian ini menekankan potensi penting penggunaan Sel Stem Mesenkimal dari Darah Tali Pusat Manusia (hUCB-MSC) dalam meregenerasi tisu kartilaj yang rosak, terutamanya dalam konteks osteoartritis. Objektif penyelidikan merangkumi pencirian morfologi permukaan nanopartikel emas (AuNPs) terikat menggunakan Mikroskopi Elektron Imbasan, analisis kebolehhidupan dan penjalaran hUCB-MSC selepas rawatan dengan pelbagai jenis AuNPs yang telah difungsikan menggunakan ujian pengecualian Trypan Blue dan RT-PCR, serta penyiasatan perbezaan hUCB-MSC yang telah menerima rawatan AuNP menggunakan pewarnaan imunofluoresen dan ujian pengenalan fungsi MSC. Penyelidikan ini mengkaji peranan AuNPs hidrofobik dan hidrofilik, dan pengaruh internalisasi AuNPs yang mungkin memberi kesan kepada hUCB-MSC, dan kesan jarak AuNP terhadap adhesi fokal dan perbezaan sel. Pencirian nanopartikel emas terikat dapat dicapai dengan menggunakan teknik mikroskopi elektron imbasan berpasangan dengan analisis perisian perisian ImageJ. Oleh kerana kultur sel yang tidak berjaya, objektif penyelidikan lanjut tidak dapat dilaksanakan.

GOLD NANOPARTICLE-INDUCED CHONDROGENIC DIFFERENTIATION OF HUMAN UMBILICAL CORD BLOOD MESENCHYMAL STEM CELLS (hUCB-MSC) FOR OSTEOARTHRITIS THERAPY

ABSTRACT

The pressing need for new solutions in osteoarthritis therapy is highlighted by its global impact on millions, healthcare resources, and daily activities, alongside the difficulty of cartilage healing and limitations of age-related bone marrow derived MSCs. This research highlights the significant potential of using human Umbilical Cord Blood Mesenchymal Stem Cells (hUCB-MSCs in regenerating damaged skeletal tissues, particularly in the context of osteoarthritis. The research objectives encompass the characterization of the surface morphology of immobilized AuNPs using Scanning Electron Microscopy, analysis of hUCB-MSC viability and proliferation following treatment with various types of functionalised AuNPs using Trypan Blue exclusion assay and RT-PCR and the investigation of hUCB-MSC differentiation in the presence of AuNP treatments using immunofluorescence staining and MSC functional identification assay. The research delves into the role of hydrophobic and hydrophilic AuNPs, and influence of AuNP internalisation which may impact hUCB-MSC, and effects of AuNP spacing towards focal adhesion and cell differentiation. Characterisation of immobilised gold nanoparticles was able to be achieved by using scanning electron microscopy technique paired with ImageJ software analysis. Due to unsuccessful attempts of cell culture, further research objectives were not able to be performed.

CHAPTER 1
INTRODUCTION

1.1 Research Background

1.1.1 Skeletal Disorder

Skeletal disorders are a significant cause of disability worldwide, impacting millions of people. Using stem cells to repair tissue has shown great promise in across medical fields, including skeletal disorders. Mesenchymal stem cells (MSCs) are multipotent stromal cells initiated from the mesoderm and the neural crest. Their unique properties make them highly attractive for regenerating damaged skeletal tissues. These cells have the potential to aid in the repair and regeneration of skeletal tissues through various mechanisms, such as homing to the injury site, promoting new blood vessel formation, differentiating into specific cell types, and responding to inflammatory conditions. In an *in vitro* setting, these cells can undergo differentiation into osteoblasts, chondrocytes, adipocytes, and myocytes. MSCs sourced from different origins exhibit a wide range of capabilities when it comes to secreting various cytokines, growth factors, and chemokines. This diversity in their secretory profiles leads to variations in the therapeutic effects of MSCs from different sources in terms of repairing and regenerating injured skeletal tissues. Considering the prevalence of skeletal diseases in the aging population, these conditions are major contributors to disability and morbidity. The skeletal system is susceptible to several common disorders, including intervertebral disc issues (IVDs), osteoporosis, bone fractures, osteogenesis imperfecta (OI), osteoarthritis (OA), and rheumatoid arthritis (RA). To address these conditions, stem cell therapy has emerged as a promising treatment option. Stem cells are utilized to repair, replace, and manage defects in tissues, either alone or in conjunction with external gene modifications. These stem cells can be obtained from a person's own body (autologous) or from a donor (allogeneic). They can be employed as either naïve or primed lineages (Kangari et al., 2020).

1.1.2 Osteoarthritis

Once considered a condition resulting from gradual erosion of cartilage, osteoarthritis (OA) is now identified as a complex disorder with diverse pathophysiological effects on multiple joints and joint structures. The definition of OA by the Osteoarthritis Research Society International reflects this understanding: it starts as an abnormality at the molecular level, affecting joint tissue metabolism, and subsequently progresses to anatomical and physiological changes characterized by degradation of cartilage, bone remodelling, osteophyte formation, joint inflammation, and loss of normal joint function. This disease can lead to significant disability and is estimated to affect around 240 million people worldwide, limiting their daily activities. The knee and hip joints are commonly affected by OA, with approximately 30% of individuals over 45 years old showing radiographic evidence of knee OA, and about half of them experiencing knee-related symptoms. Similarly, symptomatic radiographic hip OA is having 10% of the prevalence of (Katz et al., 2021).

Furthermore, OA imposes a significant burden on healthcare resources and finances. Research has indicated that OA is linked to a higher risk of hospitalization and increased emergency department expenses for individuals seeking treatment for other conditions in the emergency room. The direct cost of managing OA in Canada has risen substantially from $577 to $811 per patient per year between 2003 and 2010, primarily due to the expenses associated with joint replacement surgeries (Vina & Kwoh, 2018).

1.1.3 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) have garnered significant attention due to their easy isolation from various sources, making them ethically unproblematic. Unlike embryonic stem cells (ESCs), which have faced ethical controversies due to concerns about human embryo violation, MSCs do not raise such issues. Moreover, MSCs present a limited risk of uncontrollable growth. Even after cryopreservation at 80° C, MSCs retain their regeneration capacity. They exhibit rapid proliferation and have the capability to differentiate into multiple cell types. Additionally, MSCs

demonstrate minimal or absent immunoreactivity, making them less likely to provoke adverse reactions in hosts. Finally, MSCs have "homing" capabilities, allowing them to move towards the location of the injury. This cellular behaviour is facilitated by a variety of inflammatory or chemotactic substances; for example, vascular endothelial growth factor and hepatocyte growth factor, both of which are secreted at the site of damage, may actually attract MSCs (Cofano et al., 2019). The quantity, capacity for transformation, and extended viability of autologous MSCs derived from human bone marrow undergo a notable reduction as the donor age further. Moreover, the retrieval of bone marrow involves an exceedingly invasive process (Wang et al., 2009).

Human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) have shown great potential as a source for repairing articular cartilage. Healing cartilage defects is a significant challenge in orthopedics, as mature cartilage has limited regenerative capacity. Therefore, developing new tissue engineering strategies is crucial for cartilage repair. hUCB-MSCs, in particular, offer promise for in vivo cartilage repair due to their non-invasive collection, high proliferation rate, low immunogenicity, and ability to differentiate into chondrocytes *in vitro*. However, using hUCB-MSCs alone for cartilage regeneration faces challenges as they have a low induction efficiency for chondrogenesis in the absence of growth factors or gene delivery systems. To optimize their therapeutic application, an approach is needed that combines these abundant stem cells with prochondrogenic signals and cell adhesions to promote successful cartilage repair (X. Li et al., 2016).

1.1.4 Cell Surface

The cell surface is an amazing, complex structure with distinct physical characteristics. In order to swiftly change their shape and carry out specialised tasks including cell motility, polarity control, cell retraction, and attachment to the extracellular environment, cells may rapidly adjust the mechanical properties and forces at their outer edges. Recent research has also shown that cell identification may be impacted by surface dynamics. The plasma membrane's 2D structure residing inside a 3D area is a crucial factor influencing changes in membrane tension propagation in many studies. Membrane deformations, also known as membrane curvature, have been

thoroughly studied in in vitro experiments, notably in the context of numerous membrane-binding and -remodelling proteins. In these condensed *in vitro* systems, the link between tension and curvature has been well known. Notably, interactions with actin and curvature-sensing binding partners allow some membrane curvature-associated (MCA) proteins, such as Ezrin, to alter their capacity to tether membranes. Therefore, it is feasible that changes in the curvature landscape within an in vitro system may affect the distribution and binding of obstacles, which in turn would affect how membrane tension spreads. However, when it comes to *in vitro* study, membrane tension and curvature relationship is far more intricate than simplified experiments (Sitarska & Diz-Muñoz, 2020).

A critical step called differentiation allows stem and progenitor cells to express certain cellular machinery, enabling them to carry out specialised tasks and give rise to different cell types. To support the various forms and activities of differentiated cell types, such as secretory cells, neurons, immune cells, and epithelial cells with apicobasal polarity, this process also entails modifications in membrane trafficking and cell surface dynamics. For these specialised cells to operate properly, particular membrane trafficking mechanisms and surface characteristics are needed. It's interesting to note that current research suggests membrane transport and cell surface dynamics also influence how cells acquire their fates and differentiate during development (J. H. Li et al., 2023).

1.1.5 Gold Nanoparticles

Due to their distinctive physicochemical characteristics, gold nanoparticles are the second most favoured form of nanoparticles and are thus quite popular. Spherical gold nanoparticles are the most frequent among a variety of forms for gold nanoparticles. Capping and stabilising compounds are essential for lowering their toxicity, increasing their bioavailability and biocompatibility inside of live cells, and enabling practical uses (Mikhailova, 2021).

1.1.6 Problem Statement

The pressing need for new solutions in osteoarthritis therapy is highlighted by its global impact on millions, healthcare resources, and daily activities, alongside the difficulty of cartilage healing and limitations of age-related bone marrow derived MSCs, emphasizing the significance of innovative approaches. This research studied the hUCB-MSC differentiation after treatment with functionalised gold nanoparticles in two forms; immobilised and in suspension to understand the rate of proliferation and type of cell differentiation induced in order to improve current osteoarthritis treatment involving the transplantation of stem cell and the use of biomaterials with different wettability that regulate cartilage-and-bone regeneration.

1.2 Research Objectives

1.2.1 General Objectives

1. To characterize the surface morphology of immobilised Gold Nanoparticles on glass coverslips.
2. To analyse hUCB-MSC viability and proliferation after treatment with immobilised and suspension of Gold Nanoparticle.
3. To analyse hUCB-MSC differentiation after treatment with immobilised and suspension of Gold Nanoparticle.

1.2.2 Specific Objectives

1. To characterize the surface morphology of immobilised Gold Nanoparticles on glass coverslips at concentrations of 0.1 nM, 0.5 nM and 1.0 nM using Scanning Electron Microscopy.
2. To analyse hUCB-MSC viability and proliferation after treatment with immobilised and suspension of Gold Nanoparticle in Citrate, Gold Nanoparticle functionalised with carboxyl-terminated PEG and Gold Nanoparticle functionalised with methyl-terminated PEG at concentration of 0.1 nM, 0.5 nM and 1.0 nM using Trypan Blue and RT-PCR assay.
3. To analyse hUCB-MSC differentiation after treatment with immobilised and suspension of Gold Nanoparticle in Citrate, Gold Nanoparticle functionalised with carboxyl-terminated PEG and Gold Nanoparticle functionalised with methyl-terminated PEG at concentration of 0.1 nM, 0.5 nM and 1.0 nM using Immunofluorescence staining and MSC Functional Identification assay.

CHAPTER 2
LITERATURE REVIEW

2.1 Osteoarthritis

2.1.1 Pathogenesis of Osteoarthritis

Previously, osteoarthritis was attributed to mechanical damage or the repetitive use of a joint, commonly associated with the natural aging process. However, it is now evident that osteoarthritis is way more intricate than a simple wear-and-tear condition. Several elements such as inflammation, metabolism, and biochemical processes are recognized as significant contributors to its development. Additionally, factors like aging, obesity, joint injuries, and engaging in high-impact activities have been identified as risk factors that contribute to the onset of osteoarthritis. Consequently, osteoarthritis is presently understood as a complex, multifactorial disease involving both local and systemic factors with various underlying mechanisms. Hence, when exploring new treatment approaches for osteoarthritis, it is imperative to consider these various contributing factors (Z. Li et al., 2021).

Degenerative knee joint disease is a prevalent medical condition that ranges from isolated defects in the articular cartilage to advanced osteoarthritis (OA). The presence of articular cartilage abrasions can escalate the risk and advancement towards end-stage OA, which affects 5 to 30% of the general adult population. Treating these conditions from a biological perspective poses challenges, as chondrocytes (cartilage cells) have limited inherent regenerative abilities, especially as age advances. Nevertheless, the field of regenerative medicine has made significant strides in recent years, offering promising approaches for treating degenerative cartilage diseases. One such advancement is stem cell therapy, which has shown huge promise in both preclinical and clinical studies as a readily accessible treatment option (Arshi et al., 2020).

In the diarthrodial joints, articular cartilage plays the important role in aiding lubrication by providing a smooth, lubricated surface and conveying loads, distributing them across the impacted surface. Articular cartilage lack of blood vessels, lymphatics and nerves thus causing it to have

limited capability to heal or repair itself intrinsically. Hence maintaining articular cartilage is detrimental to joint health. Chondrocyte is a fixed cell on articular cartilage, which differs in shape, number and sizes depending on the region of articular cartilage its residing on. Chondrocyte do not have extensive replication potential, a factor that further inhibit healing or the articular cartilage (Sophia Fox et al., 2009). In endochondral ossification, the growth plate is divided into three regions: resting, proliferating and hypertrophic zone. Antiangiogenic factors and extracellular matrix (mainly collagen type II, Hyaluronan (HA) and proteoglycans) are produced by the chondrocytes in resting zone. In proliferating zone, chondrocytes proliferation occurs while they form columns. Lastly, in hypertrophic zone, chondrocytes are influenced by factors like growth factor-1 (IGF-1), Wnt/β-catenin, runt-related transcription factor 2 (RUNX2), COL10, MMP13, transforming growth factor-beta (TGF-β) family members, bone morphogenetic protein (BMP), and Indian hedgehog (IHH) to undergo hypertrophy. Hyaluronan main receptor is the CD44 surface antigen and interaction between the two contributes to cartilage homeostasis (Park et al., 2021, Wu et al., 2013).

Osteoarthritis most notable feature is the progressive damage to the articular cartilage layer that is made up by organic extracellular matrix components such as type II collagen, aggrecans and other proteoglycans. Metalloproteinases (MMPs), including MMP-1, MMP-13, MMP-3 and MMP activator, along with A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) families are the enzymes that degrade the cartilage matrix (Jang et al., 2021). Major biochemical hallmark of the disease would be the proteoglycan and collagen degradation, compromising articular cartilage functions in absorbing impact and tension at the joint which eventually causes articular chondrocytes to undergo apoptosis and dissipates further (Hartmann et al., 2020, Xia et al., 2014). Diagnosis of the disease is usually done by plain radiography that illustrates degradation of smooth cartilage at the end of long bones, the articular cartilage (Abramoff & Caldera, 2020).

2.1.2 Current Treatment Availability and Limitations

The main underlying process of osteoarthritis (OA) includes significant degeneration of the articular cartilage structures, causing extreme pain and inhibit mobility. Unfortunately, current treatment options for OA are primarily focused on managing symptoms rather than providing a cure. These approaches range from non-surgical measures to surgical interventions.

Both non-pharmacological and pharmacological techniques fall within the category of non-surgical therapies. Awareness, self-management, physical activity, and weight loss are the primary non-pharmacological therapy for osteoarthritis patients. Cane use and biomechanical modifications like brackets and orthotics are further non-pharmacological approaches for treating knee osteoarthritis. The use of acetaminophen, topical or oral non-steroidal anti-inflammatory medications (NSAIDs), or intra-articular corticosteroids are all examples of pharmaceutical treatment. For patients with advanced knee osteoarthritis, surgery is a last resort. Total knee arthroplasty (TKA), which is the most effective surgical procedure, is followed by rehabilitation to speed up recovery. Despite these therapies, each strategy continues to have difficulties and restrictions (Kan et al., 2019; Loo & Wong, 2021).

Surgical interventions are additional treatment options that go beyond pharmacological and non-pharmacological measures and are typically reserved for patients who do not respond to other forms of treatment. These surgical interventions can be more costly and are often limited to cases where other treatments have not provided sufficient relief. As the elderly population has increased, there has been a growing demand for surgical procedures like joint arthroplasty to address OA, leading to an increase in associated costs (Kamaruzaman et al., 2017).

The original idea behind cell therapy for cartilage regeneration was to employ implanted cells to restore damaged articular cartilage, potentially dispensing with osteoarthritis (OA) medications and surgical procedures. The first cells for this purpose to be considered were autologous chondrocytes. Researchers have since looked into other therapeutic cell sources, especially mesenchymal stem cells (MSCs), due to drawbacks in their efficiency, such as the chondrocyte

reduction features, and increased morbidity linked with collecting them. Early experimental research on the regeneration of cartilage using stem cells centred on stimulating chondrogenic differentiation in the stem cells. The ideal mix of growth factors or gene transfers to encourage stem cells chondrogenesis was the subject of several investigations. In addition, several studies investigated the use of biomechanical stimuli to improve the induction of chondrogenesis from stem cells (Im & Kim, 2020).

In the early stages of mesenchymal stem cells (MSCs) chondrogenesis, the appearance of hypertrophic markers like type 10 collagen and Runx-2 distinguished them from articular chondrocytes, which do not express these markers. This led researchers to focus on finding ways to suppress MSC chondrogenesis hypertrophy. However, these attempts would be pointless and result in no healing if the cells injected into the joint could not last long enough to develop into articular chondrocytes. Recently, cell-based therapies, particularly using mesenchymal stem cells (MSCs), have been utilized to alleviate and slow the progression of degenerative osteoarthritis (OA). MSCs have demonstrated superior regenerative potential for damaged cartilage and have shown clinically significant pain relief. The therapeutic effects of MSCs are attributed to their ability to differentiate into chondrocytes and optimize the intra-articular environment. Strategies to enhance chondrogenic differentiation involve the use of various growth factors, chemical materials, and scaffolds. Additionally, MSCs' paracrine effects, without direct contact, have shown promising potential in promoting cartilage regeneration. It has been shown in several meta-analyses of randomised controlled studies that MSC treatment is helpful in easing pain and enhancing clinical symptoms of OA. Nevertheless, despite the therapeutic potential of MSCs, the therapy approaches used in their present clinical applications are inconsistent and heterogeneous. Various approaches, including cell source selection, preparation, delivery methods, lesion site preparation, and concomitant treatments, have been attempted, leading to a lack of standardized criteria for clinical processes and difficulty in integrating and comparing research results (J. S. Lee et al., 2021).

There has been a lot of study done on how illness and aging affect stem cell capabilities in relation to cartilage regeneration. Adult stem cells, such as mesenchymal stem cells (MSCs) derived from bone marrow, adipose-derived stem cells (ASCs), and synovium-derived stem cells (SDSCs), have demonstrated the potential to differentiate into cartilage when exposed to chondro-inductive substances like transforming growth factor-beta (TGF-) and bone morphogenetic proteins (BMPs). The stem cells that are usually used in this research are derived from young, healthy donors, which could not accurately reflect the features of proliferation and differentiation of stem cells from OA patients who require autologous stem cell therapy. MSCs from OA patients may be isolated, grown, and differentiated towards the chondrocyte lineage, according to recent research. However, patient variables other than OA, including age and obesity, may impair stem cell quality. Age has been found to impact stem cell characteristics, with reduced single cell cloning efficiency and proliferation rate observed in older patients. However, no significant correlation has been found between age and chondrogenic differentiation. Nonetheless, there is considerable donor variability, highlighting the importance of patient selection in stem cell-based therapies. Stem cells from obese patients may be compromised by low-grade systemic inflammation associated with obesity. Obese-derived ASCs have shown reduced proliferation rates, increased cell senescence, and diminished differentiation potential, including chondrogenesis. Understanding the alterations in stemness networks in obese-derived stem cells may provide insights for overcoming the reduced chondrogenic potential (García-Álvarez et al., 2011; Hare et al., 2009; Labusca et al., 2012).

Inflammatory cytokines play a significant role in OA progression. Their presence in the joint space can interfere with chondrogenic differentiation and lead to the degradation of cartilage. Developing scaffold designs or genetic engineering to protect engineered tissue from inflammatory cytokines may be crucial for successful cartilage repair. Achieving phenotypic stability of chondrocyte-like cells over time remains a challenge. Maintaining a reduced oxygen tension in the environment has shown promise in limiting the transition to hypertrophic chondrocyte phenotype. Additionally, scaffold designs with mechanical properties similar to

native cartilage may facilitate successful cartilage repair by providing initial mechanical integrity. Composite constructs that combine cartilage and bone layers offer potential for osteochondral integration. Endogenous stem cells may now differentiate in a way that is spatially regulated by manipulating the distribution of chondrogenic and osteogenic induction agents in scaffolds. Direct infusion of stem cells into the joint may offer a secure substitute for slowing or stopping the course of OA. Delivery of stem cells during the earliest phases of the disease process may maximise their anti-inflammatory effects (Diekman & Guilak, 2013).

MSCs may be isolated from bone marrow or adipose tissue using a number of techniques. These techniques include bone marrow aspiration concentrate (BMAC), adipose-tissue-derived MSCs (Ad-MSCs), and adipose-tissue-derived stromal vascular fraction (ADSVF). However, getting an acceptable amount or quality of stem cells via these methods may be difficult since they include intrusive procedures. In contrast, the collection process for human umbilical cord blood-derived MSCs (hUCB-MSCs) presents fewer issues. It is less ethically contentious than using embryonic stem cells, and hUCB-MSCs exhibit superior differentiation capacity compared to adult stem cells. Consequently, researchers in across medical fields are progressively directing their attention to hUCB-MSCs as a promising alternative. Moreover, hUCB-MSCs have been widely studied for cartilage repair in animal models, and their potential has been recognized to the extent that they have received approval as a medical product and are already being used in clinical practice under the name Cartistem® (Medipost Inc., Sungnam, Gyeonggi-do, South Korea). Clinical studies on Cartistem® are also ongoing in the United States. But, the use of Cartistem® is currently limited to Korea, and long-term follow-up data after its application is lacking. Hence, further research and data collection are necessary to establish its efficacy in other countries and ensure its safety in the long run (D. H. Lee et al., 2022).

2.2 Cord Blood Mesenchymal Stem Cells (hUCB-MSC)

2.2.1 Application of Cord Blood Mesenchymal Stem Cells (hUCB-MSC)

Nowadays, regenerative medicine in the stem cell field is widely attractive to scientists, with stem cell therapy showing promise as a potential therapy method for certain disorders. Various sources

of stem cells are available, each with specific applications for treating particular diseases. One such source is mesenchymal stem cells (MSCs) derived from human umbilical cord blood (hUCB-MSCs), which are multipotent, non-hematopoietic, and possess the capability for self-renewal and differentiation. MSCs can be isolated from different sources, including bone marrow, cord blood, placenta, adipose tissue, and liver (Zarrabi et al., 2014).

Before the 1990s, human umbilical cord (hUC) and the blood it derives from were regarded as medical waste. But nowadays, the removal of hUCB-MSCs is seen as a non-invasive treatment and is not fraught with ethical issues. Surprisingly, hUCB-MSCs have outstanding qualities such as quick self-renewal, minimal oncogenicity, and weak immunogenicity as a result of their low expression of MHC class I and class II proteins. As a result, hUCB-MSCs are a crucial source for allogeneic transplantation treatment since there is no chance of immunological rejection when using them. Obtaining hUCB-MSCs also poses no comorbidity risk to the patient because the cell source is heterologous, making them a better option for both auto- and allo-transplantation. A number of techniques have been developed throughout time to isolate hUCB-MSCs, including those that use Wharton's jelly, arteries, or veins. Thus, hUCB-MSC-based cell therapy has been used in a variety of medical specialties, especially in regenerative and immunomodulatory therapies, including gynaecology. Despite their potential, numerous therapies utilising hUCB-MSCs continue to raise questions regarding their long-term safety and experimental character. As a result, scientists have been looking into novel approaches, concentrating in particular on how hUCB-MSCs produce and secrete chemokines, growth factors (GFs), blood extracts, biomolecules, and hormones, which can have an influence on neighbouring cells through paracrine signalling. These elements are essential for stimulating angiogenesis, anti-inflammation, immunomodulation, anti-apoptosis, and anti-fibrosis activities, all of which help the healing of damaged tissues. Exosomes and microvesicles, extracellular vesicles (EVs) that carry intracellular components such proteins, messenger RNAs, microRNAs, and bioactive lipids to target cells with certain surface receptors and affect their phenotypic and function, are notable examples (Rodríguez-Eguren et al., 2022).

The earliest and most rudimentary mesenchymal stem cells are found in human umbilical cord blood, which is easily acquired at birth without intrusive treatments, making it an ethically uncontroversial approach. After delivery, hUCB may be collected, frozen, and kept in banks for potential therapeutic use while retaining its viability and functioning. After clinical transplantation, hUCB also has a minimal risk of spreading viral infections and somatic mutations. Around twenty public cord blood banks exist globally, and both private and public cord blood banks have been developed to hold hUCB for potential uses. The main benefits of hUCB-derived MSCs are attributed to their characteristics, which include self-renewal, multipotency, hypo-immunogenicity, non-tumorigenicity, and immunomodulation. These attributes contribute to their vast therapeutic potential. Although MSCs produced from hUCBs and adult sources have a comparable spindle-shaped morphology, hUCB-derived MSCs have distinct and important benefits. The most extensively researched and collected MSCs are bone marrow derived stem cells, however obtaining MSCs from hUCB is simpler. Comparing hUCB-derived MSCs to bone marrow-derived stem cells, they show greater rates of proliferation and yield per unit volume. There are also less immune system incompatibilities after transplantation of MSCs produced from hUCB, such as graft-versus-host disease (GvHD) (Um et al., 2020).

For patients with hematologic disorders who lack HLA-identical related or unrelated donors and need hematopoietic stem cells (HSCs), umbilical cord blood transplant (UCBT) is an efficient alternate source of HSCs. Although the National Marrow Donor Programme (NMDP) and linked registries have over 39 million donors registered, many patients, particularly those from underrepresented groups, may find it difficult to locate a qualified, unrelated donor in the required amount of time. Patients from various racial and ethnic origins are now eligible for transplantation thanks to hUCB's efforts, both in the US and abroad. Hematopoiesis takes place in intraembryonic (yolk sac) and extraembryonic (ventral aortic artery) locations throughout foetal development before moving to the liver and bone marrow-derived stem cells. HSCs are typically present in bone marrow-derived stem cells after birth, with little blood circulation. Because cord blood stem cells have better proliferative properties than other stem cells, such as the ability to create

autocrine growth factors, greater levels of proliferative potential, and longer telomeres, research into these cells has increased (Laue et al., 2023; Sanchez-Petitto et al., 2023).

2.2.2 Cord Blood Mesenchymal Stem Cells (hUCB-MSC) Chondrogenic Capability

Hematopoietic stem and progenitor cells (HSPCs) generated from umbilical cord blood (UCB) have been shown in both *in vitro* and in vivo investigations to have more proliferative and expansion capacity than their adult bone marrow (BM) counterparts. This greater potential may be explained by the fact that UCB cells have longer telomeres than BM cells and leave the cell cycle's G0/G1 phase more quickly than adult BM progenitors. It is noteworthy that UCB is a rich source of hematopoietic progenitor cells (HPCs) and hematopoietic stem cells (HSCs), as well as a significant supply of B lymphocytes with immunoregulatory features. In comparison to healthy donors or patients before to the transplant, patients who receive umbilical cord blood transplants (UCBT) have greater frequencies and absolute quantities of Bregs (regulatory B cells) that produce IL-10 after recovery. In vitro testing of these reconstituting Bregs revealed they had a strong suppressive impact on allogeneic CD4+ T cells. The Bregs were discovered to be lacking in individuals with chronic graft-versus-host disease (GVHD). In UCB, the presence of IL-10-producing B cells may provide defence against persistent GVHD after UCBT. Additionally, T cells from UCB have shown enhanced antitumor responses in a murine model of B-cell lymphoma when compared to peripheral blood (PB) T cells. This antitumor activity is associated with increased tumor-homing of CCR7high UCB CD8+ T cells and a rapid acquisition of cytotoxic and T-helper (Th) 1 function. These findings might contribute to the lower relapse rate observed in patients positive for minimal residual disease (MRD) before undergoing UCBT (Zhu et al., 2021).

Ha et al., 2015, looked at the potential of mesenchymal stem cells obtained from human umbilical cord blood (hUCB-MSCs) as a new cell source for cartilage repair. Based on earlier studies, the effectiveness of a 4% hyaluronic acid (HA) hydrogel for cartilage healing was also assessed. Promising results were obtained in rat and rabbit models, where the transplantation of hUCB-MSCs with 4% HA hydrogel showed superior cartilage repair both grossly and histologically

compared to control groups (HA only and defect). The umbilical cord provides an abundant source of hUCB-MSCs, which can be isolated from the Wharton's jelly surrounding the arteries and vein within the cord. These primitive stromal cells have the ability to differentiate into various cell types, including chondrocytes, making them suitable for cartilage regeneration. hUCB-MSCs offer several advantages over other tissue sources, such as ease of collection, non-invasive procurement, avoidance of ethical concerns, and convenient storage for future use. Moreover, hUCB-MSCs are more primitive and possess a higher frequency of mesenchymal progenitor cells compared to bone marrow-derived MSCs. Their higher proliferative capacity and reduced graft-versus-host reactivity make hUCB-MSCs a promising alternative for allogeneic cell therapy. Although previous *in vitro* studies have demonstrated the chondrogenic potential of hUCB-MSCs, there have been limited in vivo studies with sufficient evidence of cartilage regeneration using these cells. Previous rat model study showed favorable cartilage repair with a composite of hUCB-MSCs and HA hydrogel. The current study, conducted in minipigs, yielded consistent results with the previous rat model, representing the first in vivo study for cartilage repair using hUCB-MSCs in large animal models. These findings indicate that hUCB-MSCs hold significant potential as an alternative source for cartilage regeneration and could be a viable option for future human clinical trials.

2.3 Gold Nanoparticles (AuNP) and their properties

2.3.1 Application of Gold Nanoparticles in Medical

Historical references to "soluble gold" date back to China and Egypt in the 4th or 5th century BC. Gold has long been used for both medicinal and aesthetic purposes. In the Middle Ages, gold was valued as a treatment for cancer, heart and infectious ailments, and other illnesses because it was thought to have magical and therapeutic characteristics. Robert Koch, a famous microbiologist, discovered the antibacterial properties of gold in 1890 while researching the impact of low potassium cyanide concentrations on *Mycobacterium tuberculosis* bacilli. Faraday, who focused on the optical characteristics of suspended gold microparticles in a colloidal solution, initiated the study of gold nanoparticles, or AuNPs, in 1857 (Mikhailova, 2021).

Nanotechnology, an interdisciplinary field encompassing materials science, physics, and chemistry, finds applications in various branches of science and technology. Nanomaterials, based on their components, properties, morphology, and size, can be categorized into polymer nanomaterials, carbon nanomaterials, metal nanomaterials, lipid nanomaterials, and semiconductor nanomaterials. Inorganic nanoparticles, exhibiting unique physical, chemical, and biological properties compared to their bulk counterparts, have become a subject of growing interest. Notably, noble metal nanoparticles, such as gold nanoparticles, have drawn attention due to their shape and size-dependent electromagnetic, optical, and catalytic properties. Consequently, extensive research is focused on developing synthesis methods to control their shape and size for various nanotechnology applications. Metal nanoparticles, including gold nanoparticles, are gaining popularity in biomedical fields due to their small size-to-volume ratio, functionalization capabilities, stability, and ease of detection. Gold nanoparticles, in particular, have distinct physical and chemical properties that render them suitable for a wide range of applications, particularly in therapies, detection and diagnostics, and precise drug delivery (Milan et al., 2022).

At the nanoscale, gold nanoparticles (AuNPs) have distinctive optical and electrical characteristics that may be changed by adjusting their size, shape, surface chemistry, or aggregation state. These AuNPs are capable of effectively and quickly absorbing visible, UV, and NIR light and converting it to heat energy. AuNPs also have a number of helpful qualities, such as low toxicity, high stability, straightforward production, and the capacity to conjugate with certain biomolecules. AuNPs are a priceless nanoplatform for several biological applications, including photothermal- and immuno-therapies, radiation, and drug administration, because to these exceptional physicochemical properties. They may be supplied to cancer areas with precision by functionalizing the surface of AuNPs with targeting molecules, offering tremendous promise for the treatment of cancer (Bloise et al., 2022).

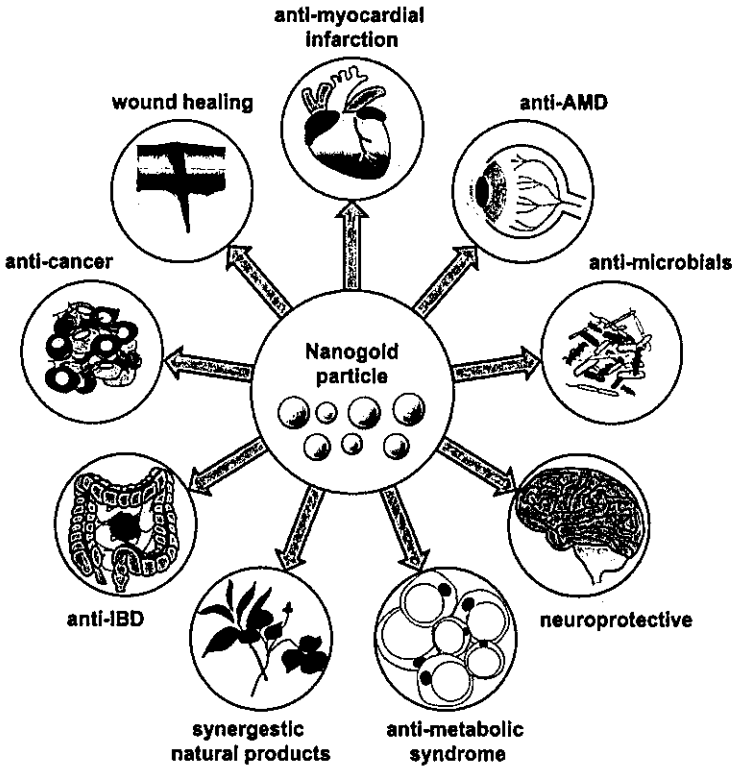

Figure 2.1. Varied Application of Gold Nanoparticles (Ko et. al, 2022)

2.3.2 Gold Nanoparticles Immunogenicity and Safety

The efficient recognition of nanoparticles by macrophages depends on a number of proteins. For instance, it has been demonstrated that IgG opsonins and fibrinogens promote phagocytosis and make it easier for nanoparticles to be eliminated from the body, but dysopsonins, such albumins, cause nanoparticles to circulate in the bloodstream for an extended period of time. The procedure of "PEGylation" has been developed by scientists to avoid immunological identification of nanoparticles. This method involves the surface of the nanoparticles being covered with a coating of PEG, as seen in Figure 2.2, to mask their identity. This shielding technique aids in avoiding immune recognition, which prolongs the time that the nanoparticles are circulated in the blood. PEG chains can be covalently bonded, trapped, or adsorbed onto the surface of nanoparticles to produce PEGylation (Singh et al., 2018).

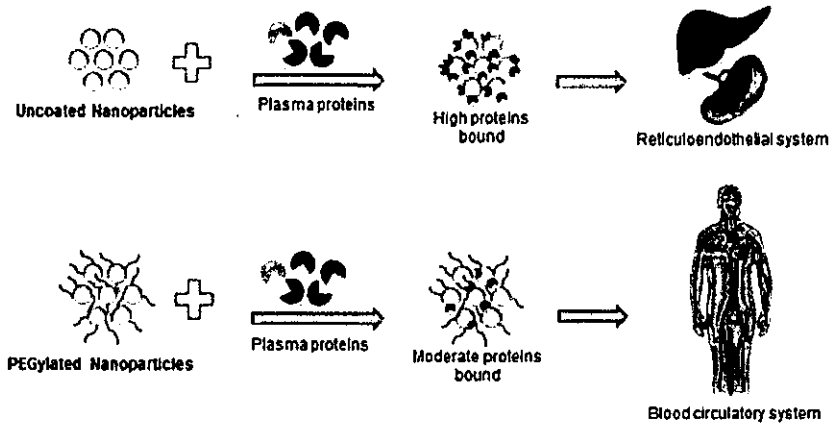

Figure 2.2 PEGylation is able to reduce inflammatory response (Singh et al., 2018).

Due to its high water solubility, biocompatibility, and favourable tolerance in the human body, PEG is a synthetic polymer that is frequently employed in biomedical applications. PEG-conjugated medicines have been widely used in a variety of biomedical sectors, including drug transport, bioconjugation, imaging, biosensing, and tissue engineering, thanks to the FDA's permission for their safe use in humans. PEG can be directly bonded to pharmaceuticals or connected to the surface of drug-encapsulating nanomaterials in drug delivery and bioconjugation, increasing their stability and solubility in vivo, lowering clearance rates, and increasing medication effectiveness. Different PEGylation techniques have been used over time for diverse biomedical goods.

The in vivo stability of micelles, liposomes, dendrimers, gold nanoshells, quantum dots, and polymeric nanoparticles has been significantly increased by PEGylation, resulting in better therapeutic effects. The attachment of opsonins (serum proteins) necessary for phagocytosis is prevented or minimised by PEG-modified nanoparticles' hydrophilicity and nearly zero zeta potential. PEGylated nanoparticles can thereby avoid being recognised by the mononuclear phagocyte system, prolonging their stay in circulation. High levels of hydration in PEG chains further enhance the hydrodynamic size of PEG-modified nanoparticles, preventing renal clearance and preventing access by antibodies and proteolytic enzymes. This considerably

lengthens the period that PEGylated nanoparticles are in the bloodstream, which is advantageous for any medications that are enclosed in PEG-based delivery systems.

The accelerated blood clearance (ABC) phenomenon is caused by the formation of anti-PEG antibodies (anti-PEG immunoglobulin M (IgM)) and immunological responses, which may be induced by the administration of PEGylated medicines. The PEGylated therapeutic drugs are recognised and removed by the mononuclear phagocyte system in the liver and spleen, which results in fast clearance and decreased effectiveness with consecutive dosages. In order to sustain PEGylated drug delivery systems' long-term efficacy, anti-PEG IgM has been found as a crucial marker and a significant contributor to the ABC of PEGylated nanoparticles (Thi et al., 2020).

2.3.3 Functionalization of Gold Nanoparticles Surface

PEGylation is commonly used to functionalize gold nanoparticles (GNPs). These GNPs feature a PEG coating, either by itself or in combination with other molecules such as biotin, peptides, or oligonucleotides, which facilitates the absorption of the GNPs by the target cells. This functionalization enables PEGylated GNPs to behave as efficient drug transporters by adhering to cell membranes. Different PEGylated GNPs have been developed, and they are efficient for intracellular and cellular targeting of biological components. They include biomolecules including lectin, lactose, and biotin. Hetero-bifunctional PEGylated GNPs with a thiol group on one end and a fluorescent dye (coumarin) on the other were created to make it simple to monitor the nanoparticles inside cells. The stability and functionality of PEGylated GNPs are influenced by the PEG molecular weight, the attached functional groups, the ligands utilised for PEGylation, and the size of the GNPs used. Researchers tested the efficacy of a collection of PEGylated GNPs linked with Thioctic Acid for tumour ablation in mice. How successfully these functionalized GNPs were internalised depended on the size of the nanoparticles, the molecular weight of the PEG, and the ligands used for PEGylation. Furthermore, the physicochemical properties of these GNPs affected their distribution inside different cells (Tiwari et al., 2011).

Amino acid and peptide functionalization of nanoparticles has been shown to be a highly successful method for increasing the specificity and effectiveness of nanoparticle-based delivery systems. In order to transfer genes more effectively while avoiding toxicity, gold nanoparticles (GNPs) functionalized with amino acids including lysine, polylysine, and glycine have shown improved DNA binding. These amino acids have a stronger ability to attach to the cationic groups on DNA due to the presence of primary ammonium groups. Particularly for the expression of the reporter -galactosidase gene, it has been discovered that lysine dendrons are preferable than polylysine. Due to their bioinert, non-toxic, readily synthesizable, and functionalizable nature, gold nanoparticles present an interesting platform for the development of transfection agents. They also offer a versatile platform appropriate for both therapeutic and diagnostic purposes. These nanoparticles may be effectively functionalized to preferentially aggregate at tumour areas using targeting ligands, which makes them an effective tool for cancer gene therapy (Ghosh et al., 2008).

2.4 Influence of Nanoparticles on Stem Cells Differentiation

Due to its superior physical qualities, such as biocompatibility with minimal cytotoxicity and great control over particle parameters, gold nanoparticles (AuNPs) have become a viable material for regenerative medicine. AuNPs are very adaptable due to the negative charge on their surface, which enables functionalization with different biomolecules, medications, DNA, antibodies, and functional peptides/polymers for application in biomedical research and therapy. In earlier research, chitosan-conjugated AuNPs and other AuNPs functionalized with polymers were created to encourage enhanced differentiation of human mesenchymal stem cells (hMSCs). The aminated polysaccharide chitosan, which is frequently employed in bone tissue engineering, has characteristics with glycosaminoglycan, a crucial part of the extracellular matrix (ECM) that is involved in cell adhesion. Chitosan polymers can stimulate osteogenic differentiation by activating the Wnt/-catenin signalling pathway, according to studies. It's interesting to note that the protein kinase 38 (p38) mitogen-activated protein kinase (MAPK) pathway has been shown to promote osteogenic differentiation of human stem cells (hMSCs) in response to AuNPs. AuNPs charge and moiety have an impact on cellular reactions and trigger osteogenesis (Yi et al., 2010).

2.5 Molecular Mechanism in Chondrogenic Differentiation

Articular cartilage is a dense connective tissue that serves as a load-bearing, cushioning, and protective structure during joint movement. It lacks nerves, blood vessels, and lymphatic vessels. Chondrocytes, which make up around 1% of the cartilage tissue, are the sole cell type responsible for regulating the synthesis of growth factors and enzymes that control the formation of the extracellular matrix (ECM). The ECM, primarily composed of collagen II and aggrecan (ACAN), is crucial for absorbing mechanical stress, facilitating chondrocyte adhesion, and regulating intracellular signaling. Chondrocytes originate from bone marrow mesenchymal stem cells (BMSCs). Initially, BMSCs aggregate and differentiate into chondroprogenitor cells, which further mature into chondrocytes through a series of differentiation steps, eventually leading to the formation of hypertrophic chondrocytes. During endochondral ossification, the cartilage matrix undergoes partial calcification, and chondrocytes are eventually replaced by osteoblasts through apoptosis.

However, in the case of cartilage damage or osteoarthritis (OA), chondrocyte hypertrophy and apoptosis can accelerate the progression of the disease. *In vitro* culture of chondrocytes can also lead to imbalances in the growth and differentiation regulation system, resulting in cell aging and differentiation. Chondrocyte differentiation is characterized by changes in the fibrous phenotype, decreased expression of collagen II, and increased expression of collagen I, matrix metalloproteinase 13 (MMP-13), and nitric oxide synthase (NOS) (Chen et al., 2021).

Tensile strain has been demonstrated to have a positive effect on various markers related to ligamentous/fibrogenic, osteogenic, and chondrogenic differentiation in mesenchymal stem cells (MSCs). The application of cyclic tensile strain to MSCs has been shown to increase the expression of osteogenic genes and promote calcium deposition. The MAP kinase pathway appears to play a significant role in mechanotransduction during osteogenic differentiation under tensile strain.