Development and Characterization of Mesenchymal Stem Cell Secretome-infused Hydrogel

Submitted by:

Ifrah Taqdees (00000358068)

Memoona Abdullah (00000356662)

M. Abdur Rehman (00000357923)

Supervisor:

Dr. Hussain Mustatab Wahedi

Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad

May, 2024

Development and Characterization of Mesenchymal Stem Cell Secretome-infused Hydrogel

Submitted by:

Ifrah Taqdees (00000358068)

Memoona Abdullah (00000356662)

M. Abdur Rehman (00000357923)

This thesis is submitted to the National University of Sciences and Technology, Islamabad, in partial fulfillment of the requirements for the degree of Bachelor of Sciences in Applied Biosciences

Supervisor:

Dr. Hussain Mustatab Wahedi

Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad

May, 2024

THESIS ACCEPTANCE CERTIFICATE

This document certifies that this is the final copy of BS FYP Thesis written by **Ms. Ifrah Taqdees** (Reg No. 358068), **Ms. Memoona Abdullah** (Reg No. 356662), and **Mr. Muhammad Abdur Rehman** (Reg No. 357923) of **Atta-Ur-Rahman School of Applied Biosciences (ASAB)**. The undersigned has thoroughly reviewed and found that the document complies with all regulations set by the National University of Sciences and Technology (NUST). It is confirmed to be free of plagiarism, errors, and mistakes, and is accepted as a partial fulfillment for the Bachelor of Sciences degree in Applied Biosciences. Additionally, all necessary amendments suggested during the final presentation have been included in the thesis.

Signature: _____

DR. HUSSAIN M. WAHEDI
Associate Professor
Deptt of Healthcare Biotechnology
Atta-Ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Name of Supervisor: **Dr. Hussain Mustatab Wahedi**

Date: 16-05-2024

Signature (HOD): _____

Dr. Hussain Mustatab Wahedi (HoD)

Head of Department Biomedicine: **Dr. Hussain Mustatab Wahedi**

Date: 16-05-2024

Signature (Dean/Principal): _____

Principal ASAB: **Dr. Muhammad Asghar**

Date: 16-05-2024

DECLARATION

We affirm that the research work titled **"Development and Characterization of Mesenchymal Stem Cell Secretome-infused Hydrogel"** authored by **Ifrah Taqdees**, **Memoona Abdullah**, and **Muhammad Abdur Rehman**, is our original creation. This work has not been presented elsewhere for evaluation. The research was conducted during our undergraduate studies at Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), under the guidance of Dr. Hussain Mustatab Wahedi. Any material borrowed from external sources has been appropriately cited and acknowledged.

Ifrah Taqdees

Memoona Abdullah

Muhammad Abdur Rehman

CERTIFICATE FOR PLAGIARISM

It is certified that this Undergraduate Thesis Titled "**Development and Characterization of Mesenchymal Stem Cell Secretome-infused Hydrogel**" written by **Ms. Ifrah Taqdees** (Reg No. 358068), **Ms. Memoona Abdullah** (Reg No. 356662), and **Mr. Muhammad Abdur Rehman** (Reg No. 357923) of **Atta-Ur-Rahman School of Applied Biosciences (ASAB)**, has been examined by me.

I undertake that:

a. The thesis contains original contributions and novel insights not found in previously published works or those currently being reviewed for publication elsewhere. No exact sentences, equations, diagrams, tables, paragraphs, or sections have been directly copied from prior sources without proper quotation marks and citations.

b. The work presented is original and own work of the authors i.e., there is no plagiarism.

c. There is no falsification of data or misrepresentation of research findings in the records. The thesis has undergone inspection using TURNITIN (an originality report is attached), confirming compliance with the HEC plagiarism policy and relevant guidelines.

Signature of Supervisor

Dr. Hussain Mustatab Wahedi
Head of Department (HoD)
Atta-ur-Rahman School of Applied Biosciences (ASAB)
NUST Islamabad

Dr. Hussain Mustatab Wahedi

HOD Biomedicine

Associate Professor

Atta-ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

"And those who strive in Our (cause), - We will certainly guide them to our Paths: For verily Allah is with those who do right."

Quran 29: 69

DEDICATION

"This work is dedicated to our beloved parents, siblings and teachers. Their unwavering support, encouragement and understanding have been our motivation to strive and move forward. We would like to dedicate our research to our friends Mr. Murtaz Aziz Ahmad (late) and Ms. Arooba Rashid (late) who left us too soon. Their love, kindness and cheerful smiles will remain in our hearts forever. Further, this study is dedicated to M. Abdur Rehman's grandmother (late) who was the happiest on his admission to NUST on her last day."

ACKNOWLEDGEMENTS

All praises and glory are for the Highest God, the most beneficent, the most merciful who blessed us with the sight to observe, the mind to think, the courage to work more and the light when we were in dark.

We feel great pleasure to have the opportunity to express our sincerest gratitude to our supervisor **Dr. Hussain Mustatab Wahedi** for providing us his supervision. He connected us with all the right people who helped us run and conclude our project successfully.

We would like to appreciate **Prof Dr. Muhammad Asghar**, Principal, Atta-ur- Rahman School of Applied Biosciences (ASAB) for his efforts to maintain the institution's mission for research and study. We also want to acknowledge the efforts of the faculty of ASAB for mentoring us during our bachelor's degree. We would like to thank **Dr. Bilal Khan Niazi**, Associate Professor, School of Chemical and Materials Engineering (SCME), NUST for guiding us in the field of biomaterials engineering.

We are grateful to our seniors, **Ms. Aasiya Hameed, Ms. Mariam Ahmed, Ms. Wajeeha Mateen** and **Ms. Mahzeb Saleem** for their guidance and support in this project.

We are extremely grateful to **Huzaifa Ali Ahfaz** and **Muhib ur Rehman Farooqui** for their reliability and invaluable support throughout our project. Their presence has been a source of strength, providing us assistance whenever we encountered obstacles. A special thanks to **Dania Ashfaq, Rameesha Khursheed, Saad bin Saeed** and **Muhammad Saad Zafar** for listening to our continuous rants the entire duration of our project.

We would also like to acknowledge **Hira Javed Bajwa, Numra Tanveer, Zainab Irfan, Nimra Abaid, Laraib Hassan** and **Aleeza Arshad** for their constant emotional support.

Table of Contents

List of Tables

List of Figures

LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
3D	Three-dimensional
a.u.	Arbitrary Unit
CS	Chitosan
ECM	Extracellular Matrix
FTIR	Fourier Transform Infrared Spectroscopy
HLA	Human Leukocyte Antigen
HSCs	Hematopoietic Stem Cells
HUCESCs	Human Umbilical Cord-derived Mesenchymal Stem Cells
iPSCs	Induced Pluripotent Stem Cells
MSCs	Mesenchymal Stem Cells
PBS	Phosphate Buffered Saline
PSCs	Pluripotent Stem Cells
PVA	Polyvinyl Alcohol
RPM	Revolutions Per Minute
SEM	Scanning Electron Microscope
V	Vanillin
w/w	Weight by Weight
w/v	Weight by Volume
XRD	X-ray Diffraction

ABSTRACT

The secretome of mesenchymal stem cells (MSC) has been found to have healing properties, but a cell-free vehicle for its targeted delivery is needed. Hydrogels are biomaterials that can hold significant amounts of water and are widely used in the healthcare industry, and they can be used as a vehicle for the targeted delivery of MSC secretome to wounds. Chitosan/PVA/Vanillin hydrogels were formed. Secretome was infused by mixing it in the Chitosan/Vanillin solution. Scanning Electron Microscopy (SEM) was used to assess the surface morphological characteristics of hydrogels and X-Ray Diffraction (XRD) was used to determine their crystalline structure. The chemical bonds in hydrogels were analyzed by Fourier Transform Infrared Spectroscopy (FTIR). Water Uptake Analysis was performed to check for the hydrogels' hydrophilicity. SEM analysis revealed that the secretome constituents were evenly spread out in the hydrogel. XRD analysis saw a slight distortion of the indicative peaks of Chitosan/PVA/Vanillin hydrogel and revealed new peaks, suggesting proper infusion of the stem cell secretome. FTIR analysis saw an increase in the amine peaks, suggesting the integrity of the secretome and its proper infusion. Water Uptake Analysis revealed less hydrophilicity of secretome-infused hydrogels than the control hydrogels, suggesting a thorough infusion of the secretome. Overall, our study optimized the protocol for stem cell secretome infusion in hydrogels as a novel drug delivery approach and revealed the compatibility of secretome with Chitosan/PVA/Vanillin hydrogels.

Keywords: Mesenchymal Stem Cell (MSC) Secretome, Hydrogels, Wound Healing, Regenerative Medicine, SEM, XRD, FITR

INTRODUCTION

Regenerative medicine is a rapidly growing field of medicine which has the potential to heal or replace disease, or trauma, and to correct congenital defects. Traditional therapies include tissue and organ transplantations for clinical treatments. However, the use of methods such as transplantation are disadvantaged by limited donor availability and significant immune complications. Regenerative medicine can provide the solutions to these challenges by employing various strategies, including the use of materials and newly generated cells, either separately or in combination, to replace missing tissues both structurally and functionally, or to aid in tissue repair. (Mao & Mooney, 2015).

Stem cells have become a focal point of research in modern regenerative medicine, which can also be referred to as stem cell therapy. Stem cells are defined by their ability of self-renewal and differentiation into several specialized cell types. Stem cell therapy is utilized with the aim to enhance the body repair machinery via stimulation, modulation, and regulation of native stem cells or through the introduction of external cells into the patient's body to restore tissue balance and promote healing. (Hoang et al., 2022).

Mesenchymal stem cells (MSCs), a specific type of stem cell, are identified as non-hematopoietic cells with the ability to differentiate into multiple lineages, such as mesodermal, ectodermal, and endodermal lineages. They are a promising option for cell therapy and human tissue reformation multipotent differentiation, self-renewal ability, long-term proliferation outside the body, paracrine functions, and immunoregulatory effects. Despite their great potential, they also possess certain limitations.

The number of MSCs isolated from bone marrow is significantly lower than the quantity required for clinical treatments, necessitating ex vivo expansion, typically in bioreactors. Post-expansion, MSCs can be administered locally or systemically. However, local injection often results in MSCs being washed away, engulfed by phagocytes, or undergoing necrosis, with less than 5% persisting in the tissue a few hours after transplantation. Systemic administration usually leads to MSC accumulation in the lungs and clearance by monocytes within 24 hours, requiring multiple doses for clinical efficacy, thereby increasing reliance on in vitro expansion (Wechsler et al., 2021).

The necessity to exploit the advantageous properties of MSCs remains critical, thus prompting the development of a cell-free alternative. MSCs secrete a broad range of bioactive molecules collectively known as the secretome, in response to the surrounding environment (Ahangar et al., 2020). The secretome is mainly composed of a significant number of proteins and some additional small molecules. The proteins which make up the composition of the secretome include cytokines, chemokines, growth factors. Metabolites, ions, peptides, microvesicles, and exosomes are the small molecules present in the mix. Research on proteins secreted by the stem cells has shown that these factors, when used alone without the cells, can initiate the healing process in damaged tissues or organs (Md Fadilah et al., 2022) This has led to the solution of a cell-free alternative derived from MSCs as a promising therapeutic approach in tissue regeneration (Ma et al., 2023).

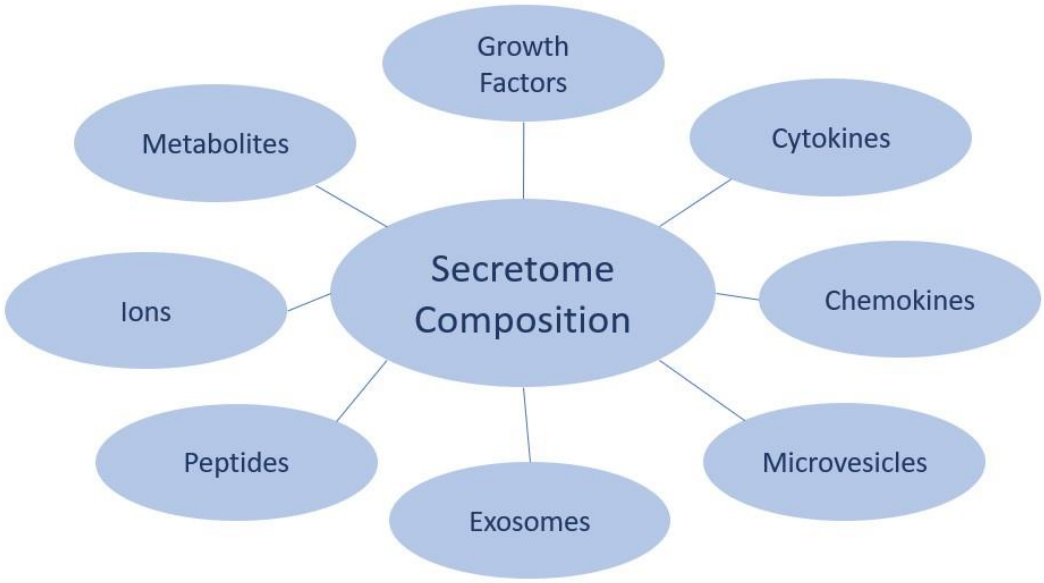

Figure 1 Composition of Secretome (Md Fadilah et al. 2022)

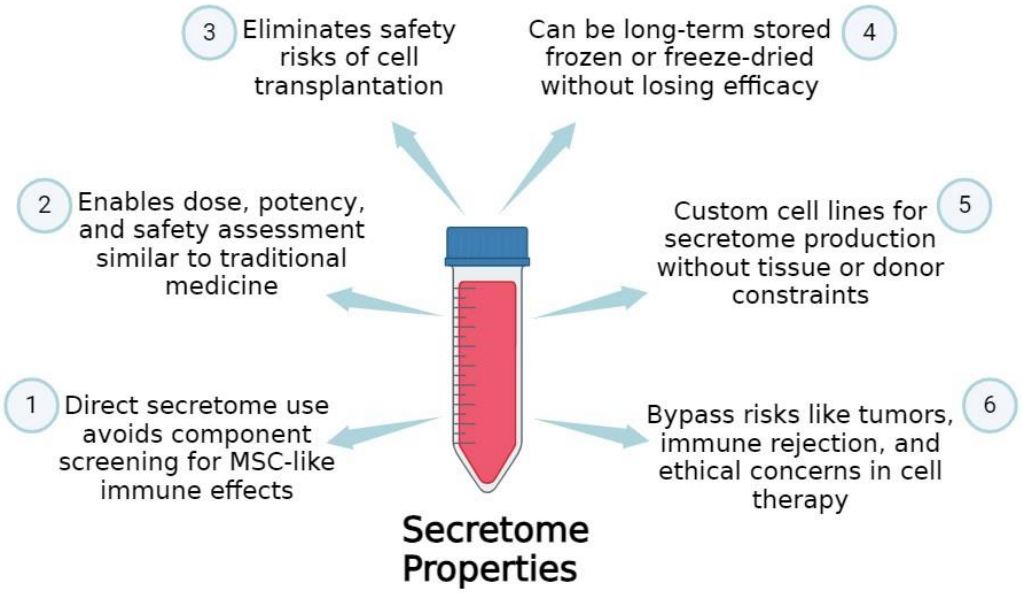

Figure 2 Advantageous properties of secretome (Su et al. 2023; Arifka et al. 2022)

The secretome requires a suitable environment for its sustained release for biomedical applications, to allow targeted delivery to damaged tissues, which can be provided by its incorporation in an appropriate biomaterial. The American National Institute of Health defines a biomaterial as "any substance or combination of substances, other than drugs, synthetic or natural in origin, which can be used for any period of time, to augment or replace partially or totally any tissue, organ, or function of the body, in order to maintain or improve the quality of life of the individual".

An ideal biomaterial for tissue healing should meet some requirements, such as non-toxicity, optimal rate of biodegradability, non-immunogenicity, tissue biocompatibility and adequate mechanical properties and morphology. By adjusting some of these physical properties, biomaterials such as hydrogels, can provide satisfactory anti-inflammatory, antibacterial, and adhesive properties. (Ijaola et al., 2022; Kaur et al., 2022).

Hydrogels are characterized by their three-dimensional network of cross-linked polymers that can hold a significant amount of water while maintaining their structural integrity after swelling. Their beneficial features include 3D structures like natural tissues, elasticity, a moist environment, porous structures that allow gaseous exchange and prevent anaerobic bacterial growth, acting as a barrier against bacterial infections, and enhancing epithelialization as well as cell migration. Such properties render hydrogels highly promising materials for tissue regeneration (Asadi et al., 2021).

1.1 Research Objectives

Our research objectives have been demonstrated, keeping in view the extensive literature. These include:

- Development and optimization of protocol for Chitosan/PVA/Vanillin hydrogel to ensure preparation of a hydrogel with suitable properties for secretome infusion.
- Development and optimization of protocol for Secretome-infused Chitosan/PVA/Vanillin hydrogel to enhance the therapeutic efficacy of the hydrogel.
- Characterization of hydrogels to confirm the incorporation of secretome and its biocompatibility.

LITERATURE REVIEW

2.1 Stem Cells

The classification of these cells is based on their potential to differentiate into totipotent, pluripotent, multipotent, and unipotent cells, and based on their origin which can be embryonic, adult or can be sourced the umbilical cord or placenta. Notably, induced pluripotent stem cells (iPSCs) are a distinct type generated from adult somatic cells by introducing specific genes encoding for transcription factors (Kucharzewski et al., 2019).

Numerous studies have shown significant advancements in cellular therapy due to the ability of stem cells to self-renew and differentiate into all cell types, playing a crucial role in physiological regeneration. Various sources of stem cells, which include adult and pluripotent stem cells (PSCs). These further include embryonic stem cells (ESCs) as well as induced pluripotent stem cells (iPSCs), which are utilized. PSCs are particularly valued for their high potential for pluripotency and self-renewal, making them a promising option for treating diseases. However, ethical issues arise with the use of ESCs, which involve the destruction of embryos at the blastocyst stage. Research has demonstrated the regenerative capabilities of iPSCs in preclinical settings, with the first clinical study conducted for treating age-related macular degeneration. Despite this, the risk of tumorigenicity remains unresolved.

These limitations have led to a surge of interest in adult stem cells which are the multipotent cells found in the tissues and organs of adults. Different research has shown that stem cell therapy can restore and heal injured organs in situ by stem cell differentiation and the formation of new specialized cells. Furthermore, some studies have demonstrated that cultured adult stem cells secrete a host of factors that are anti-apoptotic, immunoregulatory, angiogenic, and chemoattractant which in turn speed up the regeneration process. Hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) are among the most commonly used adult stem cells, mainly because they can be derived from donors with diseased conditions.(Margiana et al., 2022, 2022)

2.2 Mesenchymal Stem Cells

MSCs were initially considered "stromal" cells rather than stem cells. Some researchers proposed renaming MSCs to "medicinal signaling cells" because of their role in secreting metabolites at sites

of disease, injury, and inflammation. Further studies revealed that MSCs can release prostaglandin E2 (PGE2), which is crucial for their self-renewal ability, immunomodulation, and initiation of a cascade of events that highlight their stem cell properties. Consequently, the term "mesenchymal stem cells" was found to be appropriate (Margiana et al., 2022). To meet MSC classification criteria, these cells must exhibit expression of CD73, CD90 and CD105 markers, while lacking the expression of CD14, D19, CD34, CD79a, CD45, CD11b and HLA-DR molecules (Kucharzewski et al., 2019). Although the conventional source of MSCs is the bone marrow, MSCs or MSC-like cells can be isolated from almost any human tissue. MSC-like cells have been identified in various tissues across different developmental stages including adipose tissue, amniotic fluid, brain tissue, compact bone, dermal tissue, dental pulp, gingiva, fetal liver and lung tissues, islets of Langerhans, placenta, skeletal muscle, synovium, umbilical cord tissue, peripheral blood, among others (Fan et al., 2020).

Mesenchymal stem cells (MSCs) are being increasingly recognized as a promising therapeutic solution for various injuries or diseases lacking effective treatments due to their ability to exert reparative, regenerative, and immunomodulatory effects via paracrine signaling (Ahangar et al., 2020; Huang et al., 2020). Consequently, extensive research involving animal models has been conducted to explore the therapeutic efficacy of MSCs and their secretions (such as secretome, extracellular vesicles, exosomes, etc.) for challenging wounds. As our understanding of repair mechanisms grows, MSC-based therapy has progressed from preclinical animal studies to clinical trials. (Huang et al., 2020).

2.3 Mesenchymal Stem Cell Secretome

The anti-inflammatory properties of Secretome are at least in part conducted via the soluble immunoregulatory molecules. It is also known that the therapeutic efficacy of hUCESC-secretome in promoting the healing of epithelial cells in a murine model of dry eyes after introducing alkaline corneal epithelial ulcers. hUCESC-secretome treated eyes restored epithelium in dry eye condition after injury. Many research works have shown that the secretome contains growth factors that promote cell proliferation and the regeneration of damaged organ tissues, particularly in the case of the latter. In the last decade, many studies have confirmed the secretome's neuroprotective and neurotrophic features. MSC secretome is reported to contain a number of neurotrophic factors, and in several experimental studies the application of MSC has been shown to improve the prognosis

in the models of nerve injury. Furthermore, several researches have been conducted which have proved that the secretome of MSCs is highly effective in various steps of angiogenesis. For example, it has been noted that different MSC populations have been found to induce proliferation and migration of endothelial cells, promote tube formation, and prevent cell apoptosis in vitro.

2.4 Biomaterials

Biomaterials have the potential to deliver therapeutic molecules directly to the specific wound site. Over the recent years, there an increase in the number of patients has been observed experiencing challenges with healing and managing chronic wounds, burns, and ulcers which prove resistant to conventional medical interventions. The advancements made in the field of biomaterials have consistently addressed the challenges associated with the treatment of such complicated wounds (Kaur et al., 2022).

2.4.1 Polymeric Biomaterials

Polymers have been widely utilized for the regenerative and engineering purposes of different diseased or damaged tissues, such as of the muscles, bones, heart, brain, and skin. They offer a remarkable adaptability in their ability to adjust the chemical and physical properties of their surface. Properties of polymers, such as pore size, biodegradability, and mechanical characteristics, can be exactly controlled, making them ideal for scaffold fabrication.

2.4.2 Ceramic Based Biomaterials

These are used in dental and orthopedic applications. For example, metals and their alloys do not achieve the desired aesthetic quality, and porcelain-fused-to-metal lacks the overall transparency needed for dental biomaterials. Furthermore, the insufficient mechanical strength of polymers may render them unsuitable for skeletal regeneration applications. Numerous studies have employed carbon, bioactive, and bioresorbable ceramics in various regenerative medicine applications.

2.4.3 Composite Biomaterials

A composite material consists of two or more materials combined in a heterogeneous arrangement on a macroscopic scale. The advantage of using biomaterial composites lies in joining the best properties of their individual components, reducing the disadvantages of using each component alone, and often unveiling new properties not present in the individual components. Moreover, designing composite systems offers flexibility, allowing the properties of the final product to be

easily modified by adjusting the concentration and characteristics of the components. In the biomaterials field, composites have emerged to enhance the mechanical properties of polymers and ceramics (Rahmati et al., 2018). Hydrogels can be classified as composites when they are combined with various components like particles, fibers, or nanocellulose to create materials with unique, synergistic properties3. Composite hydrogels are often synthesized by incorporating different polymers, monomers, and additives to generate materials with enhanced mechanical properties and functionality compared to single-polymer hydrogels (Zhang et al., 2024).

2.5 Hydrogels

Hydrogels offer a wide range of design options concerning the type of molecules they consist of and their functionalities. These functionalities include the simultaneous delivery of multiple drugs, combined imaging and therapeutic capabilities, programmable and controlled release mechanisms, accessing different microenvironments and nano environments (hydrophobic or hydrophilic), and numerous other capabilities like ligand conjugation, integration with nanoparticles, drug incorporation and more. These attributes collectively make hydrogels a versatile platform for drug delivery, with some already utilized in clinical settings (Dreiss, 2020).

The capacity of hydrogels to encapsulate drugs helps in slowing or preventing their degradation and aggregation, thereby prolonging their lifespan. Furthermore, hydrogels enable sustained drug release, which can be regulated through diffusion from the matrix, or remotely triggered by external or internal factors (Dreiss, 2020).

Hydrogels exhibit varied architectures, sizes, physical properties and functions influencing drug encapsulation and release. They can be bulk/macroscopic for different delivery routes, microgels (micrometers) for specific routes or nanogels (10-10 nm) for systemic or intracellular delivery. Pore size affects deformability and cell diffusion, while mesh size (hundreds of nm) controls drug release through diffusion, modifiable by matrix degradation, swelling, and interactions such as covalent conjugation or physical bonds (hydrophobic, van der Waals, electrostatic, etc.) (Dreiss, 2020).

2.6 Hydrogel Composition

There are multiple methods for synthesizing hydrogels, broadly categorized into chemical crosslinking and physical crosslinking. Chemical hydrogels are covalently crosslinked through

processes such as by grafting, by using radical polymerization, carrying out enzymatic reactions, gelation via heat, or radiation crosslinking. Additionally, introducing cations like Ca2+, Mg2+, or Zn2+ to the hydrogel precursor can induce gelation through ionic bond formation with polymeric anions like alginate, which holds groups with negative charges. Conversely, natural hydrogels are primarily made by assembling themselves via mechanically forming crosslinks, which involves alterations ionic crosslinking and other intermolecular interactions such as hydrophobic interactions and the bonding of hydrogen. These processes are typically achieved by altering the thermal conditions of the hydrogel precursor, either raising it to 37°C or lowering it to -20°C or -80°C. Various parameters can be adjusted or controlled during the gelation process to achieve the desired hydrogel structure. Additionally, a combination of crosslinking that can be done by either chemical or physical aspects to form hydrogels.

2.6.1 Chitosan

Chitosan, derived from chitin, a natural biopolymer, is extensively used in the biomedical domain due to its effective film-forming characteristics. Apart from its applications tissue regeneration, chitosan demonstrates potential to be made a supplementary component in drug delivery systems attributing to each property such as biodegradability, compatibility with biological systems, enhancement of permeation, hemostatic properties, and abilities to combat bacteria and fungi. Altering the amino and hydroxyl groups in chitosan can improve its properties and introduce specific biological functions such as solubility, mucoadhesion, and bio-adhesivity. Various methods can be employed to modify the chitosan structure to tailor its polysaccharide network for specific purposes (Karakurt et al., 2021).

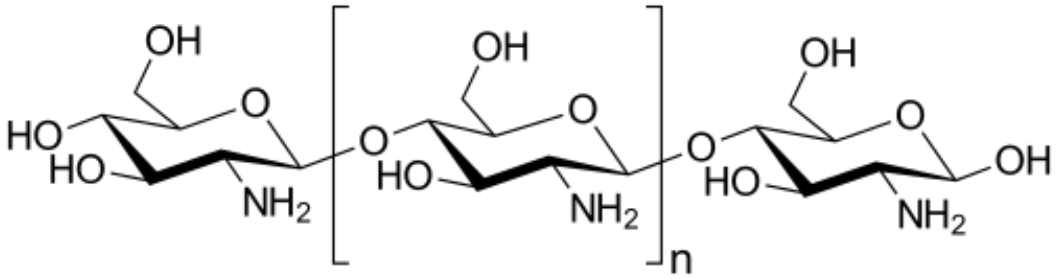

Figure 3 Chemical Structure of Chitosan

2.6.2 Vanillin

The cross-linking technique is a common method that is applied to modifying the structure of chitosan, creating a three-dimensional network and decreasing the mobility of the polymer chains. This process has a significant influence on various properties such as hydrophilicity, swelling behavior, degradation rate, stability, dispersibility, controlled release, drug targeting, and mechanical characteristics. Conventional cross-linkers like glutaraldehyde, formaldehyde, tripolyphosphate, and glyoxal are mostly used to achieve these features. On the contrary, the possible negative outcomes and environmental impact of nanotechnology have restricted its use in the pharmaceutical industry. Hence, there has been rising interest in the use of environment-friendly and natural cross-linking agents such as polyphenols and aldehyde compounds which are derived from plant extracts.(Karakurt et al., 2021).

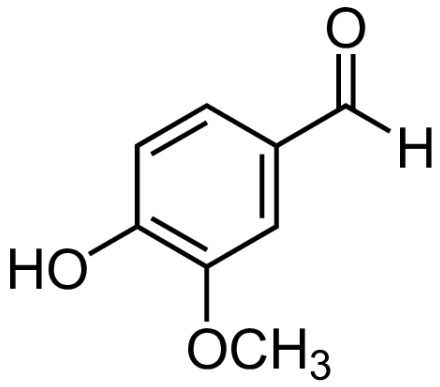

Figure 4 Chemical Structure of Vanillin

Vanillin, extracted from vanilla seedpods (*Vanilla planifolia*), is widely employed for flavoring and as a preservative agent in various industries. It serves as a significant bio-monomer with properties which show its effect as an anti-tumor, in reducing inflammation, and playing its role as an antioxidant. The aldehyde group in vanillin, qualifying it as a natural crosslinker, forms a bond with the amino groups of chitosan, therefore known as Schiff-base, and the hydroxyl group present in the crosslinker can engage with the amino or hydroxyl groups of another chitosan molecule via hydrogen bonding. This hybrid network structure hinders drug molecule mobility in the chitosan matrix, improving the stability, dispersivity, and control over the drug release in localized delivery system (Karakurt et al., 2021).

2.6.3 Poly-vinyl Alcohol

The inherent brittleness observed in many polysaccharide-based films such as chitosan restricts their practical applications. To address this drawback, blending chitosan with a compatible and non-immunogenic copolymer such as Poly-vinyl Alcohol (PVA) can prove can offer a solution. The appropriate amount of PVA contributes in allowing flexibility in the chitosan matrix, and permitting its elongation, eliminating the need for additional plasticizers that have been used previously (Karakurt et al., 2021).

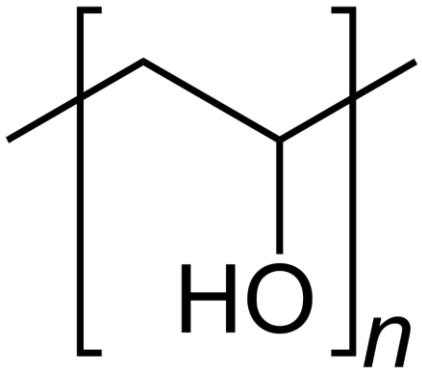

Figure 5 Chemical Structure of Polyvinyl Alcohol

The CS/PVA/Vanillin hydrogel and its preparation methods are simple, controllable, and cost-effective, making it ideal for practical use in wound dressings (Xiong et al., 2022).

METHODOLOGY

3.1 Materials

Mesenchymal stem cell secretome (eight 50ml falcon tubes) was acquired from National University of Medical Sciences, Pakistan.

Table 1 List of Chemicals

S No.	Chemicals	Manufacturers
1.	Chitosan (high viscosity, >400 mPa.s)	Macklin Chemicals
2.	Vanillin (4-Hydroxy-3-methoxybenzaldehyde)	Sigma-Aldrich
3.	Polyvinyl Alcohol (+% hydrolyzed)	Sigma-Aldrich
4.	Ethanol (100%)	Sigma-Aldrich
5.	Phosphate Buffer Saline (PBS)	Sigma-Aldrich
6.	Acetic Acid (1% w/w)	Sigma-Aldrich

Table 2 List of Instruments utilized

S No.	Instruments	Manufacturers
1.	Scanning Electron Microscope (JSM-6490A)	Jeol Ltd.
2.	D-8 Advanced XRD instrument	Bruker, Germany
3.	FTIR spectra-100 spectrometer	Perkin-Elmer

Table 3 List of Software

S No.	Software	Manufacturers
1.	GraphPad Prism (version 9)	GraphPad Software, Inc.
2.	OriginPro	OriginLab

3.2 Preparation of Chitosan and PVA solution

Chitosan and PVA solutions were prepared using a modified version of the protocol conducted by Xiong, Shuting (Xiong et al., 2022). Firstly, 1% (v/v) acetic acid solution was prepared by mixing 1 ml of acetic acid with 99ml of distilled water. 2g of fine powdered chitosan was added into 100ml of 1% acetic solution to obtain a 2% (w/v) chitosan solution. A magnetic stir bar was put in the solution before placing it on the magnetic stirrer. The solution was mixed at 350-400rpm for 8 hours until homogeneity was obtained. To make the co-polymer PVA solution, 100ml of distilled water was heated up to 90 °C. Then 10g of PVA was added to the heated water and temperature was brought down to 35-40 °C. This solution was then mixed on the magnetic stirrer for 3-4 hours until PVA was properly dissolved.

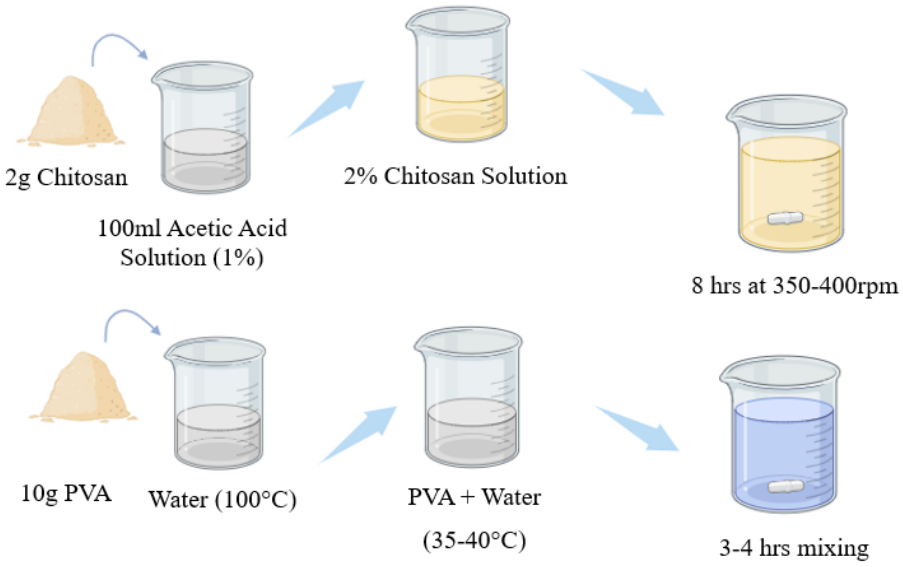

Figure 6 Preparation of Chitosan and PVA Solution

3.3 Synthesis of Chitosan/PVA/Vanillin Hydrogel

Vanillin was used as a cross-linker for the polymeric hydrogel. The amount and ratio of cross-linker solution was determined from the protocol described by (Amir et al., 2024). 0.3g of vanillin was added into 4ml of 70% ethanol and the beaker was shaken gently for 1 minute to obtain a clear solution. 10ml of the 2% chitosan solution previously prepared was added into the cross-linker and placed on the magnetic stirrer for 3 hours before adding 10ml of PVA in it. The chitosan and PVA are always added in 1:1 ratio. The PVA/Vanillin/Chitosan solution was again mixed on the magnetic stirrer for 3-4 hours until a clear solution was obtained. The hydrogel was synthesized by using the solution casting technique. 25ml of the final solution was poured into a Teflon plate of 8.5cm diameter and the plate was placed inside the drying oven at 40 °C for two days. Once the hydrogel dried, it was lifted off the plate using a scalpel and stored in a plastic bag at room temperature.

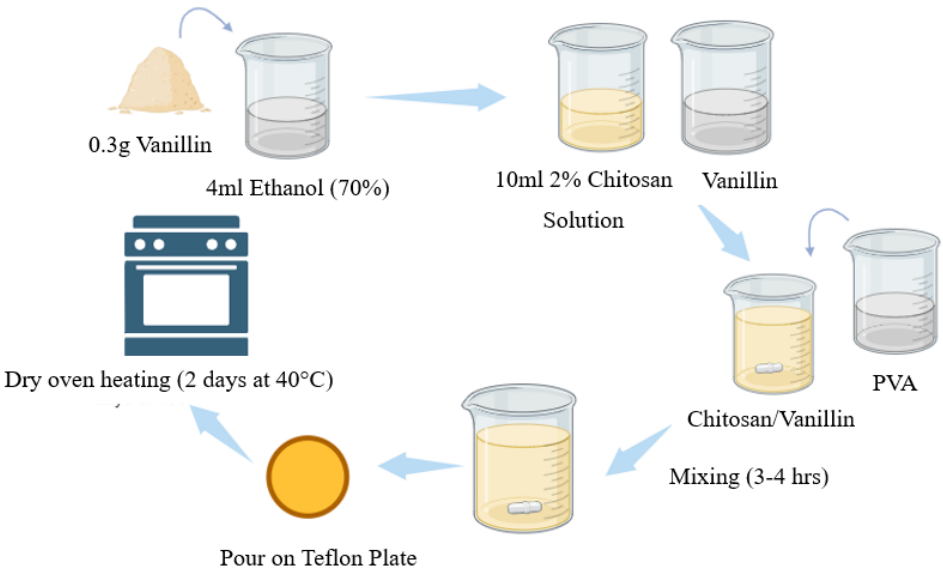

Figure 7 Synthesis of Chitosan/PVA/Vanillin Hydrogel

3.4 Synthesis of Secretome-Infused Hydrogel

To infuse MSC secretome into the Cs/PVA/V hydrogel, the previously described protocol was slightly modified. Secretome was added and mixed with CS + V solution before adding the PVA to maintain its viability and minimize exposure to environmental factors (Jammes et al., 2023). The secretome was added to CS + V solution in the ratio of 1:10 and mixed for 1-2 hours on the magnetic stirrer. Then, PVA was added to this solution and mixed for another 3 hours. Throughout the mixing, the temperature on the stirrer was kept 4 °C to protect secretome from temperature fluctuations. After mixing, the solution was poured into a Teflon plate and placed in the drying oven at 37 °C for two days. The dried secretome-infused hydrogel was lifted from the plate and stored at 4 °C in the refrigerator.

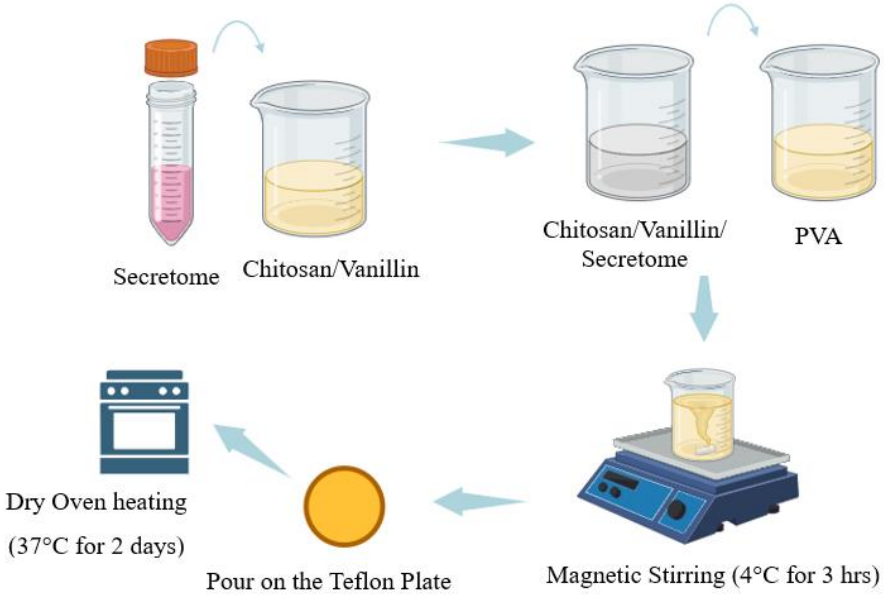

Figure 8 Synthesis of Secretome-Infused Hydrogel

3.5 Scanning Electron Microscopy

To assess the surface morphological characteristics of simple and infused hydrogel, samples were subject to Scanning Electron Microscopy (SEM) analysis. SEM provides high-resolution imaging of the surface structure of materials with detailed information on their topography, porosity, and morphology (Pourjavadi et al., 2017). The samples were prepared by drying them completely to the point there is no moisture left as moisture can interfere with the instrumental analysis. SEM analysis was carried out on JSM-6490A analytical scanning electron microscope present in the SEM lab at School of Chemical and Materials Engineering (SCME), NUST. Both the samples were coated with gold as a conductive material to enhance image quality.

3.6 X-ray Diffraction (XRD)

X-ray diffraction (XRD) is an analytical technique used to determine the crystalline structure, phase composition and molecular arrangement of materials in a non-destructive manner. To investigate the crystallinity, degree of polymer interaction and incorporation of bioactive components within the hydrogel matrix, the samples were subjected to XRD analysis. The analysis was carried out using D8-Advanced XRD instrument (Bruker, Germany) present in advanced

energy and materials lab at U.S.-Pakistan Center for Advanced Studies in Energy (USPCASE), NUST. The samples were completely dried before sending for analysis and secretome infused hydrogel was transported in an ice box to maintain its temperature. The XRD patterns were obtained using a current of 40mA, acceleration voltage of 40kV and scan speed of 0.5s.

3.7 Fourier Transform Infrared (FTIR) Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy is an analytical technique used to identify and characterize chemical bonds and functional groups in compounds. It gives distinct peaks at specific wavenumber range for specific functional groups (Ortega-Sánchez et al., 2024). The dried samples were sent to FTIR instrument present in the advanced energy and materials lab at U.S.-Pakistan Center for Advanced Studies in Energy (USPCASE), NUST. The FTIR spectra was obtained in the range of 400-4000 cm^{-1} and transmittance ranges of 20-110 a.u. This analysis was used to compare the changes in the specific functional group peaks of chitosan and PVA after the incorporation of secretome's bioactive compounds into the hydrogel matrix.

3.8 Water Uptake Analysis

Water uptake analysis is a crucial characterization experiment used to check hydrogel's hydrophilicity and swelling behavior. The experiment was carried out by cutting non-infused and secretome-infused hydrogels into uniform 1cm x 1cm sample sizes and calculating their dry weights. Then, the samples were submerged in phosphate buffer saline (PBS) and the pH of solutions was measured. Then regular time intervals were established, and after each interval each of the submerged sample was weighed again. The swelling ratio was calculated using the equation described below (Umer Shahzad et al., 2023):

$$\text{Water Uptake } \% = \frac{W_s - W_d}{W_d} \times 100$$

RESULTS

4.1 Scanning Electron Microscopy Analysis

The surface morphology and structure analysis of the developed PVA/CS/V hydrogel, and a variant infused with MSC secretome were investigated using scanning electron microscopy (SEM). Images of the fabricated hydrogels are presented in the figures below. Figure 9 shows a membrane surface that is compact yet slightly porous which is similar to native tissues when magnified to 2,500 and 10,000 times, indicating strong biocompatibility. This outcome is attributed to polymer blending, which addresses the drawbacks of single-material hydrogels. Overall, it can be observed that the PVA/CS/V hydrogel surface appears rough. However, figure 10 shows the SEM analysis of the PVA/CS/V hydrogel loaded with secretome shows a smooth and uniform surface without any visible pores or cracks, also magnified 2,500 and 10,000 times. This indicates a homogeneous distribution of the chitosan, PVA, vanillin and secretome components in the hydrogel matrix and this outcome is desirable for wound dressing applications as it provides a suitable environment for wound healing without causing any irritation. It appears that there is still some distortion present in the morphology, likely due to the absence of nanoparticles that would have contributed to a smoother and more uniform structure. This observation aligns with the findings of Shoma Suresh et al. 2020 and Amir Niazi et al. 2024.

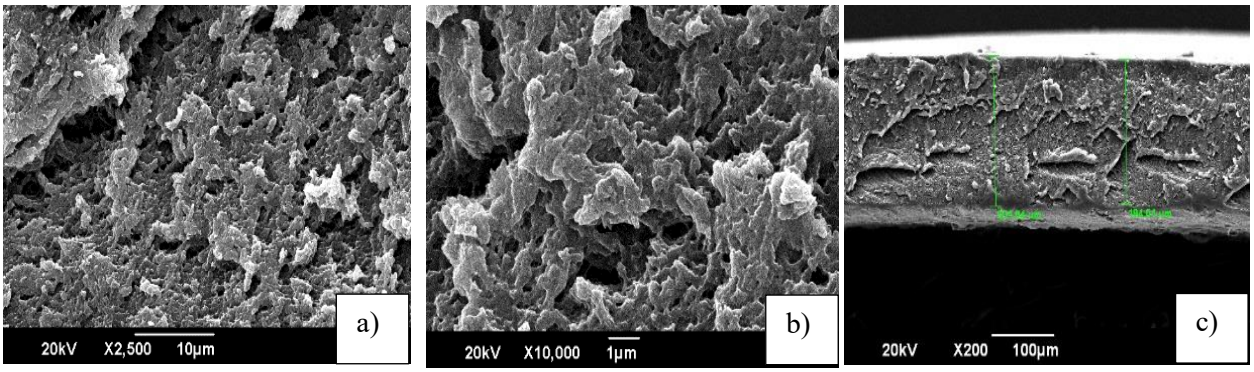

Figure 9 SEM images depicting the surface morphologies of Chitosan/PVA/Vanillin Hydrogel (from L-R) a) At 2500X Magnification b) At 10,000X c) Cross Sectional View

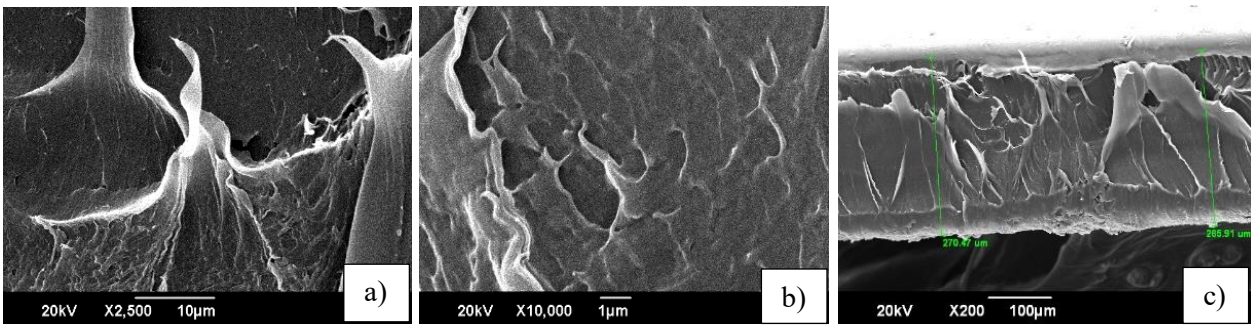

Figure 10 SEM images depicting the surface morphologies of Secretome-infused Hydrogel (from L-R) a) At 2500X Magnification b) At 10,000X c) Cross Sectional View

4.2 XRD Analysis

XRD analysis provides detailed insight into specific crystal structures, orientation and symmetry of crystal planes in materials. It is integral in the quality check of hydrogels as it facilitates identification of purity and structural integrity of individual components used in the hydrogel by providing sharp intensity peaks at corresponding angles. The XRD patterns depictive of chitosan and PVA were obtained as shown in Fig. 11 The sharp diffraction peak observed at $2\theta = 20°$ is characteristic of (110) diffraction plane of chitosan. The sharpness and intensity of this peak showed that chitosan retained its crystalline structure and chemical properties, suggesting a degree of order in the hydrogel matrix. The small shoulder at $2\theta = 13.5°$ is attributed to (101) diffraction plane of chitosan, this suggested a minor crystalline phase of chitosan in the hydrogel. The presence of both of these peaks collectively indicated a high degree of crystallinity in the matrix (Altinisik & Yurdakoc, 2013). The peak at $2\theta = 40°$ is attributed to the (200) diffraction

plane of PVA, showing that PVA was contributing to the overall crystallinity of the chitosan/PVA hydrogel network. These results were in agreement with the findings of Chen and Jiao (Chen et al., 2018) and confirmed the successful fabrication of CS/PVA/V hydrogel that had retained its chemical structure without any major crystallinity loss.

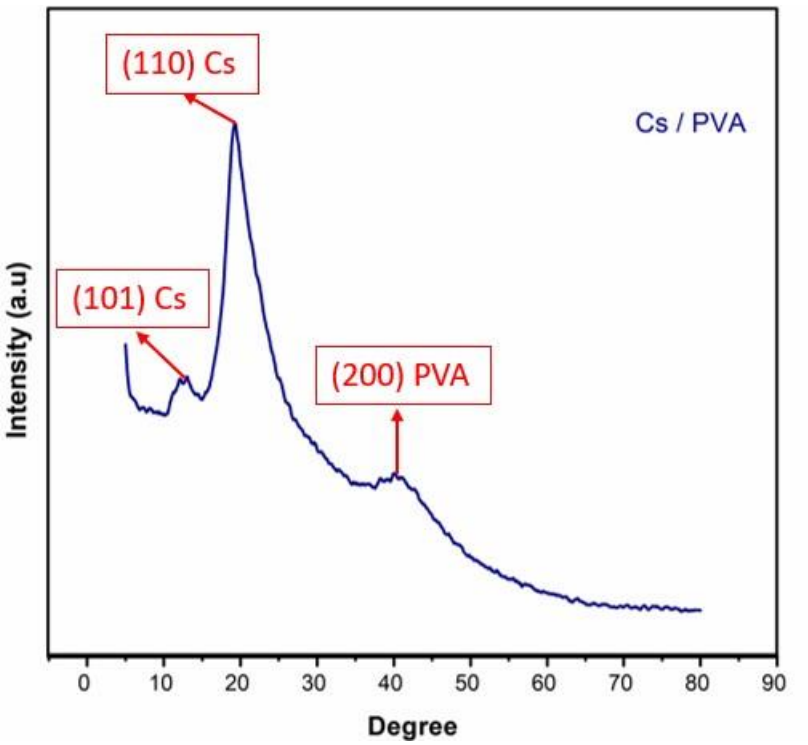

Figure 11 XRD Patterns of Cs/PVA Hydrogel

The XRD patterns of secretome-infused hydrogel were significantly different than the pure, non-infused hydrogel, as shown in figure 12. The distortion of the characteristic $2\theta = 20°$ peak of chitosan indicated that the infusion of secretome in the hydrogel causes a significant change in the overall chemical structure and crystallinity. The peak at $2\theta = 14°$ was not depictive of either of chitosan or PVA, and suggested the incorporation of secretome proteins. The small peak at $2\theta = 40°$ of PVA was observed in this graph as well. These findings collectively confirmed the incorporation of secretome in the hydrogel and also suggested that the infusion causes distortion in the overall crystalline structure of the hydrogel matrix, but only to a reasonable extent, while retaining its integrity.

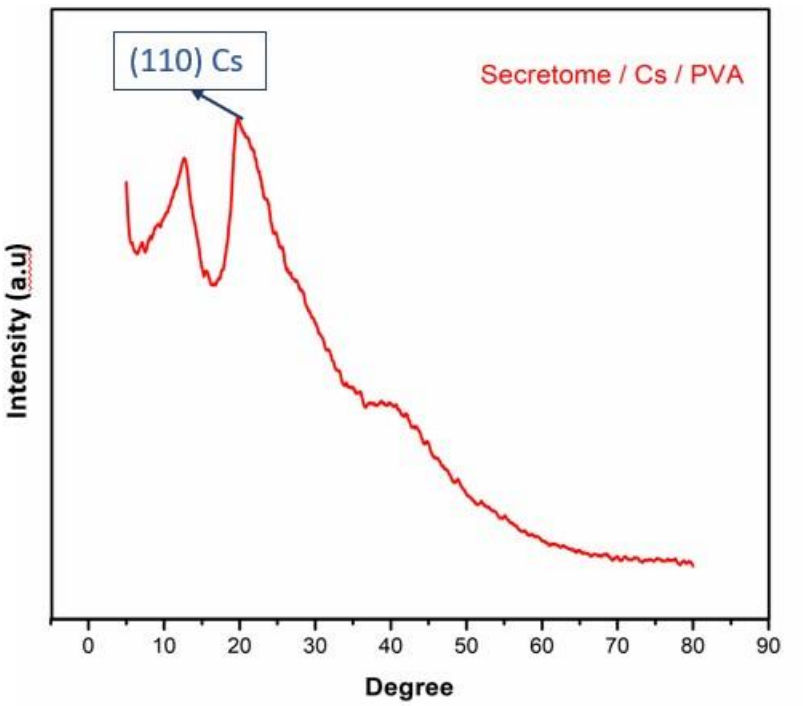

Figure 12 XRD Patterns of Secretome-infused Hydrogel

4.3 FTIR Spectroscopic Analysis

FTIR spectroscopy is an analytical technique used to identify the chemical structure of materials and provide characteristic peaks for functional groups present in the compounds. The FTIR spectra of non-infused and infused hydrogel are shown in figures 13 and 14 respectively. In figure 13, the first sharp peak at 3400 cm^{-1} is of hydroxyl group (OH stretching vibration). The second smaller peak at 2800 cm^{-1} is characteristic of CH stretching vibration. Both these peaks were indicative of the presence of chitosan and PVA in the hydrogel, as they both have CH- and OH groups in their chemical structure. In accordance to existing literature, the sharpness of these peaks indicate structural integrity and stability of the hydrogel. The peak at 1100 cm^{-1}, indicating CO- stretching vibration, suggested the presence of ether linkages, which are integral for crosslinking. Smaller peaks in the 1200-1300 cm^{-1} range correspond to C-O and C-N stretching vibrations which further solidified the presence and structural integrity of chitosan, PVA and vanillin. The stark difference observed in the secretome infused hydrogel was the change in the hydroxyl group peak at 3400 cm^{-1}. The peak lost its sharpness, which indicated that secretome components have interacted with the OH groups of chitosan and PVA, forming bonds which disrupted the original crystallinity structure. Multiple peaks in the 1400-1600 cm^{-1} range are indicative of C=O stretching vibration

of amide groups present in chitosan and secretome components. These findings confirmed the successful infusion of secretome in the hydrogel matrix as well as the biocompatibility of individual components of the infused hydrogel with each other.

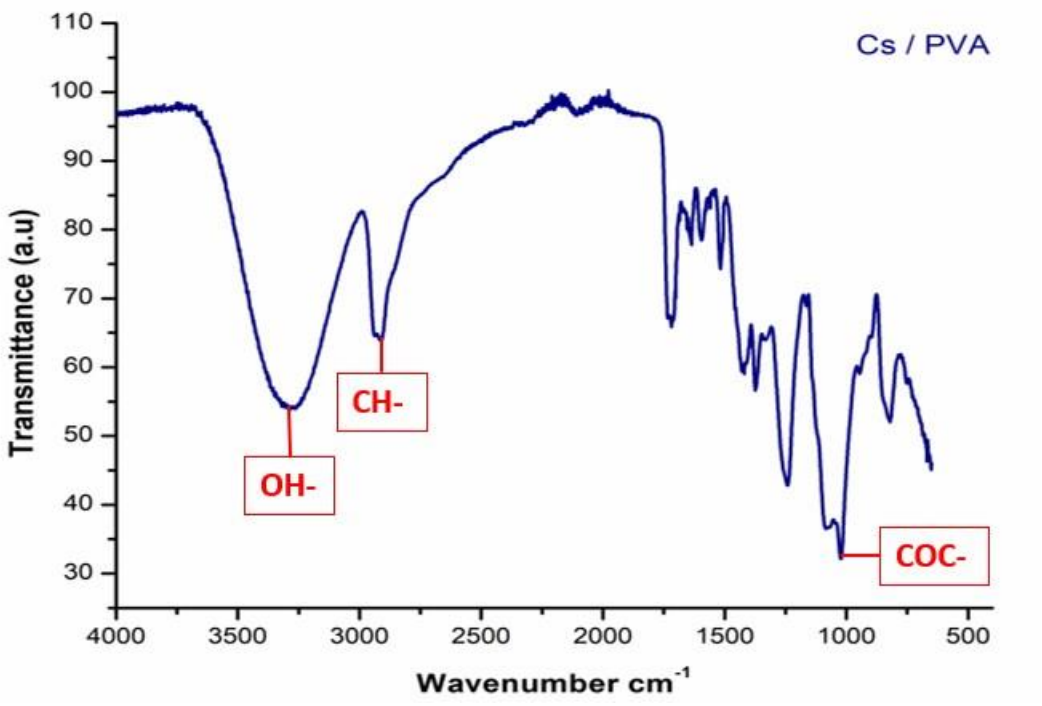

Figure 13 FTIR Spectra of Cs/PVA Hydrogel

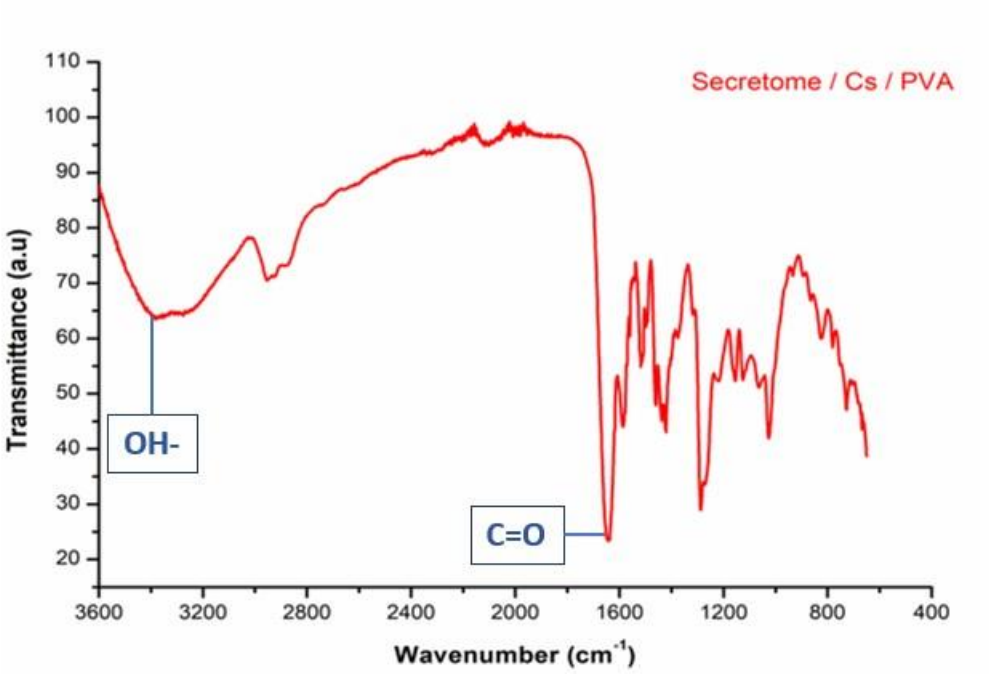

Figure 14 FTIR Spectra of Secretome-infused Hydrogel

4.4 Water Uptake Analysis

Water uptake analysis is an important factor that impacts the infiltration, mechanical characteristics, and adhesion of cells in hydrogel membranes. Chitosan, with its positively charged chains, forms an extensive hydrated layer that efficiently absorbs water molecules in humid conditions. To analyze the water uptake of our non-infused and infused hydrogels, they were submerged in PBS, for a set duration, and their respective weights were measured after specific intervals. Chitosan with its positively charged chains, forms an extensive hydration layer that efficiently absorbs water in humid conditions. To analyze the water uptake of our non-infused and infused hydrogels, they were submerged in PBS, for a set duration, and their weights were measured after specific intervals.

Figure 15 represents the water uptake analysis of our non-infused (H) and secretome-infused (SH) hydrogels at various time intervals. The results show an overall drastic increase in weights of both hydrogels with increasing time. The absorption ratios substantially surged in the initial intervals as can be seen in the graph and the increase in weight gradually slowed down towards the later intervals. The SH although swelled significantly, but their overall water uptake ratio was much lower than the H. This finding showed that secretome had been successfully infused in the

hydrogel. The secretome bio-components, when interacting with hydrogel matrix, take up more space and inhabit the pores that otherwise absorbed water more efficiently.

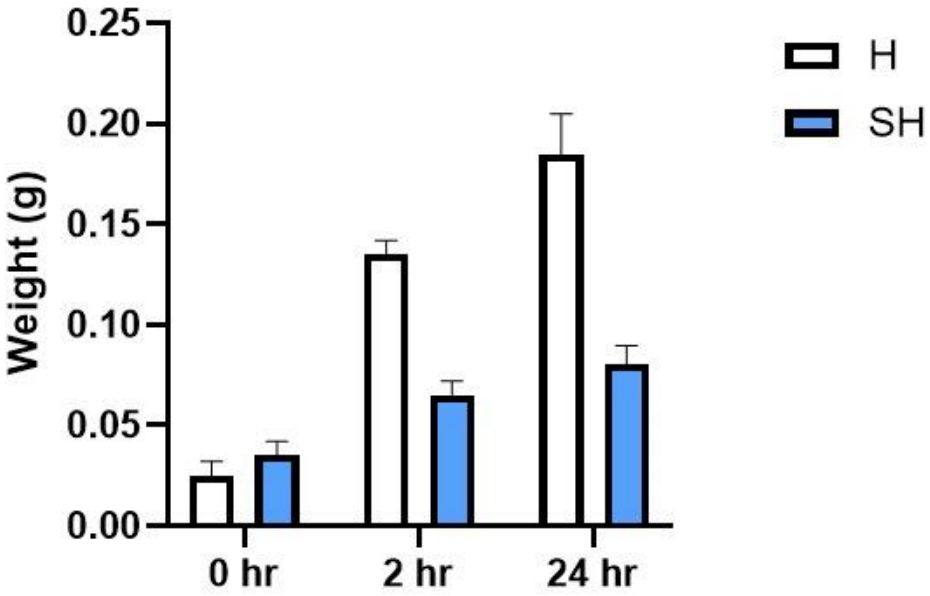

Figure 15 Water Uptake Analysis at periodic time intervals

DISCUSSION

This study was aimed to develop an optimized protocol for the fabrication of chitosan/PVA/Vanillin hydrogel and infusion of MSC secretome in the fabricated hydrogel. This study further sought to characterize the developed secretome-infused hydrogels for their potential in regenerative biomedical applications.

Mesenchymal stem cells have been extensively used for their therapeutic potential but their difficulty in isolation, expansion and culture maintenance limits their ease of use, establishing a need for cell-free therapies. The secretome of MSCs is a compelling alternative, offering distinct advantages over conventional MSC-based therapies. It can be readily obtained from conditioned media or exosome-rich fractions, circumventing the logistical complexities associated with cell harvesting and expansion (Phinney & Pittenger, 2017). Moreover, the MSC secretome exhibits remarkable stability and shelf-life, rendering it amenable to storage and transportation, thereby facilitating its widespread clinical deployment.

The inherent properties of the MSC secretome confer several therapeutic advantages. the secretome contains a diverse repertoire of bioactive factors, including growth factors, cytokines, and extracellular vesicles, which collectively carry out tissue repair and regeneration (Monsel et al., 2015). The immunomodulatory effects of secretome attenuate inflammatory responses and foster a regenerative microenvironment conducive to tissue healing, with little to no immunogenicity (Golchin et al., 2020). However, secretome cannot be used as it is and needs a delivery system for its sustained and localized release profile.

Hydrogels are three-dimensional, hydrophilic polymer networks capable of absorbing and retaining large amounts of water while maintaining their structural integrity. They serve as suitable drug delivery systems owing to their biocompatibility and tunable porosity. The hydrogel composition used for this study was chosen according to the biodegradability, cytotoxicity, biocompatibility and mechanical properties of the individual constituents. Chitosan, as a natural polymer, provides optimal conditions for cellular proliferation and differentiation, complemented by Polyvinyl Alcohol (PVA) which reinforces structural integrity (Firzanah Hisham et al., 2023). Vanillin is an effective cross-linker with low cytotoxicity that ensures stability of the hydrogel components (Bezerra et al., 2016).

Existing literature suggested the healing properties of secretome as well as its potential to be compatible with biomaterials. Based on that, we hypothesized that successful incorporation of secretome into CS/PVA/V hydrogel can increase the collective biocompatibility and synergistically enhance their respective healing and immunomodulatory properties.

The development and optimization of protocols for both the Chitosan/PVA/Vanillin (CS/PVA/V) hydrogel and its secretome-infused counterpart involved a systematic exploration of various parameters. The published protocols for similar experiments were thoroughly studied to help develop the most effective protocol. This involved adjustments in component concentrations, drying times, temperatures, and ratios to achieve the desired structural integrity, mechanical properties, and secretome viability. Each step, from polymer dissolution to crosslinking and incorporation of the MSC secretome, was methodically refined to ensure reproducibility and efficacy in facilitating therapeutic delivery.

The successful fabrication of control as well as infused hydrogels was checked through characterization techniques such as SEM, XRD and FTIR. Each of the analysis provided valubale insight, confirming the objectives of this study. SEM analysis revealed a porous and interconnected structure in both control and infused hydrogels. The incorporation of the MSC secretome did not induce substantial alterations in surface morphology, suggesting the preservation of the hydrogel's architecture.

XRD analysis provided characteristic peaks associated with the crystalline structures of chitosan (110) and PVA (200) within the hydrogel matrix. Notably secretome slightly altered the crystalline structure, indicating successful integration of the secretome into the hydrogel matrix. FTIR analysis identified the presence of functional groups linked to chitosan, PVA, and vanillin within the hydrogels such as OH-, CH-, COC and more. Introduction of the MSC secretome led to the emergence of new peaks associated with secretome biomolecules, affirming successful integration into the hydrogel matrix.

Overall, the results of this study confirmed the successful fabrication of both control and MSC secretome-infused hydrogels through comprehensive characterization techniques including SEM, XRD, and FTIR analysis. These findings fulfill the identified need for an effective delivery system for MSC secretome, addressing the limitations of cell-based therapies.

CONCLUSION

The development and characterization of the Chitosan/PVA hydrogel, followed by the infusion of MSC secretome, has been successfully demonstrated, showcasing a novel approach in biomaterial design. XRD, FTIR, and SEM analysis revealed that the components of mesenchymal stem cell secretome are compatible with chitosan and PVA.

FUTURE PROSPECTS

The future prospects for this research can be accomplished in several critical areas of investigation. Antibacterial testing of both non-infused and secretome-infused hydrogels can be performed to assess their capacity to prevent bacterial growth. *In vitro* cytotoxicity and cell viability assays can be carried out on skin fibroblasts and other cell lines to guarantee the biocompatibility and safety of the hydrogels. Additionally, the composition of the secretome and the characterization of its components can be executed to identify the precise factors that are responsible for the therapeutic effects. Lastly, in vivo studies can be conducted using mice models to assess the wound healing abilities of the secretome-infused hydrogels, thus providing a full spectrum of the clinical applications of the hydrogels.

REFERENCES

Ahangar, P., Mills, S. J., & Cowin, A. J. (2020). Mesenchymal Stem Cell Secretome as an Emerging Cell-Free Alternative for Improving Wound Repair. *International Journal of Molecular Sciences*, *21*(19), 7038. https://doi.org/10.3390/ijms21197038

Altinisik, A., & Yurdakoc, K. (2013). Chitosan/poly(vinyl alcohol) hydrogels for amoxicillin release. Polymer Bulletin, 71(3), 759–774. https://doi.org/10.1007/s00289-013-1090-1

Amir, F., Muhammad, Umer Shahzad Malik, Jahan, Z., Saadia Andleeb, Ahmad, T., & Zartasha Mustansar. (2024). A multifunctional vanillin-infused chitosan-PVA hydrogel reinforced by nanocellulose and CuO-Ag nanoparticles as antibacterial wound dressing. International Journal of Biological Macromolecules, 258, 128831–128831. https://doi.org/10.1016/j.ijbiomac.2023.128831

Asadi, N., Pazoki-Toroudi, H., Del Bakhshayesh, A. R., Akbarzadeh, A., Davaran, S., & Annabi, N. (2021). Multifunctional hydrogels for wound healing: Special focus on biomacromolecular based hydrogels. *International Journal of Biological Macromolecules*, *170*, 728–750. https://doi.org/10.1016/j.ijbiomac.2020.12.202

Bezerra, D. P., Soares, A. K. N., & de Sousa, D. P. (2016). Overview of the Role of Vanillin on Redox Status and Cancer Development. Oxidative Medicine and Cellular Longevity, 2016. https://doi.org/10.1155/2016/9734816

Chen, Y.-N., Jiao, C., Zhao, Y., Zhang, J., & Wang, H. (2018). Self-Assembled Polyvinyl Alcohol–Tannic Acid Hydrogels with Diverse Microstructures and Good Mechanical Properties. ACS Omega, 3(9), 11788–11795. https://doi.org/10.1021/acsomega.8b02041

Dreiss, C. A. (2020). Hydrogel design strategies for drug delivery. *Current Opinion in Colloid & Interface Science*, *48*, 1–17. https://doi.org/10.1016/j.cocis.2020.02.001

Elisabetta, M., Shaw, G., Carluccio, S., Reverberi, D., Sercia, L., Persano, L., Pisignano, D., Cortese, K., Barry, F. P., Murphy, J., & Gentili, C. (2020). The Secretome Derived From Mesenchymal Stromal Cells Cultured in a Xeno-Free Medium Promotes Human Cartilage Recovery in vitro. 8. https://doi.org/10.3389/fbioe.2020.00090

Fan, X.-L., Zhang, Y., Li, X., & Fu, Q.-L. (2020). Mechanisms underlying the protective effects of mesenchymal stem cell-based therapy. *Cellular and Molecular Life Sciences*, *77*(14), 2771–2794. https://doi.org/10.1007/s00018-020-03454-6

Firzanah Hisham, Akmal, M., Ahmad, F., Ahmad, K., & Noorasikin Samat. (2023). Biopolymer chitosan: Potential sources, extraction methods, and emerging applications. Ain Shams Engineering Journal, 102424–102424. https://doi.org/10.1016/j.asej.2023.102424

Golchin, A., Seyedjafari, E., & Ardeshirylajimi, A. (2020). Mesenchymal Stem Cell Therapy for COVID-19: Present or Future. Stem Cell Reviews and Reports, 1–7. https://doi.org/10.1007/s12015-020-09973-w

Hoang, D. M., Pham, P. T., Bach, T. Q., Ngo, A. T. L., Nguyen, Q. T., Phan, T. T. K., Nguyen, G. H., Le, P. T. T., van Hoang, T., Forsyth, N. R., Heke, M., & Nguyen, L. T. (2022). Stem cell-based therapy for human diseases. *Signal Transduction and Targeted Therapy*, *7*(1), 272. https://doi.org/10.1038/s41392-022-01134-4

Huang, Y.-Z., Gou, M., Da, L.-C., Zhang, W.-Q., & Xie, H.-Q. (2020). Mesenchymal Stem Cells for Chronic Wound Healing: Current Status of Preclinical and Clinical Studies. *Tissue Engineering. Part B, Reviews*, *26*(6), 555–570. https://doi.org/10.1089/ten.TEB.2019.0351

Ijaola, A. O., Akamo, D. O., Damiri, F., Akisin, C. J., Bamidele, E. A., Ajiboye, E. G., Berrada, M., Onyenokwe, V. O., Yang, S.-Y., & Asmatulu, E. (2022). Polymeric biomaterials for wound healing applications: A comprehensive review. *Journal of Biomaterials Science. Polymer Edition*, *33*(15), 1998–2050. https://doi.org/10.1080/09205063.2022.2088528

Jammes, M., Romain Contentin, Fabrice Audigié, Frédéric Cassé, & Philippe Galéra. (2023). Effect of pro-inflammatory cytokine priming and storage temperature of the mesenchymal stromal cell (MSC) secretome on equine articular chondrocytes. Frontiers in Bioengineering and Biotechnology, 11. https://doi.org/10.3389/fbioe.2023.1204737

Karakurt, I., Ozaltin, K., Vargun, E., Kucerova, L., Suly, P., Harea, E., Minařík, A., Štěpánková, K., Lehocky, M., Humpolícek, P., Vesel, A., & Mozetic, M. (2021). Controlled release of enrofloxacin by vanillin-crosslinked chitosan-polyvinyl alcohol blends. *Materials Science & Engineering. C, Materials for Biological Applications*, *126*, 112125. https://doi.org/10.1016/j.msec.2021.112125

Kaur, G., Narayanan, G., Garg, D., Sachdev, A., & Matai, I. (2022). Biomaterials-Based Regenerative Strategies for Skin Tissue Wound Healing. *ACS Applied Bio Materials*, *5*(5), 2069–2106. https://doi.org/10.1021/acsabm.2c00035

Kucharzewski, M., Rojczyk, E., Wilemska-Kucharzewska, K., Wilk, R., Hudecki, J., & Los, M. J. (2019). Novel trends in application of stem cells in skin wound healing. *European Journal of Pharmacology*, *843*, 307–315. https://doi.org/10.1016/j.ejphar.2018.12.012

Ma, H., Siu, W.-S., & Leung, P.-C. (2023). The Potential of MSC-Based Cell-Free Therapy in Wound Healing-A Thorough Literature Review. *International Journal of Molecular Sciences*, *24*(11), 9356. https://doi.org/10.3390/ijms24119356

Mao, A. S., & Mooney, D. J. (2015). Regenerative medicine: Current therapies and future directions. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(47), 14452–14459. https://doi.org/10.1073/pnas.1508520112

Margiana, R., Markov, A., Zekiy, A. O., Hamza, M. U., Al-Dabbagh, K. A., Al-Zubaidi, S. H., Hameed, N. M., Ahmad, I., Sivaraman, R., Kzar, H. H., Al-Gazally, M. E., Mustafa, Y. F., & Siahmansouri, H. (2022). Clinical application of mesenchymal stem cell in regenerative medicine: A narrative review. *Stem Cell Research & Therapy*, *13*(1), 366. https://doi.org/10.1186/s13287-022-03054-0

Md Fadilah, N. I., Mohd Abdul Kader Jailani, M. S., Badrul Hisham, M. A. I., Sunthar Raj, N., Shamsuddin, S. A., Ng, M. H., Fauzi, M. B., & Maarof, M. (2022). Cell secretomes for wound healing and tissue regeneration: Next generation acellular based tissue engineered products. *Journal of Tissue Engineering*, *13*, 20417314221114273. https://doi.org/10.1177/20417314221114273

Monsel, A., Zhu, Y., Gennai, S., Hao, Q., Hu, S., Rouby, J.-J., Rosenzwajg, M., Matthay, M. A., & Lee, J. W. (2015). Therapeutic Effects of Human Mesenchymal Stem Cell–derived Microvesicles in Severe Pneumonia in Mice. American Journal of Respiratory and Critical Care Medicine, 192(3), 324–336. https://doi.org/10.1164/rccm.201410-1765oc

Ortega-Sánchez, C., Yaaziel Melgarejo-Ramírez, Rodríguez-Rodríguez, R., Jorge Armando Jiménez-Ávalos, Giraldo-Gomez, D. M., Gutiérrez-Gómez, C., Jacobo Rodriguez-Campos, Luna-Bárcenas, G., Velasquillo, C., Valentín Martínez-López, & García-Carvajal, Z. Y. (2024). Hydrogel Based on Chitosan/Gelatin/Poly(Vinyl Alcohol) for In Vitro Human Auricular Chondrocyte Culture. Polymers, 16(4), 479–479. https://doi.org/10.3390/polym16040479

Phinney, D. G., & Pittenger, M. F. (2017). Concise Review: MSC-Derived Exosomes for Cell-Free Therapy. STEM CELLS, 35(4), 851–858. https://doi.org/10.1002/stem.2575

Pourjavadi, A., Tavakoli, E., Motamedi, A., & Salimi, H. (2017). Facile synthesis of extremely biocompatible double-network hydrogels based on chitosan and poly(vinyl alcohol) with enhanced mechanical properties. Journal of Applied Polymer Science, 135(7), 45752. https://doi.org/10.1002/app.45752

Rahmati, M., Pennisi, C. P., Budd, E., Mobasheri, A., & Mozafari, M. (2018). Biomaterials for Regenerative Medicine: Historical Perspectives and Current Trends. *Advances in Experimental Medicine and Biology*, *1119*, 1–19. https://doi.org/10.1007/5584_2018_278

Umer Shahzad, Duan, Q., Muhammad, Jahan, Z., Usman Liaqat, Sher, F., Gan, Y., & Hou, H. (2023). Vanillin cross-linked hydrogel membranes interfacial reinforced by carbon nitride nanosheets for

enhanced antibacterial activity and mechanical properties. Chinese Chemical Letters/Chinese Chemical Letters, 34(4), 108071–108071. https://doi.org/10.1016/j.cclet.2022.108071

Wechsler, M. E., Rao, V. V., Borelli, A. N., & Anseth, K. S. (2021). Engineering the MSC Secretome: A Hydrogel Focused Approach. *Advanced Healthcare Materials*, *10*(7), e2001948. https://doi.org/10.1002/adhm.202001948

Xiong, S., Li, R., Ye, S., Ni, P., Shan, J., Yuan, T., Liang, J., Fan, Y., & Zhang, X. (2022). Vanillin enhances the antibacterial and antioxidant properties of polyvinyl alcohol-chitosan hydrogel dressings. *International Journal of Biological Macromolecules*, *220*, 109–116. https://doi.org/10.1016/j.ijbiomac.2022.08.052

Zhang, P., Qi, J., Zhang, R., Zhao, Y., Yan, J., Gong, Y., Liu, X., Zhang, B., Wu, X [Xiao], Wu, X [Xiuping], Zhang, C., Zhao, B., & Li, B. (2024). Recent advances in composite hydrogels: Synthesis, classification, and application in the treatment of bone defects. *Biomaterials Science*, *12*(2), 308–329. https://doi.org/10.1039/D3BM01795H

TURNITIN PLAGIARISM REPORT

Group 8.docx

ORIGINALITY REPORT

14%	9%	10%	5%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Submitted to Higher Education Commission Pakistan Student Paper	2%
2	dokumen.pub Internet Source	1%
3	Fatima Amir, Muhammad Bilal Khan Niazi, Umer Shahzad Malik, Zaib Jahan, Saadia Andleeb, Tahir Ahmad, Zartasha Mustansar. "A multifunctional vanillin-infused chitosan-PVA hydrogel reinforced by nanocellulose and CuO-Ag nanoparticles as antibacterial wound dressing", International Journal of Biological Macromolecules, 2024 Publication	1%
4	Ria Margiana, Alexander Markov, Angelina O. Zekiy, Mohammed Ubaid Hamza et al. "Clinical application of mesenchymal stem cell in regenerative medicine: a narrative review", Stem Cell Research & Therapy, 2022 Publication	1%
5	prr.hec.gov.pk	