Study of bone formation properties of tricalcium phosphate and insulin-infused hydrogel- An in-vitro study

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ABSTRACT

Background

Insulin-infused hydrogel represents a cutting-edge approach in the field of bone tissue engineering, offering a platform for promoting bone regeneration and repair. Insulin has been shown to induce the formation of new blood vessels, which is important in providing nutrients and oxygen to the developing bone tissue and supporting its growth. For the product to be successful, it has to have a good osteogenic potential as well as biocompatibility.

Aim

We aim to develop a scaffold that can promote bone regeneration and improve outcomes in regenerative medicine. The objective is to study the biocompatibility and osteogenic properties of the developed scaffold.

Methods

The study was carried out in BPG research laboratory of Saveetha Dental College, Chennai from March 2024 to June 2024. The study included assessing the biocompatibility and osteogenic properties of the bioscaffold infused with TCP and insulin.

To assess cell viability, MTT assay was performed. MG 63 cells were seeded in a 96-well plate at an appropriate density, ensuring even distribution by gently rocking the plate back and forth, and incubated them for 18-24 hours at 37°C with 5% CO2 to allow them to adhere and grow. Finally, they measured the absorbance of the solubilized formazan product at 540-595 nm using a microplate reader, with absorbance values directly proportional to the number of viable cells.

Quantitative PCR (qPCR) analysis was done to analyse the bone forming potential by quantifying the osteogenic markers, which are critical indicators of bone formation and differentiation.

Results

Morphological evaluation of the Biocompatibility (Phase contrast) and cell proliferation levels (Live/Dead) with and without insulin loaded CS+SA+βTC with control group for different time point of incubation, using phase contrast microscopy & Fluorescent microscopy (20x objective) indicated that the number of dead cells are far lesser in the hydrogel scaffold with insulin than there are in the bioscaffold without insulin. The expression of Osteogenic-specific mRNA and protein was assessed by qRT-PCR, analysis of which indicated that
the prepared bioscaffold with insulin has the osteogenic property comparable to that of the time-tested Bio-oss.

Conclusion

From the results obtained, it can be inferred that the developed hydrogel scaffold with insulin has a high osteogenic potential. It maintains the cell viability which proves its property of biocompatibility.

INTRODUCTION

The composite scaffold developed can only provide bone generation and improve patients' outcomes when it has antioxidant properties and hemocompatibility. Antioxidants play a crucial role in cell proliferation by protecting cells from oxidative stress. Antioxidants protect cells from oxidative damage, support DNA integrity, regulate cell signaling pathways, maintain mitochondrial function, and control inflammation, all of which contribute to creating a conducive environment for cell proliferation. Hemocompatibility refers to the compatibility of a material or product with blood and the circulatory system. It is essential to assess the hemocompatibility of medical devices, implants, and other products that come into contact with blood to ensure that they do not cause adverse effects such as thrombosis, hemolysis, platelet activation, or inflammation [1].

MATERIALS AND METHODS

The study was carried out in BPG research laboratory of Saveetha Dental College, Chennai from March 2024 to June 2024. The study included assessing the biocompatibility properties and hemocompatibility of the bioscaffold infused with TCP and insulin.

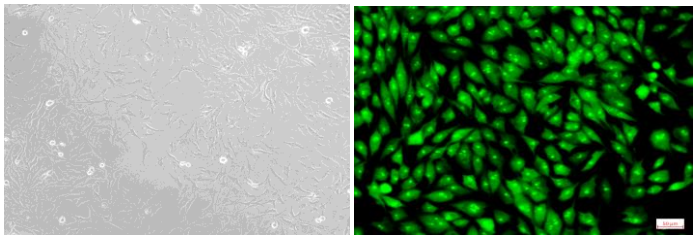
Quantitative PCR (qPCR) analysis was done to quantify the expression levels of osteogenic markers, which are critical indicators of bone formation and differentiation. The process began with the extraction of total RNA from cells, which was followed by synthesis of complementary DNA (cDNA) through reverse transcription. Specific primers were used for target osteogenic genes such as Runx2, OCN, and COL1A1. The reaction involved amplifying the cDNA with these primers, which was further detected in real-time through fluorescence detection.

To check cell morphology and viability using fluorescent microscopy in the presence of the prepared bioscaffold, the cells were seeded onto the scaffold and were allowed to adhere and grow for a specified period. The cells were stained using fluorescent dyes, such as Calcein-AM for live cells, which showed green fluorescence. The cells were then fixed using paraformaldehyde. Following this, the stained scaffold with cells was mounted onto a microscope. A fluorescent microscope to capture images of the cells on the scaffold, adjusting the microscope settings to visualize the different fluorescent signals. Finally, the images were analysed to assess cell morphology and viability, where live cells exhibited green fluorescence.

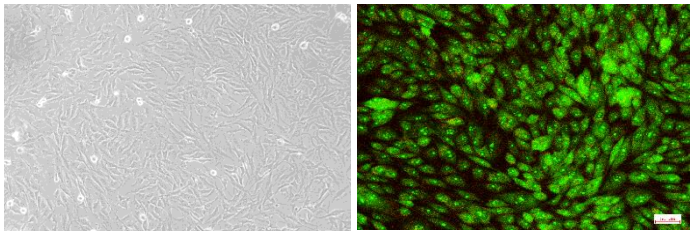
RESULTS

CELL MORPHOLOGY STUDY USING PHASE CONTRAST MICROSCOPY

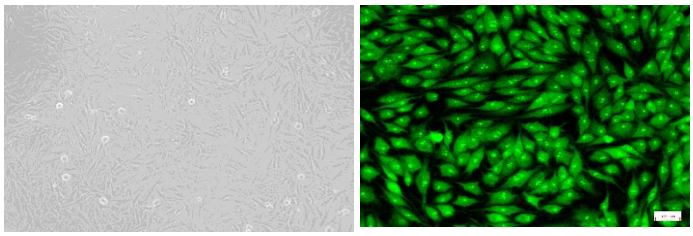
Control



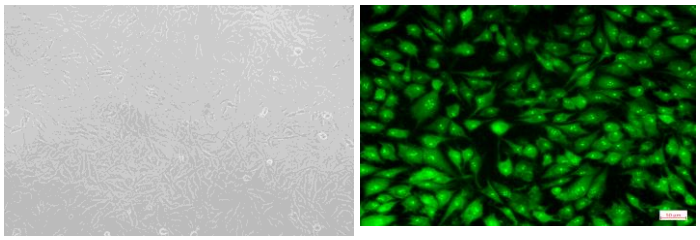
Bio - Oss



CS+SA+βTC

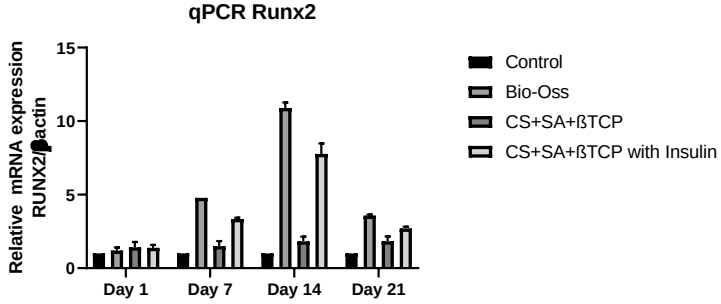


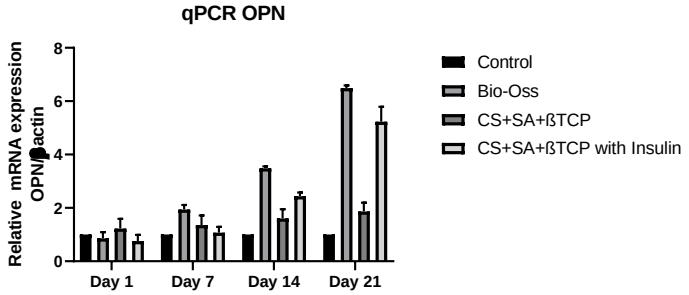
CS+SA+βTCP+insulin

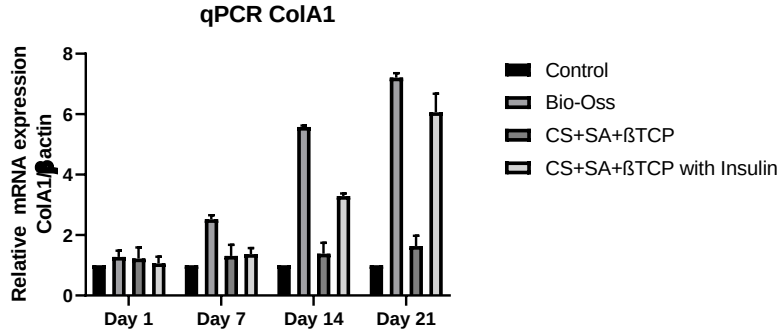


Morphological evaluation of the Biocompatibility (Phase contrast) and cell proliferation levels (Live/Dead) with and without insulin loaded CS+SA+βTC with control group for different time point of incubation, using phase contrast microscopy & Fluorescent microscopy (20x objective)

Number of dead cells are far lesser in the hydrogel scaffold with insulin than there are in the bioscaffold without insulin







Osteogenic markers levels by qPCR analysis

The expression of Osteogenic-specific mRNA and protein was assessed by qRT-PCR

From the graphs we can make out that the prepared bioscaffold with insulin has the osteogenic property comparable to that of the time-tested Bio-oss.

DISCUSSION

Quantitative PCR (qPCR) analysis is a vital technique in tissue engineering for measuring the levels of osteogenic markers, which are key indicators of bone formation and differentiation. This method allows for the precise quantification of gene expression, making it an essential tool in evaluating the effectiveness of various materials and conditions used in bone tissue engineering [1]. The process of qPCR analysis begins with the extraction of total RNA from the cells or tissues of interest. RNA extraction is a crucial step, as it ensures the purity and integrity of the RNA, which is necessary for accurate downstream analysis. Once the RNA is extracted, it is reverse transcribed into complementary DNA (cDNA) using reverse transcriptase enzymes. This step is essential because qPCR relies on the amplification of DNA, rather than RNA [2].

Specific primers are designed for the target osteogenic markers, which include genes such as ALP (Alkaline Phosphatase), BMP2 (Bone Morphogenetic Protein 2), Runx2 (Runt-related transcription factor 2), OCN (Osteocalcin), and COL1A1 (Collagen Type I Alpha 1) [3]. These markers are critical for bone formation and differentiation, and their expression levels provide valuable insights into the osteogenic potential of various treatments and materials [4]. The qPCR reaction is set up by mixing the cDNA with the primers and a qPCR master mix, which contains DNA polymerase, nucleotides, and a fluorescent dye or probe. The qPCR machine then amplifies the cDNA through repeated cycles

of heating and cooling, allowing the DNA polymerase to synthesize new DNA strands [5]. The fluorescent dye or probe binds to the newly synthesized DNA, and the increase in fluorescence is measured in real-time. This real-time measurement enables the quantification of gene expression levels, with higher fluorescence indicating higher levels of gene expression [6].

Data analysis in qPCR involves comparing the amplification curves of the target genes to a standard curve or using the ΔΔCt method for relative quantification. The standard curve method involves creating a series of dilutions of a known quantity of DNA and plotting the resulting amplification curves [7]. The unknown samples' amplification curves are then compared to the standard curve to determine their concentration. The ΔΔCt method, on the other hand, involves normalizing the target gene expression to a reference gene and comparing the expression levels between different samples or treatments [8]. In tissue engineering, qPCR analysis of osteogenic markers is used to evaluate the effectiveness of different scaffold materials, growth factors, and other treatments in promoting bone formation. By measuring the expression levels of these markers, researchers can determine the osteogenic potential of various materials and conditions [9]. For example, in bone tissue engineering, the qPCR analysis can reveal how well osteoblasts (bone-forming cells) and other cell types involved in bone regeneration respond to different scaffold materials. This information is vital for optimizing scaffold properties to enhance bone tissue formation and integration with the host tissue [10].

Additionally, qPCR analysis is valuable in cytotoxicity testing of materials used in tissue engineering. It helps in identifying any toxic effects that materials might have on cells, ensuring that only biocompatible materials are used in the development of tissue-engineered products. This ensures the safety and efficacy of the materials used in regenerative therapies [11]. Overall, qPCR analysis of osteogenic markers is an indispensable tool in tissue engineering, providing essential data on gene expression levels, cell viability, and cytotoxicity [12]. Its applications in evaluating scaffold materials, growth factors, and bioactive molecules contribute significantly to the advancement of regenerative medicine and the development of effective tissue-engineered products [13].

Fluorescent microscopy is an invaluable technique in cell biology for assessing cell viability, enabling researchers to distinguish between live and dead cells with precision. This method utilizes fluorescent dyes that target specific cellular components, allowing for clear visualization under a microscope. Among the commonly used dyes is Ethidium Homodimer-2, which selectively enters cells with compromised membranes, typically dead cells, and emits a bright red fluorescence. This characteristic makes it an excellent choice for identifying non-viable cells in a sample [14]. On the other hand, dyes like SYTO 10 permeate both live and dead cells but are eventually excluded from dead cells, resulting in green fluorescence in viable cells [15]. Propidium Iodide (PI) is another widely used dye that only penetrates cells with damaged membranes, marking dead cells with a distinct red fluorescence. Combining Acridine Orange (AO) with Propidium Iodide (PI) offers a comprehensive approach, as AO stains all nucleated cells to emit green fluorescence, while PI stains only the dead cells red, providing a clear distinction between live and dead cells [16].

The practical application of these dyes can be seen in the use of the LIVE/DEAD Viability/Cytotoxicity Kit, a popular assay in cell viability studies [17]. This kit leverages the differential staining properties of Calcein AM and Ethidium Homodimer-1 to provide a vivid contrast between live and dead cells. Calcein AM penetrates live cells and is converted into green fluorescent calcein by intracellular esterases, whereas Ethidium Homodimer-1 only enters dead cells and binds to nucleic acids, producing a red fluorescence [18]. This dual-staining approach allows for a straightforward and accurate assessment of cell viability under a fluorescence microscope.

Fluorescent microscopy, therefore, plays a critical role in various biological and medical research fields, from cancer research to drug development. By enabling precise and reliable visualization of

cell viability, it aids scientists in understanding cellular responses to different treatments and conditions, ultimately contributing to advancements in healthcare and biotechnology [19]. The ability to use multiple dyes and staining techniques provides flexibility and enhances the accuracy of cell viability assessments, making fluorescent microscopy a cornerstone method in modern cell biology research. Whether for routine lab assessments or sophisticated research studies, this technique offers a robust and versatile solution for discerning cell health and viability, driving forward our understanding of cellular processes and the development of new therapeutic strategies [20].

CONCLUSION

From the results obtained, it can be inferred that the developed hydrogel scaffold with insulin has a high osteogenic potential. It maintains the cell viability which proves its property of biocompatibility.

This study conducted, will allow researchers to evaluate the osteogenic potential of various scaffold materials and conditions, optimize bone tissue regeneration strategies, and ensure the biocompatibility of materials used in regenerative therapies. By providing precise and quantitative insights into gene expression, qPCR analysis significantly advances the development of effective tissue-engineered products.

REFERENCES

1. Desai TA. In the spotlight: tissue engineering--quantitative analysis of complex 3-D tissues. IEEE Rev Biomed Eng. 2009;2:21-2. doi: 10.1109/RBME.2009.2034243. PMID: 22275041.
2. Adamo A, Bruno A, Menallo G, Francipane MG, Fazzari M, Pirrone R, Ardizzone E, Wagner WR, D'Amore A. Blood Vessel Detection Algorithm for Tissue Engineering and Quantitative Histology. Ann Biomed Eng. 2022 Apr;50(4):387-400. doi: 10.1007/s10439-022-02923-2. Epub 2022 Feb 16. PMID: 35171393; PMCID: PMC8917109.
3. Song Z, Wu C, Sun S, Li H, Wang D, Gong J, Yan Z. Quantitative analysis of factors influencing tissue-engineered bone formation by detecting the expression levels of alkaline phosphatase and bone γ-carboxyglutamate protein 2. Exp Ther Med. 2015 Apr;9(4):1097-1102. doi: 10.3892/etm.2015.2259. Epub 2015 Feb 4. PMID: 25780393; PMCID: PMC4353779.
4. Hasler J, Hatt LP, Stoddart MJ, Armiento AR. Stable Reference Genes for qPCR Analysis in BM-MSCs Undergoing Osteogenic Differentiation within 3D Hyaluronan-Based Hydrogels. Int J Mol Sci. 2020 Dec 2;21(23):9195. doi: 10.3390/ijms21239195. PMID: 33276559; PMCID: PMC7729573.
5. Okamura K, Inagaki Y, Matsui TK, Matsubayashi M, Komeda T, Ogawa M, Mori E, Tanaka Y. RT-qPCR analyses on the osteogenic differentiation from human iPS cells: an investigation of reference genes. Sci Rep. 2020 Jul 16;10(1):11748. doi: 10.1038/s41598-020-68752-2. PMID: 32678244; PMCID: PMC7367276.
6. Zainal Ariffin SH, Lim KW, Megat Abdul Wahab R, Zainal Ariffin Z, Rus Din RD, Shahidan MA, Johari AN, Zainol Abidin IZ. Gene expression profiles for *in vitro* human stem cell differentiation into osteoblasts and osteoclasts: a systematic

review. PeerJ. 2022 Oct 17;10:e14174. doi: 10.7717/peerj.14174. PMID: 36275474; PMCID: PMC9583853.
7. Quiroz FG, Posada OM, Gallego-Perez D, Higuita-Castro N, Sarassa C, Hansford DJ, Agudelo-Florez P, López LE. Housekeeping gene stability influences the quantification of osteogenic markers during stem cell differentiation to the osteogenic lineage. Cytotechnology. 2010 Apr;62(2):109-20. doi: 10.1007/s10616-010-9265-1. Epub 2010 Apr 16. PMID: 20396946; PMCID: PMC2873986.
8. Combs CA. Fluorescence microscopy: a concise guide to current imaging methods. Curr Protoc Neurosci. 2010 Jan;Chapter 2:Unit2.1. doi: 10.1002/0471142301.ns0201s50. PMID: 20066655; PMCID: PMC3805368.
9. Hickey SM, Ung B, Bader C, Brooks R, Lazniewska J, Johnson IRD, Sorvina A, Logan J, Martini C, Moore CR, Karageorgos L, Sweetman MJ, Brooks DA. Fluorescence Microscopy-An Outline of Hardware, Biological Handling, and Fluorophore Considerations. Cells. 2021 Dec 23;11(1):35. doi: 10.3390/cells11010035. PMID: 35011596; PMCID: PMC8750338.
10. Lichtman, J., Conchello, JA. Fluorescence microscopy. *Nat Methods* **2**, 910–919 (2005). https://doi.org/10.1038/nmeth817
11. Riss TL, Moravec RA, Niles AL, et al. Cell Viability Assays. 2013 May 1 [Updated 2016 Jul 1]. In: Markossian S, Grossman A, Arkin M, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK144065/
12. Nozhat Z, Khalaji MS, Hedayati M, Kia SK. Different Methods for Cell Viability and Proliferation Assay: Essential Tools in Pharmaceutical Studies. Anticancer Agents Med Chem. 2022;22(4):703-712. doi: 10.2174/1871520621999201230202614. PMID: 33390140.
13. Stoddart MJ. Cell viability assays: introduction. Methods Mol Biol. 2011;740:1-6. doi: 10.1007/978-1-61779-108-6_1. PMID: 21468961.
14. Trinh, K.T.L.; Lee, N.Y. Recent Methods for the Viability Assessment of Bacterial Pathogens: Advances, Challenges, and Future Perspectives. *Pathogens* **2022**, *11*, 1057. https://doi.org/10.3390/pathogens11091057
15. Lim KK, Chen ES. Microscopy-based protocol for the quantification of cells viability for temperature-sensitive S. pombe. STAR Protoc. 2024 Jun 21;5(2):103076. doi: 10.1016/j.xpro.2024.103076. Epub 2024 May 19. PMID: 38768030; PMCID: PMC11111821.
16. Madorran E, Stožer A, Arsov Z, Maver U, Rožanc J. A Promising Method for the Determination of Cell Viability: The Membrane Potential Cell Viability Assay. Cells. 2022 Jul 27;11(15):2314. doi: 10.3390/cells11152314. PMID: 35954159; PMCID: PMC9367465.
17. Trinh KTL, Lee NY. Recent Methods for the Viability Assessment of Bacterial Pathogens: Advances, Challenges, and Future Perspectives. Pathogens. 2022 Sep 16;11(9):1057. doi: 10.3390/pathogens11091057. PMID: 36145489; PMCID: PMC9500772.
18. Divakar P, Moodie KL, Demidenko E, Jack Hoopes P, Wegst UGK. Quantitative evaluation of the in vivo biocompatibility and performance of freeze-cast tissue scaffolds. Biomed Mater. 2020 Jul 23;15(5):055003. doi: 10.1088/1748-605X/ab316a. PMID: 31295733; PMCID: PMC6954349.
19. Hussein KH, Park KM, Kang KS, Woo HM. Biocompatibility evaluation of tissue-engineered decellularized scaffolds for biomedical application. Mater Sci Eng C

Mater Biol Appl. 2016 Oct 1;67:766-778. doi: 10.1016/j.msec.2016.05.068. Epub 2016 May 16. PMID: 27287176.

20. Chen, Y.-Y.; Chiou, Y.-J.; Chang, P.-J.; Chang, W.-M.; Yeh, Y.-C.; Chen, C.-Y.; Chang, Y.-K.; Lin, C.-K. In Vitro Evaluation of Electrospun PCL Bioscaffold with Zinc-Doped Bioactive Glass Powder Addition. *Polymers* **2024**, *16*, 2811. https://doi.org/10.3390/polym16192811