Study of hemocompatibility and biocompatibility properties of tricalcium phosphate and insulin-infused hydrogel- An in-vitro study

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ABSTRACT

Background

Insulin-infused hydrogel has offered a versatile platform to promote bone regeneration and repair. Insulin has been shown to stimulate the formation of new blood vessels, which is required for supplying nutrients and oxygen to developing bone tissue and supporting its growth. For the product to be successful in doing so, it needs to be biocompatible and should show hemocompatibility. By assessing and optimizing the interaction of the scaffold with blood, researchers can develop safe and effective means of insulin delivery for bone regeneration.

Aim

We aim to develop a scaffold which can enhance bone regeneration and improve patient outcomes in orthopedic and regenerative medicine. The objective is to study the hemocompatibility and biocompatibility properties 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 properties and hemocompatibility of the bioscaffold infused with TCP and insulin.

To check the hemocompatibility, human RBCs were added to four Eppendorf tubes. Each of these samples was treated with distilled water (positive control), normal saline (negative control), hydrogel scaffold without insulin, and hydrogel with insulin (at 100 µg/mL). These samples were incubated at 25°C for 2 hours. The experiments were repeated in triplicate. Finally, the optical density of the samples was analyzed and compared.

MTT assay was used to assess cell viability. MG63 cells were seeded in a 96-well plate at an appropriate density, ensuring even distribution by gently rocking the plate back and forth, and were incubated for 18-24 hours at 37°C with 5% CO2 to allow them to adhere and grow. After the incubation period, the cells were treated with the test compounds, along with appropriate controls, including untreated cells as negative controls and cells treated with known cytotoxic agents as positive controls, and incubated them for the desired time period at 37°C with 5% CO2. Next, 10 µL of MTT solution (5 mg/mL) was added to each well. Thorough mixing was ensured. The wells were incubated for 1-2 hours at 37°C with 5% CO2. During this time, viable cells reduced the MTT to purple formazan crystals. Then carefully the media was removed and MTT solution from each well was removed without disturbing the formed formazan crystals, and 100-200 µL of DMSO was added to each well to dissolve the formazan crystals. The plate was placed on a shaker and incubated for 10 minutes in the dark to ensure complete solubilization of the formazan crystals. Finally, the absorbance of the solubilized formazan product at 540-595 nm was measured using a microplate reader, with absorbance values directly proportional to the number of viable cells. Blank wells containing only medium and MTT solution (without cells) were included to subtract background absorbance. For data analysis, the absorbance values of the treated wells were compared to the control wells to determine the percentage of viable cells, and statistical analysis was performed to determine the significance of the results. All reagents and equipment were sterile to prevent contamination, MTT solution and DMSO was handled with care as they can be hazardous, performed the assay in triplicate to ensure reproducibility and accuracy, and protected the MTT solution from light to prevent degradation.

Results

The optical density was found to be the highest of only the positive control, that is human RBCs centrifuged and intubated with only distilled water. From the MTT assay, it can be inferred that the MG63 osteoblasts showed a better survival rate when exposed to the bioscaffold with insulin. Cell degradation was seen more with the bioscaffold without insulin.

Conclusion

From the results obtained, it can be concluded that the hydrogel developed with insulin and tricalcium phosphate has antioxidant properties similar to ascorbic acid and has no negative effect on human RBCs, hence proving its hemocompatibility.

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.

To perform an MTT assay to assess cell viability, researchers first seeded the cells in a 96-well plate at an appropriate density, typically between 4,000 and 10,000 cells per well, depending on the cell type. They ensured even distribution by gently rocking the plate back and forth and then incubated the cells for 18-24 hours at 37°C with 5% CO2 to allow them to adhere and grow. Following this incubation period, the cells were treated with test compounds in triplicate, along with appropriate controls, which included untreated cells as negative controls and cells treated with known cytotoxic agents as positive controls. The cells were incubated with the test compounds for the desired time period, such as 24, 48, or 72 hours, at 37°C with 5% CO2.

Next, researchers added 10 µL of MTT solution (5 mg/mL) to each well, ensuring that the MTT solution was mixed thoroughly before addition. The plate was then incubated for 1-2 hours at 37°C with 5% CO2, during which time viable cells reduced the MTT to purple formazan crystals. After this incubation, the media and MTT solution were carefully removed from each well without disturbing the formed formazan crystals, either by using a pipette or by gently inverting the plate.

To solubilize the formazan crystals, 100-200 µL of DMSO was added to each well, with the exact volume optimized based on experimental conditions. The plate was placed on a shaker and incubated for 10 minutes in the dark to ensure complete solubilization of the formazan crystals. The absorbance of the solubilized formazan product was then measured at 540-595 nm using a microplate reader. The absorbance values were directly proportional to the number of viable cells. Blank wells containing only medium and MTT solution (without cells) were included to subtract background absorbance.

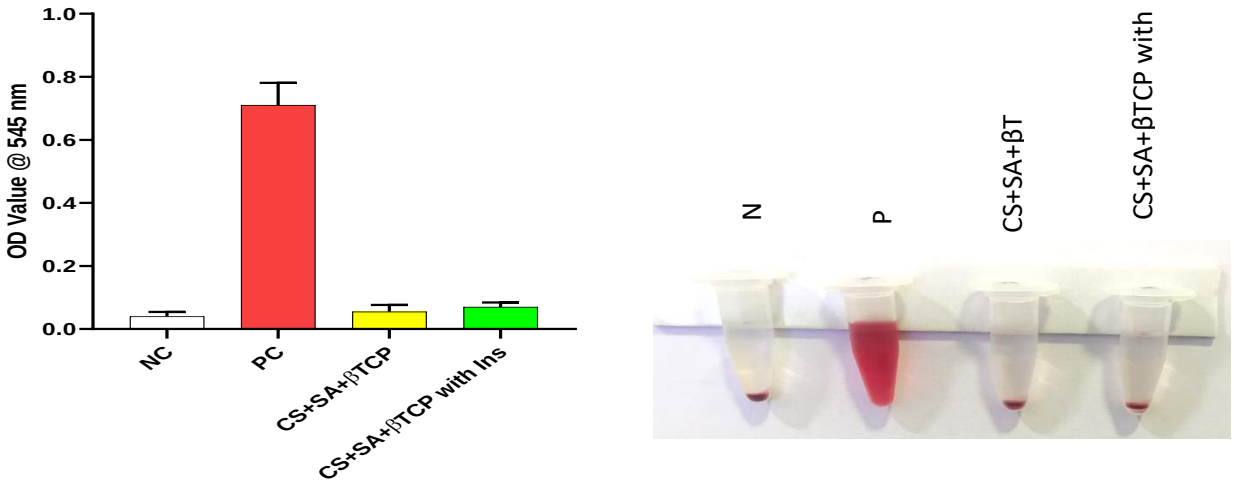
For data analysis, the absorbance values of the treated wells were compared to those of the control wells to determine the percentage of viable cells. Statistical analysis, such as ANOVA, was performed to determine the significance of the results. Throughout the process, all reagents and equipment were kept sterile to prevent contamination, and the MTT solution and DMSO were handled with care due to their hazardous nature. The assay was performed in triplicate to ensure reproducibility and accuracy, and the MTT solution was protected from light to prevent degradation.

To check the hemocompatibility, human RBCs were added to four Eppendorf tubes. Each of these samples were treated with distilled water (positive control), normal saline (negative control), hydrogel

scaffold without insulin and hydrogel with insulin (at 100 µg/mL). These samples were incubated at 25°C for 2 hours which was preceded by centrifugation. The experiments were repeated in triplicate. After this the samples were measured for their optical density. A beam of light at a wavelength of 545nm, was passed through the samples. The spectrophotometer then detected the light coming out of the samples.

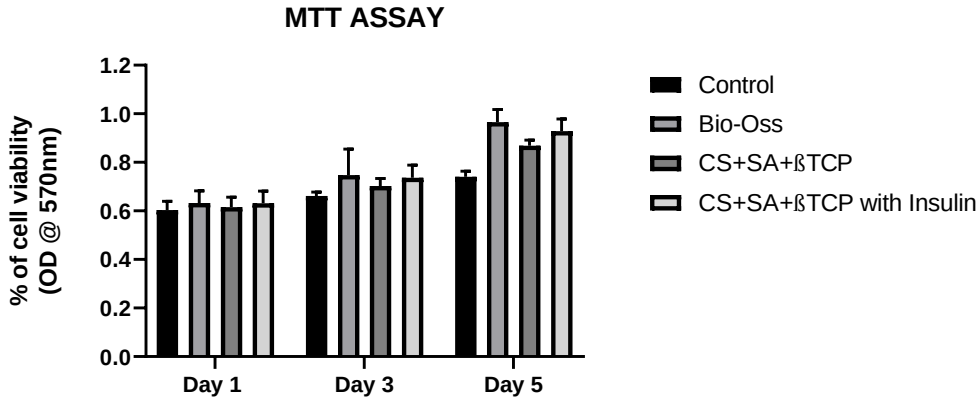
The detector measured the intensity of the transmitted light. The amount of light absorbed by the sample was calculated based on the difference in intensity between the incident light and the transmitted light. The spectrophotometer then converted the measured absorbance into optical density (OD) values using the formula: OD = -log(T), where T was the transmittance of light through each of the samples. The optical density value obtained gave us information about the concentration of the absorbing substance in the samples. Higher OD values indicated higher concentrations of the absorbing substance.

RESULTS



Distilled water served as the positive control, and Normal saline served as a Negative Control. Photographs of human RBCs treated with NC, PC, and bioscaffolds with/without Insulin at 100 µg/mL. The red color of the solution is due to the release of hemoglobin from the damaged RBCs, and the red pellets at the bottom of the Eppendorf tubes are intact RBCs precipitated by centrifugation.

As per the data obtained, the optical density was found to be the highest of only the positive control, that is human RBCs centrifuged and intubated with only distilled water.



Cell viability of osteoblast MG-63 cells after treatment with and without insulin loaded CS+SA+βTCP with control group for different time point of incubation, respectively Day 1, 3, 5.

DISCUSSION

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. Thrombogenicity refers to the tendency of a material to promote blood clot formation [2]. A hemocompatible product should minimize the risk of thrombosis, which can lead to blockages in blood vessels and impair blood flow. Testing for thrombogenicity involves assessing factors such as platelet adhesion, activation, and aggregation [3]. Hemolysis is the rupture of red blood cells, leading to the release of hemoglobin and other intracellular components into the bloodstream. A hemocompatible product should not cause significant hemolysis, as this can result in anemia, organ damage, and other complications. Hemolysis testing evaluates the impact of the product on red blood cells and measures hemoglobin levels in the plasma. Platelets play a crucial role in blood clotting and wound healing. However, excessive platelet activation can lead to thrombosis and other complications[4].

Hemocompatibility testing assesses the ability of a product to activate platelets and trigger clot formation. Inflammatory reactions can occur when the immune system reacts to a foreign material, leading to tissue damage and systemic effects[5]. A hemocompatible product should minimize inflammation to avoid adverse reactions and promote healing. Testing for inflammation involves evaluating markers of inflammation and immune cell responses [6]. The complement system is part of the immune system and plays a role in inflammation and immune responses. Hemocompatibility testing includes assessing the activation of the complement system by the product, as excessive complement activation can lead to tissue damage and other adverse effects [7]. Endothelial cells line blood vessels and play a crucial role in maintaining vascular function. A hemocompatible product should not damage endothelial cells or interfere with their function. Testing for endothelial cell compatibility evaluates the impact of the product on these cells [8].

Hemolysis testing is a common method used to assess the hemocompatibility of medical devices, materials, or products that come into contact with blood. Hemolysis refers to the rupture of red blood cells, leading to the release of hemoglobin and other intracellular components into the surrounding plasma. Excessive hemolysis can have detrimental effects on the body, such as anemia, organ damage, and inflammatory responses [9]. Hemolysis testing involves exposing red blood cells to the product or material being evaluated and monitoring the extent of cell lysis. The release of hemoglobin from lysed red blood cells is measured spectrophotometrically at a specific wavelength. The amount of

hemoglobin in the plasma is an indicator of the degree of hemolysis induced by the product [10]. There are two types of hemolysis tests, qualitative and quantitative. In qualitative tests, visual inspection is used to assess the degree of hemolysis based on the color change of the sample (e.g., red to pink or clear). Quantitative tests measure the amount of hemoglobin released into the plasma, typically using spectrophotometric methods. The results are expressed as a percentage of hemolysis relative to positive and negative controls [11].

The degree of hemolysis considered acceptable varies depending on the application and regulatory standards. Generally, a hemolysis level below a certain threshold (e.g., 2-5%) is considered safe for medical devices intended for short-term or long-term contact with blood. The results can be classified as, Non-hemolytic (Minimal to no hemolysis observed), Mild hemolysis (Low levels of hemolysis observed), Moderate hemolysis (Moderate levels of hemolysis observed) and Severe hemolysis (Significant hemolysis observed) [12]. Regulatory authorities, such as the FDA (Food and Drug Administration) and ISO (International Organization for Standardization), often require hemolysis testing as part of the biocompatibility assessment for medical devices. The results of hemolysis testing are included in the biological evaluation of the product to demonstrate its hemocompatibility. It's important to consider factors such as contact time, surface area, and mechanical stress when conducting hemolysis testing, as these factors can influence the hemolytic potential of the product. Hemolysis testing is a valuable tool for evaluating the hemocompatibility of products that come into contact with blood. By assessing the extent of hemolysis induced by a product, researchers and regulatory authorities can determine its safety and compatibility with the circulatory system [13].

Overall, assessing the hemocompatibility of a product is essential to ensure its safety and efficacy when in contact with blood and the circulatory system. Regulatory agencies often require comprehensive hemocompatibility testing as part of the approval process for medical devices and products intended for use in or on the body [14]. The MTT assay is a popular colorimetric technique for evaluating cell metabolic activity. It is based on the reduction of the yellow tetrazolium salt, MTT, to purple formazan crystals by mitochondrial enzymes in metabolically active cells. The procedure involves seeding cells in a microtiter plate, adding the MTT reagent, and incubating for a few hours. Viable cells reduce MTT to insoluble formazan, which accumulates in the cytoplasm. After incubation, the formazan crystals are dissolved using a solubilizing agent such as dimethyl sulfoxide (DMSO). The resulting colored solution is quantified using a spectrophotometer by measuring absorbance at 500-600 nm, correlating with cell viability [15]. This assay is widely used in various applications, including assessing cell proliferation, cytotoxicity of compounds, and drug efficacy. Its advantages include simplicity, sensitivity, and non-radioactive nature, making it an invaluable tool in cellular and molecular biology research [16].

The MTT assay plays a crucial role in tissue engineering, a field focused on developing biological substitutes to restore, maintain, or improve tissue function. In tissue engineering, the MTT assay is primarily used to evaluate cell viability and proliferation within scaffolds, which are structures designed to support the growth of new tissues [17]. By assessing the metabolic activity of cells, researchers can determine the effectiveness of different scaffold materials and designs in promoting cell growth and survival. One of the key applications of the MTT assay in tissue engineering is in the development of artificial skin. Researchers use the assay to test the viability of keratinocytes and fibroblasts, the primary cell types found in the skin, when cultured on various biomaterials. This helps in identifying the most suitable materials that support cell growth and mimic the natural environment of skin cells, which is essential for creating effective skin graft [18].

Another important application is in the evaluation of bone tissue engineering scaffolds. The MTT assay is used to measure the metabolic activity of osteoblasts (bone-forming cells) and other cell types involved in bone regeneration when cultured on different scaffold materials. This information is vital

for optimizing scaffold properties to enhance bone tissue formation and integration with the host tissue. The MTT assay is also employed in vascular tissue engineering to assess the viability of endothelial cells, which line blood vessels, when cultured on various biomaterials. Ensuring the survival and proliferation of endothelial cells is critical for developing functional blood vessels that can be used in vascular grafts and other regenerative therapies. In addition to evaluating scaffold materials, the MTT assay is used to test the effects of growth factors and other bioactive molecules on cell viability and proliferation. By adding these substances to the culture medium and measuring their impact on cell metabolic activity, researchers can identify the most effective combinations for promoting tissue growth and regeneration. Furthermore, the MTT assay 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. Overall, the MTT assay is an indispensable tool in tissue engineering, providing essential data on cell viability, proliferation, and cytotoxicity. 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 [19].

The MTT assay is a valuable tool for assessing cell viability, proliferation, and cytotoxicity. Its simplicity, sensitivity, and quantitative nature make it a popular choice in biomedical research. However, researchers should be aware of its limitations and consider alternative assays when necessary. The MTT assay is a widely used technique to assess cell viability and proliferation. While it offers several advantages, such as simplicity, sensitivity, and quantitative results, it also has several limitations. While the MTT assay is a valuable tool for assessing cell viability and proliferation, researchers must be aware of its limitations and potential sources of error. By carefully optimizing assay conditions and considering alternative methods, researchers can obtain more reliable and accurate results for their experiments [20].

CONCLUSION

The developed scaffold with insulin does not adversely affect human red blood cells, thus demonstrating its hemocompatibility. MTT assay results have proved the cell viability and biocompatibility of the scaffold.

Scope for future research

Ensuring the hemocompatibility of insulin and hydrogel composites is crucial for their successful application in various biomedical fields. By assessing and optimizing the interaction of these materials with blood, researchers can develop safe and effective platforms for drug delivery, wound healing, tissue engineering, and other therapeutic applications. Continued research efforts are required on refining the design of insulin-infused hydrogels to improve enhance the performance of the scaffold for bone regeneration.

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