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Production of alginate macrocapsule device for long-term normoglycaemia in the treatment of type 1 diabetes mellitus with pancreatic cell sheet engineering

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Abstract

Type 1 diabetes-mellitus (T1DM) is characterized by damage of beta cells in pancreatic islets. Cell-sheet engineering, one of the newest therapeutic approaches, has also been used to create functional islet systems by creating islet/beta cell-sheets and transferring these systems to areas that require minimally invasive intervention, such as extrahepatic areas. Since islets, beta cells, and pancreas transplants are allogeneic, immune problems such as tissue rejection occur after treatment, and patients become insulin dependent again. In this study, we aimed to design the most suitable cell-sheet treatment method and macrocapsule-device that could provide long-term normoglycemia in rats. Firstly, mesenchymal stem cells (MSCs) and beta cells were co-cultured in a temperature-responsive culture dish to obtain a cell-sheet and then the cell-sheets macroencapsulated using different concentrations of alginate. The mechanical properties and pore sizes of the macrocapsule-device were characterized. The viability and activity of cell-sheets in the macrocapsule were evaluated *in vitro* and *in vivo*. Fasting blood glucose levels, body weight, and serum insulin & C-peptide levels were evaluated after transplantation in diabetic-rats. After the transplantation, the blood glucose level at 225 mg dl⁻¹ on the 10th day dropped to 168 mg dl⁻¹ on the 15th day, and remained at the normoglycemic level for 210 days. In this study, an alginate macrocapsule-device was successfully developed to protect cell-sheets from immune attacks after transplantation. The results of our study provide the basis for future animal and human studies in which this method can be used to provide long-term cellular therapy in T1DM patients.

1. Introduction

Type 1-diabetes mellitus (T1DM) is a common autoimmune disease in childhood, characterized by insulinemia and hyperglycaemia that develops with the death of pancreatic beta cells involved in insulin production due to ongoing autoimmune or

non-autoimmune causes [1]. Many methods such as pancreas, islet, cell, and cell sheet transplantation have been tried and developed for the treatment of T1DM from the past to the present [2–4]. The most recent approach is cell sheet engineering technology, because this method greatly reduces the exposure of cells to stress during preparation for transplantation.

The cell sheets were formed by coating the surface of polystyrene tissue culture dishes with poly (*N*-isopropyl acrylamide) [5, 6]. Thus, the cells can be removed from the swollen surface with the cell sheet form without the need for enzymes. Cells can be obtained in sheets because the extracellular matrix (ECM) and cell–cell connections are intact. This cell sheet can be transferred to the tissue or organ surface in the body, or to another culture dish. They can also be obtained as a 3-dimensional structure by attaching the cell sheets on top of each other [7–11]. This method allows the target tissue to be designed in 3D.

In the literature, β -cells were used firstly to obtain cell sheet by our group [4] are a suitable source for cell sheet engineering. In our study published last year, the β -cells and MSCs were cultured in a temperature-responsive culture dish and a 3D islet-like tissue was developed. The difficulty of finding donors can be overcome by incorporating beta cell lines, which are readily available and a potential source, into cell sheet engineering applications. The biggest disadvantage of diabetic rat studies in literature is that the islet cell sheets used are not protected from immune system cells [12, 13].

New strategies have been developed in the literature to generate an artificial pancreas that would eliminate the need for immunosuppressants [14, 15]. This strategy aims to protect insulin-secreting cells within a semipermeable membrane. Therefore, the semipermeable membrane to be developed should allow the entry of nutrients and small molecules, the exit of insulin and metabolic waste products, while preventing the entry of antibodies and lymphocytes, which are elements of the immune system. Thus, even if the implants are allogeneic, they will be protected from autoimmune attacks and will show long-term survival and localization in the target region [16]. The encapsulation method not only protects cells from immune system attacks but also ensures long-term function [17].

Alginate has been preferred in most encapsulation studies because it is non-toxic, has a low immunogenic profile, non-degradable in the body environment, biocompatible, and a structural polysaccharide that supports the functions of cells. In addition, micro- and macrosized alginate particles can be prepared via microencapsulation and macroencapsulation technique respectively [18]. While cells are encapsulated individually in microencapsulation, more cell groups can be encapsulated simultaneously with macrosized materials in macroencapsulation [19]. Because the cell sheet obtained in our study was a macrosized structure, it was suitable for macroencapsulation. Macroencapsulation devices can have different geometries, such as planar membranes, polymeric hydrogel layers, or hollow bag-like structures, for high flow rates or reduced surface

areas. According to their transport mechanism, they can be classified into extravascular and vascular perfusion-based systems. The large capsule size in the vascular perfusion macroencapsulation strategy can cause blood clotting and thrombosis. However, extravascular macrocapsules do not require vascular anastomosis, the associated surgical risks are much lower than vascular perfusion [20].

MSCs with anti-apoptotic and angiogenic properties were used to create an islet microenvironment similar to that *in vivo* in a 3D structure and to support the viability of β -cells. It is also known that vascular endothelial growth factor secreted by the MSCs reduces hypoxia and ischemia in tissue [21]. Therefore, in our study, we investigated the role of MSCs in the macrocapsule in providing long-term normoglycaemia. With the cell-based treatment of pancreatic diseases, cell stratification methods, and macroencapsulation, beta cells and MSCs will be three-dimensionalized and can be transplanted into the patient as subcutaneous implants with a very minimally invasive method in the future [3, 12, 13].

The aim of this study was to ensure the long-term survival of the obtained sheet at the transplantation site in cell sheet engineering, which is the most current approach for the treatment of T1DM. In the literature review, cell sheets have been obtained for the treatment of many disease models [10–12] other than diabetes using various cells on temperature-responsive culture dishes, namely [poly-(*N*-isopropylacrylamide)-PIPAAM]. However, the sheets obtained were macroencapsulated with alginate for the first time in our study to protect them from the immune system. For viability and survival to be stable in 3D structures, they need to be supported with nutrients and oxygen, similar to *in vivo* conditions. For this reason, alginate hydrogel has two important properties (i) having optimum pore size (ii) being resistant to impacts that may occur in the subcutaneous area during daily life, optimization studies were carried out. Therefore, swelling and mechanical tests were performed to determine the optimal alginate concentration for the macrocapsule material we developed. The results of our study will contribute to the treatment of diabetes through tissue and cell sheet engineering.

2. Material and methods

2.1. Culture and characterization

2.1.1. Culture and characterization of the rAT-MSCs

Isolation, culture and characterization of rat adipose tissue-derived MSCs (rAT-MSCs) were performed according to a previously published protocol [22, 23].

rAT-MSCs were cultured in T175 flasks (BD Biosciences, Bedford, MA, USA) in L-DMEM (Invitrogen, GIBCO) containing 10%

FBS (Invitrogen, Grand Island, NY, USA) and 1% penicillin-streptomycin (Invitrogen, GIBCO). During the study, the cells were examined using a phase-contrast microscope. After thawing, flow cytometric analysis was performed to assess whether MSCs retained their markers and determine their immunophenotypic characteristics. To determine the differentiation ability of rAT-MSCs *in vitro*, adipogenic, osteogenic, and chondrogenic differentiation assays were performed. rAT-MSCs were characterized as described previously [4].

2.1.2. Culture and characterization of beta cell

The beta cells (BRIN BD11) used in this study were obtained from the cell bank. The beta cell (BRIN BD11) line was purchased from the European Collection of Authenticated Cell Cultures (ECACC General Cell-Collection, 10033003; Salisbury, UK). These cells are insulin-secreting cell lines obtained by the electrofusion of NEDH rat pancreatic islets and RINm5F (cell line derived from NEDH rat insulinoma). These cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Previously, a series of assays was performed to determine the viability, characterization, and activity of beta cells [4].

2.2. Fabrication of 3D cell sheets on the temperature-responsive dish

A total of 9×10^6 rAT-MSCs and 9×10^6 beta cells were seeded in 6-well UpCell (UpCell, CellSeed, Inc. Tokyo, Japan) for co-culture. The cells were incubated in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and 1% penicillin-streptomycin for 24 h at 37 °C CO₂ incubator [22]. The cells in the PIPAAm culture dish were removed after incubation at 37 °C and kept at 20 °C for 30 min. After 30 min of incubation, the cells were removed from the culture dish in sheets instead of a single-cell suspension, without the need for an enzymatic reaction [4]. To transfer the grown cells as a cell sheet to the target area, a support membrane made of PVDF is used. The support membrane is first placed on the cells, the temperature difference causes the detachment of cells from the surface without any additional treatment, and cells could be removed from the culture dish in the form of a sheet without folding. This method was describe previously studies [4, 11, 24].

2.3. Macrodevice fabrication and characterization

2.3.1. Macrocapsule fabrication

Alginate macrocapsules were prepared by adapting a protocol known in the literature [25]. According to this protocol, alginate macrocapsules were prepared by adding sodium alginate solution (A1112-Alginic Acid Sodium Salt From Brown, Sigma-Aldrich) to the moulds on an agarose gel mixed with CaCl₂ (C7902-Calcium Chloride Dihydrate, Sigma-Aldrich). To

obtain an agarose gel mould, 2% CaCl₂ was added to a 1% agarose mixture in 100 ml distilled water. The purpose of adding CaCl₂ to the agarose solution was to facilitate the diffusion of Ca²⁺ ions from the agarose mould to alginate and cross-link with alginate and Ca²⁺. This mixture was heated in a microwave oven to completely dissolve the agarose gel and CaCl₂ in the water. The resulting agarose gel was poured into a 1.3 cm deep container to obtain a 5 mm thick first layer agarose mould (figure 1(A)). Three agarose moulds were prepared, two of which were used directly, and only one had round wells with a diameter of 0.4 cm. To obtain the strongest form of alginate, two alginate types with different viscosities (low and medium) were used at two different concentrations (2% and 4% w/v alginate/water). The mould with the well was placed on the first mould, 60 µl of alginate was added to the well, then the cell sheet was placed on the alginate in the well, and 20 µl of the medium was placed on it. After 60 µl sodium alginate was added on the cell sheet, a final agarose mould was placed on top (figure 1(F)) and incubated at 37 °C for 30 min to cross-link Ca²⁺ ions in the mould with the alginate by displacing the Na⁺ ions in the alginate. The strongest form of the macrocapsule was obtained using 2% low-viscosity alginate. All materials used in the production of capsules were sterilized by autoclaving and UV sterilization, and then the alginate solution was filtered through a 0.22 µm (Minisart) filter to form sterile capsules. The study was carried out in a biosafety cabinet.

2.3.2. Characterizations of macrocapsule

2.3.2.1. Gel degradation studies

The degradation behaviour of macrocapsules made with different concentrations (2% and 4% w/v alginate/water) of two different alginate types (low and medium viscosity) was analysed by measuring the diameter of macrocapsules of each hydrogel at days 0, 1, and 7 during incubation at 37 °C in tris buffered saline solution (TBSS). This analysis was repeated three times for each sample [26]. The degradation of the macrocapsule is shown in the graph of the initial and final diameters.

2.3.2.2. Swelling ratio

A swelling test was performed to determine the water-absorption capacity of the materials produced in this study. For the test, the dry weights of all materials were measured, and the samples were placed in distilled water. It was removed from the swelling environment at certain intervals, its moisture was removed, and the weights of the samples were measured on precision scales. The 'swelling ratio' was calculated by putting the measured wet weight (M_1) values and the initial weights (M_0) of the materials in dry state in their place in equation: Swelling ratio (%) = $(M_1 - M_0)/M_0 \times 100\%$ [27].

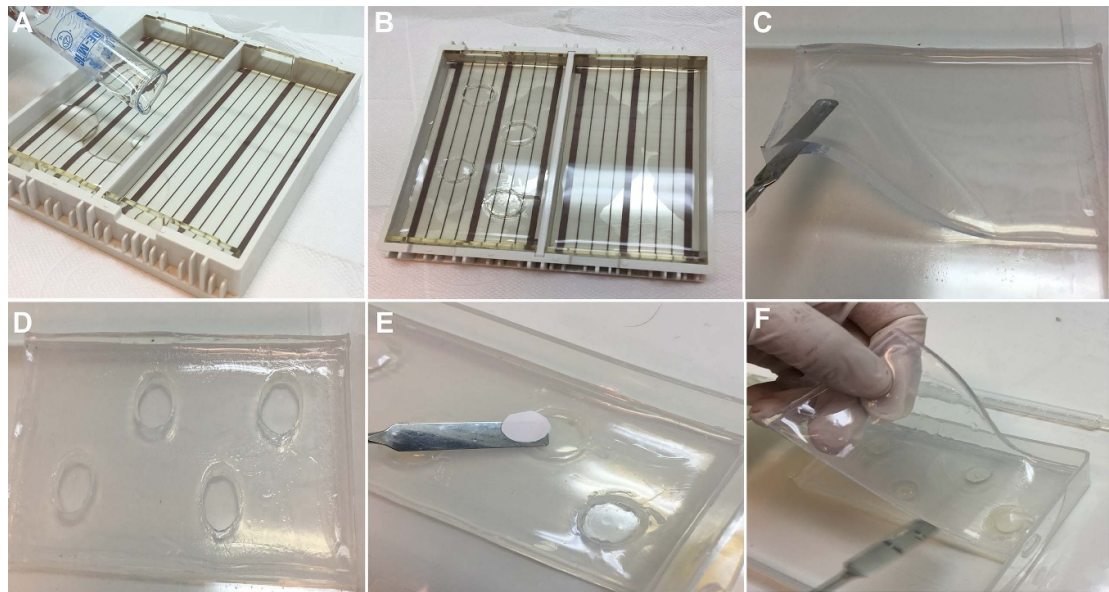


Figure 1. Representative images of the fabrication process for the gel mould and macrocapsule. (A) Pouring of 100 ml of agarose gel into the moulding tank. (B)–(D) Casting of agarose to the tank for the top, bottom and middle agarose mould. (E) Addition of alginate to the well and placement of the cell sheet. (F) Closing the final agarose mould on the alginate capsule.

2.3.2.3. Mechanical test

This test was performed to determine the mechanical properties of the capsule in tension, compression, and impact. The compression test of the capsules (4.6 mm in diameter) was carried out with an Instron Compression Device, which has a capacity of 0.5% and can apply forces between 0.02 N and 30 kN at a compression range of $0.005 \text{ mm min}^{-1}$ [27]. The lower jaw of the device is fixed, and the upper jaw is a movable part that moves up and down at a constant speed. A load was applied to the capsules to determine the permanent deformation point of the material. Three samples were tested from each macrocapsule group.

2.3.2.4. Rheological properties

The viscosities of all mixtures (2% and 4% w/v of alginate (low/medium)/water) used for the preparation of macrocapsules were evaluated on an Anton Paar rheometer with a 40 mm flat plate geometry. Viscosity behaviour was determined by dynamic shear measurements at 20°C in the frequency range of 0.01–100 Hz by dropping $500 \mu\text{l}$ onto the rheometer platform. The measurement was performed by adjusting the gap between the top plate and sample [28].

2.3.2.5. SEM

Scanning electron microscopy imaging was performed to determine the surface and pore properties of the cell-free macrocapsule material. The sample was fixed with 2% glutaraldehyde, incubated in 1% osmium tetroxide, and dehydrated by passing it through an increasing alcohol series. After drying in a desiccator for 24 h, the macrocapsule samples

were coated with Au/Pd and imaged using a JEOL JSM 6060 SEM device [28].

2.3.3. In vitro characterization of cell sheet viability and function

2.3.3.1. FITC DEXTRAN

First, to estimate the permeability of the capsules, two fluorescence (FITC) labelled dextrans were used. 4 kDa and 150 kDa FITC dextran (Sigma Aldrich, St. Louis, MO, USA) solutions were added to the macrocapsules for analysed permeability function, samples taken from capsules in 6-well culture dishes were transferred to 96-well dishes, and measurements were taken at $\text{ex} = 485 \text{ nm}$ and $\text{em} = 520 \text{ nm}$ in a microplate reader (VersaMax plus, Molecular Device, USA) [29] to determine the level of remaining FITC-dextran in the medium. Data were analysed using Student's *t*-test.

Next, 4 kDa and 150 kDa FITC dextran (Sigma Aldrich) solution were added to the macrocapsules in a 6-well culture dish and the images and thickness of the capsules were analysed in 3D (Z-stacking) under a confocal laser microscope to determine the penetration into the capsule, visually [30].

2.3.3.2. WST-1

This test was performed on days 1, 7, and 14 of culture of the macrocapsules. The living cells and cells in their early stage of apoptosis, if any, undergo are reacted with WST-1 (Roche) solution through their mitochondrion. The tetrazolium ring of WST-1 is broken down by the dehydrogenase enzymes in the mitochondrion of the cells to form orange coloured formazan crystals, while dead cells do not show any reaction. By this analysis, it was aimed to test the

viability and proliferation process of the cell sheet in the capsule. On the test days, 0.5 mg ml^{-1} WST-1 (Roche) was added to the wells containing the capsules and incubated for 2 h (37°C at 5% CO_2). After incubation, absorbance values were determined at a wavelength of 480 nm using a microplate reader with a monochromator system (VersaMax, Molecular Device, USA) [31].

2.3.3.3. LDH assay

The toxicity rates (%) of the lactate dehydrogenase (LDH) (Roche) assay were determined to analyse whether the macrocapsule samples had a toxic effect on the cells. Necrotic areas might develop on the cell sheet structure meaning that the cell viability were not supported anymore. Due to the limitations however, WST-1 analysis could not detect this loss. For this reason, LDH analysis was performed to determine whether there was necrosis on the cell sheet. The reaction mixture was then added to the macrocapsule samples containing the cell sheet and incubated for 30 min. After incubation, the stop solution was added and absorbance was measured on a microplate reader at a wavelength of 480 nm. The experiment was repeated on days 1, 7 and 14 [31].

2.3.3.4. Live/dead assay

In addition to the LDH test, a live/dead (Thermo Fisher, Oregon, USA) test was performed on day 14 to determine the *in vitro* viability of the cell sheets within the capsule [30]. In the live/dead analysis, calcein AM/ethidium staining was used to estimate whether the dead cells and necrotic areas, if any, were develop within the alginate macrocapsules. To prepare the live-dead stain, $20 \mu\text{l}$ Ethidium Bromide and $5 \mu\text{l}$ Calcein-AM were added to 10 ml PBS. The capsule was then mechanically split in half, the cell sheet was removed, and dye was added to the cell sheets and incubated at 25°C for 30 min. At the end of the experiment, the sheets were placed on a slide and examined under a confocal microscope (Leica DMI8, Wetzlar, Germany). The encapsulated and non-encapsulated cell sheets were examined for the purpose of comparison. Moreover a live/dead test were performed for the encapsulated cell sheets after transplantation. On the 60th and last days of the experiment, the graft tissue in the transplant area was removed, and live/dead staining was performed. All the samples were viewed under a confocal microscope for the viability analysis of the cells. To determine the thickness of this cell sheet, the cell sheet was placed on a slide and live/dead staining was performed. Cell nuclei were stained with DAPI and visualized by 3D (Z-stacking) analysed under a confocal microscope [32].

2.3.3.5. ATP assay

In this analysis, ATP level was determined as an indicator for the viability and functionality of cells. Besides the insulin release is an ATP dependant process, it is

also vital to investigate the sustainability of the cell sheet in the capsule through the pores, which maintained cells by means of regular supply of nutrient and oxygen while enable waste disposal, under the defined culture conditions. This test was performed to determine the viability by estimating ATP level independent of reducing agents, which might cause false positive results. Metabolic activity was determined using the Synthase-Specific Activity Microplate Assay Kit (Abcam, UK). Following the manufacturer's recommendations, supernatants containing macrocapsules in culture were collected and placed in a 96-well plate for the ATP synthesis assay at days 1, 7, and 14 after encapsulation. The absorbance was measured at 405 nm using a Versamax microplate reader. Three independent assays were performed under each condition [33].

2.3.3.6. Insulin secretion assay

The viability and function of the cell sheet inside the capsule were determined using a glucose-induced insulin release assay [34]. The encapsulated cell sheet and the non-encapsulated cell sheet as a control group were exposed to two different glucose concentrations on days 1, 7, and 14 to test whether they could secrete insulin into the medium depending on the glucose added to the medium in a 6-well culture dish, and insulin protein was determined in the collected medium. Insulin levels were measured using a Rat-InsI Insulin ELISA kit (Sigma Aldrich, St. Louis, MO, USA).

2.4. Animals

In this part of our study, male Wistar Albino rats weighing 250–300 g and at least 8–10 weeks old were used for transplantation procedures [4]. The rats used as graft recipients were obtained from the Faculty of Medicine Experimental Research Unit, Kocaeli University. Kocaeli University Animal Experiments Local Ethics Committee [Protocol No: KOU HADYEK 1/3-2019].

The weights of all rats in the experimental groups were measured before STZ injection, and the amount of STZ administered was determined according to the average body weight. All male animals ($n = 6$) received a single dose of intraperitoneal streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in citrate buffer (pH 4.5) at 60 mg kg^{-1} body weight (figure 2). Control animals ($n = 6$) received an equal volume of citrate buffer. Two days after STZ injection, an incision was made on the tail end of the rats to determine blood glucose levels, and blood was drawn from the vein and measured using a glucometer (Abbott, Optimum Neo FreeStlye, USA). Rats with fasting blood glucose levels above 250 mg dl^{-1} were considered to have developed diabetes as a result of STZ-induced beta cell destruction and were included in the experiment [20]. Experimental animals were intramuscularly administered 5 mg kg^{-1}

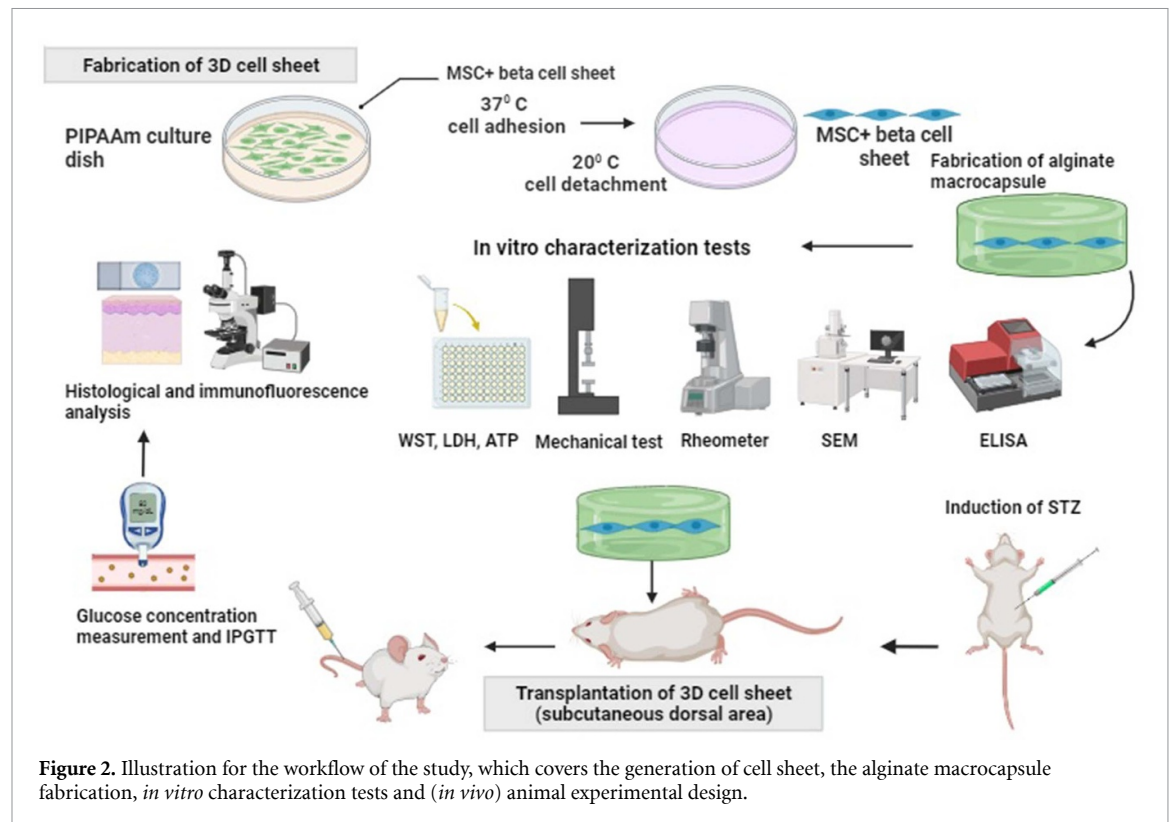


Figure 2. Illustration for the workflow of the study, which covers the generation of cell sheet, the alginate macrocapsule fabrication, *in vitro* characterization tests and *in vivo* animal experimental design.

xylazine hydrochloride and 35 mg kg⁻¹ ketamine hydrochloride. The depth of anaesthesia was monitored using palpebra, coreflexion, jaw, and skeletal muscle tone.

2.5. Transplantation

24 Wistar Albino male rats 8–10 weeks old and 250–300 g were transplanted subcutaneously after shaving the dorsal region [4]. After transplantation, rats were injected intraperitoneally with a single dose of saline. The animals were maintained at the Kocaeli University Faculty of Medicine Experimental Research Unit (DETAB).

2.6. Non-fasting blood glucose levels

Two days after transplantation, a periodic incision was made in the tail vein and blood glucose level was measured using a glucometer. The body weights of the rats were also monitored on days when the blood glucose level was measured. These procedures were repeated until the last day of the experiment [22].

2.7. Intraperitoneal glucose tolerance test

Glucose was injected intraperitoneally at 2 mg g⁻¹ body weight on days 2, 16, and 30 post-transplantation [22]. Accordingly, glucose was dissolved in water and injected into the rats at a volume of 500 μ l. Animals were fasted for 16 h before glucose injection. Blood samples were collected from the rat tail vein 30, 60, 90, and 120 min after glucose injection into the intraperitoneal region and measured using a glucometer.

2.8. Measurement of serum insulin and C-peptide level

Rat blood samples were collected from the rat tail vein on days 30, 90, and 210, and serum was obtained from these samples [22]. Insulin protein was determined in the serum samples by ELISA using the Rat Ins1 Insulin kit (Sigma Aldrich, Saint Louis, USA) and rat C-peptide kit (YL Biont, Shanghai). Protein levels were determined using a monochromator microplate reader (VersaMax, Molecular Devices, USA) following the procedures recommended by the manufacturer's instructions.

2.9. Histological and immunohistochemical analyses

On day 60, the subcutaneous tissues at the transplant site were removed and fixed with 10% formalin. Graft samples were embedded in paraffin and 5 μ m thick sections were taken for haematoxylin-eosin and immunofluorescence staining. After antigen retrieval, insulin antibody for beta cell and MSC differentiation analysis (Abcam), active-caspase 3 for apoptosis analysis (Santa Cruz Biotechnology) were diluted 1:50 with antibody diluent at appropriate ratios, and subcutaneous transplant area tissues were incubated overnight at +4 °C. The obtained tissues were analysed under a fluorescence (Leica DMI 4000 Microsystems) and confocal (Leica DMI8) microscope. Skin grafts stained with haematoxylin-eosin were coated with crytalmounting medium and analysed under a light microscope [4].

2.10. Statistical analyses

Statistical analyses of the results were performed using SPSS10.0 (SPSS, Chicago, USA). Data were tested using paired *t*-tests and ANOVA variance for multiple analyses. Each experiment was repeated at least three times. The difference between the experimental and control groups was significant when $p < 0.05$ and highly significant when $p < 0.01$ [4].

3. Results

3.1. Fabrication of beta and mesenchymal stem cell sheets

Beta-and mesenchymal stem cells (MSCs) were co-cultured at a ratio of 1:1 in a 6-well UpCell (UpCell, CellSeed, Inc. Tokyo, Japan) temperature-responsive culture dishes (figure 6(c) A–B). Since beta cells were located together with various cell groups in the islets *in vivo*, a successful culture process was achieved by complying with rAT-MSCs. MSCs showed a supporting and feeder layer effect on beta cells (figure 6(d) B–C). In cell sheet technology, a support membrane is used to transfer the obtained cell sheet to the target area. In this way, the transfer into the macrocapsule is successfully performed without folding of the sheet (figure 6(d) A) [4, 11, 24]. In our study, the obtained sheets were encapsulated with alginate, preventing unwanted folding and creasing.

3.2. Design and *in vitro* characterization of alginate macrocapsulation device

3.2.1. Rheology measurements

The viscosities of all solutions (2% and 4% low viscosity alginate, 2% and 4% medium viscosity alginate) were measured on an Antoon Paar rheometer and compared. As a result of data analysis, a decrease in viscosity was observed in four different materials due to increasing shear rate (figure 3(a)). Low 2% alginate, which is less viscous, has Newtonian flow properties, the shear stress does not change the viscosity and shows flexible behaviour.

3.2.2. Macrocapsule size, gel degradation studies

The degradation capacities of the macrocapsules fabricated from alginate, 2% low alginate, 4% low alginate, 2% medium alginate, and 4% medium alginate were evaluated in terms of diameter change during incubation in TBSS at 37 °C. If the diameter of the macrocapsules increased over the incubation time compared to the initial diameter (on day 0), we considered that degradation of the hydrogel macrocapsules occurred [26]. The capsule diameters were compared between all groups. It was determined that a 2% low concentration of alginate polymer capsule preserved its structure without degradation for 7 d in culture *in vitro* (figure 3(b)). The diameter of 2% low concentration alginate analysed that the diameter size increased from 0.73 cm to 0.74 cm on the 1st day and then the size increased to 0.78 cm on

the 7th day. The hydrogel that absorbed the largest amount of liquid was found to increase in diameter and further degraded. As shown in figure 3(b), the 2% low-alginate macrocapsules showed lower degradation than the high-concentration alginate macrocapsules, owing to the high amount of crosslinking during the partial oxidation of alginate.

Due to the fact that having higher amounts of alginate or being higher viscous in 4% low alginate, 2% medium alginate, and 4% medium alginate macrocapsules can lead to rapid degradation in TBSS, this macrocapsule showed a higher degree of degradation. This result can be attributed to the release of more alginate, which remains uncross-linked in the high viscous alginate 4% low alginate, 2% medium alginate, and 4% medium alginate macrocapsules. It was therefore shown that high-concentration and high-viscous compositions exhibited higher *in vitro* degradation compared to 2% low-concentration alginate. Degradation of biomaterials is important for encapsulation applications in tissue engineering and bioengineering. Since the aim of our study was to protect the graft against immune system attacks, degradation of the alginate macrocapsule was not desired. According to the results of this experiment, the macrocapsule to be placed in the subcutaneous region was determined to be 2% low alginate, which absorbs the least amount of liquid and degrades the least amount.

3.2.3. Swelling ratio

The swelling properties of alginate macrocapsules were evaluated in terms of the capacity of the capsules to hold liquid in the culture medium for 25 h and the corresponding change in to the subcutaneous tissue. The entry and exit of molecules such as food, water, and waste through the pores must be continuous and balanced (figure 3(c)). The 2% low alginate solution showed a lower swelling rate than the 4% low alginate, 2% medium alginate, and 4% medium alginate solutions. In addition, the water uptake ability of the scaffolds increased gradually with increasing alginate concentration. Low alginate (2%) was the only group that achieved swelling equilibrium among the other groups. This can be explained by the high cross-linking density in its structure and good pore connection. Since 2% low alginate can remain stable in the target area for a long time by minimal swelling after settling in the subcutaneous region, the experiment continued with this group.

3.2.4. FITC dextran

The FITC Dextran permeability test was applied to test whether the obtained pore diameters of the capsules did not allow the immune system cells to enter and whether the insulin protein synthesized from the beta cells in the capsule came out of the capsule to participate in circulation. The absorbance value of

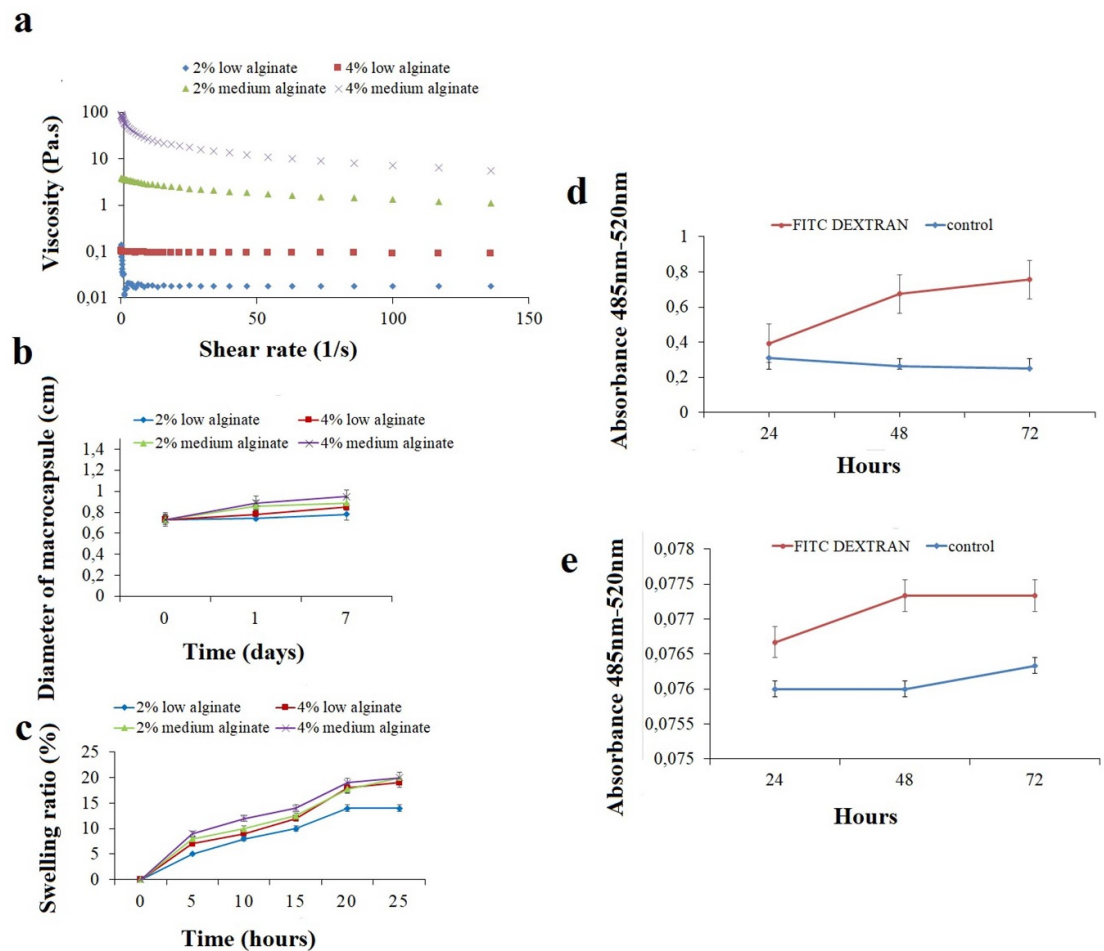


Figure 3. (a) Rheometric analysis of alginate at different concentrations. (b) Degradation profile. (c) Swelling ratio (%) in TBSS as a function of incubation time of different macrocapsules fabricated from alginate, 2% low alginate, 4% low alginate, 2% medium alginate, and 4% medium alginate hydrogels of different compositions. (d) Absorbance values on the microplate reader for the 4 kDa FITC Dextran. At the 48th and 72nd hours, the absorbance value increased, and it was considered significant that dextran penetrated the pores ($n = 3$, mean $\pm$ SD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). (e) Absorbance values on the microplate reader for the 150 kDa FITC Dextran. No significant increase in absorbance was observed at 48 and 72 h, indicating that dextran could not enter the pores ($n = 3$, mean $\pm$ SD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

4 kDa FITC DEXTRAN increased gradually after 24, 48, and 72 h, and thus dextran entered the capsule (figure 3(d)). It was determined that there was no significant increase in the absorbance value of 150 kDa; thus, dextran could not enter the pores of the capsule (figure 3(e)). The overall macrocapsule thickness were estimated as 55 μm in 3D (Z-stacking) under a confocal laser microscope (figure 6(d) D).

3.2.5. SEM

SEM was used to visualize the porous and fibrous microstructures of the capsules, pore size distribution, and surface topography of the scaffolds. As a result of the examination, the pore diameter of the capsules was found to be 0.174–1 μm (figure 4(g)). The results of our study showed that this pore size is suitable for the exit of insulin protein from the pore, and that T lymphocytes from immune system cells, cytokines secreted by them, and antibodies produced by B lymphocytes cannot enter the capsule. The macrocapsule obtained in our study enabled us to achieve

successful results in terms of both pore size and size, as in similar studies [26–28].

3.2.6. Mechanical test

A mechanical compression test was conducted to investigate the elastic moduli of the capsules. The compressive strength of the capsules was determined from the stress-strain curve by applying a load until the capsules were crushed. The elastic modulus of the capsules was and measured as 8.9 N from the slope of the linear part of the stress-strain curve formed by the elongation (mm) and force (figure 4(b)).

After the analysis, it was determined that the sample, whose yield point was determined, left the elastic deformation zone and passed into the plastic deformation zone. It has now been determined that the capsule will not return to its original shape, even when the applied load is removed after this point. The strain percentages of the cellular and empty capsules are shown in figure 3(b). The initial capsule

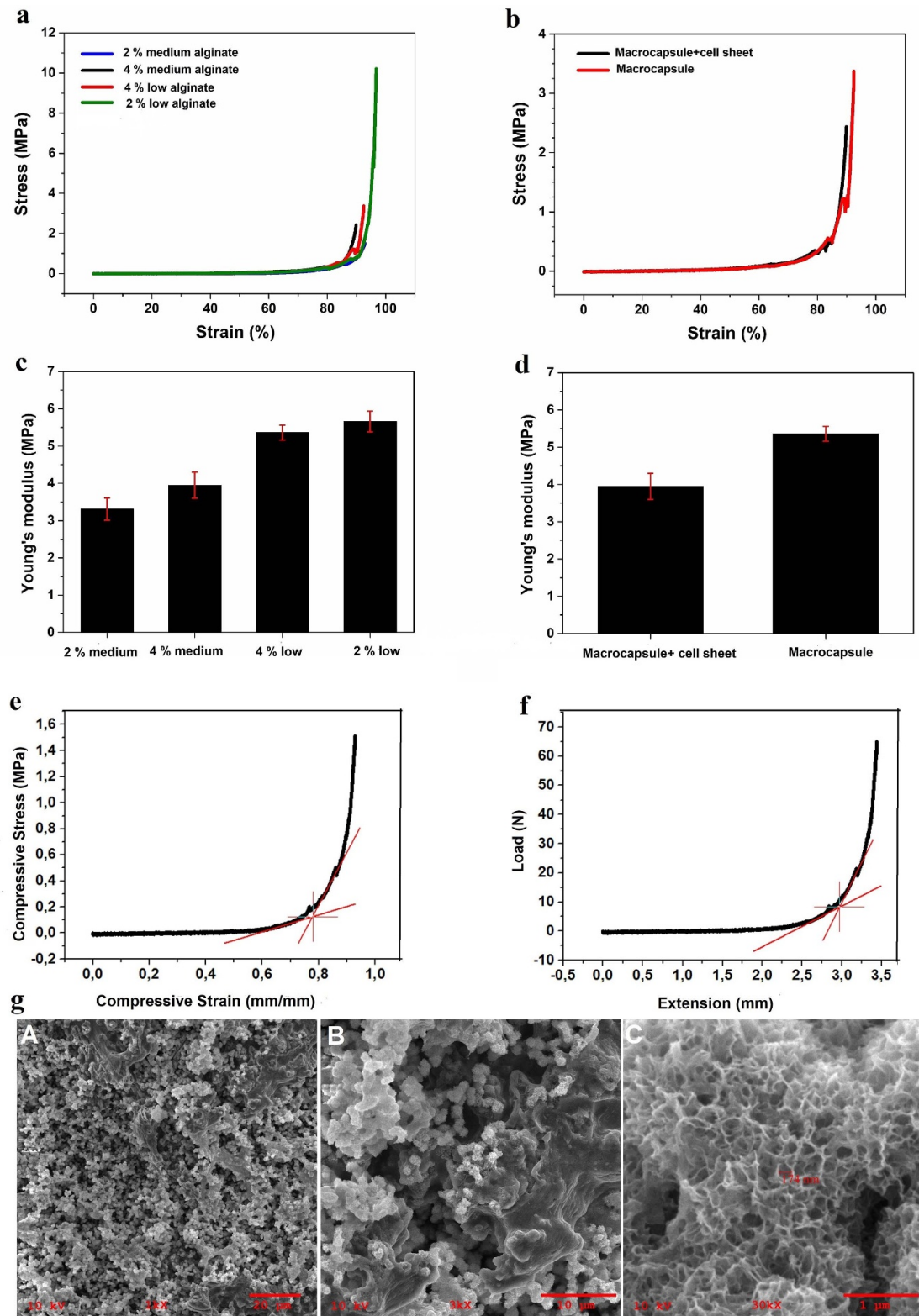


Figure 4. Optical images of 2% low-alginate hydrogel mechanical test profile of scaffolds. (a) Stress–strain curve of different alginate concentration macrocapsules and (b) Stress–strain curves of cellular and empty macrocapsules after the compression test. (c) Elastic modules of alginate at different concentrations. (d) Elasticity of cellular and empty macrocapsule. (e) Compressive strength. (f) Extension, elongation and compressive strain graphs of capsules after mechanical testing. The elongation plot shows the transition region of the 2% low-alginate capsule to plastic deformation after a force of 8.9 N. (g) SEM image of %2 low alginate macrocapsules. (Scale bar A: 20 μ m, B: 10 μ m, C: 1 μ m).

height was 4.63 mm at the beginning, and the height at the point of plastic deformation was 1.20 mm. This plastic deformation point was determined to be 8.9 N

force (figures 4(a) and (b)). The capsule reached the plastic deformation point after a force of 8.9 N and underwent permanent deformation.

It was observed that the 2% low-alginate hydrogel supported a compressive stress of 100%. The hydrogels in the other groups were found to support 80%–95% compressive stress. After the compression test, 2% low-alginate from the hydrogels exhibited excellent elastic properties owing to its spongy structure.

The compressive modulus of soft tissues has been reported by Ruiz *et al* between 2.11 and 2.21 MPa. Good mechanical properties provide mechanical support for new tissues, whereas high porosity provides a suitable 3D environment for beta cell growth and nutrient transport. Therefore, 2% low-alginate hydrogel has significant potential as a scaffold for pancreatic tissue engineering. The Young's modulus of 2% low alginate was calculated as 5.20 ± 0.20 MPa.

After the materials were characterized, it was decided to experiment with 2% low alginate, and the compression test was repeated by placing a cell sheet inside the macrocapsule. It was observed that the cell sheet placed macrocapsule decreased by 87% and Young's modulus decreased to 3.78 ± 0.13 MPa (figures 4(c) and (d)).

3.2.7. Fabrication of macrocapsule

It has been determined that this technique (figure 1) [25] allows the development of alginate capsules in standard sizes with approximately the same dimensions. Alginate capsules with an average size of 7.3 mm in diameter and 4.63 mm in height were obtained (figure 6(c)). After the preparation of different concentrations of alginate used in the production of capsules, the experiment was continued with the group that showed the strongest behaviour under *in vitro* culture conditions (2% low alginate).

3.3. Characterization of *in vitro* cell sheet performance in macroencapsulation device

3.3.1. Analysing the effect of alginate macrocapsule on cells

3.3.1.1. WST-1

Experiments were designed with the same number of cells in all experimental groups. No capsule and macrocapsule groups were included in the WST-1 test on the 1st, 7th and 14th days of culture. The groups were compared on days 1, 7 and 14. No significant change was observed in the statistical analyses performed for the non-capsule and macrocapsule groups. Cells were measured as viable and proliferative in all non-capsule and macrocapsule groups. It has been determined that nutrient and oxygen entry into the capsule is provided to a great extent through the pores so that the encapsulated cells can remain alive.

3.3.1.2. LDH assay

LDH test toxicity rates (%) were analysed to determine whether the macrocapsule samples had a toxic effect on cells. As a result of the tests performed on

the 1st, 7th, and 14th days of the samples, the percentage of viability was calculated as 94.61% on the 1st day, 93.75% on the 7th day, and 94.43% on the 14th day on the capsule group. The groups were compared among themselves on the 1st, 7th, and 14th days (figure 5(c)). As a result, it was determined that there was a significant minimally decrease in the viability rate as $*P < 0.05$ in all groups. It was analysed from day 1 to day 7 as $*P < 0.05$ in the capsule group, and from day 7 to day 14 as $**P < 0.01$. It was determined that alginate, which is the capsule material, did not have a toxic effect on cells.

3.3.1.3. Live/dead

In order to determine the viability of the cell sheet in the capsule, the live/dead (thermo fisher) test was applied on the 14th day of culture in addition to the WST-1 test. The capsule was mechanically divided into two and the cell sheet was removed and incubated with live/dead dye. At the end of the time, the sheet was examined under a confocal microscope (Leica) on the slide. In the macrocapsule group, dead cells were found in certain regions. It was determined that the live cell area was higher than the dead cell area (figure 5(e)). The thickness of the beta + MSC sheet was measured as 12 μm , as seen on the figure 6(d) E.

3.3.1.4. Metabolic activity assay

Control groups (noncapsule) were compared with capsule groups on days 1, 7 and 14 (figure 5(b)). In the following days of the experiment, the encapsulated cells were able to supply nutrients and oxygen depending on the adequate capsular pore size. It was determined that they were able to maintain their viability and functions, which are metabolic activities, as in the control (unencapsulated) groups.

3.3.1.5. Static glucose-stimulated insulin secretion (GSIS) assay

Maintaining the viability and function of the beta and MSC sheet is the most important feature of our macrocapsule design. For this reason, it was evaluated whether the encapsulated beta + MSC sheet (macrocapsule) continued to function for 14 d in culture.

Macrocapsule and noncapsule groups were included in the insulin secretion test on the 1st, 7th, and 14th days of culture. Groups were compared for days 1, 7, and 14 (figure 5(d)). On the 7th and 14th days of the experiment, a significant increase was detected in insulin secretion in response to the changing glucose levels in the noncapsule and macrocapsule groups ($**P < 0.01$, $***P < 0.001$). It was determined that insulin production continued in the cell in all of the noncapsule and macrocapsule groups. It was determined that this insulin protein released from the capsule pores and passing into the medium was an indication that the pore size was at the

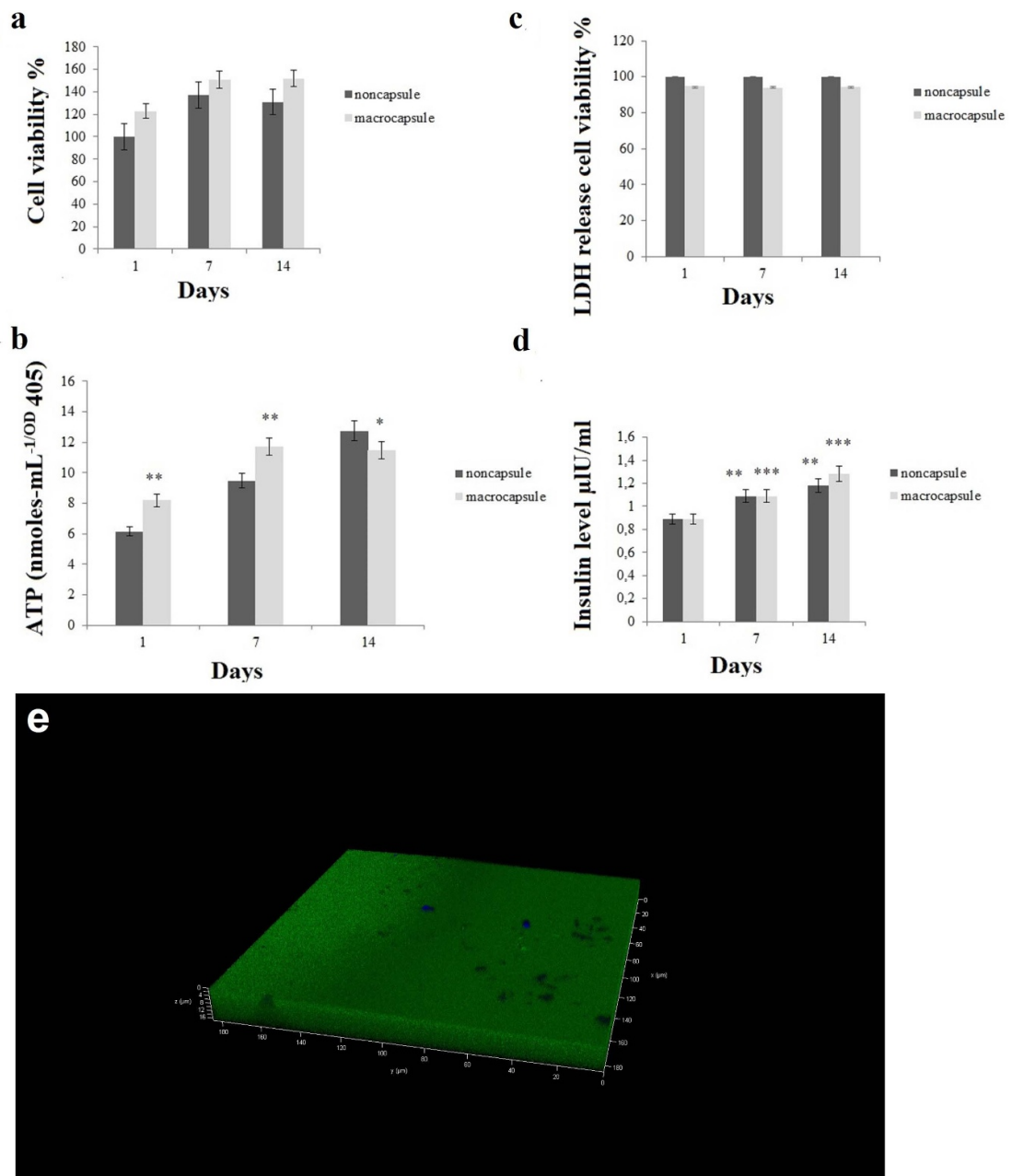


Figure 5. Analysis of permeability of the capsule, the cells in the capsule maintained their viability by successfully absorbing nutrients and oxygen from the medium they were in, just as in the groups without capsules. (a) WST-1 analysis results were compared days 1, 7 and 14 in all groups. ($n = 3$, mean $\pm$ SD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). (b) ATP synthase values of cells in *in vitro* experimental groups were measured in noncapsule and macrocapsule media throughout culture. (c) Cytotoxic effects of alginate macrocapsule on the cell sheet. The groups were compared among themselves on the 1st, 7th, and 14th days. ($n = 3$, mean $\pm$ SD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). (d) Insulin secretion and response of cells to various concentrations of glucose added to the medium were compared. ($n = 3$, mean $\pm$ SD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). (e) Live/dead staining images in macrocapsule group. Dead cells were stained by ethidium homodimer (red). Living cells were stained by Calcein AM (green).

desired level. It has been determined that the nutrient and oxygen entry into the capsule is provided to a large extent through the pores, and thus the encapsulated cells can also survive, and accordingly, they can secrete insulin in response, even if they are exposed to varying glucose concentrations ($n = 3$, mean $\pm$ SD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Therefore it was determined *in vitro* that the nutrient and oxygen uptake from the pores of the capsule was successful.

3.4. Body weights

The body weights of the rats were measured on the 2nd day after transplantation. Every other day weight measurement was done for 210 d. Whether there was a change in the weighing result depending on diabetes and the treatment applied was analysed with the student's *t* test (figure 6(a)).

The body weight was decreased in all groups except the control group in the first 10 d after transplantation. In the control

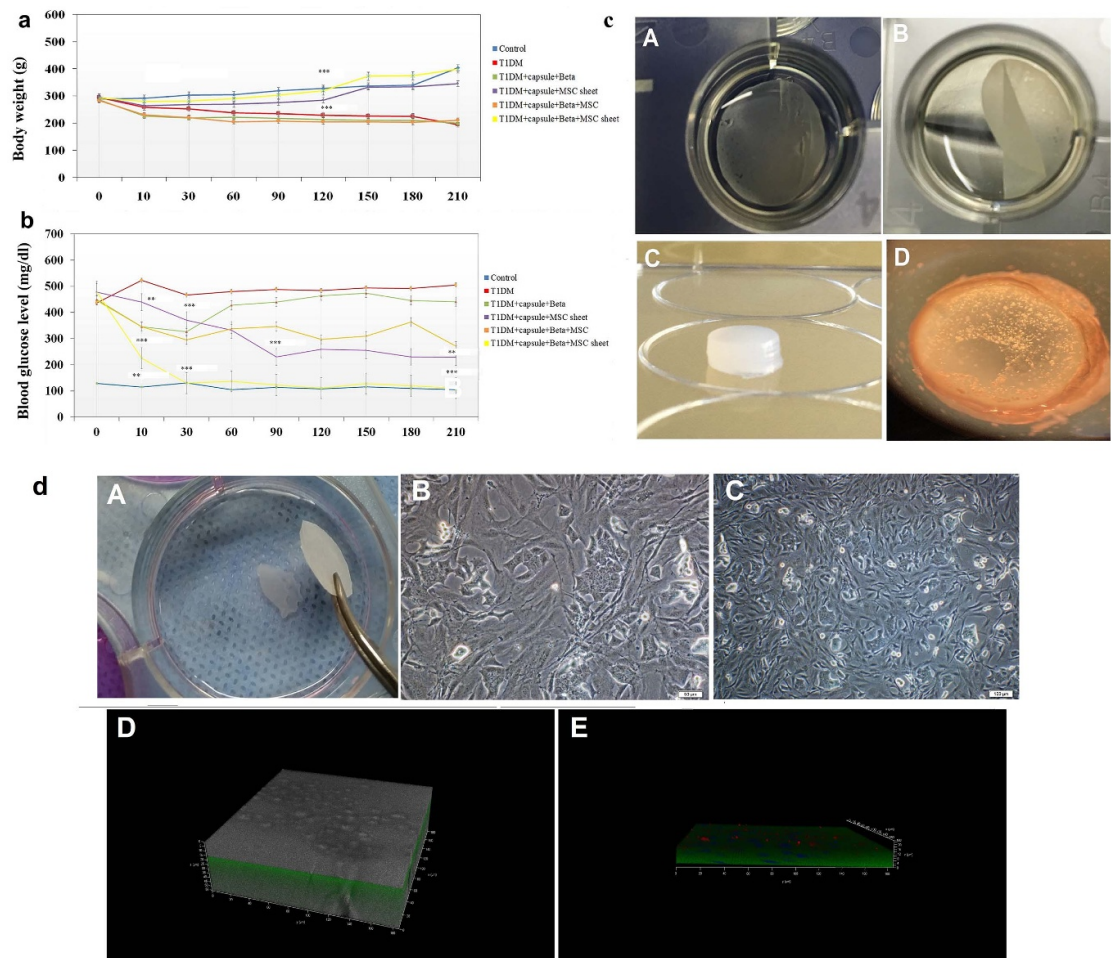


Figure 6. (a) Body weight change by day for 210 d in all groups. ($n = 6$, mean $\pm$ SD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). (b) Measurement of the blood glucose level of diabetic rat after transplantation. (c) (A) and (B): The images showed the co-culture of mesenchymal stem cells and beta cells sheet. (C): Macrocapsule image (D): Stereo microscope image of macrocapsule. (d) A: The cell sheet is harvesting from the culture dish and transported into the macrocapsule via the support membrane. (B) and (C): Phase-contrast microscope images of co-cultured beta cell and MSC (scale bars, B: 50 μm , C: 100 μm). (D): Confocal microscope (Z-stacking) image thickness of the macrocapsule (55 μm). E: The cell sheet thickness (12 μm) image at confocal microscope (Z-stacking).

T1DM + Capsule + Beta + MSC and T1DM + Capsule + Beta + MSC sheet group, a regular increase in weight was observed in the following days due to feeding. On the 130th day, body weight was evaluated significantly increase in the T1DM + Capsule + Beta + MSC and T1DM + Capsule + Beta + MSC sheet group ($***P < 0.001$). In the T1DM + Capsule + Beta and T1DM + Capsule + Beta + MSC groups, body weight decreased despite feeding, since blood sugar did not return to normal due to the lack of expected response to treatment.

3.5. Non-fasting blood glucose

From the second day after transplantation to rats, blood samples taken from the tail vein for 210 d from all groups were measured with a non-fasting blood glucose glucometer device (figure 6(b)). It decreased significantly from 490 mg dl⁻¹ to 310 mg dl⁻¹ on the 2nd day in the T1DM + Capsule + Beta + MSC sheet

group. When evaluated statistically, it was considered significant since $***P < 0.001$. The blood glucose level of this group was measured as 225 mg dl⁻¹ on the 10th day and 168 mg dl⁻¹ on the 15th day to reach the normoglycaemia value. On the 30th day, this value was measured as 129 mg dl⁻¹ and when analysed statistically, it was considered significant as $***P < 0.001$. On the 210th day of the experiment, this value was measured as 118 mg dl⁻¹ and the target value was reached.

In the T1DM + Capsule + Beta + MSC group, blood sugar, which was measured as 402 mg dl⁻¹ on the 2nd day, increased to 531 mg dl⁻¹ on the 5th day and 345 mg dl⁻¹ ($***P < 0.001$) on the 10th day. It was determined that it decreased to 295 mg dl⁻¹ on the 30th day and to a maximum of 271 mg dl⁻¹ ($***P < 0.001$) at the end of 210 d. The desired normoglycaemia target was not achieved in this group.

In the T1DM + Capsule + Beta group, 517 mg dl⁻¹ was measured on the 2nd day, and blood

glucose remained in the range of 400–450 mg dl⁻¹ for 210 d. The blood value was similar to the T1DM group, and normoglycaemia could not be achieved.

In the T1DM + Capsule + MSC group, blood glucose was measured as 458 mg dl⁻¹ on the 2nd day, 439 mg dl⁻¹ on the 10th day, and 370 mg dl⁻¹ (***P* < 0.001) on the 30th day. At the end of 210 d, it was determined that it decreased to 229 mg dl⁻¹ (***P* < 0.001) at the most. In this group, the response to treatment developed more than expected and it is thought that the MSC may have differentiated into an insulin-secreting beta-like cell in a high glucose environment. At the same time, more normoglycaemia was achieved in the T1DM + Capsule + MSC sheet group than in the T1DM + Capsule + Beta + MSC and T1DM + Capsule + Beta groups, since MSCs were transplanted in the sheet.

3.6. IPGTT blood glucose level

In order to evaluate the glucose response of cell sheets transplanted into diabetic rats, a comparative analysis was performed from the rat tail vein blood was collected at 30, 60, 90, and 120 min on fasting rats after intraperitoneal glucose injection at 30th day. (figure 7(a). T1DM + Capsule + Beta + MSC, blood glucose level remained consistently high (>400 mg dl⁻¹) after glucose administration, as in non-transplanted diabetic rats in the T1DM + Capsule + Beta group. In the T1DM + Capsule + Beta + MSC sheet group, the blood glucose level evaluated its maximum (198 mg dl⁻¹) 30 min after glucose administration, and then gradually decreased to normal levels (127 mg dl⁻¹) at the 60th minute. At 120 min, it showed a value similar to control (healthy) group rats, it was showed that successful beta-MSC cell sheets transplanted into T1DM + Capsule + Beta + MSC sheet group. Other groups were compared with the control group on 90th minutes. The results was considered significant in the comparative analysis between the T1DM + Capsule + MSC sheet and T1DM + Capsule + Beta + MSC sheet group (**P* < 0.05, ***P* < 0.01).

3.6.1. Serum insulin levels

In the T1DM + capsule + Beta, T1DM + capsule + Beta + MSC, and T1DM groups that did not respond to treatment, the serum insulin concentration was 0.25 l µIU ml⁻¹. In the T1DM + capsule + Beta + MSC sheet group, the serum insulin level increased significantly to a level similar to that of the control (healthy) rats (1.04 µIU ml⁻¹) after transplantation. On days 90 and 210, serum insulin concentrations were 1.06 µIU ml⁻¹ and 1.16 µIU ml⁻¹, respectively. In the T1DM + capsule + Beta, and T1DM + capsule + Beta + MSC groups, there was no change in serum insulin concentration compared to the pre-transplant level. In these groups, insulin

levels remained consistently low for at least 210 d after transplantation (figure 7(b)).

3.6.2. Serum C-peptide levels

In the analysis performed after the glucose challenge test, on the 90th and 210th days, T1DM + Capsule + Beta, (0.202 pg ml⁻¹ T1DM + Capsule + Beta + MSC (0.225 pg ml⁻¹, rat C-peptide levels in the group, T1DM + Capsule + Beta + MSC) sheet (0.883 pg ml⁻¹) was significantly lower than that of the group. These results clearly showed that groups with sheet MSCs were much more effective normoglycaemic level than in only beta cell and non-sheeted beta-MSC groups (figure 7(c)).

3.7. Histological analyses

On the 60th day, subcutaneous tissues were taken from the transplant area. Skin grafts were fixed in 10% formalin. As a result of haematoxylin-eosin staining, the images of the cells inside the grafts were analysed under a light microscope. Beta and MSCs were stained blue with haematoxylin. The homogeneous structure of the alginate was stained with eosin (figures 7(a)–(d)). A homogeneous eosinophilic alginate capsule is seen in all groups.

3.7.1. Live/dead staining

Live/dead staining was performed to examine the viability of the cell sheet in the capsule removed from the transplant site. These tissues were not fixed because were stained directly with live/dead and analysed under a confocal microscope. In each of the sheet groups of T1DM + Capsule + Beta, T1DM + Capsule + Beta + MSC, and T1DM + Capsule + Beta + MSC, dead cells were stained red, and live cells were stained green. Dead cells were found in less area in the T1DM + Capsule + Beta + MSC sheet group compared to the other groups (figure 7(k)).

3.7.2. IF staining

Insulin staining was done to observe the status of beta cells in the capsule after 60 d transplantation and analysed fluorescent microscope. Insulin stained positive (figures 8(E)–(H)). After the IF analysis performed in the MSC sheet transplanted group, it was observed that the cells in the capsule staining insulin was positive. This result suggested that under high glucose condition, MSCs differentiated into insulin-producing cells (figure 8(H)). According to the results of active caspase 3 staining performed to investigate whether MSCs prevent damage to beta cells, protect the graft and protect the cells from apoptosis, more active-caspase 3 positive areas were seen in the T1DM + Capsule + Beta group compared to the T1DM + Capsule + Beta + MSC, T1DM + Capsule + Beta + MSC sheet and T1DM + Capsule + MSC sheet groups (figures 8(M)–(P)).

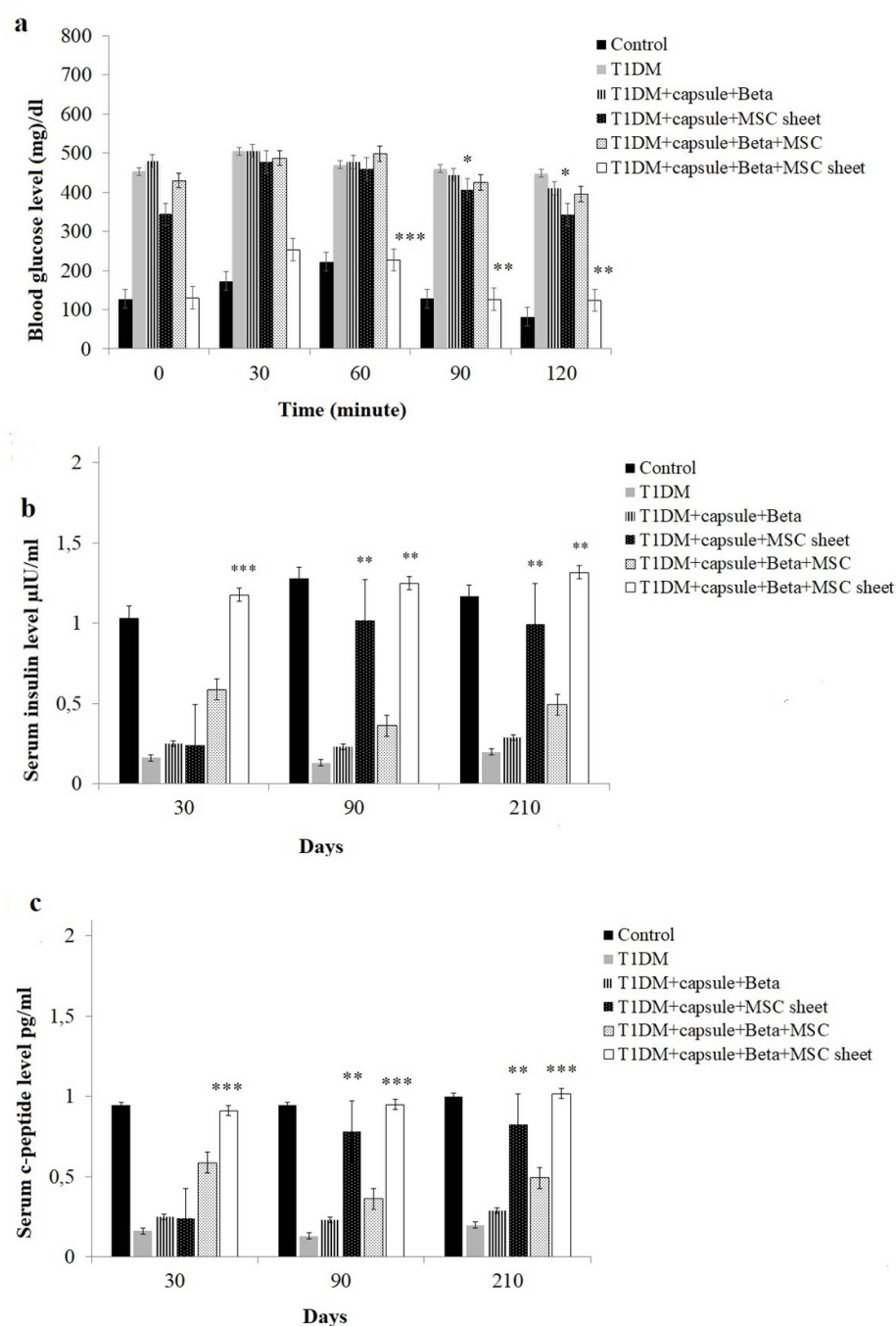


Figure 7. The IPGTT results after cell sheet transplantation in diabetic rat. (a) The blood glucose level was monitored up to 120 min after glucose administration. (b) Serum concentrations of insulin protein in diabetic rats. Insulin protein was determined by ELISA from blood samples collected from the tail vein after IPGTT assay on the 30th, 60th, and 210th days after transplantation ($n = 3$, mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) (c) Serum c-peptide level was investigated in the diabetic rats.

4. Discussion

Tissue engineering and cellular therapy approaches have come to the fore in order to prevent the use of insulin and provide type 1 diabetic patients with a better quality and comfortable life. In 2011 Saito *et al* cultured pancreatic islets in a temperature-responsive culture dish and obtained them in sheets, then succeeded in placing them under the skin of diabetic rats and providing normoglycaemia for 60 d [11]. Afterward, in order to develop a longer and

more effective treatment, Hirabaru *et al* modified the method, with the addition of MSCs as a difference to the culture medium together with the islets and managed to provide normoglycaemia in rats for 84 d [12]. The disadvantage of these two studies is the use of islets due to the difficulties of finding an islet donor and the need for huge amounts of islets to achieve normoglycaemia in a T1DM patient. Donor shortage in pancreas and islet transplants has led researchers to obtain insulin-secreting beta-like cells from different sources. The beta cell line is a more readily available

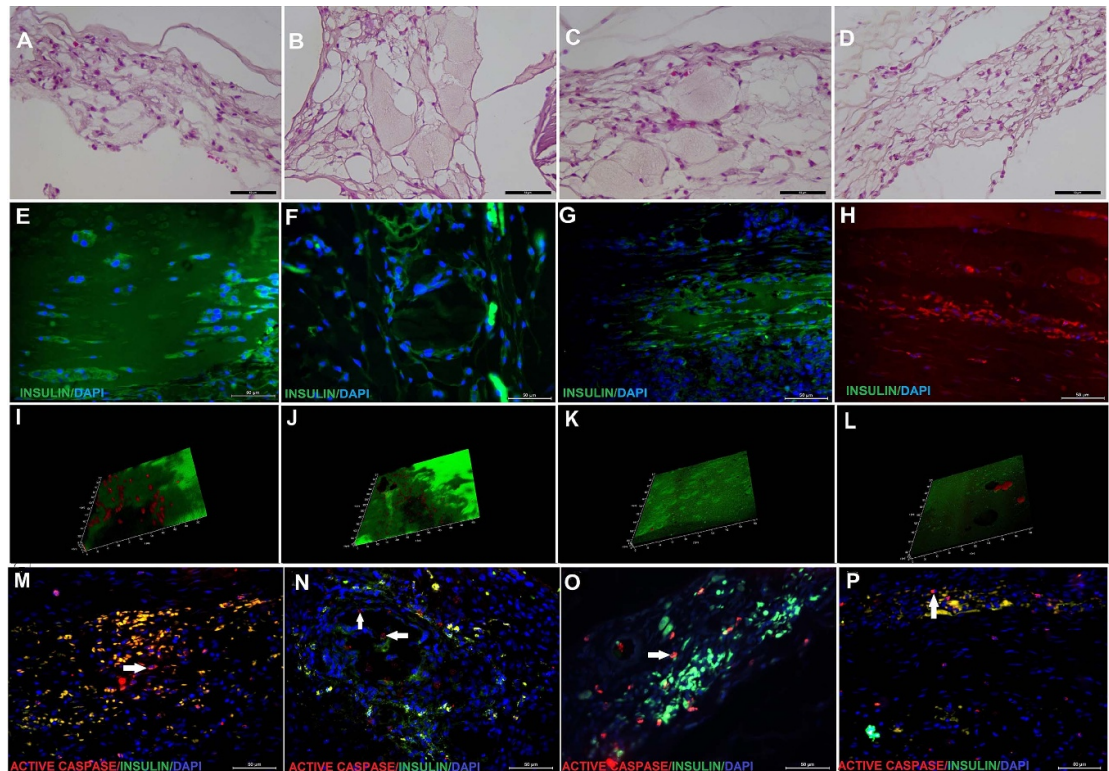


Figure 8. HE stained image of the graft in the transplant area. Beta and MSC stained with haematoxylin in alginate are seen. A homogeneous eosinophilic alginate capsule is seen. A: Beta cells in the transplanted macrocapsule are seen at larger magnification. (A): T1DM + Capsule + Beta (B): T1DM + Capsule + Beta + MSC and (C): T1DM + Capsule + Beta + MSC sheet (D): T1DM + Capsule + MSC sheet group (Scale bars, (A)–(D): 50 μ m). Staining images of beta cells in the capsule with cell immunofluorescent antibody. (E): T1DM + Capsule + Beta (F): T1DM + Capsule + Beta + MSC and (G): T1DM + Capsule + Beta + MSC sheet (H): T1DM + Capsule + Beta + MSC sheet group. (E)–(G): Insulin (Green), H: Insulin (Red). Nuclei were stained with DAPI (Blue). (Scale bars, 50 μ m). H. In the IF analysis, it was observed that differentiated MSCs were stained positive for insulin. The graft in the transplantation area was removed on the 60th day and the viability of the cells in the capsule was observed. It was examined live/dead staining in 3D with a confocal microscope and images were photographed in 3D. Dead cells were stained by ethidium homodimer (red). Living cells were stained by Calcein AM (green). (I): T1DM + Capsule + Beta, (J): T1DM + Capsule + Beta + MSC, (K): T1DM + Capsule + Beta + MSC sheet (L): T1DM + Capsule + MSC sheet group. Double immunofluorescent staining images of cell apoptosis in grafts after transplantation. Active caspase 3 antibody was analysed by immunofluorescent staining method in capsule grafts. Double staining was performed to observe insulin producing cells. (M): T1DM + Capsule + Beta (N): T1DM + Capsule + Beta + MSC, (O): T1DM + Capsule + Beta + MSC sheet, T1DM + Capsule + MSC sheet group. Active caspase 3 (Red), Insulin (Green). The white arrow show that apoptotic cells in the graft. (Scale bars, 50 μ m). The white arrow show that apoptotic cells in the graft.

and accessible cell source and beta cell lines, beta-like cells that secrete insulin are used in studies [15, 17]. Pancreatic beta cell line are a suitable resource for cell sheet engineering study and was also used in our study [4] because of these reasons. It was used for the first time in our previous study [4] and provided 30 d of normoglycaemia. Beta cells and MSCs were cultured in a temperature-responsive culture dish, and a 3-dimensional islet-like tissue could be constructed. This system, the difficulty of finding donors can be overcome and beta cell lines, which are easily available and a potential source, should be included in the application areas of cell sheet engineering. Beta and MSCs were incubated in RPMI 1640 medium by direct co-culture method, because co-transplantation of pancreatic islets and MSCs has positive effects on islet viability and function, in parallel with previous studies [22, 23, 35]. According to the results of other studies, adipose tissue derived MSCs might be considered the ideal cell of choice for cell-based treatment

of type 1 diabetes. Because the co-transplantation induces the differentiation of MSCs into insulin-producing cells [22].

Cell therapies as a potential treatment for type 1 diabetes remain of great interest. Studies have turned to cellular therapies to prevent insulin addiction and lead a comfortable life [2]. Although islet transplantation is the first cell that comes to mind in many studies from the past to the present, unfortunately, it should not be seen as a potential cell source due to reasons such as donor limitation and difficulties during their isolation in the present and in the future. However, islet transplants are difficult to replicate in animal experiments as a large number of islets are required to maintain normoglycaemia. Therefore, new sources should be sought for beta cells or insulin-producing cells. Significant efforts have been made on protocols to generate glucose-sensitive and functional beta cells from induced pluripotent stem cells or human embryonic stem cells from living

donors [36]. Studies in this direction have shown that several growth factors, small molecules, nutrients and hormones moderately promote beta cell proliferation and neogenesis [37]. In another study, insulin-producing beta-like cells were obtained from human embryonic stem cells and normoglycaemia was provided to rats for 174 d after transplantation [36]. However, since the use of embryonic stem cells is limited due to ethical problems and is prohibited in some countries, which negatively affects the study potential, beta cell line was preferred in our study. The BRIN BD 11 cell line has a high insulin releasing potential and is glucose sensitive and has been applied in *in vivo* transplant studies [38]. In one study, BRIN BD 11 cells transplanted into the mouse intraperitoneal region showed active insulin release for 28 d [39]. This short time is due to the small number of BRIN BD 11 cells being transplanted and aggregated at the transplant site. In our previous study, we think that, unlike this study, giving twice as many beta cells and transplanting the cells by embedding them in matrigel prevents aggregate formation and suppresses hypoglycaemia and hyperglycaemia [4]. With this approach, surrounding an unlimited source of beta cells with a permeable membrane, which would allow active insulin release but eliminate the use of immunosuppressants, has been a glimmer of hope for preclinical and clinical studies.

The immune system protection of pancreatic islets using an alginate capsule was first demonstrated in rats by Lim and Sun [40]. Although there are various biomaterials used in cell encapsulation; the application of alginate macrocapsule, which is a natural polymer, provided long-term survival of the graft by containing more cells, protecting these cells from immune reactions, and showing high biocompatibility with living organisms. In our study, we designed the pore size capsule we wanted using alginate and observed the capsule structure *in vitro* and *in vivo* analyses where antibodies and immune system cells could not enter, but nutrient, oxygen, and insulin input, and output were successfully achieved. In the analyses applied for the viability and function of the cells in the capsule *in vitro*, it has been proven that alginate does not have a toxic effect on the cells, as in other studies [30]. Alginate, an impact resistant and biocompatible polymer, was chosen for the capsule we placed under the skin. It has an elastic characteristic that can stretch up to a certain point of force application and can return to its original state in a very short time when released. In addition, the elastic modulus was found to be close to the elastic modulus of the scaffold in the literature in the study, in which a natural scaffold in gel form was shown [41].

Devices used in cell encapsulation today can be broadly classified as nano-scale, micro-scale, and macro-scale devices, which are implanted in extravascular or intravascular regions anywhere in the body. The targeted cell source in macrocapsules offers the

opportunity to process a large number of cells at once, not individually as in a microcapsule. Since we used a cell sheet in our study, we were able to protect a macro-sized structure from immune system cells and antibodies only by macroencapsulation.

Intravenous devices contain islets/cells encapsulated in tubes or hollow biocompatible fibres connected to the recipient's vasculature. They offer distinct advantages over extravascular macrocapsules such as better access to oxygen and nutrients, higher diffusion facilitated by blood flow, and immediate recognition of changes in glucose. However, their need for systemic anticoagulation and propensity to develop thrombosis at anastomotic sites make them undesirable devices for widespread use in clinical islet/cell transplantation [42]. Based on these disadvantages, scientists have turned to research on extravascular macrocapsulation devices [30]. These devices can be broadly classified according to their morphology as tubular and planar devices. Hollow fibre devices, on the other hand, are durable, injectable, easily adaptable, and easily retrieved for subcutaneous implantation. However, to achieve full insulin independence they require high doses and islets that are highly susceptible to damage after *in vivo* transplantation, limiting their frequent applicability. The macrocapsule we obtained in our study, both pore size and size, as in similar studies [30].

The microcapsule, which we have developed, acts as an immune isolation device, restricting the entry of immune cells and antibodies, while allowing passive diffusion of nutrients, oxygen and glucose into the capsules and insulin from the capsules into the body. Therefore, the immune protective barrier must sustain minimal thickness to ensure adequate flow of essential nutrients. In our study, we focused on the development of a permeable macrocapsule that maximizes the intra-capsule oxygen supply and at the same time induces neovascularization. As shown by Colton and Avgoustiniatos (1991), even a barrier with 25 μm thickness, through which oxygen must pass, reduces the oxygen tension by 50% in immune-isolated islet encapsules [43]. If the oxygen concentration supply drops below a certain level, cells would die. For this reason, the microcapsule thickness was restricted to 55 μm , in our study (figures 6(d) (D)). This thickness is even thinner than the smallest microcapsules required for oxygen flow. However, there is also a study where the capsule was 250 μm and normoglycaemia was achieved in diabetic canines after 84 d [44]. We achieved normoglycaemia in rats after 210 d by placing the cell sheet we obtained in the microcapsule with the minimum possible thickness.

The thickness of a tissue or cell sheet to be transplanted should not exceed 100–150 μm for nutrition and oxygenation [45]. The thickness of the beta + MSC sheet we produced in our study is 12 μm as shown on the figures 6(d) (E). In a study on cell sheets, the thickness of the double-layered cell sheet

we obtained was measured as 16 μm [32]. In another study on diabetes, the thickness of the sheet was reported to be 50–70 μm [3].

Encapsulation studies were first designed in the micro dimension. In these applications, called microencapsulation, the large volume ratio/surface area has been advantageous for the bulk transport of microcapsules, but this technology has certain limitations. Most importantly, a large transplant area is needed. Limitations such as the presence of the sufficient number of capsules to meet the daily insulin requirement, a suitable microvascular region providing rapid nutrient access, the difficulty of removing the microcapsule if necessary, and the insufficient survival rate of islets have been noted in some studies [46–48]. Although microcapsule applications have been tried in experimental animals and human subjects, difficulties such as cell sources, implant location and vascularization have not been resolved [49, 50]. In the experimental studies of the microcapsule, transplantation operations were performed to regions such as the kidney subcapsular, and liver portal vein. The hepatic portal vein is an invasive procedure and the sub-renal capsule has a poor microenvironment for oxygen supply.

One of the critical points of our study is that the capsule material is mechanically stable, biocompatible and well optimized in terms of pore size. After research, alginate has been widely preferred for use in microencapsulation due to its stable architecture, superior hydrophilicity, simple gelation process, biocompatibility, abundant availability, relative ease of supply, long-term stability, and low production costs *in vivo* [51]. Alginate was also used in the macroencapsulation method with too many islets [14–16]. In alginate hydrogels, it has been shown that the chain ratio of mannuronate to guluronate (M:G ratio) determines the toughness, the length and order of the guluronate, and the mechanical strength, swelling, permeability, and elasticity properties of the alginate [40]. They also have high stability and mechanical strength, which causes macrophage activation after transplantation, resulting in cellular overgrowth antigenicity and a dense fibrotic growth surrounding the capsules. Since the biggest aim of our study was to provide long-term normoglycaemia in rats, it was to prevent early graft loss due to macrophage, fibrosis tissue formation and antigenicity activation. We achieved our goal by optimizing and using the alginate material in the most appropriate concentration.

It is also vital to select the suitable pore size for the success of a bioartificial macrocapsulation device. Extremely small pore sizes can inhibit the inward diffusion of nutrients and oxygen from the beta cell-containing interior space of the planar device and the outward diffusion of insulin (7 kDa) and metabolites [45]. Conversely, a particularly large pore size may allow unwanted other cytotoxic chemokines

and immunoglobulins (150 kDa) to enter capsule pore, leading to cell destruction. Therefore, one of the most important criteria in designing a functional macrocapsulation device is the suitable pore size [51]. The pore size of the macrocapsule we obtained in our study was 6.92 μm and showed the selective permeability required to prevent the entry of immune system cells and IgG into the capsule.

The water uptake and swelling ratio of macrocapsule is necessary for diffusion. Also, early swelling prevents cellular proliferation and viability. However, the experiment was continued with 2% low alginate, which has a minimum swelling capacity, since excessive swelling will cause the material to degradation [52]. In this study, the water uptake ability of the macrocapsule is due to the difference in average porosity and pore size between the groups. Another possible reason is the decrease of polymer content of macrocapsule because macrocapsule swelling ratio mainly is due to the bonding of polymer chains with H_2O molecules.

According to the results of our study, the degradation ratio of macrocapsules was proportional to their swelling ratio. In this way, lower degradation ratio during 7 d depended on 2 low-concentration alginate and the higher degradation ratio during 7 d depends on 4% low alginate, 2% medium alginate, and 4% medium alginate. Therefore, the addition of 2% low-concentration alginate prevents rapid degradation of the capsule and ensures long-term disintegration. It seems that high concentration alginate decreases macrocapsule hydrophilicity and also increases the viscosity of macrocapsule, as a result, faster down its degradation. As a rule degradation ratio swelling ability, and porosity of macrocapsule could also affect their mechanical strength [53].

The Young's modulus of the %2 low concentration alginate was significantly higher than that of the 4% low alginate, 2% medium alginate, 4% medium alginate, due to the high cross-linking density of the %2 low alginate. Higher levels of cross-linking form more bonds, stabilizing the low 2% alginate skeleton and increasing modulus [54].

The ATP assay, Live/Dead assay, LDH assay and WST-1 analysis were performed in parallel to validate the analysis results of each other. These assays are critical to determine whether the viability of the cell sheet in the capsule is affected by both biomaterial and culture conditions in the *in vitro* culture condition from the production stage to the transplantation process. In the WST-1 analysis method, the number of viable could be estimated, but due to the nature of the reaction, it could not be possible to detect any death cells that might be formed within the necrotic zones. To determine any increase in the cell death (toxicity) or any formation of necrotic zones, LDH and live/dead staining analysis were performed.

For this reason, LDH and live/dead analysis were included in our study to determine whether there

was any necrosis formation in the cell sheet. In the live/dead assay, calceinAM/ethidium staining was performed to estimate whether alginate macrocapsules affected the number of dead cells increased or necrotic areas developed.

By measuring the ATP level, the viability and functionality of cells were determined regardless of the presence of reducing agents. The presence of any reducing agent leading a redox reaction would cause a false positive result in WST-1 assay. Further, it is important to investigate the regularity of the cell sheet in the capsule through the pores in the functioning of the process of nutrient, oxygen supply and waste disposal under culture conditions.

In our study, in which we aimed at long-term protection of the viability and function of the cell sheet in the macrocapsule produced from 2% low concentration alginate, the results we obtained were similar to those of the studies in the literature [28, 30]. The microcapsule produced was proven to increase cell viability and to maintain ATP levels while it limits the formation of apoptosis and necrosis.

One of the important points for the effective insulin release of the graft obtained using alginate is the transplant site. From a surgical perspective, the ideal site should allow easy application to minimize potential implantation complications [55]. A transplant region requiring the minimal number of cells would be advantageous to decrease the need for high transplant volumes or for large numbers of donors and infusions. Therefore, in our study, it was decided that the subcutaneous (subcutaneous) area would be appropriate as the transplant site. While the subcutaneous site is a good vascular resource, the islet is the new study area as a transplant site, and good accessibility of transplanted tissue to ease of monitoring would also be beneficial. In a study in the literature, cell sheets were placed on the liver surface as a transplant site [3]. However, this method was not considered appropriate in our study because it is a much more invasive procedure compared to the subcutaneous region. Considering the potential of the macrocapsule to provide engraftment to the transplanted area in our study, good results were obtained in the subcutaneous region as the area where the vascular regions are dense.

Although the location of the transplant site is important for the study, another important issue for us is the preservation of the integrity of the transplanted cells and the fact that the cells do not undergo apoptosis. For this reason, in our study, the design of functional islet-like systems by forming beta + MSC cell sheets based on the principle of cell-sheet engineering and the transfer of these systems to the subcutaneous region took place. There are various studies in which cell sheet systems are used for the treatment of diabetes. Such as abdominal, subrenal, subcutaneous, and site transfers [3, 11, 12, 56]. It has been reported that these 3D cell sheets

are effective by maintaining their viability in the subcutaneous region and providing normoglycaemia [11, 12]. However, in subcutaneous studies, normoglycaemia was achieved in rats for a maximum of 60 d with islet transplantation [11]. In another study, normoglycaemia was provided in the abdominal region for 84 d [12]. The limitation of these studies is the lack of an application that will protect the transplanted graft from immune attacks. Based on this deficiency, we aimed to macroencapsulate the cell sheets, to protect the graft from immune attacks and to provide longer-term normoglycaemia in rats. Our study showed that rats blood glucose level decreased to normal levels in post-transplant blood samples taken during the 210 day follow-up period.

MSCs are seen as a potential source for cellular therapies. MSCs show the ability to differentiate into cells of many tissues different from the tissue from which they originate, their plasticity feature. In our study, beta cells and isolated rAT-MSCs were cultured together in temperature-responsive culture vessels and formed as a PIPAAm sheet. In this way, since the cells are in contact with each other as in their niches *in vivo*, and the ECM structure is preserved in cell-cell and cell-surface interactions, their viability in the capsule lasted longer. In addition, this method also eliminated the harmful effects of enzymes used to remove cells with traditional methods on the cell structure [57, 58]. In addition, the study of macroencapsulation and transplantation of a cell sheet is a first in the literature.

Our study showed that rats blood glucose level decreased to normal levels in post-transplant blood samples taken during the 210 day follow-up period. However, when the experimental groups were compared, it was shown that the blood glucose level of the beta-MSC sheet group was closer to the healthy control group than the beta-MSC transplant group. This showed that the cell sheet in the macrocapsule device can insulin secretion and insulin production into the systemic blood circulation. The activity of cell sheet in macrocapsule device was demonstrated by IPGTT. In cell sheet and transplantation studies performed with temperature-responsive dishes, the preservation of ECM contributed to the activity of the cells as well as being morphologically healthy [57, 58].

In summary, the most important and different others studies feature of our study is that transplantation of beta cells together with MSC is more successful in the normalization of blood glucose in rats than only beta cell transplantation. To the best of our knowledge, there is no study in the literature in which beta cells were cultured in thermosensitive dishes and the resulting cell sheet was encapsulated with alginate and transplanted to diabetic rats. However, there are studies in which pancreatic islets are enzymatically digested and a single cell suspension is made and this cells obtained is cultured in temperature-responsive dishes [11, 56]. As it is known, 80% of pancreatic

islets are composed of beta cells, while 20% of other endocrine cells, vascular and stromal cells of the islet. Therefore, beta cells could be supported morphologically and functionally after transplantation, since especially vascular and stromal cells were found to support beta cells in these studies.

In the pancreatic islet microenvironment, beta cells are found together with many other cell types of islet cells. Based on this fact, we designed an *in vivo* experiment by co-culturing beta cells with MSCs. Our findings led us to conclude that, just as in our study and that of others [4, 12, 22], MSCs support these insulin secreting beta cells. Data of the comparative study obtained from *in vivo* analyses (blood glucose, IPGTT blood glucose, serum insulin & c-peptide ELISA) performed on rats by transplanting beta cells and beta + MSCs, it was found that normoglycaemia was maintained by significantly improving the blood glucose level in the beta cell experimental groups transplanted together with the MSCs (figures 6(b) and 7(a)–(c)).

Active caspase 3 staining was performed to show that cytokines and growth factors secreted by MSCs to prevent damage to beta cells in the transplantation area, to protect the graft and to block apoptosis of cells [59]. According to the results, it was observed in microscopic analyses that beta cells transplanted with MSCs underwent apoptosis compared to beta cell transplantation alone. This result can be explained by the cytokines and growth factors TGF β and IL-6 secreted by MSCs, which protect the graft and prevent apoptosis of beta cells due to their anti-apoptotic and anti-inflammatory properties [60]. These results are consistent with the results of other MSC studies [61, 62].

One of the most important features of MSCs is their differentiation into various tissues such as muscle, cartilage, bone, liver, neurons, heart, adipose tissue and into insulin producing cells (IPCs) both *in vivo* and *in vitro* [22, 35]. During the study, interesting results were found after the insulin staining of transplanted grafts. In the T1DM + Capsule + MSC sheet transplanted group, insulin staining of the cells in the capsule was positive, after immune fluorescence analysis. The positive cells found in this group suggest that under high glucose condition, MSCs might differentiate into IPCs (figure 8(H)). Another result that correlates with this conclusion is that the blood glucose level of the T1DM + Capsule + MSC sheet group decreased from hyperglycaemic level to 229 mg dl⁻¹ (normoglycaemia) in *in vivo* experiments (figure 6(b)).

We considered that long-term normoglycaemia in the macrocapsule group that we support with MSCs is due to the anti-apoptotic, immunosuppressive, and immunomodulatory effects of MSCs. When the findings of our study are evaluated, the presence of MSCs together with beta cells in extravascular alginate macrocapsules in the subcutaneous region in order to

provide long-term cellular therapy in T1DM patients is the best and long-acting treatment method, and it lays the groundwork for future clinical studies in which this method will be used.

5. Conclusions

In our study, our goal of designing the most effective macrocapsule in formation and providing long-term normoglycaemia was successful at the end of the project.

If we consider the reflection of our study on the clinic, the capsules showed successful survival in the subcutaneous region, which is the most suitable region for the least invasive intervention to the patient as the transplant site. The fact that the capsule material we obtained is biocompatible and shows maximum performance in terms of mechanical durability is an indication that we have made good progress in our study. In addition, the subcutaneous region is a very potential region in terms of vascularization, and nutrition and oxygenation of our cells in the capsule can be ensured. Thus, the cells in the capsule were able to survive and function for 210 d.

It was observed that when beta and MSCs were transplanted individually in suspension, they underwent apoptosis at the target site more than when transplanted in sheets. Therefore, in our study, we observed that the survival and insulin secretion potential of the 3D macroencapsulated tissue, which we created by sheeting transplantation of beta cells and MSCs using cell sheet technology, is more successful than the transplantation of individual islet capsules.

The cellular treatment of pancreatic injuries will be able to be transferred to the patient with a minimally invasive method in the form of subcutaneous/subcutaneous implants in the future, by macroencapsulation by making beta and MSCs three-dimensional with the cell sheet engineering method. In this way, even if the implants are allogeneic, they will be protected from autoimmune attacks and will show long-term survival and placement in the target area.

These results lay the groundwork for future clinical studies in which these formulations will be used to provide autoimmune animal models and long-term replacement therapy for type 1 diabetes.

In parallel with the successful results of our study, when a functional islet is formed in a different region other than the pancreas-liver, it will find application in the treatment of type 1 diabetes in the clinic. It is a minimally invasive method to transfer the tissue we have obtained subcutaneously, instead of transferring it directly to the pancreas-liver region by encapsulating it. We think that this tissue can be turned into an implant/preparation/chip that can be easily placed under the skin with the minimal invasive method in

the future. In this respect, our study will progress in a direction that can easily reach the clinical trial stage.

Data availability statement

All data that support the findings of this study are included within the article.

Statement of ethics

Experimental Animal Center of Kocaeli University and Ethical Board of the Medical Hospital of Kocaeli University, Turkey [Protocol No: KOU HADYEK 1/3-2019] according to the institutional guidelines and national animal welfare with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences (Institute for Laboratory Animal Resources 1996).

Declaration of interest

The authors have no conflicts of interest to declare. The authors alone are responsible for the content and writing of the paper.

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Author statement

Y Y and B D designed the study and supervised the experiments, Z H designed and apply all *in vivo* experiments, S M contributed in biomaterial optimization procedures, A Ö, G G and G D contributed in stem cell procedures. All these authors have substantial contributions to the final manuscript and approved this submission. All authors are aware of the order of authorship and that no further change in authorship will be performed after submission, excepting those previously authorized by the editor-in-chief.

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