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Construction of Mesenchymal Stem Cell-Derived Artificial Human Urinary Bladder: A Preliminary Study

Mezenkimal Kök Hücre Kaynaklı Yapay İnsan Mesanesi Geliştirilmesi: Bir Ön Çalışma

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ABSTRACT

Objective: The present study aimed to obtain the required cells and select a suitable scaffold material for constructing an artificial bladder using the tissue engineering approach.

Materials and methods: The convenience of obtaining human adipose tissue-derived stem cells (hADMSCs) was used in this study. It was attempted to differentiate these cells into smooth muscle cells (SMC), which are present along the wall of the bladder. Urothelial cells were enzymatically isolated from tissue biopsies. Synthetic (polylactide co-glycolic acid, PLGA) and natural (chitosan) polymers were used in scaffold fabrication using a tissue engineering approach.

Results: In the cellular experiments, urothelial cells couldn't be cultured in polystyrene culture vessels *in vitro* and required a support material to maintain viability. Better results were obtained with the feeder layer. The hADMSCs exhibited the expected morphological changes in the serum-rich medium content in the SMC differentiation experiments. Chitosan, biocompatible and biodegradable, was mixed with PLGA as an alternative scaffold combination.

Conclusions: This study indicated that hADMSCs-derived smooth muscle cells and biopsy-isolated urothelial cells cultured on hybrid chitosan–PLGA scaffolds with appropriate physical properties could serve as a suitable model for tissue-engineered artificial bladder construction.

Keywords: Mesenchymal stem cell, tissue engineering, urinary bladder, urothelial cells

ÖZ

Amaç: Bu çalışmada, doku mühendisliği yaklaşımıyla yapay mesane yapımı için gerekli olan hücrelerin elde edilmesi ve uygun iskele malzemesinin seçilmesi amaçlanmıştır.

Materyal ve Metot: Bu çalışmada kolaylıkla elde edilebilen insan yağ doku kökenli kök hücreler (hADMSCs) kullanılmıştır. Bu hücrelerin mesane duvarı boyunca yerleşmiş olan düz kas hücrelerine (SMC) farklılaştırılmasına çalışılmıştır. Ürotelyal hücreler ise doku biyopsi örneklerinden enzimatik aktivite ile izole edilmişlerdir. Doku iskelesi yapımında, doku mühendisliği yaklaşımı kullanılarak, sentetik (Poli-laktid-ko-glikolik asit, PLGA ve doğal (kitosan) polimerler kullanılmıştır.

Bulgular: Hücresel deneylerde, ürotelyal hücreler polistiren kültür kaplarında in vitro olarak kültüre edilememiş ve canlılıklarını sürdürmek için bir destek malzemesine ihtiyaç duydukları belirlenmiştir. Bu aşamada, besleyici hücre tabakası ile iyi sonuçlar elde edilmiştir. Ayrıca hADMSC'ler, SMC farklılaşma deneylerinde yüksek serum içeriğine sahip ortamda beklenen morfolojik değişiklikleri sergilemiştir. Biyouyumlu ve biyolojik olarak parçalanabilen kitosan alternatif iskele kombinasyonu olarak PLGA ile karıştırılmıştır.

Sonuç: Bu çalışma, uygun fiziksel özelliklere sahip hibrit kitosan-PLGA yapı iskeleleri üzerinde çoğaltılmış hADMSC türevli düz kas hücreleri ve mesane biyopsisinden izole edilmiş ürotelyal hücrelerin, doku mühendisliği ile yapay mesane üretimi için iyi bir model olabileceğini göstermiştir.

Anahtar Kelimeler: Doku mühendisliği, mesane, mezenkimal kök hücre, ürotelyal hücreler

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INTRODUCTION

The bladder is an important organ in the human body and is not directly involved in affecting vital activities. Any major problems in the bladder, such as urinary incontinence, congenital urological anomalies, and cancer formation, could reduce the quality of life of an individual and even result in death in some instances.[1]

In the cases where bladder repair cannot be performed, such as cancers, congenital anomalies (bladder exstrophy, posterior urethral valve stenosis, etc.) or dysfunctions, and organ losses, organ transplantation is considered a solution. However, difficulty finding a suitable donor and the development of complications after transplantation limits the application of this approach. Moreover, complications are encountered upon curating certain critical urinary system problems, such as inflammation, neoplasm formations, immune rejection, etc. Therefore, it is imperative to search for novel treatment methods for the above conditions related to bladder issues.[2,3]

The development of various biomaterials and tissue engineering methods in recent years has led to increased use of alternative techniques, achieving considerable success in eliminating bladder dysfunctions.[4]

In the present study, biocompatible and biodegradable chitosan[1,5] was mixed with another FDA-approved PLGA polymer to create an alternative tissue scaffold.[1,6] Thus, an alternative tissue scaffold was created for this study. Chitosan was selected as previous studies have demonstrated that this biocompatible and biodegradable material[7] stimulated angiogenesis during artificial tissue/organ production.[1,8] The other biodegradable and FDA-approved polymer PLGA was selected for similar reasons.[1,9,10]

Another important factor determining success in tissue engineering applications is the use of cells with appropriate properties. In various tissue engineering applications, cells are obtained from different sources depending on the specific requirements.[11] Stem cells are present in various tissues of an adult organism as undifferentiated cells with self-renewing abilities and the potential to differentiate into several cell types.[12] These stem cells formed the cellular basis of the present study.[13,14]

Since smooth muscle cells are one of the main cell types in the structure of the bladder, it was decided to obtain these cells by differentiating the human adipose tissue mesenchymal stem cells (hADMSCs).[15] Urothelium cells were the other type of cells required in the present study and were attempted to be isolated from bladder biopsy samples.[1,16,17]

The present study aimed to obtain the required cells for constructing a complete artificial bladder using the tissue engineering approach and then assemble these cells on a suitable scaffold material to achieve the appropriate bladder anatomy/physiology.

MATERIALS AND METHODS

Ethics Committee Approval: The tissue biopsy samples required for the isolation of urothelial cells, which were to be used in the cellular experiments in the study, were obtained from the surgical interventions performed at the Department of Pediatric Surgery, Faculty of Medicine, Manisa Celal Bayar University. The protocols used in the study were approved by the Ege University Clinical Research Ethics Committee (Date: 11.01.2010, decision no: 09–12/1). All experimental studies were conducted at the Animal Cell Culture and Tissue Engineering Laboratories, Department of Bioengineering, Faculty of Engineering, Ege University. The study was carried out by the Declaration of Helsinki.

Construction of Tissue Scaffolds: In the first stage of the tissue scaffold experiments conducted within the scope of the present study, scaffolding was performed using the polymer PLGA (poly-lactide co-glycolic acid)[18] and chitosan,[19] which have been used for constructing artificial bladder in previous studies as well. First, a chitosan scaffold was constructed to be used as the control. Porous tissue scaffolds were prepared by freeze-drying a 10 mg/mL chitosan solution (C3646, Sigma, USA) in 0.2 M acetic acid. The solution of PLGA (503H, 50:50, Boehringer Ingelheim Chemicals, USA) was prepared in 15% dichloromethane. Afterwards, the above chitosan solution was mixed with 4% PVA (Polyvinyl alcohol, 363138, Sigma, USA) in the ratios of 3:1 and 10:1, forming two different mixtures. PVA was used to support homogenization by forming an interface between chitosan and the PLGA solution, which are present in two separate phases under normal conditions and, therefore, cannot form a homogeneous mixture in such conditions. Hybrid scaffolds were prepared by freeze-drying the solution formed by mixing chitosan and PLGA with PVA. All prepared tissue scaffolds were examined under a scanning electron microscope (SEM, Jeol JSM-5200, Japan).

Cell Culture Experiments

Differentiation of hADMSCs into Smooth Muscle Cells: Smooth muscle cells are one of the basic cell types in the structure of the bladder. Therefore, in the present study, these cells were obtained by differentiating hADMSCs. The hADMSCs population from the cell culture stocks available at the Animal Cell Culture and Tissue Engineering Laboratories, Department of Bioengineering, Faculty of Engineering, Ege University was used. The details regarding

the isolation, culture, and characterization methods of the hADMSCs are available in the previous report published by our research group.[15] The differentiation of hADMSCs, which formed another basis of the present study, into smooth muscle cells (SMCs) was attempted using different methods. DMEM containing 5% horse serum and 50 µM hydrocortisone (H4001, Sigma, USA) was used to differentiate adipose tissue-derived mesenchymal stem cells into smooth muscle cells (SMCs).[20] In this process, various surface coatings (0.1–2% gelatin, FBS) were also utilized to support the differentiation. The hADMSCs were plated on these surface coatings at an initial cell concentration of 3000 cells/cm^2, followed by incubation in the differentiation medium for eight weeks. Afterwards, the cells were labelled with the α-smooth muscle actin antibody (ab15267, Abcam, USA) using immunocytochemistry for characterization.

Isolation and Culture of Urothelial Cells: The urothelial cells lining the inner surface of the bladder were isolated from the ureter or bladder biopsy samples using 25% (w/v) dispase (17105, Invitrogen GIBCO, USA) and Trypsin-EDTA solution (L2163, Biochrom AG, Germany).[21,22] The isolated cells were then cultured in serum-free keratinocyte medium (KSFM,17005034, Invitrogen GIBCO, USA) enriched with the epidermal growth factor (EGF), bovine pituitary extract (BPE) (37000-015, Invitrogen GIBCO, USA) and 30 ng/ml cholera toxin (227036, Calbiochem, Germany). In the above culture process, the urothelial cells were supported with epithelial characteristics using the feeder cell layer formed by 3T3 mouse fibroblast cells, which were generated using mitomycin C (A2190, Applichem, Germany).[22,23] The isolated and successfully cultured urothelial cells, as described above, were characterized using immunocytochemistry by the anti-cytokeratin7 antibody (ab9098, ABCAM, USA).

RESULTS

The SEM image of the porous tissue scaffold prepared through freeze-drying from the 10 mg/mL solution of chitosan in 0.2 M acetic acid is depicted in Figure 1a. The examination of the SEM images obtained after the formation of the porous tissue scaffold from the chitosan–PLGA solution mixed with PVA in a ratio of 3:1 using the freeze-drying method revealed that chitosan and PLGA did not mix up homogeneously with each other (Figure 1b).

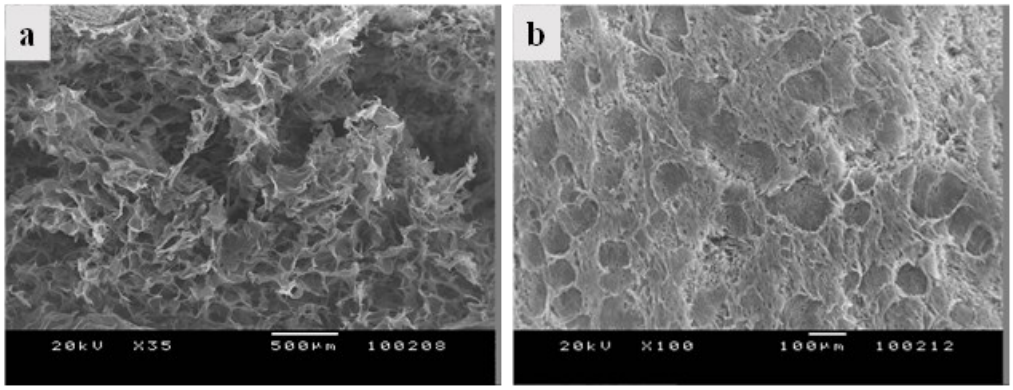

Figure 1. SEM images of the tissue scaffolds prepared using the freeze-drying method. a-Chitosan scaffold; b-Chitosan–PLGA scaffolds.

In the present study, different surface coatings were used to differentiate hADMSCs into smooth muscle cells. The control and experimental wells, with 0.1% gelatin coating, presented differences in the differentiation of hADMSCs into SMCs (Figures 2a-2d). In the differentiation (exploratory) wells, the cells were longer and closer to the SMC morphology (Figures 2b, 2d, and 2f). In addition, after eight weeks, a slight labelling with the α-smooth muscle actin antibody was observed in the differentiated cells (Figures 2d and 2f).

Additionally, the various surface coatings did not produce a significant difference in differentiation but instead resulted in almost equal levels of antibody labelling.

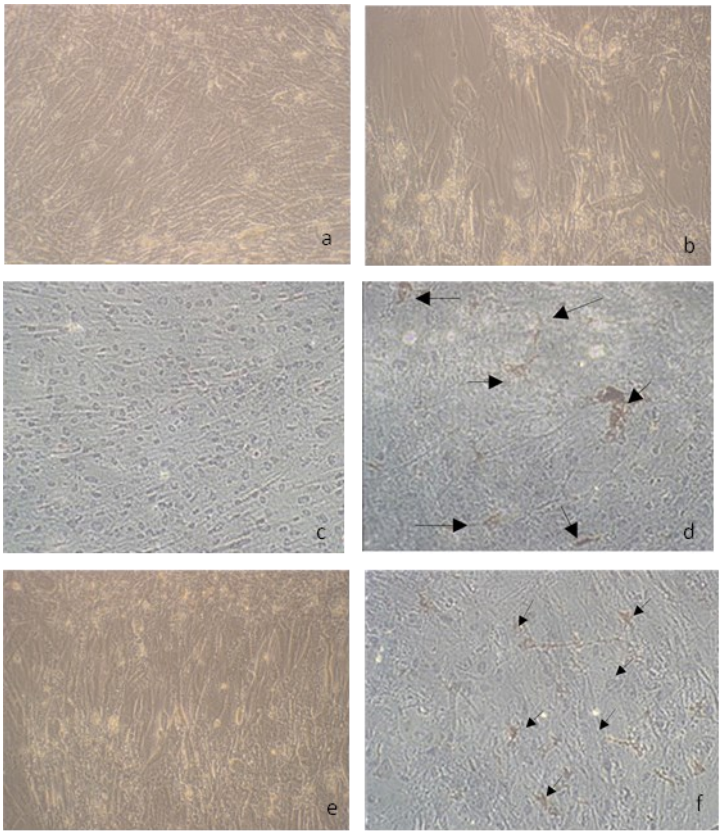

Figure 2. Inverted light microscopy images of ADMSCs in the SMC differentiation medium in the polystyrene wells with 0.1% gelatin coating (a, b, c, and d) and without the uncoating (e and f). The left column and the right column depict the control and the differentiation wells, respectively. The micrographs presented in d and f depict the actin-labelled α-smooth muscle cells (the arrows indicate the antibody-labelled regions).

To isolate urothelial cells, dispase was used for tissue digestion, and after nearly seven days, the resulting culture contained both epithelial and fibroblast cells. It was expected that epithelial cell colonies would be formed with regular medium change (serum-free keratinocyte medium). However, it was observed that as time progressed, at approximately the 9th day, both cell types had lost their viability in the culture. This indicated that the urothelial cells required a supporting layer to survive and proliferate in the culture dish. Therefore, a feeder cell layer was formed, resulting in success in the cell culture using this modified method. The urothelial cells obtained using the above procedure were characterized using the anti-cytokeratin 7 antibody. As depicted in Figure 3, the isolated urothelial cells were positive for the antibody.

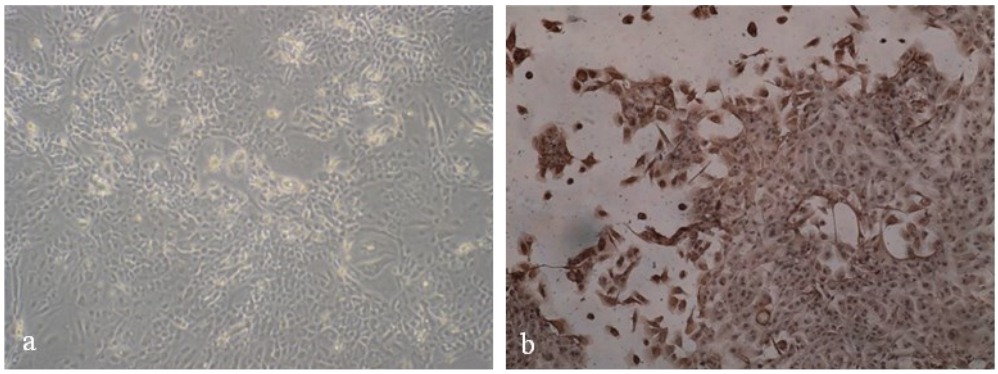

Figure 3. Cultured urothelial cells. a- The urothelial cells cultured on the feeder cell layer; b- The cells labelled with the anti-cytokeratin 7 antibody.

DISCUSSION AND CONCLUSION

The present study involved using cell culture and scaffolding methods to construct the mesenchymal stem cell-derived artificial human bladder. Alternative cell culture and scaffolding experiments were conducted to resolve issues such as fibrotic tissue formation,[24] scaffold rejection, and difficulty reaching the autologous somatic cells,[3] of which have been encountered in previously reported studies on artificial bladder development.

Three different types of biomaterials are generally used for modelling the extracellular matrix to repair or reconstruct the bladder using tissue engineering techniques – natural materials, decellularized tissue matrices, and synthetic polymers. Several scholars have reported using decellularized tissue matrices such as bladder submucosa (BS) and small intestine submucosa (SIS). However, considerably negative effects of using cell-free matrices were stated.[24] Therefore, SIS or BS were not used in the present study. In 1998,[25] new-generation synthetic polymers entered the phase of clinical trials, owing to great advancements in the field of biomaterials. In this study, a three-dimensional structure prepared by placing the urothelial cells and smooth muscle cells isolated from the dog bladder on a bladder-shaped PGA tissue scaffold was implanted in dogs. The success of the afore-stated study also accelerated the human trials, and a pilot study was conducted with seven patients.[26] In this trial, the bladder wall piece was prepared by placing the urothelial cells on the inner surface of the collagen-PGA composite and smooth muscle cells on the outer surface were implanted back into the defective bladder region in the patients. Successful results were achieved in this human study as well. Different polymers have since been used to construct artificial bladder tissues, with proven effectiveness. In this context, the present study aimed to use the biocompatible and biodegradable chitosan, which was demonstrated to exert an angiogenesis-stimulating effect by Drewa and his research group[27] in a mixture with PLGA, which is another biodegradable and FDA-approved polymer, to construct an alternative scaffold for artificial tissue formation. In addition, the present study aimed to select a suitable tissue scaffold type with appropriate properties for artificial human bladder studies. A hybrid scaffold type was selected for use in the study as it could provide support to different cell types in terms of different aspects, such as flexibility, durability, etc., for the repair of structures containing various cell types, such as the bladder. A current study in the literature also supports the accuracy of this choice.[9] In this publication, PLGA-based fibrous tissue scaffolds were evaluated in terms of biodegradability and mechanical strength and their suitability for the bile duct was examined. In this context, information that may support that PLGA is suitable for the bladder, which has a multilayer feature similar to the bile duct.

Mesenchymal stem cells are undifferentiated cells with self-renewal abilities and the potential to differentiate and transform into different cell types. These cells formed the cellular basis of the present study. It is reported that these multipotent cells present in various tissues of an adult organism, such as adipose, bone marrow, liver, and skin, are capable of rapid multiplication in a suitable culture medium.

Adipose tissue is used widely as an effective source of mesenchymal stem cells owing to its widespread presence in an organism and easy accessibility without requiring highly invasive techniques. In addition, MSCs are often preferred to be obtained from the adipose tissue owing to the high efficiency of the process (i.e., numerous stem cells are obtained from a small amount of tissue). Since the present study aimed to establish a scientific basis for the construction of the artificial bladder using adipose tissue-derived mesenchymal stem cells, subcutaneous and visceral adipose tissue biopsy samples ($\approx$1 cm^3) from children were used as the source of stem cells in the present study.[15] Consequently, the result data were consistent with those reported in the literature.[28]

Smooth muscle cells (SMCs) formed an important part of the present study and were preferred to be obtained from hADMSCs through differentiation using specific media rather than the SMCs isolated from a biopsy sample obtained from a donor. Previous studies have demonstrated that the SMCs obtained from a healthy somatic tissue have limited cleavage capacity. However, it is also reported that SMCs may be differentiated and reproduced in the desired amount *in vitro*, which is quite advantageous for tissue engineering studies. It is also reported that using the SMCs derived from hADMSCs in tissue engineering applications minimizes or could even eliminate the possibility of immune rejection, and this was one of the main reasons for preferring this approach in the present study.[18] The literature reports different approaches for the *in vitro* differentiation of hADMSCs into smooth muscle cells. However, different from the general literature, in the present study, which involved the use of tissue engineering, a smooth muscle differentiation experiment was conducted by referring to the study reported by Yoon Ghil and his research group.[29] In the present study, a medium with high serum content and 10% FBS was used, together with horse serum and hydrocortisone, which have been reported to induce myoblast and myotube formation in several previous studies. The enzymatic method was used in this study to isolate the urothelial cells lining the inner surface of the bladder. Specifically, the dispase en-

zyme was used as it is reported widely in the relevant literature.[16,17] However, it was observed that the urothelial cells isolated using this method retained their viability only for a short duration in the traditional polystyrene culture plates, and the sustainability of the culture could not be ensured. Therefore, a feeder cell layer was introduced in the present study to support the culture of the isolated urothelial cells. This modification of dispase and the supporting layer resulted in urothelial cells that exhibited a higher growth rate and a healthier morphology in the culture medium. This observation was consistent with previously reported studies.[22,23] The urothelial cells in the culture were then characterized using immunohistochemistry and the anti-cytokeratin 7 antibody, and results consistent with previous studies were obtained.[30]

In conclusion, hADMSCs-derived smooth muscle cells and biopsy-isolated urothelial cells cultured on hybrid chitosan–PLGA scaffolds with appropriate physical properties could serve as a suitable model for tissue-engineered artificial bladder construction. However, various optimisation studies should be carried out to achieve higher success during the differentiation of stem cells to SMC. If necessary, the microenvironment should be supported with various growth factors. Similarly, to increase the cultivation success of biopsy-isolated urothelial cells and ensure their survival in culture for a more extended period, extra surface modifications should be made, and the process must be supported mechanobiologically. It should be noted that culture conditions need to be improved for both cell types to better contribute to artificial bladder construction, which requires dynamic conditions. On the other hand, optimization studies need to be continued for hybrid chitosan–PLGA tissue scaffolds to gain mechanical properties more suitable for the multilayer bladder structure. In this context, improving all processes and disseminating stem cell-based artificial organ designs is recommended.

Ethics Committee Approval: Ethical committee approval was received from the Ege University Clinical Research Ethics Committee (Date: 11.01.2010, decision no: 09-12/1). Written informed consent was obtained from patients who participated in this study.

Conflict of Interest: No conflict of interest was declared by the authors.

Author Contributions: Concept - SET, AŞ, SİDG; Supervision - SET, AŞ, SİDG, EM; Materials - SET, AŞ, SİDG, EM; Data Collection /or Processing: SET, AŞ, SİDG; Analysis and/or Interpretation-SET, AŞ, SİDG; Writing - SET, AŞ, SİDG, EM.

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