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Novel multilayer meniscal scaffold provides biomechanical and histological results comparable to polyurethane scaffolds: An 8 week rabbit study

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ABSTRACT

Objective: The aim of this study was to evaluate the meniscal regeneration and arthritic changes after partial meniscectomy and application of either polyurethane scaffold or novel multilayer meniscal scaffold in a rabbit model.

Methods: Sixteen NewZealand rabbits were randomly divided into three groups. A reproducible 1.5-mm cylindrical defect was created in the avascular zone of the anterior horn of the medial meniscus bilaterally. Defects were filled with the polyurethane scaffold in Group 1 (n:6) and with novel multilayer scaffold in Group 2 (n:6). Rabbits in Group 3 (n:4) did not receive any treatment and defects were left empty. All animals were sacrificed after 8 weeks and bilateral knee joints were taken for macroscopic, biomechanical, and histological analysis. After excision of menisci, inked condylar surfaces and tibial plateaus were evaluated for arthritic changes. Digital photographs of excised menisci were also obtained and surface areas were measured by a computer software. Indentation testing of the tibial condyles and compression tests for the relevant meniscal areas was also performed in all groups. Histological analysis was made and all specimens were scored according to Rodeo scoring system.

Results: No signs of inflammation or infection were observed in any animals. A significant difference was observed between meniscus surface areas of the multilayer scaffold group (20.13 ± 1.91 mm^2) and the group with empty meniscus defects (15.62 ± 2.04 mm^2) ($p = 0.047$). The results of biomechanical compression tests revealed a significant difference between the Hayes scores of the second group (1.728) and the empty defect group (0,467) ($p = 0.029$). Intact meniscal tissue showed higher mechanical properties than all the defected samples. Multilayer scaffold group demonstrated the closest results compared to healthy meniscus tissue. Tibia indentation tests and histological evaluation showed no significant differences between groups ($p = 0.401$ and $p = 0.186$ respectively).

Conclusions: In this study, the initial evaluation of novel multilayer meniscal scaffold prevented the shrinkage that may occur in the meniscus area and demonstrated superior biomechanical results compared to empty defects. No adverse events related to scaffold material was observed. Besides, promising biomechanical and histological results, comparable to polyurethane scaffold, were obtained.

Introduction

With the high risk of osteoarthritis after partial or total meniscectomies, preservation and repair of meniscal tissue are strongly

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recommended.[1–3] However, repair of some large meniscal tears especially those located on the avascular inner portions, is not possible. Allograft applications, which have been reported to cause immune response, disease transmission and recurrence of meniscal tears, have also been shown to not be able to stop the degenerative process.[4–6] Several materials have been developed to replace the resected meniscal tissue. In the search for optimum scaffold material, several types of biomaterial have been studied. Even though silk implants led to cell growth and matrix accumulation in vitro, its biomechanical properties must be adapted to reach those of healthy meniscus cartilage.[7,8] With the advantages of being inexpensive and promoting cell migration, cellulose scaffolds also demonstrated inferior compression test results.[9] Although polyurethane scaffolds provided satisfactory tissue ingrowth and pain relief; its disadvantages include synovitis induced by carbon particles, release of potentially carcinogenic compounds from Estane, compressive characteristics incomparable to native meniscus and inability to protect the articular cartilage compared to meniscectomy. Recent research have shown significant revision rates, and inability of polyurethane scaffolds to provide a substitute equivalent to meniscus. Clinical indication of this material is being seriously questioned and considered as a "more flop than fit" technique.[10] Yet, none of these materials have proven to be completely successful in the sense of serving as an identical replacement of resected meniscal tissue, nor proven to be capable of fully protecting the cartilage.[7,11]

Therefore, in the present study, a novel multilayer meniscal scaffold, produced with different materials on each layer, was developed and investigated. The multilayer scaffold consists of durable poly 3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) on the superficial layer, osteoconductive strontium ranelate on the bottom surface and cellulose derived from Luffa cylindrica (a plant known as sponge gourd, with the same shape and size as a cucumber) on the middle layer to provide superior resistance against compressive forces. The hypothesis developed for this study is that superior biomechanical results and a histologically improved quantity and quality of meniscal healing would be achieved with the multilayer meniscal scaffold. The aim of this study was to evaluate the meniscal regeneration and arthritic changes after partial meniscectomy and application of two different scaffolds in a rabbit model, and to compare the macroscopic, histological and biomechanical results of the developed novel multilayer meniscal scaffold with the clinically used polyurethane scaffold.

Methods

The multilayer scaffold was composed of three layers, each with different material properties. The uppermost layer of the scaffold was made of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), and the bottom layer, neighboring the tibial plateau, contained hydroxyapatite nanopowders and strontium ranelate, which provided osteoconductive properties. Between these two layers the middle layer was augmented with durable cellulose fibers derived from Luffa cylindrica to ensure resistance against compressive forces (Fig. 1).

Characteristics of the polyurethane scaffold

The other scaffold used in the present study was a biodegradable, synthetic, acellular polyurethane scaffold (**Actifit**, *Orteq Ltd, London, UK*).[12] Urethane segments are more stable than polycaprolactone segments, and over time they are phagocytized by macrophages and integrate into the surrounding tissue.[13,14] A porous structure constitutes 80% of the scaffold, and the remaining 20% is absorbable aliphatic polyurethane.

Experimental protocol

Animals and anesthesia. A total of 32 knee joints of 16 adult, male (average 12 months old) New Zealand white rabbits with weights of between 2600 and 3500 grams were included in the study. The weights of the 16 male New Zealand rabbits (12 months old on average) were similar, and there was no statistically significant difference between the three groups ($p = 0{,}727$).

Subjects were kept in standard laboratory conditions (under daylight for 12 hours, 50–60% humidity, and 20–22 °C room temperature). 16 rabbits were randomly assigned into three groups. Group 1 consisted of six rabbits which received the polyurethane scaffold, the multilayer scaffold was applied to six rabbits in group 2; four rabbits were taken as the control group and the created meniscal holes were left empty. All rabbits were administered an intramuscular injection of 5 mg/kg of xylazine and 35 mg/kg of Ketamin preoperatively. In addition, 50 mg/kg of cefazolin sodium was administered for surgical prophylaxis.

Surgical procedure. Under sterile conditions, knee joints were approached via an anterior longitudinal incision, followed by a medial parapatellar approach (Fig. 2). After lateral dislocation of the patella, the knee was hyperflexed and a full thickness meniscal defect was created on the avascular, inner 2/3 of the medial meniscus with a 1.5 mm diameter, sharp biopsy cannula, as described in the literature[15–17] (Fig. 3). The twelve defects in group 1 were filled with polyurethane scaffolds prepared in cylindrical shapes with a diameter of 1.5 mm; whereas the defects in group 2 received the novel multilayer meniscal scaffold with the same diameter. The eight knees in the control group were not applied any treatment, and the defects were left empty. After reduction of

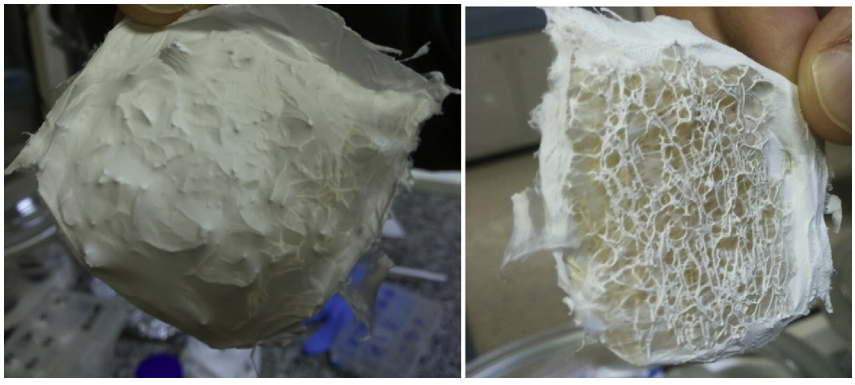


Fig. 1. The appearance of the upper and lower surfaces of the multilayer meniscal scaffold.

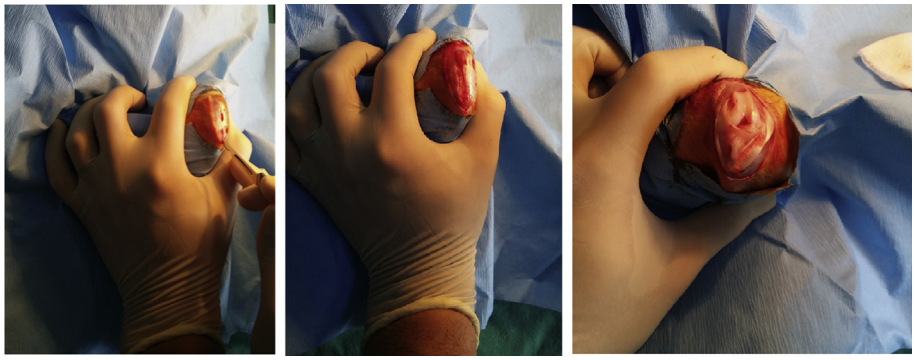


Fig. 2. Steps of surgical procedure. Anterior longitudinal incision (left), medial parapatellar approach (middle) and lateral dislocation of patella (right).

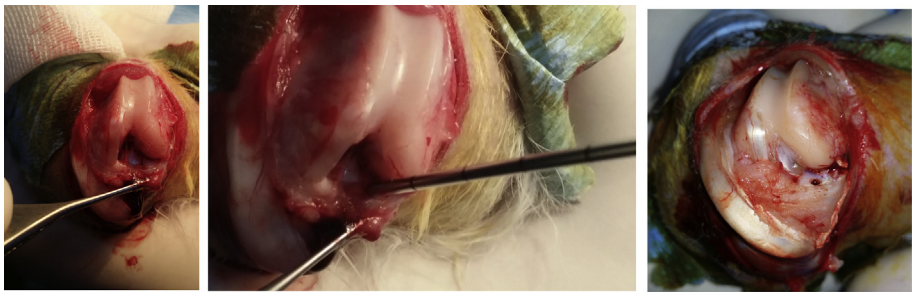


Fig. 3. Visualisation of the medial meniscus anterior horn after hyperflexion of the knee joint (left), creation of a 1,5 mm-diameter full thickness meniscus defect on the avascular inner 2/3 of medial meniscus (middle), appearance of the meniscal defect (right).

patella, all knee joint capsules were sutured with 4/0 Vicryl using standard primary sutures (Fig. 4).

All animals were allowed to move freely in their cages and were fed ad libitum for eight weeks. Subjects were sacrificed with a high dose of thiopental sodium at eight-week end point. The medial menisci harvested from the left knees of each animal were fixed in formaldehyde for 48 hours for histological investigation, while the tibial plateaus involving the menisci of the right knees were prepared for biomechanical testing.

Macroscopic evaluation. All the knee joints were examined to determine any changes in morphological characteristics, such as local temperature increase, color changes, or fistula, before sacrification; all the sacrificed subjects were evaluated for findings of infection; and all femoral condyles and right tibial plateaus were immersed in Indian Ink to observe osteoarthritic changes more clearly (Fig. 5). The carbon content of the ink bonded more to degenerated areas than to smooth cartilaginous surfaces, making arthritic changes more visible. Evidence of degeneration in each femur and tibia was noted.

Digital photographs were taken of each medial meniscus, with a scale (ruler) placed beside it on the table. Surface areas of the medial menisci were calculated on computer software (Image J version 1.46, National Institute of Health, Bethesda, MD) (Fig. 6).

Histological evaluation. The scoring system described by Rodeo et al was used to evaluate meniscal healing of the specimens stained with hematoxylin eosin.[18] Each sample was given a score ranging

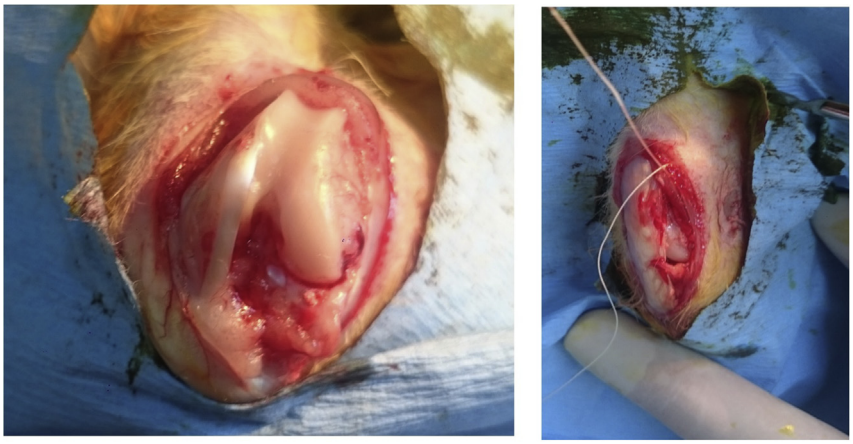


Fig. 4. Implantation of 1, 5 mm-diameter cylindirical scaffolds into the defects (left), suturation of the joint capsule after reduction of patella (right).

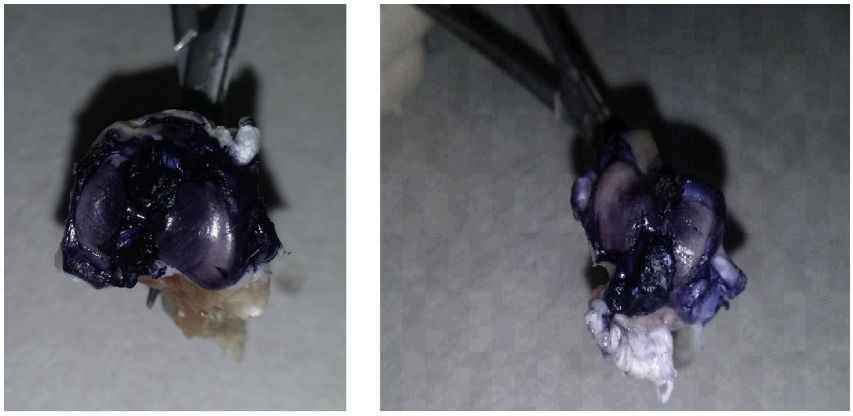


Fig. 5. Appearance of tibial plateaus from above after immersing in Indian ink, in order to visualize the degenerative changes more obviously.

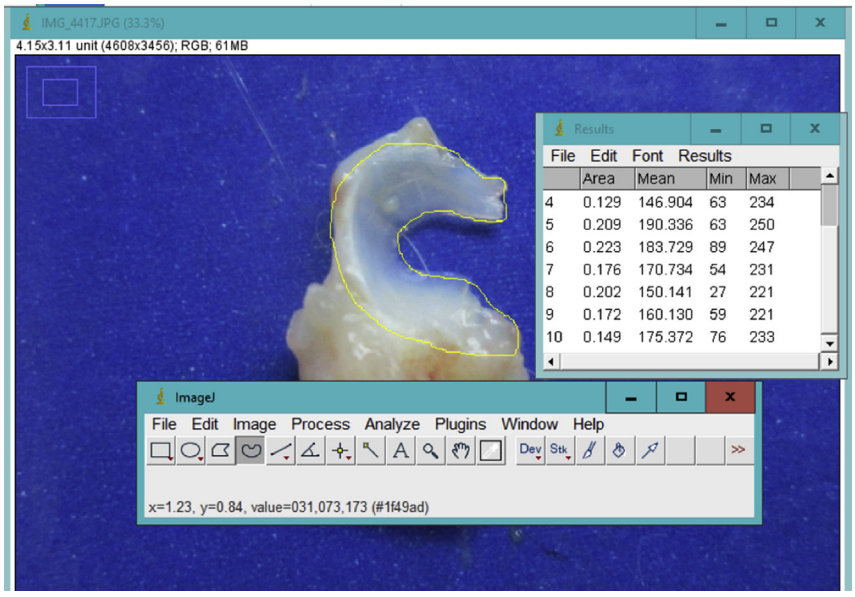


Fig. 6. Screenshot of the computer program (Image J version 1.46, National Institute of Health, Bethesda, MD) used for calculation of meniscal surface areas.

from 0 to 6 according to cell quality and quantity, collagen, matrix morphology and dominant cell type.

Biomechanical evaluation. The right knees of each rabbit were prepared for biomechanical testing. Medial menisci were dissected from tibia, and two 2.5 mm-diameter samples for each meniscus were taken; one from the healthy middle part and another involving the defected areas of each medial menisci. Cylindrical samples for indentation testing were obtained. After measuring their thickness, all samples were placed in a specially designed 2.5 mm-diameter steel pot. The specimens were subjected to compression load with a flat tipped indenter of the same diameter. All tests were performed under axial loading with an electromechanical actuator (5 kN AG-X; Shimadzu, Kyoto, Japan) (Fig. 7).

Determination of mechanical properties. The thicknesses of all the meniscus specimens were measured with a digital caliper. Hayes scores were calculated for each sample using their thicknesses and the results of compression tests. Elasticity (Young modulus) calculations were performed with the formula "$E = P(1-v^2)/2a \cdot \omega \cdot k$" using the previously described methods (P: applied load-N, ω: axial displacement of indenter-mm, a: diameter of indenter-mm, v:

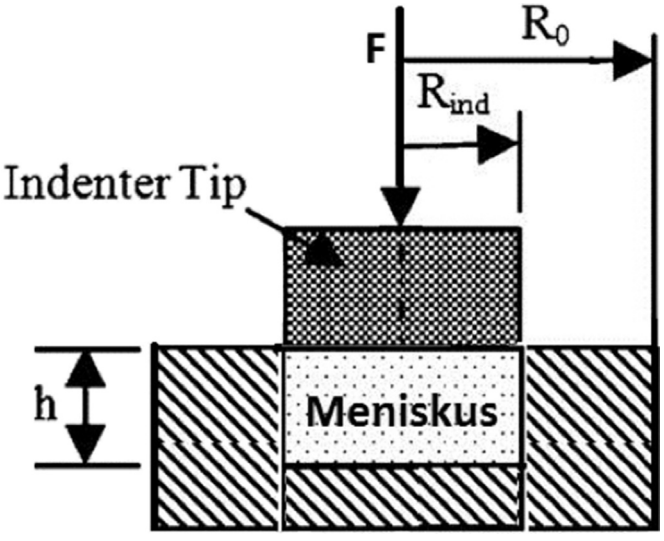


Fig. 7. Schematic view of indentation tests.

Poisson's ratio-0.45, h: cartilage thickness; K constant was calculated according to a/h for each sample).[19]

After removal of menisci, the tibial plateaus were fixed with a clamp mechanism to apply tibial indentation tests. Two different tibial articular cartilage spots (one under the defected meniscus and another under the healthy meniscus area) were subjected to compression tests (Fig. 8).

Statistical analysis. Statistical analysis was performed using SPSS 22.0. A p value of <0.05 was considered statistically significant. Parametric distribution of quantitative data was evaluated by the Shapiro–Wilk test, and in cases of normal distribution (i.e. tibial indentation, thickness, meniscus area and Hayes scores of meniscus), the difference between three groups was evaluated by ANOVA analysis. For non-normally distributed data or data obtained by scoring (i.e. weight of rabbits, Hayes scores of scaffolds and histological results) the difference between groups was compared with Kruskal–Wallis test.

Results

No animals died or were excluded from the study. All animals were observed to be healthy during their eight-week follow-up period. There were no wound problems, such as redness, swelling, heat, disruption or abscess, which would suggest infection, and all wounds healed uneventfully. After sacrification, no evidence of inflammation was observed in all three groups.

After immersion in Indian ink in order to see degenerative changes more clearly, the tibias and femurs of the rabbits in all three groups macroscopically did not show any signs of remarkable articular cartilage degeneration.

A statistically significant difference was found between the meniscus surface areas of multilayer scaffold group and the empty defect group ($p = 0.047$). Although group 1 and group 2 had a similar mean area value, there was no significant difference between the polyurethane scaffold group (group 1) and the control group with empty defects (Table 2).

A statistically significant difference was found regarding the thickness of the defected areas between the multilayer scaffold and the empty defect group ($p = 0.043$) (Table 3).

Comparison of mechanical properties using Hayes scores and the results of compression tests, showed that there was a statistically significant difference between the multilayer scaffold and empty defect groups ($p = 0.029$). Although the median Hayes scores of the second group (1.728) nearly doubled the first group (0.896), this difference was not statistically significant ($p = 0.745$). Healthy meniscal tissue samples obtained from all three groups were found to have the highest Hayes scores in the compression tests, and were similar for all groups (2.21 ± 0.67; 2.58 ± 0.98 and 2.85 ± 0.45 respectively) (Tables 1 and 4).

Indentation tests for the tibial plateaus after removal of the menisci showed that the area below the empty defected meniscus area had the lowest values (264.84 ± 20.25); and the areas under the scaffold-applied meniscus had higher values, being (343.65 ± 130.88) and (299.74 ± 81.98) for group 1 and 2, respectively. The indentation results for tibial cartilage under healthy meniscus in all three groups were higher than these defected areas (368.49 ± 143.19; 401.80 ± 109.00; 374.65 ± 93.08). However, the statistical analysis of all these data revealed no significant differences in results of the indentation tests between tibial cartilage under healthy meniscus (p: 0.882) or defected areas (p: 0.401) among the three groups (Table 1).

The histological evaluation of meniscus healing using the scoring system described by Rodeo et al revealed no significant differences between groups regarding structural characteristics, dominant cell type, collagen regulation, matrix morphology or the histological scores obtained by their summation ($p = 0.186$) (Table 5).

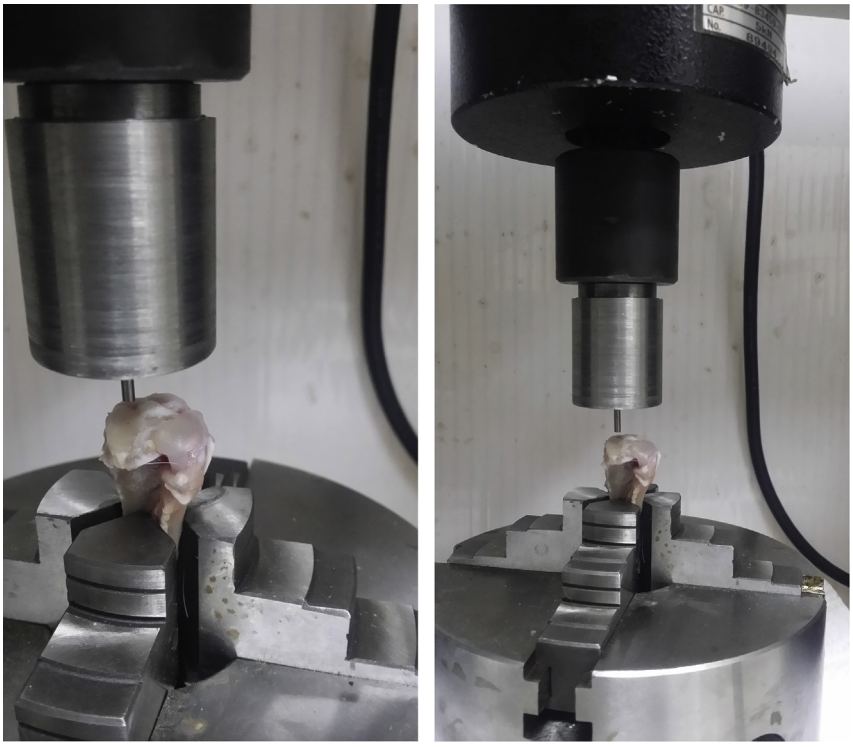


Fig. 8. Application of tibial indentation tests for two different spots of tibial plateau.

Table 1
Comparison of all parametric and non-parametric data between the three groups.

	Group 1	Group 2	Group 3	p	p[c]
	mean ± SD	mean ± SD	mean ± SD	ANOVA	postHoc
TibiaIndentation_ menisci	368.49 ± 143.19	401.80 ± 109.00	374.65 ± 93.08	0.882[a]	n/a
TibiaIndentation_scaf	343.65 ± 130.88	299.74 ± 81.98	264.84 ± 20.25	0.401[b]	n/a
Thickness_scaf	0.95 ± 0.20	1.08 ± 0.29	0.68 ± 0.10	**0.043**[a]	$P_{1\text{-}2} = 0.962$ $P_{1\text{-}3} = 0.238$ $\mathbf{P_{2\text{-}3} = 0.043}$
Thickness_menisc	1.32 ± 0.35	1.35 ± 0.16	1.33 ± 0.26	0.977[a]	n/a
Hayes_menisc	2.21 ± 0.67	2.58 ± 0.98	2.85 ± 0.45	0.396[b]	n/a
Meniscus_area	19.2 ± 3.34	20.13 ± 1.91	15.62 ± 2.04	**0.047**[a]	$P_{1\text{-}2} = 1.000$ $P_{1\text{-}3} = 0.152$ $\mathbf{P_{2\text{-}3} = 0.047}$
	Group 1	Group 2	Group 3	p[d]	p**
	median (min–max)	median (min–max)	median (min–max)	KW	postHoc
Weight	2900 (2500–3500)	2700 (2600–3400)	2625 (2500–3450)	0.727	n/a
Hayes scaffold	0.8965 (0.75–2.53)	1.728 (1.57–3.63)	0,467 (0.14–0.88)	**0.005**	$P_{1\text{-}2} = 0.745$ $\mathbf{P_{2\text{-}3} = 0.029}$ $P_{1\text{-}3} = 0.590$
cell quality and quantity	1 (1–2)	1.5 (0–2)	0 (0–1)	0.056	n/a
dominant cell type	1 (1–2)	1 (1–2)	0.5 (0–1)	0.074	n/a
Collagen organization	0 (0–1)	0 (0–1)	0 (0–1)	0.812	n/a
Matrix morphology	0 (0–0)	0 (0–1)	0 (0–1)	0.336	n/a
Histological score	2 (2–5)	2.5 (2–6)	0.5 (0–4)	0.186	n/a

***postHoc Dunn test.
[a] ANOVA F test.
[b] ANOVA Brown Forsythe test.
[c] PostHoc Bonferroni test.
[d] Kruskal Wallis test.

Table 2
Mean meniscus areas of the three groups.

Discussion

The multilayer meniscal scaffold developed in the present is believed to be the first meniscal scaffold that contains different material features pooled together for different purposes. The newly-developed multilayer meniscal scaffold application was found to be superior to leaving the defect empty regarding the protection of the meniscus area. Biomechanically a significant difference was seen between the group subjected to multilayer scaffold and the group with empty defect. The results of the compression test for the group subjected to the multilayer scaffold were closer to those obtained from a healthy meniscus.

Particularly for the treatment of injuries in the avascular regions, where repair often fails, replacement of the damaged meniscal tissue with allografts or scaffolds has been suggested. A number of different biomaterials have been developed to replace the resected meniscal tissue. Among these materials, meniscal scaffolds, which were initially designed to relieve pain after meniscectomy, were later used to prevent cartilage destruction and subsequent osteoarthritis. In the search for optimum scaffold material, none have proven to be a completely satisfying identical replacement of resected native meniscal tissue.[7,11]

Besides the development of new scaffold materials, reinforcement of present scaffold materials are also being studied. Patel et al

Table 3
Thickness of defected and healthy meniscus areas in each group.

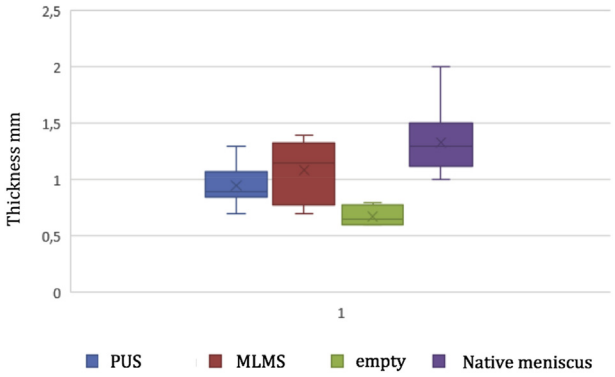


Table 4
Comparison of the distribution of Hayes scores of defected areas in each group and the healthy meniscal tissue.

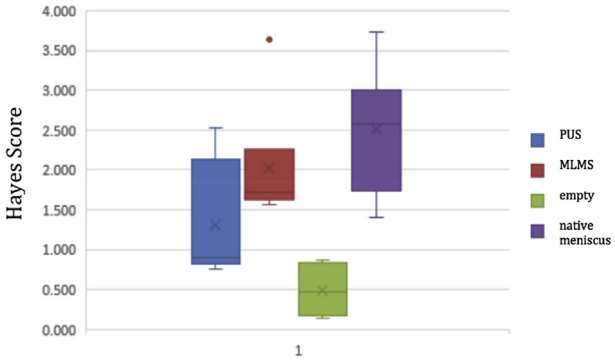
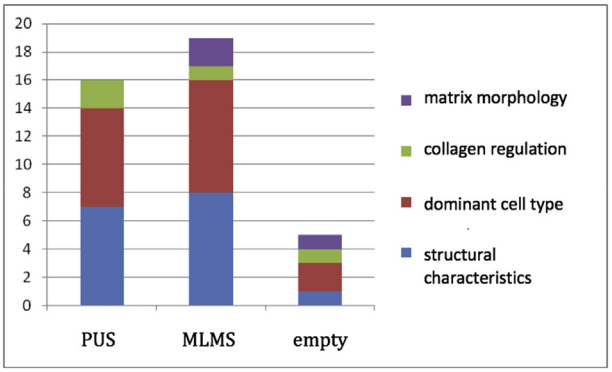


Table 5
Distribution of histological data according to groups.



reported induced formation of neomeniscus tissue with collagen scaffolds reinforced with synthetic polymer fibers.[20] In another study; fiber reinforcement of decellularized extracellular meniscus matrix and polycaprolactone with electrospinning resulted in superior mechanical properties.[21] Also addition of biological agents such as stem cells have also been tried. Recently; bone marrow stem cell impregnated silk fibroin scaffolds showed better feasibility in terms of structure and function in a rabbit model.[22] Koch et al also reported accelerated meniscal healing withpolyurethane scaffolds loaded with mesenchymal stromal cells.[23] Similarly platelet derived growth factor (PDGF)-coated meniscus scaffolds demonstrated increased migration of meniscal cells and consequently improved biomechanical properties.[24]

PHBV, a polymer often used in osteochondral or osseous defects, has been observed, in vivo and in vitro, to be effective on the proliferation and differentiation of osteoblasts.[25,26] Strontium ranelate

is an oral agent used in the treatment of osteoporosis, as it has anabolic and antiresorptive effects on bone. Strontium-doped calcium phosphate scaffolds have been used in bone regeneration studies.[27,28] In the present study, Sr was used to obtain an osteoconductive nanofibrous sheet at the lowest layer of the novel scaffold developed. On account of its advantages, which include being cheap and stimulating cell migration, Bodin et al attempted to use bacterial cellulose, produced in a liquid corn cell culture, as meniscal implants.[9] Although still being lower than the results of native meniscus, the cellulose scaffold demonstrated superior compression test results compared to collagen implants. In order to increase the compressive strength of the novel multilayer scaffold developed for the present study, cellulose in the form of Luffa cylindrica fibers was used in the middle layer.

To the best of our knowledge, the novel multilayer meniscal scaffold is the first scaffold to be produced using different materials on each layer, as no such scaffold has been reported in the literature. This novel multilayer scaffold consists of durable PHBV on the superficial layer, osteoconductive strontium ranelate on the bottom surface and cellulose derived from Luffa between these layers to provide superior resistance against compressive forces. The only layered scaffold structure encountered in the literature was the silk-based meniscal scaffold developed by Mandal et al.[8] However, unlike the scaffold developed in the present study, Mandal's scaffold was composed of the same material with different pore sizes on each layer. The scaffold described in the present study is the first meniscal scaffold to accommodate different material properties brought together for different purposes.

In the present study, a 1.5 mm diameter, full thickness defect on the internal 2/3 avascular area was created with a sharp biopsy punch to ensure defect standardization. This localization on the central meniscus is particularly preferred in scaffold studies owing to its avascular nature.[29] Several authors have investigated the effects of allogenic stem cell application, hydrogel compounds with platelet-rich plasma and marrow stimulation on meniscal defects of the same size and location as created in the present study.[15,17,30] Oda et al evaluated the relationship between meniscal healing and infrapatellar fat pad with a collagen scaffold application on 2.0 mm diameter defects located in the same area.[16] Although this kind of a cylindrical defect does not represent the linear meniscal tears frequently encountered in clinics, it still provides a reproducible, standard defect that both lacks inter-subject differences and allows for easier assessment of healing properties.

After immersion in Indian ink in order to see degenerative changes more clearly, the tibias and femurs of the rabbits in all three groups macroscopically did not show any signs of remarkable articular cartilage degeneration. This could be attributed to the short follow-up time of our study and could be improved with a longer end point.

There was no statistically significant difference between the histological scores of the group with empty defect and those of the first two treatment groups. This could be attributed to the fact that the scores given for different histological characteristics varied in a quite narrow interval (0–1 in two parameters, 0–2 in the other two parameters); and accordingly, very close values were found.

Another important limitation of the present study was the morphology of the defect. The cylindrical full-thickness defect formed in these experiments has a very different structure than the linear tears seen in clinics. Moreover, its non-sutured form creates a significant difference. Other shortcomings of the study include the following: absence of a greater number of subjects and groups with different follow-up times, the use of a rabbit model the use of a cylindrical rather than segmental defect, no sutures were used, this is a non-clinically applicable technique, there were a limited number of specimens and only one early follow up interval, no imaging studies were performed, limited immunohistochemistry was done.

Conclusions

The newly-developed multilayer meniscal scaffold application prevented the shrinkage that may occur in the meniscus area and demonstrated superior biomechanical results compared to empty defects. Macroscopic, histological and biomechanical results comparable to polyurethane scaffolds used in clinics were obtained; while no adverse events related to the scaffold material was observed.

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