Journal of Plastic, Reconstructive & Aesthetic Surgery (2019) 72, 1555–1563

JPRAS
An International Journal of Surgical Reconstruction
www.JPRASurg.com

Long-term studies on the integration of acellular porcine dermis as an implant shell and the effect on capsular fibrosis around silicone implants in a rat model

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Received 10 August 2018; accepted 23 April 2019

KEYWORDS
Capsular fibrosis;
Capsular contracture;
Acellular porcine;
Dermal matrix;
Breast reconstruction

Summary Acellular dermal matrices have recently increasingly been used in alloplastic breast reconstruction with silicone breast implants. Among these matrices, acellular porcine dermis (APD) is frequently applied, but long-term data on tissue integration and capsular fibrosis formation are still missing.

Silicone prostheses with (group A) and without (group B) APD as an implant-covering shell were implanted in male Lewis rats. At 3, 12, and 52 weeks after implantation, the constructs were explanted. Molecular biological and immunohistochemical analyses were performed afterwards.

On comparing the collagenous layer and the newly formed myofibroblast-rich layer around the implants of both groups, it became apparent that in group A, these layers were thinner, followed by a lower expression of TGFβ1 after 12 and 52 weeks. Further, in this group, at the endpoint of 52 weeks, a lower amount of CD68-positive cells in the collagenous and myofibroblast-rich layers were observed and the expression of TNFα was reduced, while the number of Ki67-positive cells was significantly higher with time. Furthermore, MMP1 expression

Parts of this article have been presented at the following meetings/conferences: 1. 47th Congress of the German Society of Plastic, Reconstructive and Aesthetic Surgeons 8 September–10 September 2016, Kassel, Germany. 2. The 36th Congress of the German Society of Senology, 26 May–28 May 2016, Dresden, Germany.

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https://doi.org/10.1016/j.bjps.2019.04.015

in group A was lower than that in group B, and the calculated ratio of MMP1:TIMP1 expression was higher.
The long-term results clearly show a reduction in inflammatory and fibrotic tissue reaction when APD is used to cover silicone prostheses. These experimental data will be of considerable importance for implant-based breast surgery, as they indicate a potential benefit in the reduction of capsular fibrosis formation of an interposition of APD between the recipient and the silicone implant.

Introduction

Silicone breast implants are frequently used for both esthetic and reconstructive reasons. However, one of the major lasting problems in using silicone implants is the development of capsular contracture, which causes implant deformities and breast pain in advanced stages.[1,2]

Biological implants such as allogenic, porcine, or bovine acellular dermal matrices are commonly used to create a biological interface between the implant and the host tissue with the aim of reducing capsular contracture and to provide additional cover between the implant and the skin envelope.[3-5] Particularly, in alloplastic breast reconstruction after mastectomy, they are also applied to reconstruct an undefined inframammary fold.

Different hypotheses exist concerning the pathomechanism of capsular contracture, yet none of them has been clearly verified to date.[6,7] In experimental studies on human capsule explants, as well as in animal models, certain tissue reactions in the capsular tissue were described at both a molecular biological and an immunohistochemical level, whereas questions of microorganism-induced chronic inflammation in a biofilm have not yet been definitely answered for clinical daily routine. Therefore, to date, no true causal therapy or prophylaxis exists.[8-11] Use of acellular porcine dermis (APD) to overcome such problems has been investigated in a considerable number of clinical trials, case reports, and case series.[12-15] However, the number of experimental studies to clarify the influence of APD in preventing capsular contracture is still limited. In particular, no experimental longitudinal analysis exceeding a time span of more than 12 weeks has been published thus far.

The aim of this study was, therefore, to analyze molecular biological and cellular mechanisms concerning the development of capsular contracture in a rat model, using an APD as a full cover of silicone implants, and to evaluate the long-term results over a period of one year after implantation.

Materials and methods

To create an authentic model, slightly textured silicone testicular implants for pets with a length of 0.63 inches (Neuticles® UltraPLUS® XXSmall, Neuticles® CTI Corporation, Oak Grove, MO, USA) were implanted in young male Lewis rats (Charles River Laboratories, Sulzfeld, Germany). Animal experiments were approved by the Institutional Animal Care and Use Committee of the Regierungspräsidium von Mittelfranken, Germany (no. 54-2532.1-50/13).

Two experimental groups were defined. In group A, implants were completely covered by a non-cross-linked APD matrix (Fortiva®, Tutogen Medical, Neunkirchen am Brand, Germany) (Figure 1). The scaffolds were sterilized by low-dose gamma irradiation. In group B, as the control, uncovered prostheses were implanted. Two prostheses from the same group were implanted in separate implant pockets under the muscle layer on the backs of the rats, the so-called panniculus carnosus. The explantation time points were set at 3, 12, and 52 weeks. At these points, 8 samples of each group (A and B) were assured.

All operations were performed under aseptic conditions. After initiating anesthesia with isoflurane inhalation (Forene 100%, Völtar GmbH, Hamburg, Germany), the rat was placed in the prone position and the back was shaved. Skin disinfection was performed with Cutasept F (Bode Chemie GmbH, Hamburg, Germany). For analgesia, 200 μl Rimadyl® Carprofen (Pfizer GmbH, Berlin, Germany) was injected subcutaneously, as well as 0.2 ml Veracin® (dihydrostreptomycin sulfate, procaine benzylpenicillin, and benzathine benzylpenicillin, Albrecht GmbH, Aulendorf, Germany) intramuscularly as antibiotic prophylaxis. Before implantation, the APD was wrapped around the prostheses, covering the complete silicone surface, and closed by a continuous suture with 4/0 polypropylene. A pocket was prepared under the panniculus carnosus by blunt dissection, and the prostheses with or without APD as an envelope were implanted. Skin closure was done with a 4/0 polyglactin suture (Vicryl® 4/0, Johnson & Johnson Medical GmbH, Ethicon Surgical Care, Norderstedt, Germany). Aluminum spray (Alu-Spray "trocken," Pharma-Partner Vertriebs-GmbH, Hamburg, Germany) was used as a wound dressing.

In the postoperative course, analgesia was administered through drinking water with tramadol hydrochloride (Tramal® Tropfen, Grünenthal, Aachen, Germany).

Explantation

The prostheses including the surrounding tissue (skin, subcutaneous tissue, and panniculus carnosus) were explanted after 3, 12, and 52 weeks and the animals sacrificed by administering an intracardiac injection of 0.5 ml T61® (Intervet Deutschland GmbH, Unterschleißheim, Germany) in deep anesthesia.

Figure 1 Left: slightly textured silicone testicular implants (0.63 in.). Middle: acellular porcine dermal matrix. Right: testicular implant totally wrapped by APD.

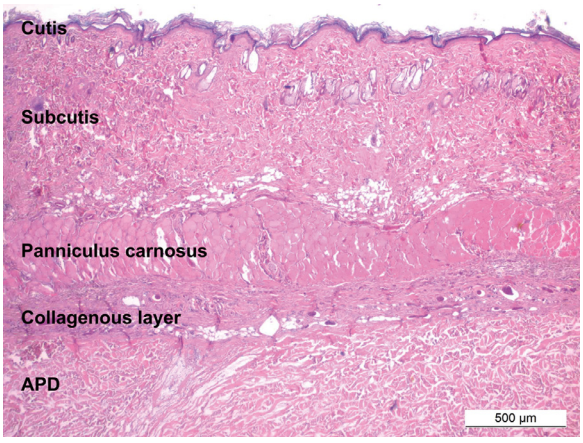

Figure 2 Histological overview in HE. From top to bottom: cutis, subcutis, panniculus carnosus, collagenous layer, and APD.

Tissue processing and histological and immunohistochemical staining

After extracting the prostheses, all samples were fixed in 4% formaldehyde (Roti-Histofix 4%, Carl Roth GmbH, Karlsruhe, Germany). Each sample was embedded in paraffin blocks after cutting into similar pieces. Using a rotary microtome (Thermo Fisher Scientific HM 355 S Automatic Microtome, Waltham, MA, USA), sections were made with a 2.5 µm layer thickness and attached to glass slides.

Histological staining with hematoxylin and eosin (HE) provided an overview for tissue/layer analysis (Figure 2). Immunohistochemical staining for cell proliferation was performed with Ki67 (Rabbit monoclonal-Anti-Ki67, Zytomed Systems GmbH, Berlin, Germany) and CD68 to visualize monocytes and macrophages as a marker of inflammation (Mouse antirat CD68, MCA341R, AbD Serotec, Raleigh, NC, USA). In addition, double staining of alpha smooth muscle actin (alpha-SMA, Mouse anti-Actin clone 1A4, smooth muscle, Zytomed Systems GmbH, Berlin, Germany) was performed and von Willebrand factor (vWF, Factor VIII, Biocare Medical Inc., Concord, CA, USA) was used for visualization of myofibroblasts and smooth muscle cells in vessel walls.

Morphometry

The results of the aforementioned staining were evaluated with a microscope system (Leica Leitz DM RBE and Leica DFC420, Leica Microsystems GmbH, Wetzlar, Germany). Furthermore, photographs were taken in 5 regions of interest using increased magnifications (Figure 3). For the histological overview and the collagenous layer in the HE staining, as well as for the vessels in the APD in the alpha-SMA and vWF staining, 100-fold magnification was used. Quantification analysis of the myofibroblast layer and CD68- and Ki67-positive cells was performed in 400-fold magnification (Figure 4). The thickness of the APD was measured in 25-fold magnification. CD68- and Ki67-positive cells were counted in each of the 5 regions of interest, and arithmetic mean was calculated for each sample followed by calculation of

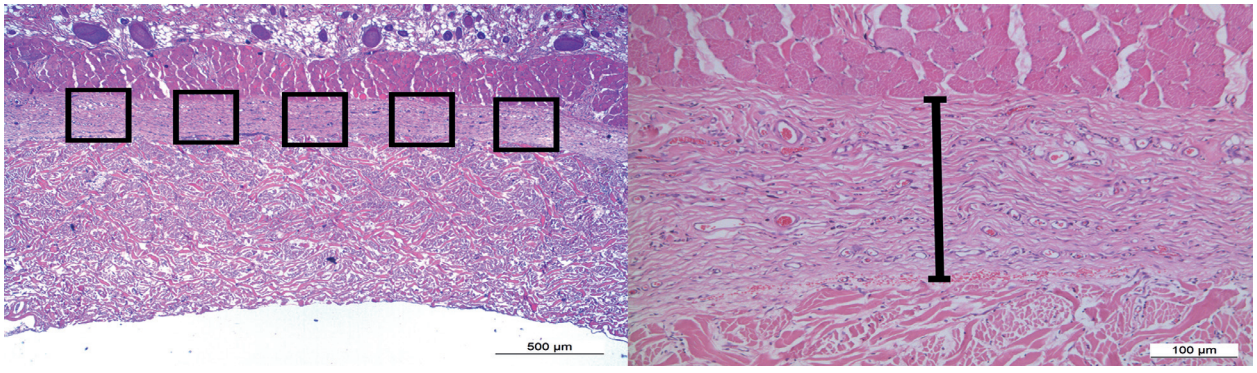

Figure 3 Left: HE staining with assessed regions of interest. Right: thickness of collagenous layer marked (HE staining).

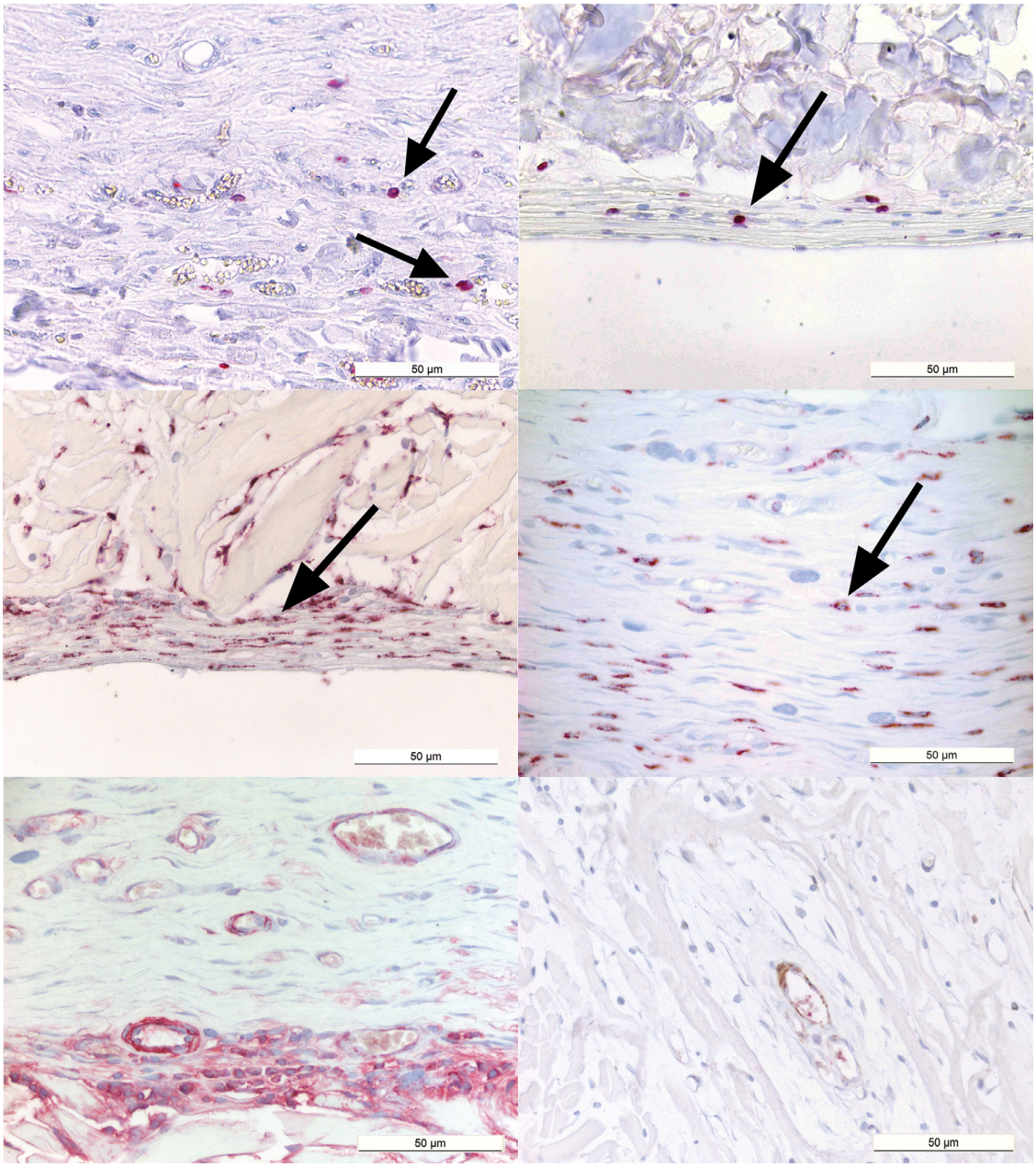

Figure 4 Top: Ki67-positive cells in the collagenous layer (left) and in the myofibroblast-rich layer (right) (arrows). Middle: CD68-positive cells in the collagenous layer (right) and in the myofibroblast-rich layer (left) (arrows). Bottom: myofibroblast-rich layer between collagenous layer (top) and APD (bottom) (left). Vessels in the APD (central) (right).

the arithmetic mean for all samples of the different study groups at the single explantation time points.

Real-time quantitative polymerase chain reaction (qPCR)

Quantification of the mRNA expression of TGFβ1, TNFα, matrix metalloproteinase 1 (MMP1), and tissue inhibitor of MMP1 was performed by qPCR. After 3, 12, and 52 weeks, total RNA was extracted from both the experimental group and the control group. For this, a deparaffinization kit (QIAGEN GmbH, Hilden, Germany) was used for the formalin-fixed paraffin-embedded blocks. The mRNA was isolated with RNeasy® FFPE Kit (QIAGEN GmbH, Hilden, Germany). Total mRNA was reverse transcribed into cDNA using the Sensiscript® Reverse Transcription Kit (QIAGEN GmbH, Hilden, Germany). All primer sequences are listed in Table 1. Real-time quantitative PCR was carried out using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) and a thermocycler (CFX96 Real-Time System C1000 Touch™ Thermal Cycler, Bio-Rad Laboratories Inc., Hercules, CA, USA). All kits were used according to the manufacturers' protocols. Samples were tested as triplicates. The expression of each gene was normalized to that of the housekeeping gene

Table 1 Primer sequences for qPCR.

Gene	Forward primer (5′—3′)	Reverse primer (5′—3′)
GAPDH	CAACGACCCCTTCATTGACC	TTCTCAGCCTTGACTGTGCC
TGFβ1	TGGAGCCTGGACACACAGTA	TGTTGGTTGTAGAGGGCAAGG
TNFα	TCAGCCTCTTCTCATTCCCG	ACAGAAGAGCGTGGTGGC
MMP1	GGAAGGTGATATTGTGTTCGCC	CTATGGTCTCCTCTGTAGAAGGC
TIMP1	ACGCTAGAGCAGATACCACG	AGAGGCCAGAGATGCAAAGG

glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the evaluation was made by the 2-deltadeltaCt method.

Statistical analysis

Mean values of collagenous and myofibroblast-rich layer thickness and cell counts of CD68- and Ki67-positive cells were compared, descriptively stratified by group and explantation time points. Multivariable analysis was performed by computing generalized linear mixed models with random effects accounting for multiple measurements on the same implant. Thickness of the collagenous and the myofibroblast-rich layer was assumed to follow a log-normal distribution, whereas cell counts of CD68- and Ki67-positive cells were modeled with a negative binomial regression model due to overdispersion. Group and explantation time points, as well as an interaction time period to model time-dependent effects, were included in the regression analysis as predictor variables. Differences between the numbers of blood vessels in the APD at different explantation time points were evaluated by the Mann–Whitney test. Owing to multiple hypotheses analyzed in this study, *p* values should be considered as exploratory. All statistical analyses were conducted using the statistical software package *R* Version 3.2.2 (The R Foundation for Statistical Computing, Vienna, Austria).

Results

In all rats, operative and postoperative phases were uneventful. In the hematoxylin- eosin stained overview, the collagenous layer, which occurred in all samples in both groups, increased until the 12-week time point. After 52 weeks, this layer became thinner than that after the 3-week time point in the experimental group (A), whereas it remained almost at the same level in the control group (B). At every time point, the collagenous layer was shown to be significantly thinner in group A than in group B ($p<0.05$) (Figure 5). Based on the alpha-SMA staining, a periprosthetic myofibroblast-rich layer was detected at the interface between the silicone implant and the APD (group A) and the collagenous layer (group B), respectively. After 12 weeks, the thickness increased in both groups but presented as 37% thicker in group B. In the long-term period, the thickness of the myofibroblast-rich layer decreased in group A almost to the initial level at 3 weeks. By contrast, this layer became consistently thicker in group B, showing a statistically significant difference ($p<0.05$) (Figure 6). The cells showed a markedly homogeneous distribution within the single myofibroblast layer and a consistent morphology. Furthermore, the relation of cells and extracellular matrix (ECM) was balanced. In addition, the same layer of myofibroblasts was seen between the APD matrix and the collagenous layer in group A at 3 and 12 weeks after implantation (peri-ADM layer). However, this peri-ADM layer was not present in all samples, and after 52 weeks, it was completely degraded.

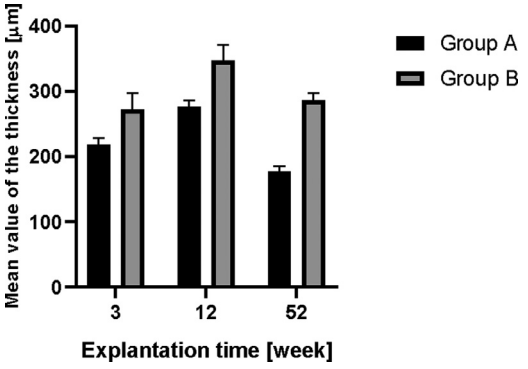

Figure 5 Results of the comparison of thickness of the collagenous layer in both groups and at 3 explantation time points from a descriptive analysis.

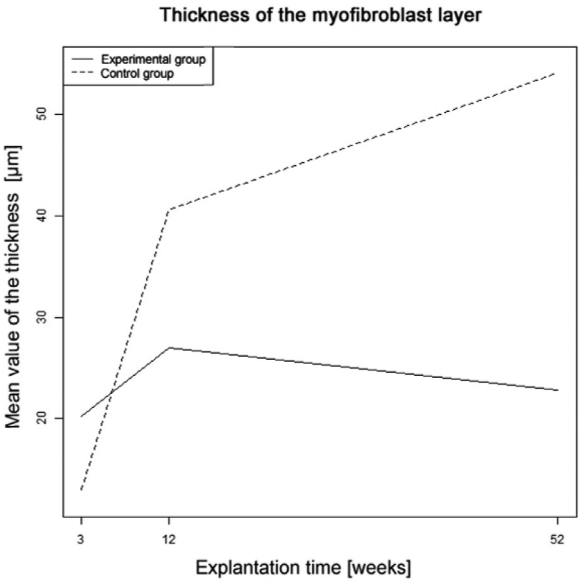

Figure 6 Results of the comparison of thickness of the myofibroblast-rich layer in both groups and at 3 explantation time points from a descriptive analysis.

The grade of proliferation shown by detection of Ki67-positive cells revealed an evident decrease in proliferating

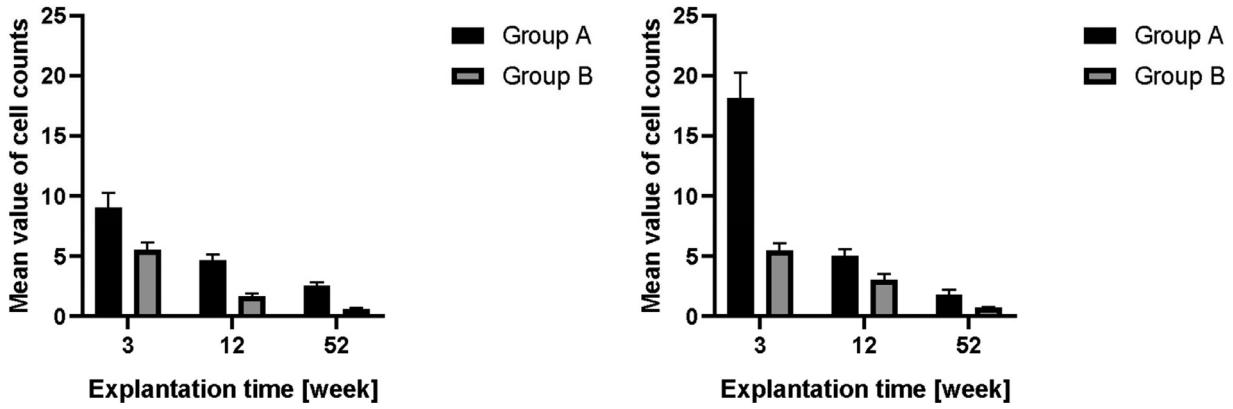

Figure 7 Results of the comparison of Ki67-positive cells in the collagenous layer (left) and in the myofibroblast-rich layer (right) in both groups and at 3 explantation time points from a descriptive analysis.

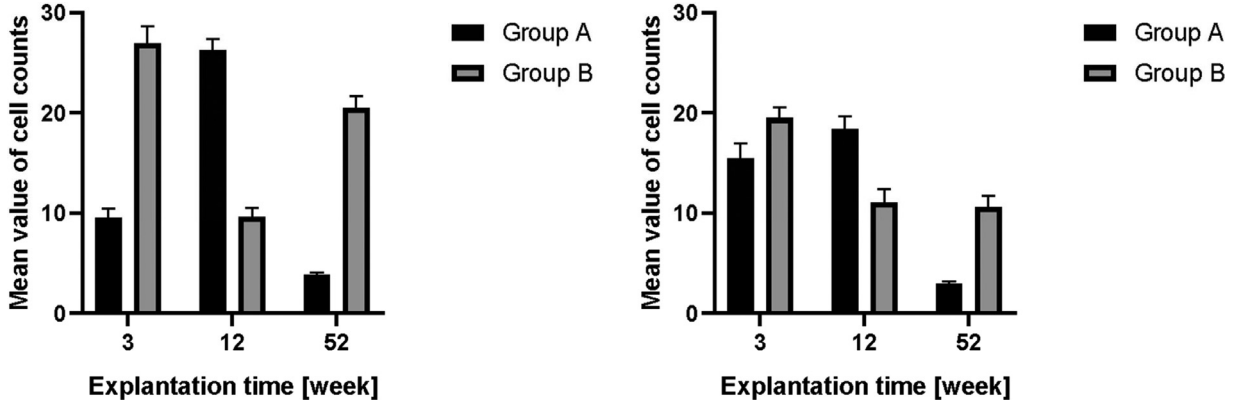

Figure 8 Results of the comparison of CD68-positive cells in the collagenous layer (left) and in the myofibroblast-rich layer (right) in both groups and at 3 explantation time points from a descriptive analysis.

cells with time, both in the collagenous and in the periprosthetic myofibroblast-rich layer in both groups ($p<0.05$) (Figure 7). The total cell count of Ki67-positive cells was higher in group A at every time point.

For the grade of inflammation shown by detection of CD68-positive cells as a representative staining for monocytes/macrophages, an opposing trend was seen in the collagenous layer. A lower cell count in group A after 3 weeks than that in group B was followed by an increase in inflammatory cells in group A and a decrease in group B after 12 weeks. Finally, the cell count in group A fell below the initial level after 52 weeks, and a strong increase in cells was observed in group B ($p<0.05$) (Figure 8). A similar development was seen in the periprosthetic myofibroblast-rich layer, with the difference that there was no significant change in cell count in group B between 12 and 52 weeks (Figure 8).

As a sign of integration, the formation of blood vessels in the APD was analyzed. Overall, there was a statistically significant increase in blood vessels in the APD around the implants at all time points, developing from the adjacent tissue in the direction of the APD ($p<0.05$) (Figure 9).

The analysis of the gene expression of the aforementioned target genes showed a distinctly lower mRNA expression for TGFβ1 in group A than in group B. With time, the expression showed a statistically significant drop ($p<0.05$)

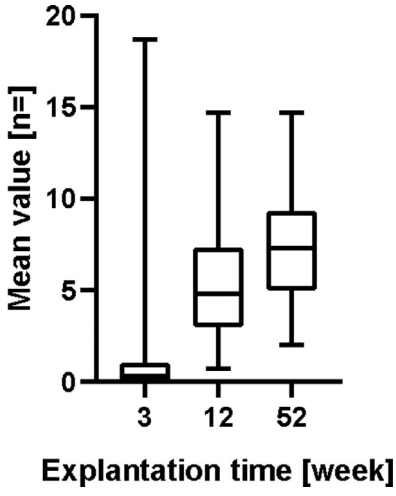

Figure 9 Results of blood vessel ingrowth in the APD after 3, 12, and 52 weeks.

(Figure 10). Similar data were collected for TNFα, where the expression rate in group A was always below that in group B (Figure 10). The expression rate of MMP1 was statistically significantly lower in group A at every time point

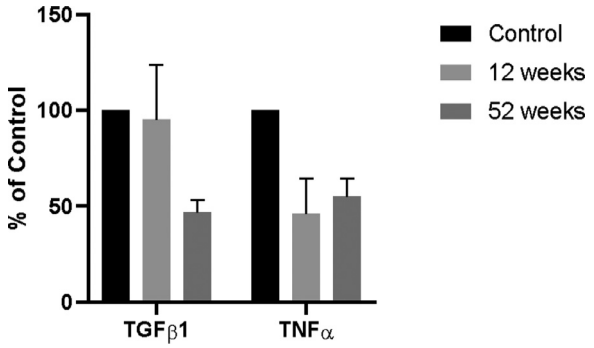

Figure 10 Results of gene expression of TGFβ1 after 12 and 52 weeks compared to the control group (left). Results of gene expression of TNFα after 12 and 52 weeks compared to the control group (right).

($p<0.05$). Tissue inhibitors of metalloproteinase 1 (TIMP1) revealed inhomogeneous data, without an obvious difference between the groups. The calculated relation between MMP1 and TIMP1 showed a higher ratio in group A with time.

Discussion

Biologicals such as acellular dermal matrices are commonly used in breast augmentation and alloplastic breast reconstruction.[16,17] A long-lasting support of the inframammary fold as well as the coverage of the lower pole of a silicone implant, along with dermal augmentation under the usually thin skin envelope, are the basic mechanisms of acellular dermal matrices, especially in reconstructive surgery after mastectomy. It has been hypothesized that a silicone implant covered by APD could prevent capsular contracture and would reduce the foreign body reaction, as well as the inflammatory response, acting as a biological barrier between the implant and the surrounding tissue.[3,8]

Because the incidence of breast augmentations is still high and alloplastic breast reconstruction remains a common alternative compared to autologous breast reconstruction after mastectomy, plastic surgeons are confronted with the unsolved problem of capsular contracture. Tissue engineering might be an alternative in the future to overcome the problem of using allogeneic, xenogeneic, or artificial materials in this context.[18,19] Clinical and observational studies emphasize the use of APD for the aforementioned indications, but long-term studies with molecular biological and immunohistochemical analysis are rare. Among the various allogeneic or xenogeneic materials that are commercially available, we chose a newly developed porcine matrix that is delivered as a sterile non-cross-linked acellular dermal matrix.

In this long-term experimental study, we analyzed multiple parameters commonly known to play a key role in the development of capsular contractures. Different cells are found to be predominant within breast capsular tissue. Particularly, the number of fibroblasts and the thickness of the collagenous layer have been reported to correlate with the Baker contraction grades.[3,20,21] Furthermore, myofibroblasts have been considered as playing an important role as progenitor cells and in stabilizing the capsular contracture.[22] TGFβ1 and TNFα are well described as the main molecules involved in the modulation and formation of this disease.[23,24] TGFβ1 acts as a potent fibrotic, angiogenic, and inflammatory mediator and plays a prominent role in fibrotic diseases, whereas TNFα is suggested as inhibiting the collagen synthesis at a transcriptional level, showing a pro-inflammatory activity.[24-27] In this context, a decreased expression of these genes is assumed to correlate with an antifibrotic tendency.

To the best of our knowledge, this is the first experimental study with a follow-up of 52 weeks in an *in vivo* animal model in this context. With time, there are several changes at the molecular and histological levels, which are of interest in this context. Our study provides further and specific information that has not been described in other studies to date, probably because of the comparatively long duration of the experimental setting.[25,28-31]

For the potential profibrotic parameters, notably, TGFβ1 and the thickness of the collagenous and the periprosthetic myofibroblast-rich layer, we found a statistically lower expression rate and development, respectively, in the experimental group (with APD). Similar results were seen for the assessed pro-inflammatory parameters. TNFα level and the number of CD68-positive cells in the collagenous and periprosthetic myofibroblast-rich layers were also statistically significant lower. Interestingly, significant and hitherto unknown differences between the groups at the histological and molecular biological levels only could be observed due to the long-term follow-up of this study. The thickness of the collagenous layer, for example, showed an increase after 12 weeks in both groups but dropped remarkably after 52 weeks, finally decreasing to a level lower than that at 3 weeks in the experimental group. CD 68-positive cells in the collagenous layer revealed an antipodal course with time in both groups. Although an increase in cell count was detected after 12 weeks in the experimental group compared to the 3-week count, CD68-positive cells in the control group initially dropped, showing a level lower than that in the experimental group. Contrary to this development, after 52 weeks, the cell count decreased to a level lower than that after 3 weeks in the experimental group and clearly increased in the control group to reach more than twice the cell count of the experimental group. Similar results were observed for the CD68-positive cells in the periprosthetic myofibroblast-rich layer after 3 and 12 weeks. Here, the cell count also showed a distinct decrease in the experimental group after 52 weeks, whereas the cell count presented as unchanged in the control group. These descriptive results underline the hypothesis of a latent chronic inflammatory influence in the etiology of capsular contracture.

The continuous ingrowth of blood vessels observed in the experimental group, together with an expansion toward the implant, may be interpreted as a clear sign for integration of the matrix. Furthermore, this could explain the higher rate of proliferating cells than that of the control group.

In addition, the role of the remodeling of ECM is known as an indicator of fibrotic activity in the development of capsular contracture in general. Matrix metalloproteinases (MMPs) are major enzymes in ECM degradation and are regulated by two major endogenous inhibitors: α_2-macroglobulin and tissue inhibitors of metalloproteinases

(TIMPs).[32–34] ECM degradation and collagenous remodeling could be considered as an important process in capsular fibrosis. Therefore, a high expression of MMPs and low expression of TIMPs would be comprehensible for active ECM remodeling. Furthermore, a pathological accumulation of collagen is attributed to the dysregulation of MMP expression.[35] Moreover, MMPs are known to exacerbate inflammation and fibrosis due to excessive and unregulated destruction and remodeling of ECM.[36,37] MMP1 as interstitial collagenase is involved in tissue maintenance and wound repair.[38–40] An elevated systemic TIMP1 concentration can be observed in severe fibroproliferative disorders.[41,42] Little is known about the activation and influence of MMP1 in capsular tissue. Serum concentrations of female patients showed no significant difference in MMP1 levels compared to those of patients after reduction mammoplasty, whereas MMP2 revealed a significant increase.[43] Kyle et al. found an upregulation of MMP12 in contracted breast capsule tissue from patients after preselection of target genes using a genome microarray analysis.[36] In their study, TIMP4 showed no significant regulation, similar to our results.

The MMP1 protein functions as an interstitial collagenase to break down interstitial collagen types I, II, and III.[44,45] Focusing on the analysis of APD and adjacent tissue next to the silicone implant, the expression of MMP1 may play a relevant role. Because MMP1 showed a level lower than that of the control group and TIMP1 revealed no remarkable difference, this could be a key finding. Although not statistically significant, the concentration of MMP1 in the experimental group may thus indicate a reduced activity due to a lower collagen deposition adjacent to the APD. Consequently, one can hypothesize that APD might lead to a lower collagen deposition and lower extracellular activity as well as inflammation, resulting in reduced or stable MMP1 expression. Assuming this, the initial trigger for an increased MMP1 activation might be disregarded.

This can also be speculated for the MMP1-to-TIMP1 ratio, which we found to show a higher expression level in the experimental group. As seen in previous studies focusing on fibrotic diseases, decreased levels were accompanied with severe fibrotic activity and increased synthesis and deposition of collagen.[32,41,43,46,47] Furthermore, Ulrich et al. described a lower MMP1-to-TIMP1 ratio in patients with capsular contracture after breast augmentation, which correlated with the Baker classification.[32] In the context of this study, the higher ratio may, therefore, be interpreted as antifibrotic tendency induced by APD.

Conclusion

In this long-term *in vivo* study, we found an antifibrotic effect by completely wrapping silicone gel implants with APD on immunohistochemical and molecular biological levels. Relevant changes in fibrotic and inflammatory markers in the surrounding tissue could be demonstrated in the long-term 1-year follow-up. Assuming a latent inflammatory etiology of capsular contracture, this study provides important results for assessing the potential benefits of APD. We conclude that further clinical and experimental studies should focus on long-term follow-ups for more detailed results.

Transferred to the clinical application of this concept, one may hypothesize that using APD together with silicone implants could have a positive impact on the development of capsular contracture. Further clinical studies are required to corroborate these experimental data in the future.

Conflict of interest statement

The authors received financial funding for this investigator-driven study by Tutogen Medical GmbH (subsidiary of RTI Surgical®, Alachua, FL, USA).

R.E. Horch has performed investor-initiated studies and served as lecturer and advisor to KCI, Baxter, and Tutogen Medical GmbH.

M. Schmitz performed investor-initiated studies for LifeCell™.

Furthermore, there are no conflicts of interest for one of the authors.

Acknowledgments

Parts of the present work were performed in fulfillment of the requirements for obtaining the doctoral title “Dr. med.”

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