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EVALUATION OF THE SHORT TERM HOST RESPONSE AND BIOMECHANICS OF AN ABSORBABLE POLY-4-HYDROXYBUTYRATE SCAFFOLD IN A SHEEP MODEL FOLLOWING VAGINAL IMPLANTATION

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Abstract

Objective: To compare the host and biomechanical response to a fully absorbable poly-4-hydroxybutyrate (P4HB) scaffold to the response to PP mesh in an animal model of vaginal POP surgery. Design: A study employing a sheep model Setting: KU Leuven Center for Surgical Technologies Population: 14 parous female Mule sheep Methods: P4HB scaffolds were surgically implanted in the posterior vaginal wall of sheep. The comparative PP mesh data were obtained from an identical protocol. Main outcome measures: Gross necropsy, histological and biomechanical evaluation of explants, and the in vivo P4HB scaffold degradation were evaluated at 60- and 180-days post-implantation. Results: Gross necropsy revealed no implant related adverse events using P4HB scaffolds. The tensile stiffness of the P4HB explants increased at 180-days (12.498 ± 2.66 N/mm (P=0.019)) as compared to 60-days (4.585 ± 1.57 N/mm) post-implantation, while P4HB degraded gradually. P4HB scaffolds exhibited excellent tissue integration with dense connective tissue and a moderate initial host response. P4HB scaffolds induced a significantly higher M2/M1 ratio (1.70 ± 0.67 SD, score 0-4), as compared to PP mesh (0.99 ± 0.78 SEM, score 0-4) at 180-days. Conclusions: P4HB scaffold facilitated a gradual load transfer to vaginal tissue over time. The fully absorbable P4HB scaffold, in comparison to PP mesh, has a favorable host response with comparable load bearing capacity. If these results are also observed at longer follow-up, a clinical study for vaginal POP surgery may be warranted to demonstrate efficacy. Key words: Pelvic organ prolapse, vaginal surgery, Poly-4-hydroxybutyrate, degradable scaffold, host response, biomechanics.

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P4HB scaffold induces vaginal tissue remodelling

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Abstract

Objective: To compare the host and biomechanical response to a fully absorbable poly-4-hydroxybutyrate (P4HB) scaffold to the response to PP mesh in an animal model of vaginal POP surgery.

Design: A study employing a sheep model

Setting: KU Leuven Center for Surgical Technologies

Population: 14 parous female Mule sheep

Methods : P4HB scaffolds were surgically implanted in the posterior vaginal wall of sheep. The comparative PP mesh data were obtained from an identical protocol.

Main outcome measures: Gross necropsy, histological and biomechanical evaluation of explants, and the in vivo P4HB scaffold degradation were evaluated at 60- and 180-days post-implantation.

Results: Gross necropsy revealed no implant related adverse events using P4HB scaffolds. The tensile stiffness of the P4HB explants increased at 180-days (12.498 ± 2.66 N/mm (P=0.019)) as compared to 60-days (4.585 ± 1.57 N/mm) post-implantation, while P4HB degraded gradually. P4HB scaffolds exhibited excellent tissue integration with dense connective tissue and a moderate initial host response. P4HB scaffolds induced a significantly higher M2/M1 ratio (1.70 ± 0.67 SD, score 0-4), as compared to PP mesh (0.99 ± 0.78 SEM, score 0-4) at 180-days.

Conclusions: P4HB scaffold facilitated a gradual load transfer to vaginal tissue over time. The fully absorbable P4HB scaffold, in comparison to PP mesh, has a favorable host response with comparable load bearing capacity. If these results are also observed at longer follow-up, a clinical study for vaginal POP surgery may be warranted to demonstrate efficacy.

Key words: Pelvic organ prolapse, vaginal surgery, Poly-4-hydroxybutyrate, degradable scaffold, host response, biomechanics.

INTRODUCTION

Pelvic organ prolapse (POP) is a common condition resulting from damage to the supportive structures of the pelvic floor(1, 2). The annual incidence of surgery for POP is approximately 4.9 cases per 1000 women with the overall life-time risk for POP surgery of 11%(3, 4). Synthetic permanent polypropylene (PP) meshes have been introduced to surgical repair of POP to provide mechanical support to the pelvic floor by inducing a foreign body response(3, 5). However, they have been associated with the clinical complications in long-term. Even though, PP meshes have been modified(6, 7) and resulted milder host response and better outcomes(8), the reputation is damaged. The US Food and Drug Administration (FDA) re-classified transvaginal POP meshes from Class II to Class III in 2016 and have not approved vaginal PP implants since April 2019 in some countries including the USA. The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) (2015) has recommended identification of alternatives to polypropylene, and focusing on biodegradable biomaterials for POP repair to reduce the risk of long-term complications(9).

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Our research group has identified poly-4-hydroxybutyrate (P4HB) as a candidate material for vaginal POP surgery(10) with the hypothesis that a delayed-absorbable implant will provide mechanical support while being gradually replaced by functional connective tissue. P4HB is a biologically produced biosynthetic polymer(11) which degrades to the human metabolite 4HB(12) and gets eliminated from the body completely. Several P4HB devices for soft tissue support have been cleared by the FDA(13). P4HB has also been used for many other clinical applications, including reconstructive surgery, tendon, and ligament repair.

Knitted P4HB scaffold, provide good anatomical and functional outcomes in hernia repair(14), although the applied forces are different, it still concerns a load-bearing soft tissue correction, as in the case of POP. Our previous *in vitro* study illustrated that vaginal fibroblasts on P4HB scaffolds generated a more favourable cellular proliferation, and collagen deposition than on PP(10). In addition, the knit design favouring the optimal cellular response to P4HB scaffold was identified.

These previous outcomes encouraged us that P4HB was a promising candidate material for pelvic floor surgery. Therefore we, decided to further evaluate the host response and biomechanics of fully degradable P4HB scaffold in sheep, an animal model for vaginal POP surgery(15, 16) The outcomes of P4HB scaffold were compared to data of PP mesh obtained from an identical study performed by our group.

1. **MATERIAL and METHODS**
2. **Implants**Based on our previously performed *in vitro* studies (10), we selected a knitted, monofilament P4HB scaffold design with an implant thickness of 0.28 mm, a fibre diameter of 100 µm, and a pore size of 2.22 mm^2. As comparison, light-weight polypropylene – Restorelle® (Coloplast, Minneapolis, MN, USA), with an implant thickness of 0.34 mm, a fibre diameter of 80 µm, and a pore size of 3.1 mm^2, was used.
3. **Animals, surgical procedures and study design**

Animals, anaesthesia and surgical procedures are detailed in Supplementary material 1 and 2. The animals used in this study were maintained and treated according to experimental protocols (P057/2014//P 064/2013and P051/2016) approved by the Ethics Committee on Animal Experimentation of the Faculty of Medicine, KU Leuven. 14 parous female Mule sheep (7 years old, weighing 51.5 ± 5.7 kg) were randomly divided into two groups for each time point. All sheep underwent rectovaginal surgery for the implantation of P4HB scaffold. The surgical procedure was carried out according to the previously described method (4) by the same experienced surgeon (LH). Briefly, the rectovaginal septum was dissected following hydro-dissection. P4HB scaffolds (35 x 35 mm) were fixed with interrupted non-degradable 3/0 polypropylene sutures (Prolene®, Ethicon, Zaventem, Belgium) at the corners and halfway along each side (Figure 1). The vaginal wall was closed with a running 3/0 polyglactin 910 (Vicryl) suture.

Harvesting Implants Ewes were premedicated by intramuscular administration of 1 ml/50 kg xylazine and euthanised with intravenous pentobarbital (20 ml/50 kg Release, Ecuphar, Oostkamp, Belgium)(17). Gross anatomical examination of the explanted vagina was performed using the following parameters: i) fluid collection, ii) exposure of the implant, iii) synechiae, iv) signs of infection. Any shrinkage of the implant was calculated by measuring the length and width of the scaffold. Vaginal explants (vaginal tissue/P4HB implant complex) were dissected into four pieces for assessing both active and passive biomechanical properties, in vivo degradation of the P4HB scaffold, and histomorphology.

Figure 1.

1. **Outcome measurements****Before implantation**
2. Mechanical properties of the implant before the implantation Before the implantation, P4HB scaffold and PP mesh were subjected to a uniaxial testing under dry conditions according to a standardised protocol (18). Details of the uniaxial testing was explained in supplementary material 3.**After implantation**
3. Active biomechanical properties Longitudinal vaginal strips (3 x 7 mm) from explants were dissected, weighed, and immediately suspended in individual organ baths containing fresh Krebs solution (4). Samples were pre-tensioned to 0.5 mN and equilibrated for 60 min before measurement. The samples

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were subjected to contractile stimulation by 80 mM of KCl. Contractile forces were recorded using custom-made software. Measurements were analysed using Origin software (OriginLab Corporation, Northampton, MA, USA). All values were normalised to sample weight, transducer calibration and gravitation constant.

4. Passive biomechanical properties

Uni-axial tensiometry on the vaginal explants was performed by using Zwick uni-axial tensiometer (Zwick GmbH & Co. KG, Ulm, Germany) with a 200N load cell. Posterior middle vaginal tissue was used as a control. Samples were cut longitudinally (10 x 30 mm), clamped tension free, and the zero elongation was defined as the clamp-to-clamp distance at preload (0.1 N). The samples were loaded to failure with an elongation rate of 10 mm/min. The strain was calculated by dividing the elongation by the clamp-to-clamp distance and stress dividing force by the cross-sectional area (19). The stiffness (N/cm2) of the specimens was determined with the slope of the stress-strain curve in comfort zone by using TestXpert II software (Zwick GmbH & Co) (18).

In vivo degradation of the P4HB implant

In vivo degradation of P4HB scaffolds were determined by molecular weight (Mw) change via gel permeation chromatography (GPC) analysis and by changes in scaffold morphology via scanning electron microscopy (SEM) (JEOL JSM6700F) following to vaginal tissue by digestion.(20). Details of the tissue digestion, and analysis of GPC and SEM were explained in supplementary material 4.

Histomorphology Details of histology and immunohistochemistry (IHC) staining, and scoring is given at Supplementary material 5 (Table S1, Figure S1). Tissue integration of the P4HB scaffolds was evaluated from SEM images of the vaginal explants. Histology sections were stained with Haematoxylin and Eosin (H&E) and Masson Trichrome to quantify the foreign body giant cells (FBGC), polymorphonuclear cells (PMN), blood vessels and connective tissue. IHC staining was performed for detection of neovascularisation (CD34), neuronal network (PGP 9.5), myofibroblasts and smooth muscle cells (α-SMA), leukocytes (CD45), M1 (HLA-DR) and M2 (CD163) macrophages. The M2/M1 ratio was calculated. Semi-quantitative assessment was performed by using a qualitative grading scale (4, 21) by two individual researchers blinded for both timepoints.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7.0 (GraphPad Software, Inc; La Jolla, USA). Data normality was tested by the Kolmogorov-Smirnov test. Two-way ANOVA was used for normally distributed data and multiple comparisons between individual groups using a Tukey's test. The Kruskal-Wallis followed by the Dunn's post hoc test was used for data that was not normally distributed. Data are reported as mean ± standard deviation or median and standard error of the mean as appropriate. The significance level was defined as $p < 0.05$.

RESULTS

3.1. Before Implantation

3.1.1. Mechanical characteristics of the implants

P4HB scaffold and PP mesh exhibited similar behaviour under cyclic uniaxial load (Figure 2) with a strong inelastic deformation in the first cycle, followed by a fast stabilisation. However, the membrane stiffness of the PP mesh is threefold higher compared to P4HB scaffold within ten cycles. A decrease in the stiffness of the PP mesh was observed after 10 cycles contrary to the P4HB scaffold which exhibited a relatively small increase in its stiffness after cyclic mechanical loading (17).

Figure 2.

3.2. After Implantation

3.2.1. Gross anatomical examination of the explanted vagina and the implant contraction

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All implants were well incorporated in the deeper vaginal tissue layers without any signs of encapsulation. We did not observe any exposure of P4HB scaffolds or fluid collection at 60- and 180-days post-implantation. Sheep implanted with PP mesh (n=6) developed vaginal synechiae (n=3). P4HB scaffolds were intact, without macroscopic deformation. Shrinkage (% area) of the implants after 60 days and 180 days were 52.2 ± 10.41 SD and 45.9 ± 7.95 SD, respectively. The shrinkage of the PP implants (29.6±7.8 SD and 46.7±14.2 SD) at 60 and 180 days post-implantation were reported as this previously (4).

3.2.2. Active biomechanical properties

Vaginal tissue contraction (Figure 3) in response to KCl was higher at 180 days (226.90 mN ± 79.08, SEM) in comparison to 60 days post-implantation (148.53 mN ± 22.76, SEM). Vaginal tissue exhibited higher contractile function after P4HB scaffold implantation compared to after PP mesh implantation (37.87 mN ± 18.99, SEM at 60-day and 199.49 mN ± 71.56, SEM at 180-day), however the difference at both time points was not statistically significant.

Figure 3 .

3.3.3. Passive biomechanical properties

Biomechanical properties of the vaginal explants and control tissue were compared at 60- days and 180-days post-implantation (Figure 4). The P4HB explants at 60 days, exhibited significantly lower stiffness values compared to the control tissue (4.58 ± 1.57 N/mm SEM vs 11.49 SEM, p=0.024). The stiffness of the explants after 180 days post-implantation increased significantly (12.498 ± 2.66 N/mm, 0.019) as compared to 60-days post-implantation and exhibited a comparable stiffness with the control tissue (11.343 ± 1.96 N/mm, SEM). According to the ball burst test which is not directly comparable to uni-axial tensile testing, there were no statistically significant changes in stiffness values of the PP explants at both time-points (4).

Figure 4.

3.3.4. In vivo degradation of the P4HB scaffold

The degradation of P4HB scaffold was determined by change in molecular weightover time (Figure 5A). The average Mw of the P4HB scaffold gradually decreased significantly over time, 279 ± 3 kDa (SD), 201 ± 5 kDa (SD) and 104 ± 7 kDa (SD) at 0-, 60- and 180- days post-implantation, respectively. The integrity and morphological changes of the implant was visualised by SEM (Figure 5B). The monofilament of P4HB scaffold with smooth surface pre-implantation kept its overall macroscopic integrity at both time-points. SEM images revealed the formation of fibre surface erosion over time. At 60 days, there were only superficial scratches on the fibres, which gradually progressed into surface fissuring over 180 days post-implantation.

Figure 5.

3.3.5. Histomorphology

P4HB Scaffold maintained its integrity after 60- and 180-days post implantation and showed good integration within the submucosa of the vaginal tissue (Figure 6). The P4HB scaffold could be identified in between the lamina propria and muscularis layer in the H&E-stained samples (Figure 7). There was no significant difference in the presence of FBGCs at the border of the implant between both time points (0.93 ± 0.42 SD at 60-days and 0.73 ± 0.54 SD at 180-days) (Figure 8A). A decrease in the presence of polymorphonuclear cells (PMNCs) was observed over time, however this difference was not significant (2.06 ± 0.70 SD at 60-days and 1.65 ± 0.83 SD at 180-days) (Figure 8B). There was significantly higher inflammatory cell infiltration around the P4HB scaffold as compared to the PP mesh at both 60- (P4HB: 2.06 ± 0.70 and PP: 0.60 ±0.31 SD) and 180-days (P4HB: 1.65 ± 0.83 and PP:0.32 ±0.10 SD) (Figure S2A) (4). There was no significant difference in vessel count around the P4HB scaffold at 60- and 180-days based on H&E staining (Figure 8C). However, PP mesh, exhibited a higher number of vessels as compared to P4HB scaffold at both time points (P4HB: 0.87 ± 054 SD at 60-days, 0.78 ± 0.53 SD at 180-days; PP: 1.52 ± 0.46 SD at 60-days and 1.96 ± 0.32 SD at 180-days) (Figure S2B). At 60 days, P4HB scaffolds were surrounded with newly formed

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connective tissue with aligned collagen fibres. After 180 days the connective tissue matured, indicated with darker blue, and density increased (Figure 8D).

Figure 6.

Figure 7.

Figure 8.

There was no significant difference of neovascularisation (Figure 9B) and neuronal ingrowth (Figure 9F) and myofibroblast differentiation (Figure 9H) between 60- and 180-days after vaginal implantation with P4HB scaffold. Presence of leukocytes at 180 days (score 2.7 ± 0.86 SD) was significantly higher compared to 60 days (score 2.05 ± 0.55 SD) after P4HB scaffold implantation (Figure 9D). At 180-days a more widespread infiltration area was CD45 positive, whereas at 60-post implantation this was more visible at the implant border. When compared to PP mesh, P4HB scaffold exhibited significantly ($P<0.005$) higher leukocyte infiltration (PP=1.29 ± 0.36 SD) at 60 days (Figure S3A), and significantly lower myofibroblast differentiation (PP: 2.41 ± 0.26 and P4HB: 1.62 ± 0.56 SD) at 180 days (Figure S3B).

Figure 9.

There was a statistically significant decrease observed in macrophage type-1 (M1) infiltration to P4HB scaffold between implantation times of 60- and 180-days (Figure 10B). At 60 days, M1 infiltration was more dominant (2.02 ± 0.62 SD) at the P4HB scaffold-tissue interface compared to 180 days (1.56 ± 0.59 SD). No statistically significant difference in M2 infiltration was observed between 60- (1.97±0.85 SD) and 180-days (2.24 ± 0.66 SD) post-implantation of P4HB scaffold. The M2/M1 ratio was significantly ($P<0.01$) higher at 180-days (1.70 ± 0.67 SD) as compared to 60-days (0.91± 0.60 SD) post-implantation of P4HB scaffold (Figure 10F). If compared with the retrospective PP mesh data, M2/M1 ratio at 180-days post-implantation was significantly higher with the use of P4HB scaffold (Figure S3C). (PP 0.92 ± 0.17 SD and 0.99 ± 0.78 SD at 60- and 180-days, respectively).

Figure 10.

DISCUSSION

This study is the first preclinical evaluation of P4HB scaffold in vaginal prolapse surgery using a large animal (sheep) model. We have evaluated the host inflammatory response, biomechanical properties, and degradation profile of explants over time. Here we did not observe scaffold exposure, or any other adverse events such as infection, fluid collection or synechiae at 60- and 180-days post-implantation. The stiffness of the vaginal P4HB scaffold explants increased between 60- and 180-days, while the polymer underwent significant degradation over time. P4HB scaffold resulted in a moderate host response, which is demonstrated by an increased M2/M1 ratio (remodelling), low myofibroblast differentiation and formation of well-organised collagen over time, compared to the PP mesh.

In our current study, we obtained well-remodelled vaginal tissue with enhanced biomechanical properties after transvaginal implantation of P4HB scaffold into sheep, which serve as a good translation model for women (4, 15, 22). The host response in terms of vascularisation and collagen deposition after P4HB scaffold implantation were similar to the effect of lightweight PP mesh. However, the initial inflammatory response of P4HB scaffold was greater compared to PP mesh. After 180-days the inflammatory state changed to a more calm and chronic state of the host tissue and resulted in tissue remodelling (macrophage type 2 response). Inflammatory reaction to slowly degrading hybrid implant (TIGR® Matrix) also got milder over time and resulted a thicker tissue formation, as compared to PP mesh, in a sheep abdominal hernia model (23). The P4HB scaffold were still intact and well-integrated with the vaginal tissue, without signs of encapsulation or exposure, something that was seen with the use of PP mesh. This might be related to the exceptionally low membrane stiffness of the P4HB scaffold. Constant applied load after implantation can cause an increase of the implant stiffness, which, in turn, may lead to vaginal degeneration and eventually exposure (24, 25). One can predict no or limited exposures due to the P4HB scaffold, as even after 10 cycles of mechanical

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loading, there was only a slight increase in the stiffness of the P4HB implants. Gradual absorption of P4HB provided a good balance between implant degradation and new tissue formation. Fast degradation of the implants may be associated with poor clinical outcomes due to the weakness of the remodelled tissue. For instance, it was reported that degradable polyglactin-910 implants disappeared within a 6-week period after anterior vaginal wall implantation (26), which resulted in 25% of recurrent cystoceles after 12 months. De Tayrac at al. (27) found that the PLA implant lost most of its mass already after 1.5 months *in vitro,* where degradation is slower compared to in vivo. Apart from preclinical studies for synthetic degradable materials, there are examples of clinically used degradable xenografts in transvaginal surgery such as porcine dermis or porcine small intestinal submucosa such as InteXEN® (28) and Surgisis® (29). However, due to rapid degradation of xenografts, the load bearing capacity of vaginal tissue after the surgery was insufficient in the long term due which resulted in recurrent prolapse (28). Therefore, a sufficient degradation profile of a newly designed implant is necessary to maintain pelvic floor support over time. In the case of P4HB Scaffold, contrary to the decrease in molecular weight, the stiffness of the vaginal explants was increased over time, explants had the comparable stiffness with the vaginal tissue. The stiffness of the explants were at least 10 times higher even when compared to the initial stiffness of the P4HB Scaffold, which suggests remodelling and regeneration of the vaginal tissue contributes to increased tissue stiffness (23). The tissue components such as collagen contribute to the biomechanical properties of the tissue by allowing the tissue to resist deformation under mechanical force (30). As the absorbable P4HB scaffold slowly and gradually degrades, it induced functional, viable vaginal tissue with mechanical integrity. The functionality of the vagina, which can be determined by its ability to actively contract by the smooth muscle activity, may change after implantation. The presence of an implant may induce fibrosis, or alter the collagen or elastin content which subsequently result in a decrease in the vaginal contractility (22). Despite the increase in stiffness of the P4HB scaffold explants, and no difference on the mechanical properties of the PP mesh vaginal explants over time, the contractile function of the vagina implanted with P4HB scaffold was higher than the ones with PP mesh. Implantation of P4HB scaffold resulted in a higher number of inflammatory cells compared to PP mesh, which might be attributed to a higher areal density of P4HB scaffold compared to PP mesh (4) or the ongoing degradation of the P4HB scaffold. However, cellular infiltration of inflammatory cells within the P4HB scaffold/tissue interface was decreased over time and was followed by organised connective at the later stage. The increase in CD45 positive cells at 180 days might be caused by an inflammatory process towards a more chronic state of the foreign body response with a transient presence of monocytes, FBGCs and perhaps lymphocytes (31). Implant properties influence the inflammatory cell interactions at the implant-tissue interface and lead to altered foreign body responses, may further promote greater biocompatibility upon implantation(32). Hjort et.al reported a moderate and decreased inflammatory response over time due to gradual implant degradation(23). If we look at the M2/M1 ratio, P4HB Scaffolds were dominated by M2 macrophages compared to the PP mesh at 180-days post-implantation. Transition from M1 to M2 phenotype occurs with the initiation of the remodelling phase of wound healing and enhanced tissue regeneration could be expected (33, 34). In addition, myofibroblast differentiation, which plays a key role in the existence of fibrosis (35, 36), was less pronounced in the P4HB scaffold explants at 180-days compared with PP mesh. This finding also supports the result of a mechanically stronger tissue which is created by the effective remodelling process after P4HB scaffold implantation.

We acknowledge some limitations of this study. We have used retrospective data for PP mesh, as the design of the current study was identical to the previous. This approach is in line with the aim of reducing the use of animals in research (37). The duration of the study is not sufficient to demonstrate the tissue properties after complete absorption of P4HB scaffold. To characterize the long-term performance of P4HB Scaffold, a longer-term follow-up is necessary.

On the other hand, this is the first preclinical study demonstrating the performance of P4HB Scaffold as a transvaginal implant. We used a well- established animal model used in pelvic floor research. Additionally, the P4HB scaffold was well-characterised in our recent study (10) before transvaginal implantation.

P4HB scaffold exhibits good mechanical support to vaginal tissue and results in a moderate host response in vivo without any visible implant related complications. Although six-month data shows gradual load transfer

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from the P4HB scaffold to the vaginal tissue, the biomechanics of the tissue need to be further evaluated after complete degradation to determine if restored vaginal tissue strength and stiffness is self-sufficient to withstand the loads of daily life. Based on these encouraging results, we have started a two-year follow up study in sheep, prior to introducing the P4HB scaffold clinically. The P4HB scaffold may represent a unique fully absorbable alternative to permanent polypropylene mesh for the surgical correction of POP in women.

Disclosure of interests

CMD., ZG., LH, EV, MZ, EM and JD declare that they have no competing interests. JPR declares that he received unrestricted research grants of Coloplast, Tepha Inc.

Contribution to authorship

JPR and JD planned and developed the study. CMD and LH performed the implantations, and CMD, LH, EV and ZG performed the explantations and sample collection. ZG, CMD, EV, LH, MZ, EM performed data collection and analysis. ZG was the primary author of the manuscript, and all authors revised the manuscript and approved the final version.

Details of ethics approval

This study was conducted with the approval (LA1210191, approval date January 2016) of the the Ethics Committee on Animal Experimentation of the Faculty of Medicine, KU Leuven and in adherence to experimental protocols (P057/2014//P 064/2013and P051/2016).

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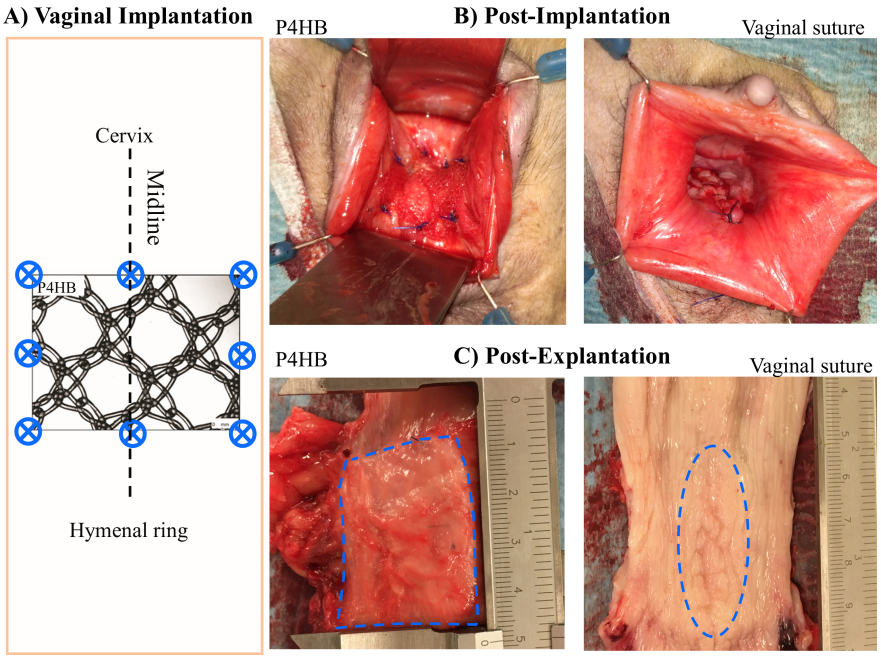

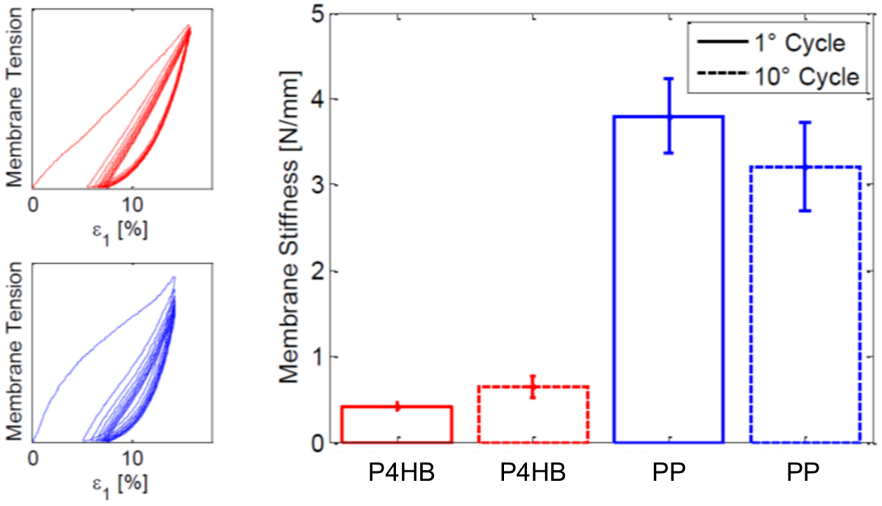

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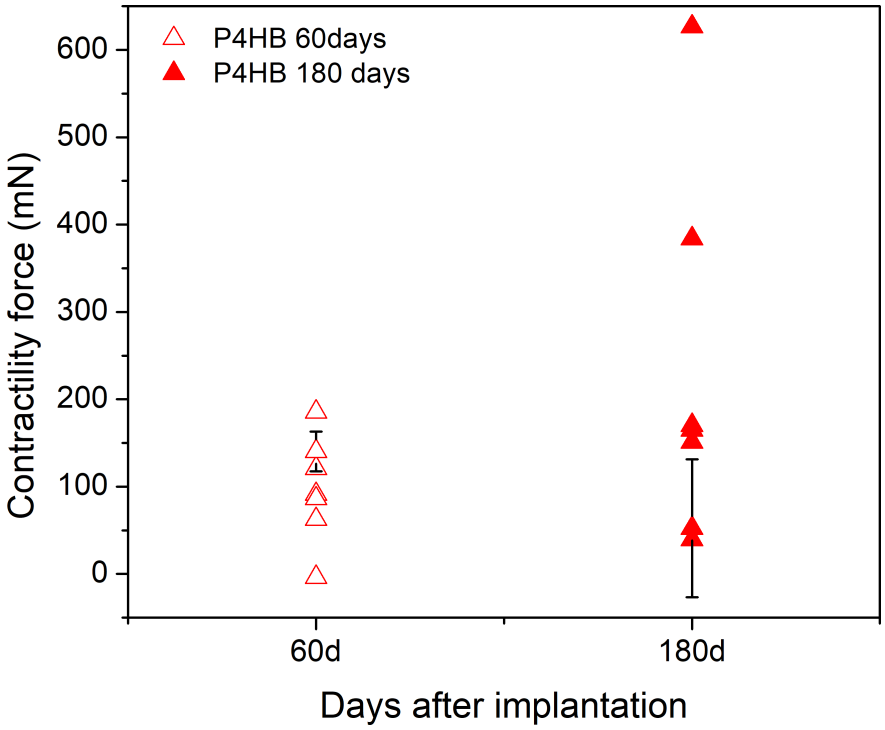

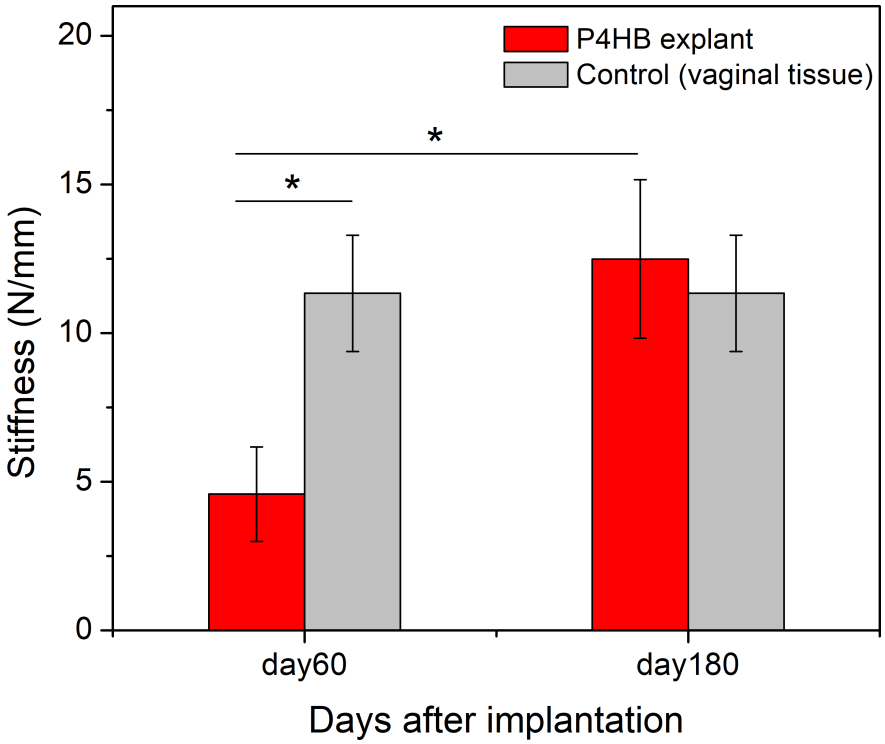

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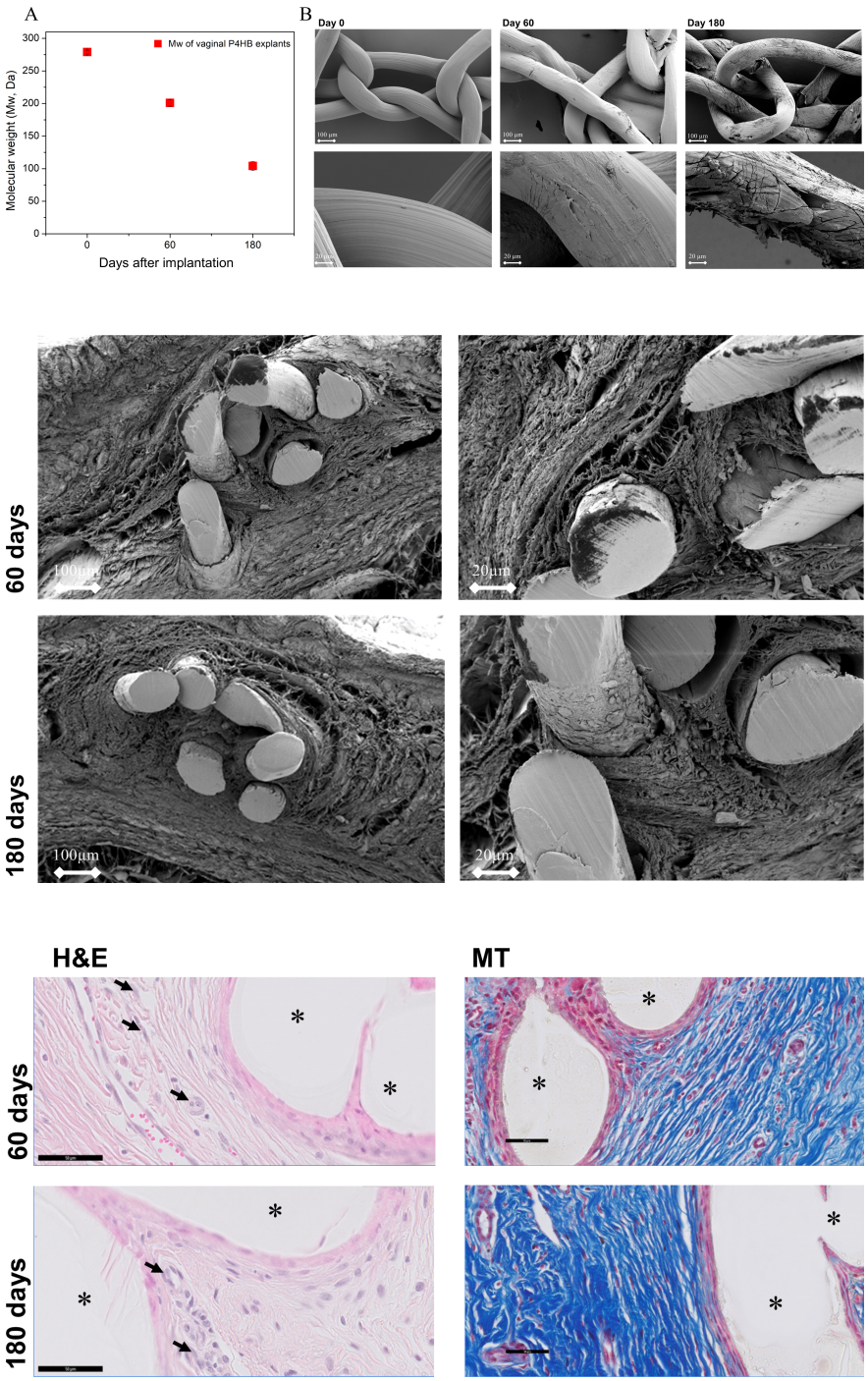

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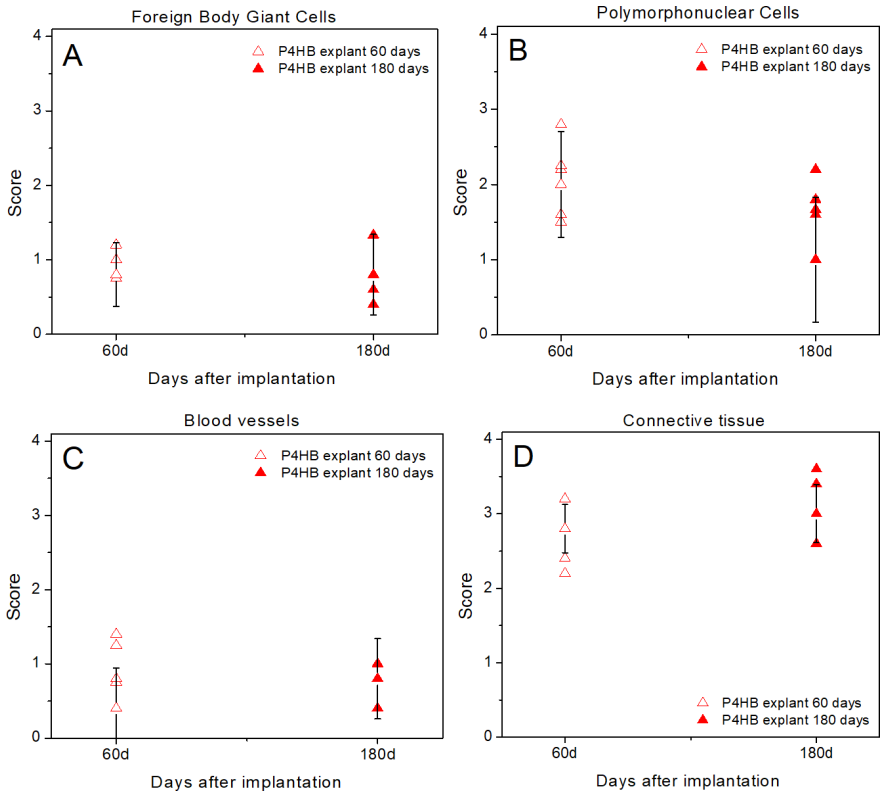
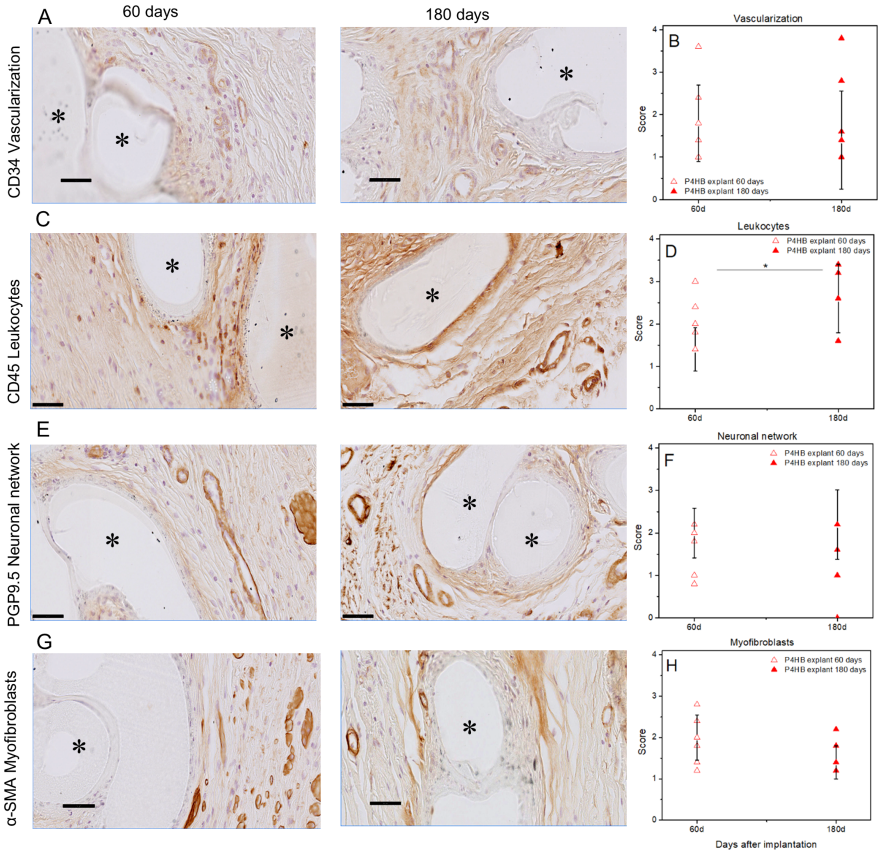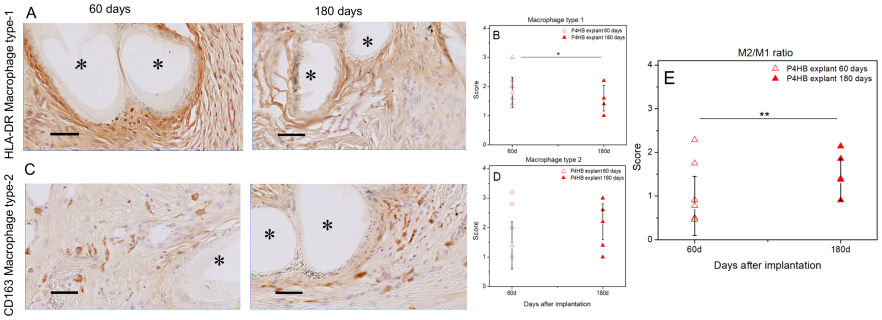
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