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Prevention of foreign body reaction in a pre-clinical large animal model

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ABSTRACT

In this work, the foreign body reaction (FBR) to small subcutaneous implants was compared between small (rodent) and large (swine) animal species for the first time. Dexamethasone-releasing poly(lactic-co-glycolic acid) microspheres/polyvinyl alcohol hydrogel composite coatings were adapted to prevent FBR to small, subcutaneous implants in a large animal model (Goettingen minipigs). The implants consisted of small silicon chips (used to mimic small medical devices) that were coated with the composite formulations. The stages of the FBR were compared with previous studies in rats (that used the same-sized implants); the onset and severity of chronic inflammation (collagen deposition) was identified as a key difference between the two species. In the absence of inflammation control, fibrosis was observed from day 7 post-implantation in minipigs, whereas in rats this did not occur until day 14. This is significant as swine skin is the most commonly used model for pre-clinical testing of dermal formulations. It was determined that for long-term prevention of the FBR (longer than 24 h), a lag phase in dexamethasone release between days 1 and 10 did not affect the anti-FBR properties of the implant in rats. However, continuous release of dexamethasone, with no lag phase, was necessary to prevent inflammation in minipigs (effective dexamethasone dose was 100 μg delivered immediately after implantation and 10 μg/day delivered continuously thereafter). This study offers significant insight into the translation of anti-FBR strategies across species, and showcases the importance of tailoring the controlled release kinetics of the formulation to the host response.

1. Introduction

Implantable biomaterials such as biosensors are recognized by the immune system as foreign; this leads to a cascade of events collectively known as the foreign body reaction (FBR) [1,2]. The FBR consists of two main phases: an acute phase, characterized by the infiltration of inflammatory cells, mainly neutrophils, and a chronic phase, characterized by the presence of active fibroblasts (cells that deposit collagen fibers around the implant) [1,3–5]. The collagen fibers will ultimately encapsulate the foreign body in a dense, fibrous collagen layer (fibrosis).

The FBR has been the focus of many research studies over the past decades. Most of the early work in this area was related to organ rejection prevention [6–8]. In recent years, the emergence of implantable medical devices has led to the FBR being investigated to extend device lifetime [9–12]. In the case of subcutaneously implanted devices, their size, shape, mechanical properties, type of biomaterial, implantation duration and even method of implantation can yield a different response. The FBR can be minimized by using materials with mechanical properties similar to those of the surrounding tissue [13–15], by incorporating hydrophilic coatings that prevent protein adsorption (biofouling) [16–20] and by using biocompatible materials that do not produce toxic or irritating by-products upon degradation [21–24]. However, these approaches only minimize the FBR but do not eliminate it altogether. The only method that has been shown to completely prevent the FBR is the use of local delivery of anti-inflammatory agents which prevent infiltration and further attraction of inflammatory cells [25–32]. Systemic administration of anti-inflammatory agents and immuno-suppressants is regularly used to prevent organ rejection [33–41] but it is not a desirable approach for medical devices and implantable biomaterials due to the high risk-to-benefit ratio.

Dexamethasone, a synthetic glucocorticoid, is the most commonly used anti-inflammatory agent to prevent FBR [26,27,29,31]. Due to its potency, only small amounts of dexamethasone are required; this is essential for small implants where space is limited. Implant coatings designed to suppress the FBR for implantable glucose biosensors have been previously reported and their efficacy has been tested in several rat models (normal, diabetic, and obese) for one and three-month implantation durations [9,11,28,42–46]. Dexamethasone was delivered throughout the implantation period by incorporation in poly(lactic-co-glycolic acid) (PLGA) microspheres that were embedded in a polyvinyl alcohol (PVA) hydrogel coating. While studying the efficacy of these coatings in small animals is necessary as proof-of-concept, it is accepted that studies in larger animals are required in order to extrapolate the results to design the first-in-human clinical trials. The Goettingen minipig,

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a breed of miniature swine, is a common animal model for dermal studies due to the similarities between human and swine skin [47–50]. The objective of the present work was to study the FBR to miniaturized implantable biomaterials in the Goettingen minipig, identify key parameters that will determine anti-FBR dosing regimens of dexamethasone, and apply the findings for long-term prevention of the FBR. To achieve this, PLGA microsphere/PVA hydrogel composites that release dexamethasone in various amounts and rates (as determined in previously published work from our group [51]) were prepared. The composites were used to coat silicon chips, mimicking an implantable biosensor, and were implanted in the subcutaneous tissue of Goettingen minipigs. The local tissue reaction to the implants was determined histologically at multiple time points after implantation.

2. Materials and methods

2.1. Materials

Dexamethasone was purchased from Cayman Chemical Company (Ann Arbor, MI). High-molecular weight poly(vinyl alcohol) (HMW-PVA, MW 30–70 kDa), was purchased from Polysciences, Inc. (Warrington, PA) and low-molecular weight PVA (LMW-PVA, 99% hydrolyzed, MW 133 kDa) was purchased from Sigma-Aldrich (St. Louis, MO). PLGA Resomer® RG503H (inherent viscosity 0.32–0.44 dl/g) was a gift from Boehringer-Ingelheim and PLGA DLG2A (inherent viscosity 0.15–0.25 dl/g), was a gift from SurModics Pharmaceuticals (Birmingham, AL). Methylene chloride and dimethyl sulfoxide (DMSO, ACS grade) were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. PLGA microsphere preparation

Dexamethasone-loaded PLGA microspheres were prepared as previously described [9]. Briefly, 2 g of PLGA was dissolved in 8 ml methylene chloride (DCM). 200 mg of crystalline dexamethasone was added to the polymer solution and the mixture was homogenized at 10,000 rpm for 2.5 min using a T 25 digital ULTRA-TURRAX® homogenizer (IKA® Works, Inc.) to obtain a homogenous suspension. The suspension was subsequently transferred to 40 ml of 1% w/v LMW-PVA aqueous solution and homogenized for 1 min at 10,000 rpm to obtain a solid–oil–water emulsion. The emulsion was transferred to 500 ml of 0.1% w/v LMW-PVA aqueous solution and stirred at 600 rpm under vacuum for 3 h to remove the DCM. The hardened microspheres were purified via three centrifugation cycles at 3500 rpm for 2 min each, freeze dried and stored at 4 °C until further use. Blank microspheres were prepared in the same way without the addition of dexamethasone. PLGA polymers of two molecular weights were used in different preparations: 25,000 g/mol (50:50 Resomer 503H) and 12,000 g/mol (50:50 DLG 2A).

2.3. Preparation of implants

Implants were of cylindrical shape and consisted of a rectangular silicon chip core ($5 \times 0.5 \times 0.5$ mm) coated with PVA hydrogel embedded with PLGA microspheres (7–11 mm length, 1.5 mm diameter when hydrated). To coat the silicon chips, 75 or 150 mg of microspheres was suspended in 1 ml of 5% w/v HMW-PVA aqueous solution. The mixture was vortexed and placed in a sonicated bath for 10 s to achieve good microsphere distribution and break any aggregates. The PVA hydrogel was formed after physical crosslinking of the HMW-PVA via three freeze–thaw cycles. First, the suspension was subjected to one freeze–thaw cycle (2 h at −20 °C and 1 h at ambient temperature). After the first cycle, the partially thickened suspension was fed in a two-piece grooved mold (grooves of 1.5 mm in diameter). The silicon chips were sandwiched between the two mold pieces that were then subjected to two more freeze–thaw cycles to complete the PVA crosslinking and form a self-supporting hydrogel around the chips. Each mold was used to coat 30 silicon chips of approximately 2 mg weight. Please note that low-molecular weight PVA was used as a surfactant to improve emulsion stability during the microsphere preparation process, while high-molecular weight PVA was used to form a hydrogel. Hydrogel strips containing the silicon chips were air-dried and cut at 7, 9 or 11 mm length implants. The implants were placed in 16 gauge needles and stored at 4 °C until further use. Different implant formulations were labeled as shown in Table 1.

2.4. PLGA microsphere characterization

Particle size: An Accusizer 780 (Particle Sizing Systems) was used to measure the particle size of the PLGA microspheres. 3–5 mg of dried microspheres was suspended in 1 ml of 0.1% w/v LMW-PVA solution, bath-sonicated for 10 s and analyzed for volume-based average size.

Dexamethasone loading: 5 mg of dried microspheres or composites was dissolved in 1 ml DMSO and then diluted 10 times in phosphate buffered saline (PBS) pH 7.4. Dexamethasone concentration was determined via RP-HPLC (PerkinElmer, Inc.). Mobile phase: acetonitrile/water/phosphoric acid (30/70/0.5%, v/v/v); column: Zorbax® C18 (4.6 mm × 15 cm); detection wavelength: 240 nm; flow rate: 1 ml/min.

2.5. In vivo pharmacodynamics study

Seven young, female Goettingen minipigs were used as a large animal model to study the inhibition of the FBR to subcutaneous implants. Minipigs were studied in iterations of 2 or 3 animals. The number of animals that were utilized for each formulation is indicated in the figure legends. Each study lasted for 30 days. The implants that were tested are shown in Table 1. They were implanted at the back of the animals on days 0, 9, 16, 23, 27, and 29. All implants were spaced at least 5 cm apart to ensure no interference. The area right above the spinal cord was not implanted. The animals were sacrificed on day 30 and the implants with surrounding subcutaneous tissue were harvested and stored in 10% buffered formalin solution (Sigma-Aldrich Co. LLC.). This resulted in implants being excised on days 1, 3, 7, 14, 21, and 30 post-implantation. All time points were ±3 days to allow for unforeseeable delays. The extracted implants that prevented the FBR were analyzed for the remaining dexamethasone content. All animal studies were reviewed and approved by the University of Connecticut's Institutional Animal Care and Use Committee (IACUC) prior to the beginning of the experiments.

2.6. Histological evaluation

Fixed tissues were processed, embedded in paraffin and sectioned in 20 μm films at the University of Connecticut's Pathobiology Department. Tissue sections were stained with Hematoxylin & Eosin (H&E) and the presence and progress of the FBR were evaluated by observation under a light microscope. Normal connective tissue appears pink, adipose tissue white, deposited collagen fibers light pink, and inflammatory cells purple. Digital images representative of the tissue reaction around the implants are presented here.

Table 1
Implant composition and size.

Implant	Microspheres	PLGA per ml PVA solution (mg)	Length (mm)
Control	Blank	75	7
R	1M	75	7
R150	1M	150	7
R9	1M	75	9
R11	1M	75	11
R2W	2W	150	7

Table 2
Physical properties of PLGA microspheres. Results are average values ± SD (n = 3).

Microspheres	Extend of polymer degradation	PLGA MW (g/mol)	Mean particle size (um ± SD)	Drug loading (% w/w ± SD)
1M	1 month	25,000	49.18 ± 5.84	10.54 ± 0.34
2W	2 weeks	12,000	38.95 ± 1.94	11.86 ± 0.13

3. Results

3.1. Microsphere characterization

Physical properties of the microspheres were measured to confirm that the formulations were consistent with the previous work [51]. The particle size and drug loading of the microspheres are shown in Table 2. Please note that due to the low solubility of dexamethasone in water and some polymer loss during microsphere preparation, the dexamethasone loading exceeded the theoretical (10% by weight).

3.2. FBR to implants with no dexamethasone

Implants coated with blank (no dexamethasone) microspheres embedded in PVA hydrogel were used to study the FBR in Goettingen minipigs. Tissue sections excised at 3, 7, 14, 21 and 30 days after implantation give a representation of the progress of the FBR (Fig. 1).

The FBR consisted of the typical acute phase observed on day 3 (indicated by the presence of purple-stained inflammatory cells around the implant), and the chronic phase with fibrous encapsulation clearly visible from day 14 onwards (observed as a light pink band with collagen fibers oriented around the implant). Collagen deposition around the implant can be clearly seen on day 7 prior to fibrous encapsulation (Fig. 2).

3.3. Effect of total dexamethasone dose

Implants coated with dexamethasone-loaded composites were used to determine whether the formulation that was effective in normal, diabetic and obese rats can be used in large animals. Implant *R* was the

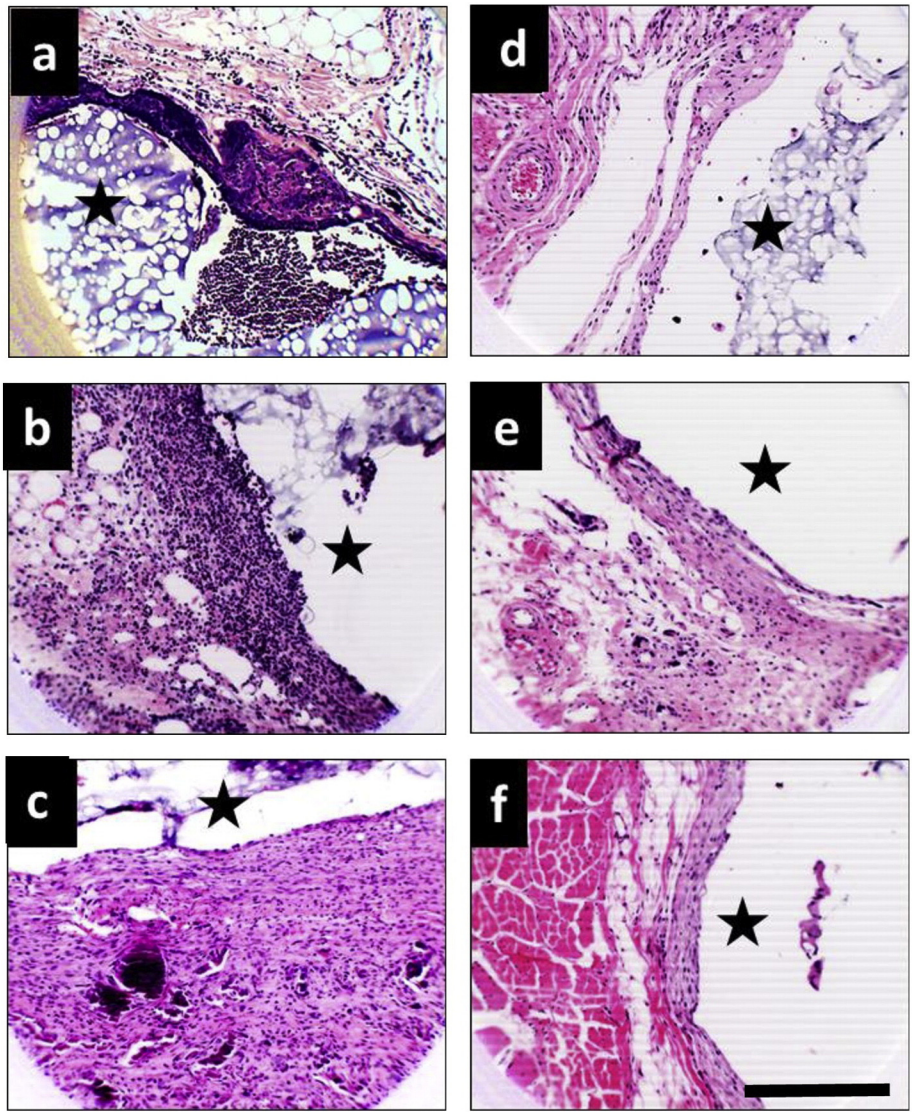

Fig. 1. Histological evaluation of the foreign body reaction to control (no dexamethasone) implants. The stars denote the implant location. Connective tissue is stained pink, collagen fibers light pink and inflammatory cells purple (H&E staining). *a*: day 1; *b*: day 3; *c*: day 7; *d*: day 14; *e*: day 21; *f*: day 30 post-implantation (n = 3). Scale bar: 500 μm.

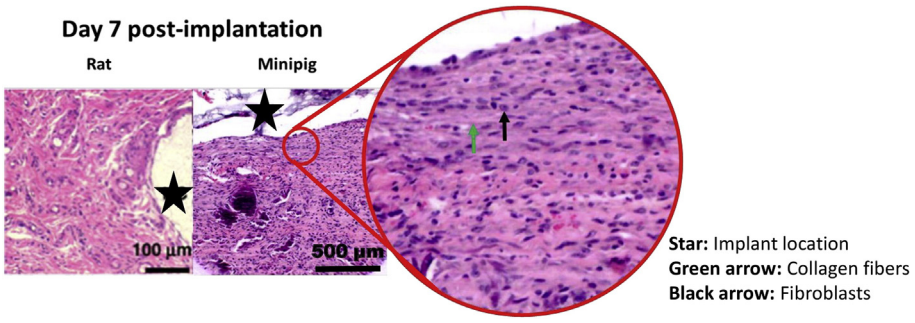

Fig. 2. Comparison of FBR in rats and minipigs 7 days post-implantation. The stars denote the implant location. Connective tissue is stained pink, collagen fibers light pink and inflammatory cells purple (H&E staining). Green arrow: collagen fibers; black arrow: fibroblasts. Rat image taken from Patil et. al. [43]. Fibroblasts were identified based on the cell morphology. Active fibroblasts have an oval shape with spherical nucleus and are characteristically positioned in-between collagen fibers which they produce.

reference formulation which had the same composition as that which was effective in all rat models [9]. Implants with higher dexamethasone doses were also tested in order to optimize the dexamethasone dosing. Two strategies to increase the total dexamethasone per implant were employed: *a)* doubling the microsphere density in the coating to 150 mg per 1 ml of PVA hydrogel (the maximum microsphere content that allows enough glucose permeability through the implant as determined in previous studies, unpublished data by Burgess et. al.); and *b)* increasing the implant length from 7 to 11 mm. All implant preparations are detailed in Table 1.

As shown in Fig. 3, the implants with the higher dexamethasone doses (implants *R150* and *R11*) completely suppressed the acute inflammation as no cell infiltration was observed on day 3 post-implantation. Implants *R* and *R9* with lower dexamethasone doses, including the reference implant, did not completely prevent the acute inflammatory phase but did reduce it significantly compared to the control. However, none of the implants prevented the chronic phase of the FBR, and collagen deposition is observed from day 7 in all cases, with the fibrous capsule being observed from day 14. The implants with the higher dexamethasone doses (implants *R150* and *R11*) showed relatively less dense fibrous capsules compared to the control implants, however, this is not satisfactory for the purpose of long-term prevention of FBR to prolong the functionality of implantable biomaterials.

3.4. Effect of PLGA molecular weight

Implants made with PLGA microspheres of lower molecular weight were tested. These microspheres release dexamethasone continuously for two weeks, with no lag phase, as previously reported [51]. A formulation with lower molecular weight PLGA microspheres loaded in PVA hydrogels with the maximum amount (150 mg per 1 ml hydrogel) was tested (implant *R2W*). As shown in Fig. 4 this formulation prevented acute inflammation (no inflammatory cell accumulation, panel A in Fig. 4) as well as chronic inflammation (no fibrous encapsulation, panel D in Fig. 4) up to day 11. Minimal collagen deposition was observed on day 14 when dexamethasone was depleted. Implants extracted 1 and 14 days after implantation were analyzed for dexamethasone content. It was determined that after 24 h, 61.56 ± 5.72% of dexamethasone remained in the implant (i.e. 35.16 ± 5.72% released during the burst phase) while 7.77 ± 1.82% remained in the implant at the end of 14 days (i.e. 92.81 ± 1.82% released). Accordingly, the effective dexamethasone dose was calculated based on 40% of the total

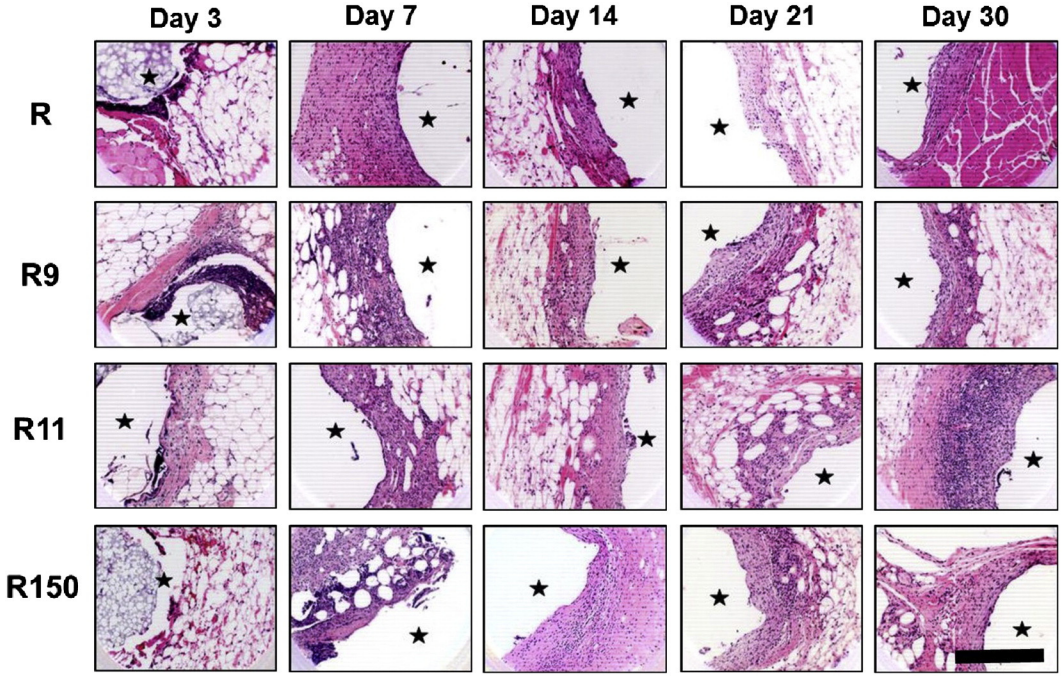

Fig. 3. Histological evaluation of the foreign body reaction to dexamethasone-releasing implants R, R9, R11 and R150 on days 3, 7, 14, 21, and 30 post-implantation. The stars denote the implant location. Connective tissue is stained pink, collagen fibers light pink and inflammatory cells purple (H&E staining) (n = 3). Scale bar: 500 µm.

dexamethasone released during the first 24 h and the rest continuously released throughout the implantation period (approximately 100 μg released the first day and 10 μg/day throughout the remainder of the implantation period).

4. Discussion

Local delivery of dexamethasone, an anti-inflammatory agent, has been successfully used to counter the FBR to subcutaneously-implanted biomaterials in normal, obese and diabetic rats. In order to translate this technology to the clinic, it was decided to bridge the gap between rats and humans by using a large animal model, Goettingen minipigs. The progress and prevention of the FBR were studied, with the intention to directly compare the finding with corresponding investigations in rats. Direct comparison was made possible by starting with implants with the same characteristics as the ones that were previously tested in rats (size, shape, mechanical properties, materials, anti-inflammatory drug) [9]. Our results reveal critical factors that need to be taken into consideration when extrapolating this type of data across different species.

It was found that while the events leading to fibrotic encapsulation are similar between minipigs and rats, their timing differs significantly. In rats, fibroblast recruitment and activation start 14 days after implantation [42], while in minipigs active fibroblasts and collagen deposition can be observed from day 7. This is shown in Fig. 1 panel c and in Fig. 2 where a dense collagen network surrounds the implant. This network will later on contract to form the fibrotic membrane isolating the implant. In the corresponding studies in rats, no dense collagen network was observed on day 7 after implantation. Instead, a loose connective tissue similar to subcutaneous tissue under normal conditions was observed. The difference in the onset of fibrosis between rats and minipigs is of great importance as it indicates a difference in the severity of the chronic inflammation between the two species and consequently the amount of drug necessary to counter it.

In transition from small to large animals during preclinical testing of drug-releasing formulations, dose is usually scaled up according to body weight with corrections based on empirical formulas. In the case of locally-acting implants, however, dose scale-up is not necessary as no systemic effect is desired. In order to prevent the FBR to devices implanted in the subcutaneous tissue, a minimum level of drug has to be maintained in the local environment throughout the implantation period. This level may vary from species to species due to differences in drug clearance from the subcutaneous tissue, drug diffusion rates through the tissue, drug metabolism and the presence of cells.

Implants made of PLGA microspheres of the same molecular weight as the ones tested in rats and same total dexamethasone loading were used as reference formulations. These implants did not prevent the FBR and the tissue surrounding them was similar to that around the

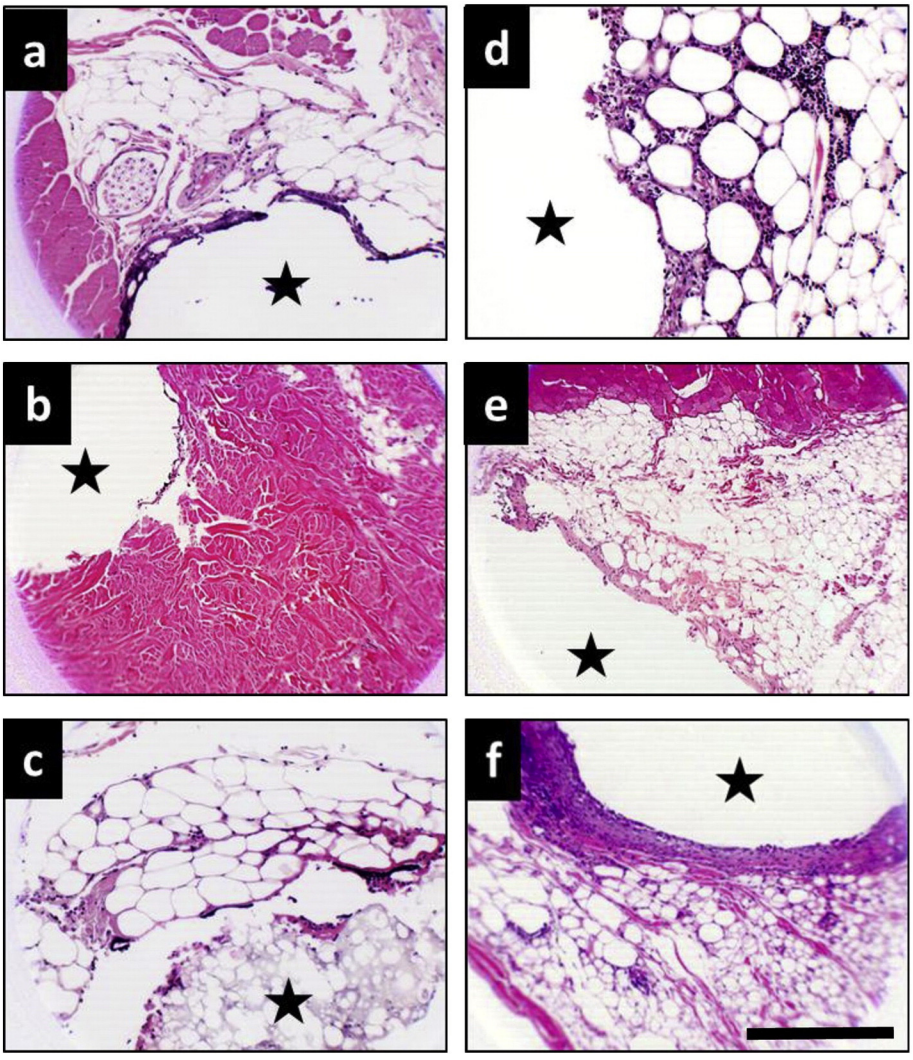

Fig. 4. Histological evaluation of the foreign body reaction to dexamethasone-releasing implants R2W. The stars denote the implant location. Connective tissue is stained pink, collagen fibers light pink and inflammatory cells purple (H&E staining). *a*: day 1; *b*: day 3; *c*: day 7; *d*: day 11; *e*: day 14; *f*: day 21 post-implantation (n = 4). Scale bar: 500 μm.

control implants. As evidenced by the absence of inflammatory cell accumulation in Fig. 3, higher dexamethasone doses prevented the acute phase of the FBR. The higher dexamethasone doses were achieved by either increasing the microsphere density in the biocompatible coating or increasing the total length of the implants. However, increasing the dexamethasone dose was limited due to the small size of the implants. The maximum amount of dexamethasone per implant that was achieved was 0.22 mg. This amount was not enough to prevent fibrous encapsulation of the implants.

Based on the in vitro dissolution testing of these implants [51], 40% of the drug is released within the first 24 h and the remaining amount is released continuously starting from about day 10 post-implantation. This leaves a nine-day period with little-to-no dexamethasone release in the tissue (lag phase). The lag phase did not interfere with the anti-FBR effect of the implants in rats. Based on the observations on the earlier timing of fibrosis onset in minipigs compared to rats, it was hypothesized that eliminating this lag phase might prevent FBR. This hypothesis was tested and confirmed using PLGA microspheres of a lower molecular weight. Implants made with these microspheres release dexamethasone continuously for about 14 days with no lag phase [51]. Once implanted in the minipigs, the implants prevented the FBR until day 11 and early collagen deposition is observed on day 14. This is likely due to the dexamethasone depletion that occurs around that time.

Based on the results discussed above, the dexamethasone dose that is effective to counter the FBR in Goettingen minipigs which is ~100 μg is released during the first day (to counter the acute inflammation cause by tissue trauma) and about 10 μg/day released continuously throughout the implantation period (to prevent fibroblast activation and collagen deposition). This was confirmed following analysis of the amount of dexamethasone remaining in extracted implants at 24 h and 14 days. This dosing regimen applies to subcutaneously-implanted materials of small size (around 7 × 1.5 mm when hydrated). It has been previously shown that the size of the implant affects the severity of the FBR in rats, so dose adjustment will likely be necessary for implants of different sizes. The release kinetics of dexamethasone, however, will have to remain the same, i.e. a burst release followed by a continuous, zero-order release throughout the implantation period.

The size of our implants was chosen to reflect the trend of miniaturization of implantable medical devices. The extent of the anti-inflammatory drug dose limitation varies from implant to implant and it depends on implant size, desired coating thickness, desired coating permeability to various tissue molecules, and the type of controlled release vesicle used to deliver the drug. In order to extend the anti-FBR effect of the biomaterials beyond the two-week period achieved here, the drug-carrier technology (in this case the PLGA microspheres) has to be optimized or replaced in order to maximize the drug loading efficiency and accommodate high dexamethasone doses in such small samples.

5. Conclusions

In the present work, the FBR to subcutaneous implants was compared between a small and large animal model for the first time. The onset and severity of fibrosis were identified key differences with minipigs, demonstrating earlier onset and more severe chronic inflammation compared to rats. It was determined that the dexamethasone release from the implant coatings must be tailored to the species-specific stages of the FBR. In order to counter the more severe chronic inflammation observed in minipigs compared to rats, dexamethasone release must be continuous, with no lag phase. The effective dexamethasone dosing regime was 100 μg during the first day and 10 μg/day thereafter; this regime is applicable to implants of similar size (7 × 0.15 mm) and can be extrapolated to longer implantation periods in minipigs. Lastly, these findings can facilitate the dose calculations in the first-in-human studies as porcine skin is the optimal model for dermal formulations.

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