The inflammatory reaction and the expression of pro- and anti-inflammatory cytokines to a novel polyester-polyurethane aortic prosthesis evaluated in dogs at 6 and 12 months post-implantation

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Abstract

The main objective of this study was to evaluate the intensity and character of the inflammatory reaction caused by an innovative polyester-polyurethane vascular prosthesis implanted into the abdominal aorta of 9 Beagle dogs aged 1-3 years. At 6 and 12 months post implantation the prostheses were removed and tissues samples were examined using 2 methods: histology and immunohistochemistry (IHC). Histology slides stained with hematoxylin and eosin (H&E) were evaluated for the intensity of inflammation by observing the density of inflammatory cells and graded 1 to 4 (1- light inflammation, 4 – severe inflammation). The pro-inflammatory mediator tumor necrosis factor-alpha (TNFα) and two anti-inflammatory mediators, interleukin 1 receptor antagonist (IL1ra), and interleukin 10 (IL10), were also assessed in the tissue samples by IHC methods. Mean (n=5) inflammation grade in H&E slides at 6 months post-implantation (6Mpost) was 2 and mean (n=4) inflammation grade at 12 months (12Mpost) was almost 3. IHC staining showed that TNFα and IL1ra in tissue samples obtained from 6Mpost dogs were expressed at the same intensity indicating equal pro- and anti-inflammatory cytokine levels. However, in the 12Mpost tissues TNFα was expressed more intensely than IL1ra and IL10. Moreover, in 2 dogs at 12Mpost, there were signs of infection assessed on the basis of neutrophil infiltration in the prostheses. In conclusion, the assessment of pro-inflammatory mediators such as TNFα and anti-inflammatory mediators, such as IL1ra and IL10, can help to interpret the intensity of the inflammatory process directed at synthetic prostheses.

Key words: polyester, polyurethane, inflammation, immunohistochemistry, aortic prosthesis
Introduction

Polyester as a synthetic polymer is a commonly used material in both human and veterinary medicine as sutures and prosthetic material for hernia meshes, vascular grafts and other medical devices (Chlupáč et al. 2009). Polyester sutures are often used for cruciate ligament repair and portosystemic shunt ligation in dogs (Hunt and Hughes 1999, Selmi et al. 2002, Mölsä et al. 2014). Polyester, polyurethane, and other synthetic polymers are non-absorbable materials which when placed in the living organism can remain unchanged for years (Schwartz and Henry 2002, Xue and Greisler 2014). However, synthetic polymer materials may initiate a foreign body reaction, characterized as chronic inflammation, creating an inflammatory environment which may alter the properties of the implanted synthetic material causing dysfunction of the prosthesis (Nappi et al. 2016).

A set of reactions in response to the implantations of sutures, stents, prostheses and other devices have been described: tissue damage, blood and synthetic material interactions, formation of temporary matrixes on the surfaces of the synthetic materials, acute and chronic inflammation, development of granulation tissue, development of foreign body reactions, and formation of fibrous tissue capsules (Diegelmann and Evans 2004, Velnar et al. 2016). The response reactions to an implanted prostheses can be local as well as systemic. The migration of polymorphonuclear cells (neutrophilic leukocytes) plays the main role in acute inflammation. The neutrophilic leukocytes release inflammatory mediators which attracts the monocytes, macrophages, lymphocytes, and plasma cells. These cells proliferate resulting in chronic inflammation. Granulation tissue begins to appear which is characterized by the infiltration of fibrocytes, fibroblasts and endothelial cells. Scar formation occurs with the development of a fibrous capsule around the foreign body, completing the healing process with wound epithelialization (Serhan et al. 2010, Velnar et al. 2016). The inflammatory reaction directed at the synthetic material is also initiated by the body’s immune system. This is characterized by monocyte/macrophage activity, inflammatory cytokine production and foreign body giant cell formation around the filaments of the implanted synthetic material (Anderson et al. 2008). Depending on the type of material and its biocompatibility properties, different macrophage populations can predominate at the site of inflammation with various cytokine production which may stimulate or inhibit proliferation and activity of leukocytes (neutrophils, monocytes, lymphocytes) and wound healing cells (fibroblasts, keratinocytes, endothelial cells) (Luttikhuizen et al. 2006).

Cytokines which activate and promote the inflammatory process are interleukin 1 (IL1), interleukin 2 (IL2), TNFα and others. Cytokines which inhibit the inflammatory process are interleukin 10 (IL10), interleukin 1ra (IL1ra), vascular endothelial growth factor (VEGF) and others (Kofler et al. 2005, Kwee and Mooney 2015).

The last decades have seen a surge of research on synthetic blood vessel prostheses in the search of more optimal materials. Despite many years of investigative studies the use of synthetic polymer prostheses for vascular reconstruction continue to result in post-operative complications. These have been often associated with the non-compliance of the biomechanical properties of the prostheses compared to the natural blood-vessel wall, the development of neointimal hyperplasia, the deformation of the prosthesis, infection, and others. Therefore, it is important to improve existing prostheses, as well as to develop novel ones, with the aim of minimizing the incidence of complications after implantation (Abbott et al. 1993, Sarkar et al. 2007). All novel vascular prostheses must be tested in vitro and in vivo before used in humans (Podlaha et al. 2009, Byrom et al. 2010, Swartz and Andreedis 2013). Dogs provide an excellent testing model having similar blood vessel biomechanical properties and are easy to work with allowing for close clinical observation and assessment of motion and pain. The aim of this study was to test the biostability and biointegration capabilities of a novel vascular material developed by the Riga Technical University (RTU), implanted in the dog’s abdominal aorta. This article describes the intensity and character of inflammation defined by the assessment of inflammatory cell quantity and the level of expression of TNFα, IL1ra and IL10 in the novel polyester-polyurethane aortic prosthesis 6 and 12 months post implantation.

Materials and Methods

The study was performed at the Latvia University of Agriculture, Faculty of Veterinary Medicine from the year 2011 to 2013. Nine female Beagle dogs, 1-3 years old, underwent retroperitoneal implantation of a polyester-polyurethane aortic prosthesis (designed at the Riga Technical University RTU), patent No. LV 14192 B, Latvia) in the abdominal aorta. The experiment was confirmed by the Food and Veterinary Service of the Republic of Latvia (No. of license 36). The innovative prostheses were made using a weaving technology to create inert, non-toxic, mechanically compatible polyester and polyurethane composite filaments which were interconnected in 3 layers creating a porous
braided material. Based on in vitro studies and on published information describing the prosthesis material, these vascular implants provide an innovative wall structure which is very similar to the structure of the vascular walls of a native blood vessel (aorta) (Lukyanchikov and Kantsevicha 2010a, b).

Dogs were divided into two groups based on the time the prostheses remained in the aorta before removal. In five dogs the prostheses were harvested at 6 months (6Mpost) post implantation and in 4 dogs at 12 months (12Mpost) post implantation. All the dogs were euthanized after implant removal.

Before surgery, all the dogs were physically examined and blood tested. They were clinically normal, and the complete blood counts (CBC), biochemistry panels and urine analyses were within normal ranges. The surgeries were performed under general anaesthesia. Pre-medications included atropine sulphate (0.02 mg/kg) and acepromazine maleate (0.1 mg/kg) given intramuscular (IM); diazepam (0.25 mg/kg) and ketamine hydrochloride (10 mg/kg) were used intravenous for induction, and Isoflurane inhalation was used for maintenance. The dogs were monitored for O2, pulse, blood pressure throughout surgery until fully awake. They were placed in a right lateral position and the surgery area (the lateral lumbar vertebral area) was prepared. The surgeries were done according to generally accepted principles of aseptic technique. The skin was cut parallel to the lumbar vertebrae below the longest dorsal muscle (m. longissimus dorsi) and caudally to the last, left rib. Abdominal external oblique (m. obliquus externus abdominis), internal abdominal oblique (m. obliquus internus abdominis) and abdominal transverse muscles (m. transversus abdominis) were cut to access the abdominal aorta which was dissected from the surrounding tissue without cutting the peritoneum. Blood flow in the aorta was stopped with two vascular clamps placed about 4 to 5 cm apart. Three minutes before cutting the aorta, heparin was administered intravenous. The aorta was perpendicularly transected, and a segment equal to prosthesis size was removed. The RTU manufactured prostheses (Fig. 1) ranging in size from 5 - 8 mm (mean 6 ± 0.05 mm) in diameter and 8 - 18 mm in length were implanted into the aorta defect. All...
prostheses were placed in the same location of the aorta between the branching of the renal arteries and the bifurcation into the common iliac arteries. The prosthesis was sutured to the aorta using non-absorbable ‘Premilene’ 7/0, muscles and subcutaneous tissue with absorbable suture ‘Serafit’ 2/0, and the skin with ‘Supramide’ 3/0 suture. The clamps were removed after bleeding was controlled at the anastomoses sites. To reduce postoperative wound infection and pain, all the dogs received the antimicrobial agent enrofloxacin (5 mg/kg IM 1x/day for 7 days), the non-steroidal anti-inflammatory meloxicam (0.2 mg/kg PO 1x/day for 5 days) and the analgesic tramadol (4 mg/kg SC 2x/day for 5 days). All the animals were clinically examined 2x/day for activity, food and water intake, urination, defecation, body temperature, pulse, breathing frequency and movement amplitude including coordination and pain of hind limbs. Supervision and care were taken in accordance with animal welfare requirements. During the post-implantation period, CBC, biochemistry panels and urine were analysed for both groups at 6 months post-surgery, and for group 12Mpost a second time at 12 months post-surgery.

All the dogs were euthanized while under general anaesthesia (described above) with “Dorminal” Na pentobarbital (200 mg/kg IV). The harvested aortic prostheses were fixed in 10% neutral formalin for 4 days. Each prosthesis was transected and longitudinally cut resulting in 4 similar tissue samples and then processed for histology slides: Each sample was sectioned into 5 μm slices for slide preparation and staining. The slides were stained with H&E for histopathology and marked for immunohistochemistry with TNFa (nr. orb7100, Biorbyt, United Kingdom, dilution 1:50), IL1ra (Q-19) (sc-8481, Santa Cruz Biotechnology Inc., USA, dilution 1:50), IL10 (nr.orb100193, Biorbyt, United Kingdom, dilution 1:50). Histology and immunohistochemistry slides were examined using a light microscope Leica CTR 500 with 400x magnification. Each slide was assessed and subjectively graded by the veterinary pathologist. Inflammation was scored on a scale of 1-4 (1 = minimal to mild; 2 = moderate; 3 = severe; 4 = very severe) (Pierce et al. 2009, Orenstein et al. 2012, Jayanth et al. 2015). Evaluation of the slides also included: 1) the endothelialization of the luminal surface of the aortic prosthesis, 2) the connective tissue proliferation between prosthetic filaments, and 3) the fibrous capsule formation around the prosthesis. In the slides stained for immunohistochemistry, the positive structures and their relative amounts were determined using the semi-quantitative census method according to M. Pilmane (Pilmane et al. 1998).

The intensity of positive structures was determined using the following graduations:

- (-) no positive structures in the field of view;
- (+) a slight amount of positive structure in the field of view;
- (+++) an average amount of positive structures in the field of view;
- (++++) many positive structures in the field of view;
- (++++++) very many positive structures in the field of view.

Five randomly positioned and representative fields of view were evaluated for each section and the averages were calculated.

Digital images were obtained with a camera (Leica DFC 490) synchronized with the computer program Image-Pro Plus 6.1.

For statistical analyses Descriptive statistics (mean ± SE), Mann-Whitney test and Pearson’s correlation were performed using MC Excel and SPSS programs with a significance set at p<0.05 (Arhipova and Bassert 2006). After the implantation surgery, activity was decreased but quickly improved after two days. Mean body temperature, appetite, drinking, urination and defecation, pulse and breathing frequency were normal shortly after surgery and during the rest of the study in both groups. During the entire study no dogs showed any clinical evidence of insufficient blood supply in the posterior part of the body. Disturbances in movement amplitude or coordination, and/or pain of hind limbs were never detected. Before surgery the mean leukocyte counts were 10.38±0.47 x10^12/l in group 6Mpost and 8.50±0.64x10^12/l in group 12Mpost. At the end of the study the mean counts were 11.63±0.77 x10^12/l in group 6Mpost and 9.65±0.67 x10^12/l in group 12Mpost (p>0.05). There were no substantial changes detected in the described blood and urine parameters before implantation and before tissue harvesting.

Results

Dog’s activity, food and water intake, urination and defecation, body temperature, pulse, breathing frequency were normal prior to implantation (Mccurnin and Bassert 2006). After the implantation surgery, activity was decreased but quickly improved after two days. Mean body temperature, appetite, drinking, urination and defecation, pulse and breathing frequency were normal shortly after surgery and during the rest of the study in both groups. Disturbances in movement amplitude or coordination, and/or pain of hind limbs were never detected. Before surgery the mean leukocyte counts were 10.38±0.47 x10^12/l in group 6Mpost and 8.50±0.64x10^12/l in group 12Mpost. At the end of the study the mean counts were 11.63±0.77 x10^12/l in group 6Mpost and 9.65±0.67 x10^12/l in group 12Mpost (p>0.05). There were no substantial changes detected in the described blood and urine parameters before implantation and before tissue harvesting.

Results of inflammation grades and IHC levels are shown in Table 1. In all slides obtained from aortic prostheses harvested 6 months after implantation (6Mpost) the luminal surfaces were completely covered with endothelial cells. The prostheses were covered by a relatively thin to a medium-thick capsule of the connective tissue. Medium-intensive formation of collagen fibres around the synthetic filaments of the prosthesis wall was detected. Six months after implantation, the
The inflammatory reaction and the expression ...

Collagen fibres were relatively mature, but the location of the fibres was less dense as compared to the collagen organization at 12 months after implantation (12Mpost). Pronounced macrophage infiltration around the synthetic filaments was found in all slides of the 6Mpost group. In some sections on the slides, macrophages had a tendency to form small aggregations enclosing bundles of synthetic filaments. The foreign body giant cells (2 to 4 cells in the field of view) were located closely around the synthetic material. Generally, lymphocytes

Table 1. Inflammation grade and frequency of immunohistochemical profiles of pro- and anti-inflammatory mediators in aortic prosthesis wall.

<table>
<thead>
<tr>
<th>Dogs number</th>
<th>Inflammation grade</th>
<th>IL 10</th>
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Fig. 2. Inflammatory cell infiltration in polyester-polyurethane aortic prosthesis wall between synthetic filaments 12 months after implantation. Hematoxylin and eosin (H&E) staining, x200.
were observed evenly spread among the prosthetic filaments. In some slides, lymphocytes were forming small aggregations with a high cell density at the periphery of the prosthesis wall and capsule. In one dog, lymphocytes were observed just below the endothelial cell layer near the anastomosis. Neutrophil leukocytes in this 6Mpost group were not detected. Summarizing, the histology results obtained from 6Mpost dogs can be divided into three grades of inflammation: grade 1 (mild) lymphocytic inflammation (1 dog); grade 2 (moderate) granulomatous inflammation (3 dogs); grade 3 (severe) lymphocytic/mild granulomatous inflammation (1 dog). A mean estimate of inflammation in the 6Mpost group was grade 2 - moderate inflammation.

The evaluation of slides of 12Mpost group showed that the luminal surfaces of the aortic prostheses were completely covered with one layer of endothelium. Collagen organization in the prostheses walls was more pronounced and the prostheses were externally covered with a relatively thick fibrous tissue capsule as compared to that of 6Mpost prostheses. In two dogs of 12Mpost group, the dominant inflammatory cells were macrophages. In both groups the macrophage and foreign body giant cell distribution was the same, but the intensity of macrophage infiltration was more pronounced in 12Mpost group (Figs. 2 and 3). Lymphocytes were evenly distributed among prosthetic filaments and did not differ from those of 6Mpost prostheses. The prostheses of two 12Mpost dogs were markedly infiltrated by neutrophil leukocytes among the synthetic filaments and in the fibrous connective tissue capsule. The slides of these dogs showed minimal macrophage infiltration and only moderate lymphocyte infiltration.

In the 12Mpost slides, 2 inflammatory groups were distinguished: grade 3 (severe) chronic granulomatous inflammation (2 dogs) and grade 4 (very severe) pyogranulomatous inflammation (2 dogs).

The positive structures of TNFα in the IHC slides of 6Mpost dogs were observed to be evenly distributed among the prosthetic fibres and in the fibrous tissue capsule. A denser expression of TNFα-positive structures was in macrophage and lymphocyte aggregations. The structures positive to TNFα were marked at inten-
The inflammatory reaction and the expression of cytokines in prosthetic tissue samples were assessed. Moderate amounts of Interleukin 10 (IL10)-positive structures were observed to be evenly distributed among prosthetic filaments and their bundles in macrophage and lymphocyte infiltration sites. A small amount of structures positive to IL10 was in the fibrous capsule.

Interleukin IL1ra-positive structures similar to IL10-positive structures, were predominantly marked among the prosthetic filaments and their bundles (Fig. 4). The number of IL1ra-positive structures was expressed relatively higher than that of IL10 positive elements, but it was still estimated as moderate in the field of view.

Characteristics and location of TNFα-, IL10-, and IL1ra-positive structures in slides from 12Mpost dogs were similar to those observed in slides from 6Mpost dogs. The average number of IL10-positive structures in 12Mpost slides (Fig. 4) did not differ from that in 6Mpost slides (p>0.05). Structures positive to IL1ra in 12Mpost slides (Fig. 5) were less numerous than those in the 6Mpost slides (p<0.05). Structures positive to TNFα were expressed at higher intensity (p>0.05) in 12Mpost than in 6Mpost slides. Numerous TNFα-positive structures were found in 12Mpost slides with neutrophil leukocyte infiltration (Fig. 6).

TNFα-positive structures had a weakly positive, but statistically significant correlation with the inflammation grade (r=0.24; p<0.05) and IL1ra-positive elements had a weakly negative statistically significant correlation with the inflammation grade (r=-0.25; p<0.05).

A positive correlation between TNFα and time (months) after implantation of prosthesis was found (r=0.40; p <0.01), but a strong negative correlation was observed between the number of IL1ra-positive structures and time after implantation of prosthesis (r=-0.62; p<0.01).

**Discussion**

In the present research it was found that the luminal surfaces of the prostheses were covered with endothelium both at 6 and 12 months after implantation.
This is an expected finding because endothelization occurs in the first months. Complete endothelization of the luminal surfaces in polyester aortic prostheses had been found three months after implantation (Ueberrueck et al. 2005). Also, collagen deposition had been observed throughout polyester-polyurethane prostheses walls at 6 weeks, and at 18 and 36 months after implantation (Wu et al. 1997, Seifalian et al. 2003, de Valence et al. 2012). The infiltration of macrophages, foreign body giant cells and lymphocytes into the prosthesis made from synthetic polymers is considered to be a consequence of the immune system’s reaction to an inert foreign material and has been detected even 50 months after implantation (Schwartz and Henry 2002, Sigler et al. 2005). In dogs Karapinar et al. (2004) found a mild to moderate degree of inflammation in polyurethane vascular prostheses 6 months after implantation, while Bashar et al. (2002) detected a moderate to severe inflammation 12 months after implantation of polyesters and stainless steel stents in the abdominal aorta. In this study, 6 months post implantation, the intensity of inflammation was estimated as moderate, but 12 months after implantation as severe, which may indicate a continuation of the foreign body’s response. Infiltration of neutrophil leukocytes in synthetic material implants more than 3 weeks after implantation indicates the presence of infection in the implant (Anderson 2001, Sigler et al. 2005, Anderson et al. 2008).

TNFα is an inflammatory cytokine released by macrophages, lymphocytes, leukocytes and other cells (Zachary and McGavin 2012). Medical synthetic polymers, including polyester and polyurethane, when in contact with monocytes/macrophages, trigger secretion of TNFα and IL1, which are increased in the presence of an infection (Cardona et al. 1992). TNFα is considered a pro-inflammatory cytokine produced by classically activated macrophages. However, IL1ra and IL10 anti-inflammatory cytokines and wound healing cytokines are produced by alternatively activated macrophages (Brodbeck et al. 2002, Boccafoschi et al. 2014). In this study the expression of the pro-and anti-inflammatory cytokines in 6Mpost dogs was equal. This may indicate that the immune system has the capacity to suppress the inflammation by inhibiting the pro-inflam-
The inflammatory reaction and the expression of inflammatory cytokines and the activation of macrophages. Six months after implantation, the inflammation in the prosthesis wall was sufficiently suppressed by anti-inflammatory cytokines. However, at 12 months, the inflammatory activity was more pronounced as found by the decrease in anti-inflammatory cytokines and an increase in pro-inflammatory cytokines.

Despite detection of an inflammatory reaction in the aortic prostheses walls in both 6 and 12 months groups, the main function of the prostheses, providing sufficient blood supply to the posterior part of the body, was not affected.

The authors conclude that the aortic prosthesis with the innovative composite structure tested in this study integrates well and provides functionality for the dog’s vascular system at up to 6 months post implantation. However, further investigations are needed to determine complete biointegration at 12 months after implantation. This study demonstrated that the activity of various inflammatory cell populations may be identified and more precisely assessed by evaluating pro- and anti-inflammatory cytokines at the inflammatory site.

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