Int Urogynecol J
DOI 10.1007/s00192-014-2529-0

ORIGINAL ARTICLE

Can highly purified collagen coating modulate polypropylene mesh immune-inflammatory and fibroblastic reactions? Immunohistochemical analysis in a rat model

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Received: 1 April 2014 / Accepted: 29 September 2014

Abstract
Introduction and hypothesis Collagen has been proposed to be a useful biomaterial, but previous attempts to combine meshes with a collagen membrane have failed. The objective was to verify the effect of high-purified collagen gel coating in the immune-inflammatory response, host collagen metabolism, and angiogenesis around polypropylene mesh.
Methods In 20 female Wistar rats were implanted, at one side of the abdominal wall, a monofilament polypropylene mesh (PP), and, on the other side, a mesh coated with a new highly purified collagen gel (PPC). The animals were divided into sub-groups and euthanized at 7, 14, 21, and 90 days after implantation. Immunohistochemical analysis was performed using interleukin 1 (IL-1), matrix metalloproteinases (MMP-2, MMP-3), surface antigen CD-31, and tumor necrosis factor (TNF-α). Objective analysis (percent reactive area, average density, and vessels concentration) was performed using AxioVision Software.
Results Comparative analysis showed: higher vessel density in the PPC group after 14 days (p=0.002); a decrease in the average density of MMP-2 in the PPC group after 21 and 90 days (p=0.046); more stability in the behavior of MMP-3 in the PPC group throughout the periods with the percentage reactive area for MMP-3 showing a significant decrease just in the PP group after 14 and 90 days (p=0.017), and also for MMP-3 average density, in which reduction was significant after 21 days in the PP group, but not until after 90 days in PPC group (p<0.001).
Conclusions Highly purified collagen coating causes significant changes in angiogenesis and in the immune reaction of metalloproteinase around mesh implants in rats. These findings can be useful for improving mesh biocompatibility for pelvic floor surgery if such effects could be properly controlled.

Keywords Collagen · Coating · Immunohistochemistry · Pelvic floor surgery · Polypropylene mesh

Introduction

Urinary incontinence (UI) and pelvic organ prolapses (POP) have become more prevalent with aging of the population. It is supposed that approximately 11 % of women will undergo surgery for POP repair in their lifetime. Also, approximately 30 % of these patients will need reoperation because of prolapse recurrence within 4 years post-surgery [1]. The poor results associated with traditional techniques based on native "poor quality" tissues have led to the spread of mesh use in urology and gynecology practice [2].

The advantages of synthetic meshes are associated with greater strength and durability, availability, and versatility. Also, mesh kits have allowed for a better standardization of the procedures, fewer time-consuming surgeries, and faster postoperative recovery. However, increasing attention has been given to the risk of complications related to mesh implants [3], especially those related to the bladder, rectum, and sexual function [4], which led to the recent warning from the Food and Drug Administration (FDA) about the high complication rates related to the use of meshes for POP repair [5, 6].

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Published online: 22 October 2014

The search for a new ideal synthetic product for better surgical results with fewer complications is an imminent research field in medicine. Studies on coated meshes have already been published. Meshes can be coated with collagen, titanium, silver, bovine pericardium, or porcine intestinal submucosa, for example [4, 7–9]. Collagen is an important immune and inflammatory modulator and has been proposed to be a useful biomaterial for increasing the efficiency of the healing process [10]. However, previous attempts to combine meshes and collagen were based on covering the mesh with a nonporous collagen membrane or cross-linked collagen preparations, which resulted in mesh encapsulation and failure [11–13]. The proposal of a polypropylene-coated mesh with purified collagen gel to modulate the initial phase of prosthesis–host integration, thus allowing for improved mesh integration with neighboring tissue, emerges as a potential evolution of such surgical materials. The aim of this study was to verify the effect of high-purified collagen gel coating on the immune-inflammatory response, host collagen metabolism, and angiogenesis around the monofilament PP mesh.

Materials and methods

Ethical aspects

The study was performed after approval by the Ethics Committee for Animal Experiments (CEEA-IB-UNICAMP) of the Institute of Biology, University of Campinas, according to protocol number 1972–1, and followed the ethical principles for animal experiments adopted by the Brazilian College of Animal Experiments (COBEA).

Sample

Twenty female Wistar rats were randomly implanted, at one side of the abdominal wall, with a 20×10 mm monofilament PP mesh, and on the other side with the same mesh, but coated with a new ,highly purified collagen gel (PPC) prepared at the Laboratory of Biopolymers and Chromatin Organization, Optical Anisotropy, Microspectrophotometry, Image Analysis, and Topo- and Cytochemistry at our institution. The animals were divided into four sub-groups with 5 animals each and were euthanized at 7, 14, 21, and 90 days after implantation.

Collagen gel

The highly purified collagen gel preparation consists of type I collagen obtained from bovine tendon, presenting two alpha-1 chains and one alpha-2 chain. The product was highly purified according to the previously described technique to eliminate the immunogenic telopeptide parts [11]. Collagen solubilization involved refrigerating the dissected tendons in an aqueous solution of 0.01 % hydrochloric acid and 1 mg of pepsin/g of tissue for 24 h. The collagen obtained was reconstituted by adding a 0.9 % NaCl solution to a final concentration of 5 %. The solution was then stabilized through extensive dialysis in distilled water for 5 to 7 days.

Mesh preparation

The mesh used in this study was donated by Promedon (Cordoba, Argentina) and is made of monofilament type I polypropylene (original weight: 85 g/m^2). It is the same as that included in NAZCA TC™ and Calistar A™ sets, currently on the market. Implants were prepared for the following groups as follows:

Group PP: mesh measuring 20 × 10 mm
Group PPC: mesh measuring 20 × 10 mm, coated in collagen gel

Both meshes (coated or uncoated) were sterilized with gamma rays (dose: 25 kGy) to preserve the structural stability of the collagen molecules [14].

Surgical technique

After anesthesia with sodium pentobarbital 3 % (0.15 mg/g), a 2-cm cross-sectional incision was made in the lower abdominal area. The mesh was implanted in the animal in a standardized manner on each side of the abdominal wall, separated by the linea alba, between the hypodermis and the anterior fascia of the abdominal muscles. PP meshes were implanted on the left side and PPC meshes on the right side. Each animal was implanted with meshes of both groups (coated and uncoated) to form its own control. The animals were divided into four subgroups of 5 animals each, according to the time interval from mesh implant to euthanasia (7, 14, 21, and 90 days). The rats were euthanized with a lethal dose of sodium pentobarbital (3 %) and the abdominal wall was removed en bloc for analysis just after the animal's death.

Immunohistochemical analysis

The samples were fixed in formalin (10 %) for 24 h and then transferred to a 70 % alcohol solution where they remained for another 48 h. Five-micron-wide histological sections were made. Four fragments of the material were placed on each slide.

The immunohistochemical analysis was carried out by using specific antibodies in order to assess: immunological response (interleukin 1− IL-1); collagen metabolism (matrix metalloproteinases 2 and 3, MMP-2 and MMP-3); angiogenesis (surface antigen CD-31); tissue necrosis/apoptosis (tumor necrosis factor alpha-receptor, TNF-α).

For histological evaluation, a Zeiss Primo Star™ microscope mounted with a Zeiss AxioCam ICC 1™ camera (Carl Zeiss Microscopy, Jena, Germany) was used. The entire slide was scanned under ×20 magnification, and three fields of each slide were randomly selected for subsequent image acquisition. Objective analysis of immunoreactive expression (percentage reactive area, average density, and vessels concentration) was performed with AxioVision Microscope V 4.8.0.0 Software (Carl Zeiss). Percentage reactive area was defined as a part of the slide, which is colored by a specific antibody that shows the extension of a specific immunoreactive expression. Average density refers to the amount of immunoreactivity per area, which represents the concentration of immunoreactive expression. The same investigator, who did not know to which animal the fragments belonged, carried out all the evaluations.

Statistical tests

The ANOVA test for repeated measures was used for comparisons between groups and among periods, followed by Tukey's multiple comparison tests to compare the four subgroups pursuant to the time elapsed since euthanasia. The contrast profile test was used to analyze both situations, at each period. Variables were rank-transformed owing to the absence of normal distribution and because they were considered using an ordinal scale. For all statistical tests a significance level of 5 % was adopted.

Results

A comparative analysis, among the four periods and between the two groups, is presented in Tables 1–3, and summarized as follows:

With regard to angiogenesis, there was a higher vessel density in the PPC group after 14 days (p=0.002) with a significant decrease after 90 days (p=0.002) in this group (Fig. 1).

With regard to collagen metabolism, there was a decrease in the average density of MMP-2 expression in the PPC group after 21 and 90 days (p=0.046). MMP-3 expression in the PPC group was stable among the periods. MMP-3 percentage reactive area showed a significant decrease only in the PP group after 14 and 90 days (p=0.017). MMP-3 expression average density showed a significant decrease after 21 days in the PP group, but just after 90 days in the PPC group (p<0.001; Figs. 2, 3).

With regard to immunological response (IL-1), no significant differences were found in average density among the periods (p=0.245) or between groups (p=0.726) and also in the percentage reactive area among periods (p=0.762) or between groups (p=0.866).

With regard to tissue necrosis/apoptosis (TNF-α), no significant differences were found in the average density among periods (p=0.155) or between groups (p=0.313) and also in percentage reactive area among periods (p=0.280) and between groups (p=0.635).

Table 1 Immunohistochemical angiogenesis evaluation (CD-31): mean of number of vessels

Period	7 days	14 days	21 days	90 days
PP	25.82	14.44	15.58	7.30
PPC	29.90	29.88	23.24	8.64

Discussion

Most of the clinical adverse events described after vaginal mesh implants are related to intense and/or prolonged inflammation and/or inappropriate collagen deposition around the meshes. Therefore, attempts to modulate both acute and chronic inflammation and to reduce and drive the fibroblast reaction around the mesh are goals to be achieved. In this scenario collagen emerges as a potentially useful biomaterial. It was demonstrated in permanent material implant experimental studies that the addition of collagen to polypropylene meshes can act as a protective factor against complications by reducing both the tendency toward encapsulation and peri-implant inflammation [8, 9].

Collagen is the most abundant protein in mammalian connective tissues. Collagen type I has been proposed for use as a coating for polypropylene mesh because of its unique biological properties with great biocompatibility and low immunogenicity [4]. Its structure is very stable and, therefore, it does not induce a powerful immune reaction, even if a xenogeneic material is used [15, 16].

Collagen matrix processed from bovine tendon has been used to treat diseases such as orthopedic lesions, axonal lesions, stress urinary incontinence, and pelvic organ prolapse. Initial tests in fibroblast culture have demonstrated that these cells recognize and adhere to collagen fibers obtained from heterologous material (type I collagen obtained from bovine tendon) [7, 17, 18]. Such qualities support the hypothesis that type I purified collagen gel may be used as a framework in conjunction with polypropylene mesh.

Collagen used in this study was obtained using an original method developed at our institution (Brazilian Patent Number 018,100,044,206) based on prolonged water dialysis and enzymatic treatment, which is quite different from the previous collagen preparations. The PP mesh was provided by Promedon (Cordoba, Argentina) and is the same as that included in the NAZCA TC™ and Calistar A™ sets, which are

Table 2 Expressions of interleukin 1 (IL-1), tumor necrosis factor alfa (TFN-α), and matrix metalloproteinase 2 and 3 (MMP2, MMP-3; area, μm^2, and density averages)

	7 days		14 days		21 days		90 days	
	Area	Density	Area	Density	Area	Density	Area	Density
IL-1								
PP	41.28	82.76	29.28	79.54	27.42	78.58	17.75	72.87
PPC	32.45	81.28	21.72	79.80	36.47	79.54	22.11	76.77
TFN-α								
PP	6.43	59.03	4.30	58.15	4.22	76.94	5.82	68.43
PPC	7.03	65.97	5.05	53.33	3.83	58.75	8.61	66.43
MMP-2								
PP	10.40	75.59	7.46	76.16	9.57	80.82	9.64	85.86
PPC	11.14	80.91	11.79	74.61	6.66	69.75	8.64	74.72
MMP-3								
PP	17.42	81.26	6.78	80.60	8.28	71.99	6.14	71.68
PPC	7.54	84.45	7.00	79.13	11.18	73.10	5.49	66.37

PP polypropylene group, *PPC* polypropylene + collagen gel group *CD-31* surface antigen CD-31

CE-marked in Europe, with regulatory approval for commercialization in Asia and Latin America.

The assembly of collagen gel used in this study was based on the principle of spontaneous self-aggregation of macromolecules, which ensures a typical supramolecular organization when fragments of original molecules are exposed to specific physical and chemical conditions. Based on this principle, in 1995, Vidal first described the acquisition of molecules with supramolecular arrangement similar to tendon collagen using a method based on prolonged dialysis with distilled water. In this process, water molecules form hydrogen bridges with collagen molecules, which facilitate fibril movement and the reorganization of a helicoid structure similar to the tendon fibril structure. The author also observed that early interruption of dialysis resulted in fiber bundles with irregular diameter and orientation [11]. In this same study, the author objectively showed that elasticity is directly proportional to the duration of collagen dialysis. Elasticity reaches its peak within 5 to 7 days. The intrinsic properties of the collagen used in the present study make it unsuitable for comparison with the other collagen coating preparations already described. Ugytex™ (Sofradim, Trévoux, France, distributed by Bard as Pelvitex™), for example, consists of a PP mesh covered by a hydrophilic film of atelocollagen, polyethylene glycol, and glycerol, which enhances chemical stability of the collagen, but can alter its biocompatibility. This is a possible explanation for the adverse events related to its use.

Table 3 The ANOVA test for repeated measures for comparison among periods (7, 14, 21, and 90 days) and groups (PP and PPC)

Variable	Comparison among periods (*p*)	Comparison between groups (*p*)
Mean vessel number	0.002[a]	0.002[d]
IL-1 area	0.762	0.866
IL-1 density	0.245	0.726
MMP-2 area	0.947	0.807
MMP-2 density	0.496	0.046[e]
MMP-3 area	0.017[b]	0.310
MMP-3 density	<0.001 [c]	0.779
TNF-α area	0.280	0.635
TNF-α density	0.155	0.313

[a] 7≠90, 14≠90, 21≠90 in PPC group/power: 95 %

[b] 7≠14, 7≠90 in PP group/power: 79 %

[c] PP≠PPC, days 21 and 90/power: 53 %

[d] PP≠PPC, day14/power: 95 %

[e] 7≠21, 7≠90, 14≠21, 14≠90 in PP; 7≠90, 14≠90 in PPC group/power: 99 %

Although mesh infection has been considered a relevant factor in complications directly related to permanent implants, the bacteriological evaluation was not the focus of this study. Pure and coated meshes were sterilized by gamma radiation to preserve collagen biological properties. Moreover, collagen preparation included an enzymatic treatment with an acid solution (pH: 1.7), which is supposed to help with collagen sterilization.

Using the collagen as a gel has been demonstrated to be a better option than the collagen membrane proposed by other researchers [19, 20]. The rationale for the use of collagen coating is that each mesh filament strongly adheres to the coating while keeping the pores free, thus allowing for host tissue integration with the formation of scarring tissue, which does not tend to encapsulate the mesh while minimizing the

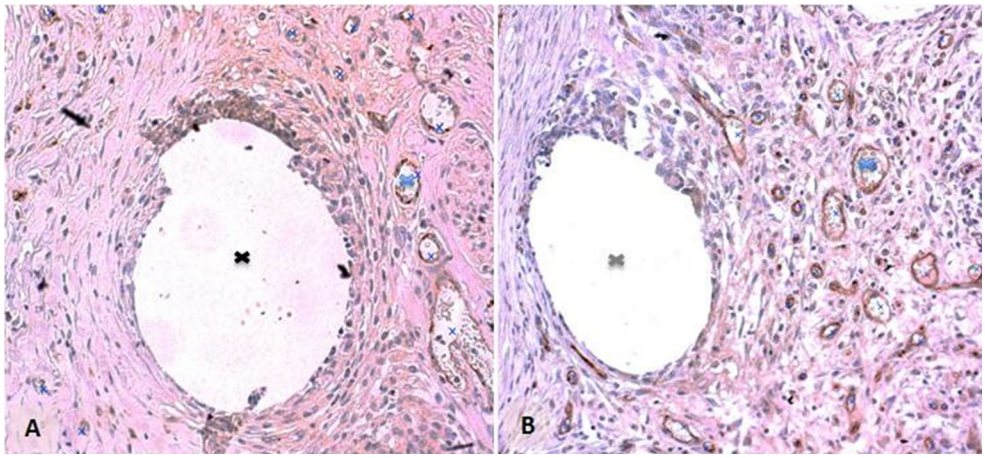

Fig. 1 Analysis of immunoreactive expression of CD-31 at 14 days post-mesh implant. The empty central areas were those previously occupied by mesh filaments (*black cross* PP, *silver cross* PPC). Expression of CD-31 has marked the vessels found in the surrounding tissue in a brownish color. Note the higher vessel density in **b** the PPC group than in **a** the PP group (mean vessels count: 29 vs 14). Images were acquired using a Zeiss AxioCam ICC 1™ camera with ×20magnification. *PP* polypropylene group, *PPC* polypropylene + collagen gel group

formation of foreign body giant cells. Another important characteristic of the present collagen gel is its capacity to be rapidly and completely degraded and absorbed after implantation and replaced by native collagen in the early phase of the integration process [13]. It can be postulated that coating synthetic mesh with type I purified collagen gel like this may improve proliferative reactions during tissue repair, contributing to prosthesis biological and functional adaptation by enhancing and augmenting the growth of mesothelial cells [21]. Also, some reports have confirmed the utility of collagen coating as such a barrier [22]. In a previous experimental study by our group, the effect of PP mesh coating with this new purified collagen gel showed an increase in the adherence of the mesh to neighboring tissue, less intense and persistent lymphocytes, plasma cells, and granulomatous reaction, and a higher birefringence level of collagen fibers, thus reflecting an improved molecular organization of newly formed collagen and a positive remodeling action in mesenchymal repair involving polypropylene mesh [23].

We used the rat subcutaneous tissue as an experimental model based on previously described experiments to evaluate graft and prosthesis integration, in which such a model had proved to be valuable [24]. Also, this model allowed for the comparison of two different treatments, as the animal served

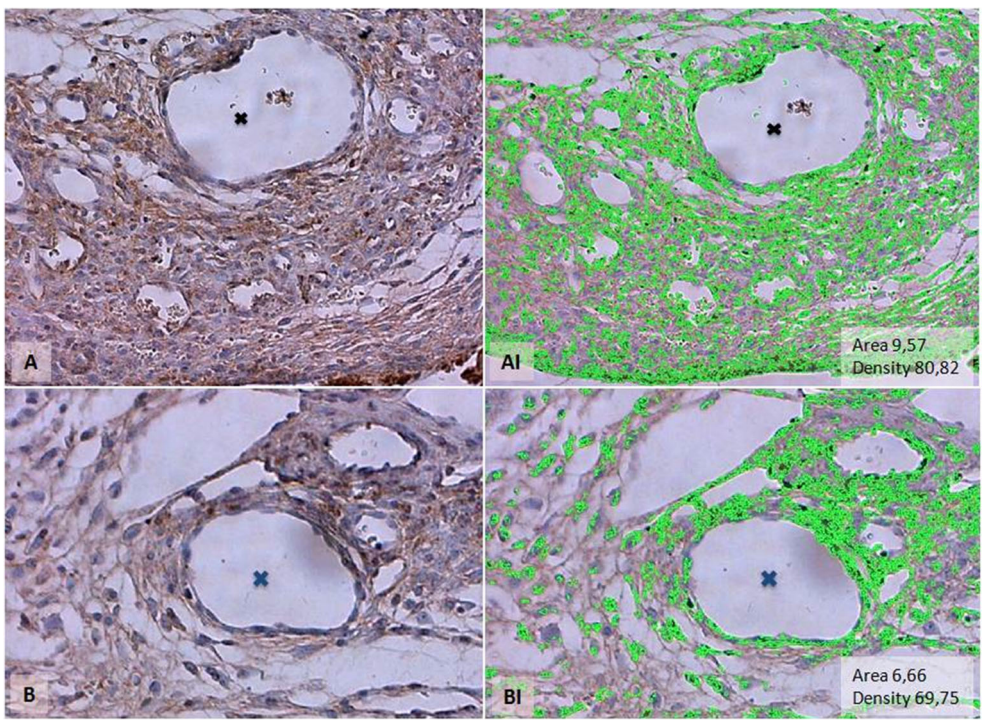

Fig. 2 Analysis of immunoreactive expression of MMP-2 at 21 days post-mesh implant. The empty central areas were those previously occupied by mesh filaments (*black cross* PP, *blue cross* PPC). The *brownish* areas in the surrounding tissue represent the immunoreactive expression of MMP-2 in **a** the PP group and in **b** the PPC group. A special feature of the AxioVision Microscope V 4.8.0.0 software highlighted the reactive area in *green* for subsequent automatic calculation of the percentage reactive area and average density in the PP group (***aI***) and in the PPC group (***bI***). Note the higher expression of MMP-2 in the PP group (**a**) than in the PPC group (**b**). Images acquired using a Zeiss AxioCam ICC 1™ camera with ×20magnification.

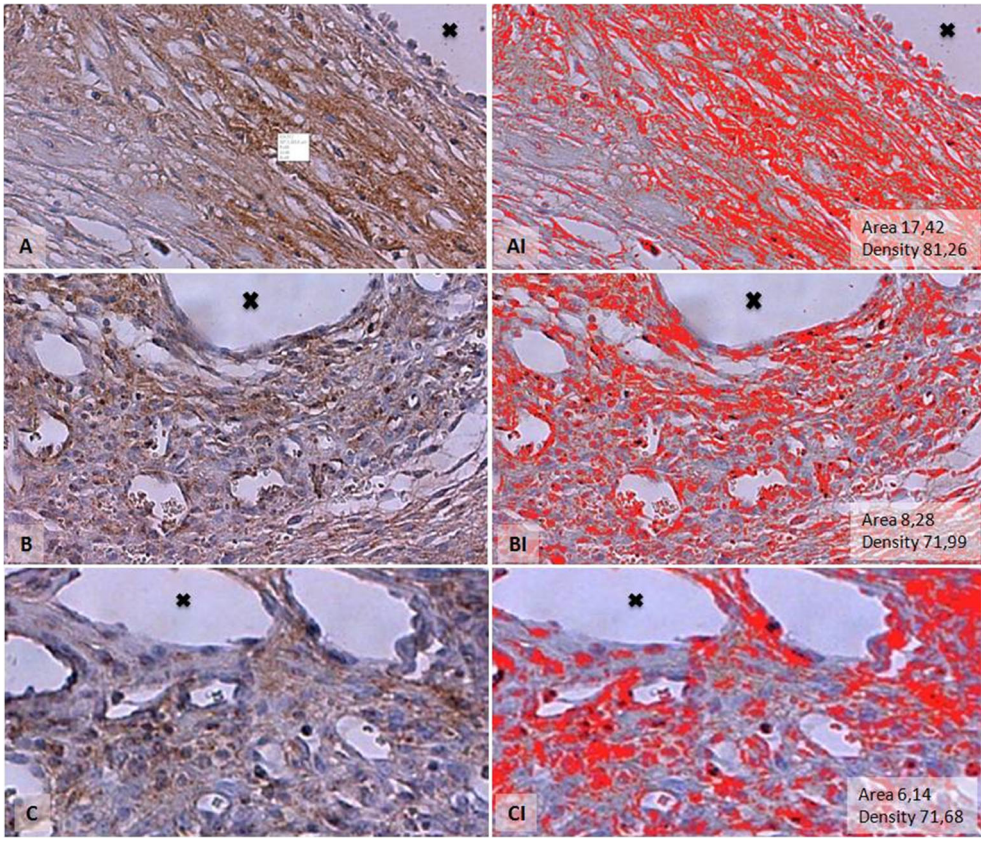

Fig. 3 Analysis of immunoreactive expression of MMP-3 in the PP group at **a** 7 days, **b** 21 days, and **c** 90 days post-mesh implant. The empty central areas marked with a *black cross* were those previously occupied by mesh filaments and the *brownish* areas in the surrounding tissue consist of immunoreactive expression of MMP-3. A special feature of the AxioVision Microscope V 4.8.0.0 Software highlighted the reactive area in *red* in the PP group at 7 days (***aI***), 21 days (***bI***), and 90 days (***cI***) for subsequent automatic calculation of the percentage reactive area and average density. Note the decrease in MMP-3 expression throughout the periods. Images were acquired using a Zeiss AxioCam ICC 1™ camera with ×20magnification

as its own control, and therefore the effects of individual variability in the mesh integration could be adequately controlled. In the current literature different models have been tested. Huffaker et al. used a rabbit vagina model to compare tissue responses to two PP meshes: one uncoated PP mesh (Gynemesh PS™) and one collagen-coated PP mesh (Pelvitex™) [8]. At 12 weeks post-implant, both meshes induced a chronic mild inflammatory response and showed similar scores of inflammation, neovascularization, and fibroblastic proliferation. Overall, low apoptotic indexes (less than 1 %) were verified in the tissue surrounding all implants, but they were significantly higher in the collagen-coated group (0.39 % vs 0.1 %; $p = 0.04$), as opposed to the present study, in which we found no difference in TNF-α tissue expression between the coated and uncoated meshes. As the methods and collagen used are quite different, comparison between the two studies was not allowed. De Tayrac et al. used a sheep vagina model to study the host response to collagen-coated and uncoated PP meshes [9] and found no differences in the inflammatory reaction at 12 weeks. In the present study, no difference was found between PP and PPC in IL-1 immunoreaction, corroborating that collagen coating does not have a significant impact on the immunological reaction to the implant.

It was described that the composition of type I collagen and its ultrastructural organization favors platelet adherence and local primary hemostasis with adhered platelets releasing fibroblast growth factor and stimulating angiogenesis factors [11–13]. In accordance with this concept, the PPC group showed an increased number of vessels 14 days after the implant (p=0.002). The improved angiogenesis in the early phase could lead to a better microenvironment for mesh integration and infection prevention.

The MMPs are part of the larger family of metalloproteinase enzymes [25]. In normal wound healing, MMPs are produced by activated inflammatory cells (neutrophils and macrophages) and wound cells (epithelial cells, fibroblasts, and vascular endothelial cells). They play key roles in debriding damaged/devitalized extracellular matrix, angiogenesis, re-epithelialization, wound contraction, and scar remodeling [26]. However, there is strong clinical evidence that chronically elevated levels of MMPs and other proteases prevent wounds from healing, and that treatments that lower MMP activity promote healing of wounds that have stalled [27, 28]. In the present study, we have demonstrated that the collagen covering changed the amount and duration of MMP-2 and MMP-3 in the tissue. It is important to understand what these findings might represent. Once there is evidence that an imbalance in favor of proteolysis contributes to the pathogenesis of poor healing [29], the finding of the average density decrease in MMP-2 in the PPC group after 21 and 90 days (p= 0.046) could represent an advantage for the PPC group. Concerning MMP-3, although no significant difference

between the groups was found at each time point, a more stable pattern was noticed in the concentration of MMP-3 in the PPC group among periods compared with the PP group, with the percentage reactive area of MMP-3 showing a significant decrease in the PP group only after 14 and 90 days (p=0.017) and also for the average density of MMP-3, in which the reduction was significant after 21 days in the PP group, but not until after 90 days in the PPC group (p<0.001). Considering the fact that, in a previous study, scar contraction was impaired and wound repair delayed in MMP-3-deficient mice [30], we could extrapolate that the stability in the concentration of MMP-3 found in the PPC group during the earlier phases could be beneficial to healing.

The present findings should be interpreted with caution because of some limitations of the study. Even though subcutaneous implantation of prostheses has already been used to analyze cicatrization, neovascularization, and collagen synthesis in periprosthetic tissue, the real response in vaginal tissue, which is thicker and more keratinized, still remains to be clarified. Moreover, as an intrinsic weakness of experimental studies, all our extrapolations regarding human patients are speculative. In addition, the analysis of immunoreactive expression is a difficult task. The use of software as a mean of increasing the objectivity of analysis is not yet a definitively established method. However, in spite of these limitations, our results represent a step forward in mesh research and should encourage further research in order to improve its biocompatibility and usefulness in pelvic reconstructive surgery.

Conclusion

Highly purified collagen coating causes significant changes in angiogenesis and in the reaction of MMP-2 and -3 around PP mesh implants in rats. These findings may be useful for improving PP mesh biocompatibility for pelvic floor surgery if such effects can be properly controlled.

Acknowledgments We thank FAPESP (Research Support Foundation of São Paulo State) for the financial support for the execution of this research project (process number 2013/05,841–3).

Conflicts of interest None.

Authors' contributions to the manuscript F.G.F. Dias: data collection, manuscript writing; A. Prudente: data collection; R.T. Siniscalchi: data collection; B.C. Vidal: project development; C.L.Z. Riccetto: project development, manuscript writing.

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