Aesth Plast Surg (2008) 32:32–40
DOI 10.1007/s00266-007-9051-4

ORIGINAL ARTICLE

Pirfenidone Prevents Capsular Contracture After Mammary Implantation

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Published online: 30 October 2007

Abstract
Background Pirfenidone (PFD), a new antifibrotic and antiinflammatory agent, prevents and resolves fibrous tissue. This study evaluated the effect of PFD on adverse events in mammary implants using an animal model. Mammary implantation, the most frequent aesthetic surgery, may present several complications after surgery such as swelling, capsule contracture, hardness, and pain.
Methods Wistar rats underwent submammary implantation with either smooth or textured silicone gel implants and were administrated 200 mg/kg of PFD daily. The control group received saline. The animals were killed at 8 weeks. The capsular tissue of both implants was removed for histologic and molecular analyses.
Results Typical postaugmentation periimplant capsules with opacity on adjacent tissues developed 8 weeks after silicone implantation. No significant differences were observed between the textured and smooth implants in any analyzed parameter. Clearly, PFD reduced capsule thickness around submmamary tissue, fibroblast-like cell proliferation, and recruitment of inflammatory cells. The total cell numbers per field were reduced as well. In contrast, the control group presented abundant mononuclear cell infiltration and fibroblast-like cell proliferation. The total content of collagen in the PFD group was 50% less than in the control group. Fibroblast cells displayed 45% less activated phenotype in the PFD group than in the control group, as determined by immunohistochemistry techniques. In the PFD animals, transforming growth factor-β (TGF-β) decreased 85% and collagen 1 gene expression 60%, compared with the control group.
Conclusion The findings show a positive effect of PFD on mammary contracture in 10 rats. Despite the small number of animals, the differences found in 10 control rats encourage the authors to propose a larger study later and to suggest PFD as a potential preventive strategy in human mammary implantation surgery.

Keywords Capsular contracture · Mammary implantation · Pirfenidone · PFD

Presented at the Latinoamerican meeting on plastic surgery. Buenos Aires, Argentina, April 2006.

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Pirfenidone (PFD) may be used as a coadjuvant mechanism to reduce undesirable events after breast implantation in humans. We support this observation on the basis of the following facts. Concepts about beauty have increased mammary surgery demand, with reconstructive and beauty goals. However, despite the great utility of this medical proceeding, swelling and capsule contracture around the implant represent infrequent postsurgery complications. These complications may cause malformation, hardness, and pain in the breast, with physical and psychological alterations in some patients.

The causes and pathogenesis of capsular contracture have not been completely understood. Different publications mention a variable incidence of capsules ranging from 0% to 74% [1], depending on the implant shell, surface texture, and anatomic implantation site (subglandular or subpectoral) [2]. The causes of these capsules could be accumulation of tissue fluids in the implant pocket, intensive inflammatory response, subclinical infection, age of the patient, foreign materials, and alteration of cellular and molecular mechanisms in the implantation area.

When an implant is placed, the body reacts by encapsulating it, starting a rejection [3–6]. This immunologic response is mediated by cytokines and growth factors such as interleukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNF-α?), platelet-derived growth factor, and transforming growth factor-β1 (TGF- β1) [7, 8]. The presence of myofibroblasts in the contracted capsule has been reported together with α-smooth muscle actin (α-SMA) production, in which capsules more severely deformed presented a higher α-SMA production, suggesting a direct role of activated myofibroblasts in the contracture development [8]. It also has been demonstrated that the number of myofibroblasts present in the tissue is proportional to the contracture thickness [9].

The aforementioned data were obtained with human beings. On the other hand, several studies have used animal models to mimic breast contracture observed in patients with postmammary implants. Animals such as pigs, rabbits, rats, and mice have been used with variable results. Some animal models involve the addition of an inductor agent to the implant to accelerate contracture development. All of them show fibrosis development, fibroblast activation, inflammation, and capsule thickness [10–16].

In previous studies using rabbit and mice models, the preinstillation of the implant pocket with sodium 2-mercaptoethane sulfonate (mesna) and mitomycin C reduced capsule thickness, the number of fibroblasts, and collagen deposition [10, 11]. Nonetheless, these drugs are not commonly used currently in clinical practice. To reduce fibroblast activation and wound retraction, steroid infiltration into the wound and the implant interior has been reported with few complications. Steroids too are not frequently used currently, nor are they recommended by the implant manufacturers. The complications include thinning skin, tissue atrophy, striations, blue skin, and implant exposition [17–25].

On the other hand, new antifibrotic drugs have been reported recently for various medical problems. Pirfenidone (5 methyl-1-phenyl-2-[1H] pyridine) (PFD), a new antifibrotic agent, has proved to be effective both *in vitro* and *in vivo*, preventing and resolving fibrous tissue in experimental models of lung fibrosis [26], peritoneal adhesion [27], human and experimental liver cirrhosis [28, 29], uterine fibromyomas [30], kidney fibrosis [31], and keloid scars [32]. In addition, PFD is able to inhibit fibroblast growth factor and transforming growth factor-β (TGF-β) production in human fibroblasts, blocking the G1 phase of the cell cycle.

Because mammary implants induce fibrosis and inflammation and PFD has been shown to have antifibrotic and antiinflammatory properties, we evaluated the effect of PFD on scar and capsular contracture of mammary implants in a rat model. This animal model of silicone implant placement for 8 weeks is similar to what others have used in the past [13]. In the current study, the concomitant use of PFD for 8 weeks after silicone implant placement caused a decrease in fibroblast activation, collagen deposition, and gene expression of profibrogenic molecules. Therefore, PFD might be used as a coadjuvant mechanism to reduce undesirable events after breast implants in humans.

Materials and Methods

Materials

Pirfenidone was provided by Cell Therapy and Technology (Mexico). Droperidol and ketamine were purchased from Virbac Inc. (France). The α-SMA antibody was obtained from Boeringer Manheim (Germany), and theel implants were provided by NAGOR GFX (Ireland).

Animals

The 20 female Wistar rats used in this study were obtained from Charles Rivers Inc. These animals, weighing 250 g, were housed according to the principles and procedures outlined in the National Institute of Health's Guide for the Care and Use of Laboratory Animals. The rats, divided into two groups of 10 rats each, underwent submammary implantation with smooth and textured silicone gel implants, each with pore size of 350 μg. A group of 10 animals was administered 200 mg/kg of PFD orally in 1 ml of volume per day, starting on the day of surgery. The control group (10 animals) was administered the same volume of vehicle. The animals were killed at 8 weeks. Capsular tissue of both implants was removed and preserved for later histologic and molecular analysis.

Surgical Procedure

The rats were anesthetized with 100 μl/100 g weight of droperidol and ketamine intramuscularly. An abdominal

paramedian incision was made next to the mammary glands, and submammary pockets were dissected. A smooth implant then was inserted into the right side and a textured one was placed in the left side. Both incisions were closed using 3-0 silk sutures. After surgery, the animals were allowed to recover and fed ad libitum.

Histologic Analysis

For histologic evaluation, tissue was fixed in formaldehyde buffered with a phosphate solution (0.1 *M*, pH 7.4) at room temperature. Next, 5-μm-thick sections were obtained, and slides were stained with hematoxylin-eosin. Histologic evaluation was determined using a computer-assisted automated image analyzer (Image-Pro Plus 4.0 Media Cybernetics Inc., MD, USA) to analyze 10 random fields in different areas per slide at ×200 magnification.

Determination of Fibrosis Index

Capsular tissue sections were immediately fixed by immersion in 10% paraformaldehyde diluted in phosphate saline buffer, dehydrated in graded ethylic alcohol, and embedded in paraffin. The 5-μm-thick sections were stained with Masson's trichrome. For these slides, the percentage of capsular tissue affected by fibrosis was determined using a computer-assisted automated image analyzer (Image-Pro Plus) to analyze 15 random fields per slide and calculate the ratio of connective tissue against the whole capsule area [33].

α-SMA Immunohistochemistry

For immunohistochemistry analysis, capsular tissue sections were mounted in silane-covered slides and deparaffinized. The endogenous activity of peroxidase was quenched with 3% H_2O_2 in absolute methanol. The slides were incubated overnight at room temperature with mouse monoclonal antibodies against α-SMA (Boeringer Manheim). The antibody was detected with peroxidase-labeled rabbit polyclonal antibodies against mouse immunoglobulins, stained with diaminobenzidine, and counterstained with hematoxylin. For quantification analysis, 10 random fields were evaluated at ×400 magnification. Immunohischemical positive and negative cells were counted by an automated image analyzer (Image Pro Plus), and the data were expressed as the percentage of positive cells.

Total RNA Extraction

Isolation of total RNA from capsular tissue was performed according to the modified method described by Chomczynski and Sacchi [34]. Briefly, capsular tissue containing collagen-producing cells was obtained and homogenized

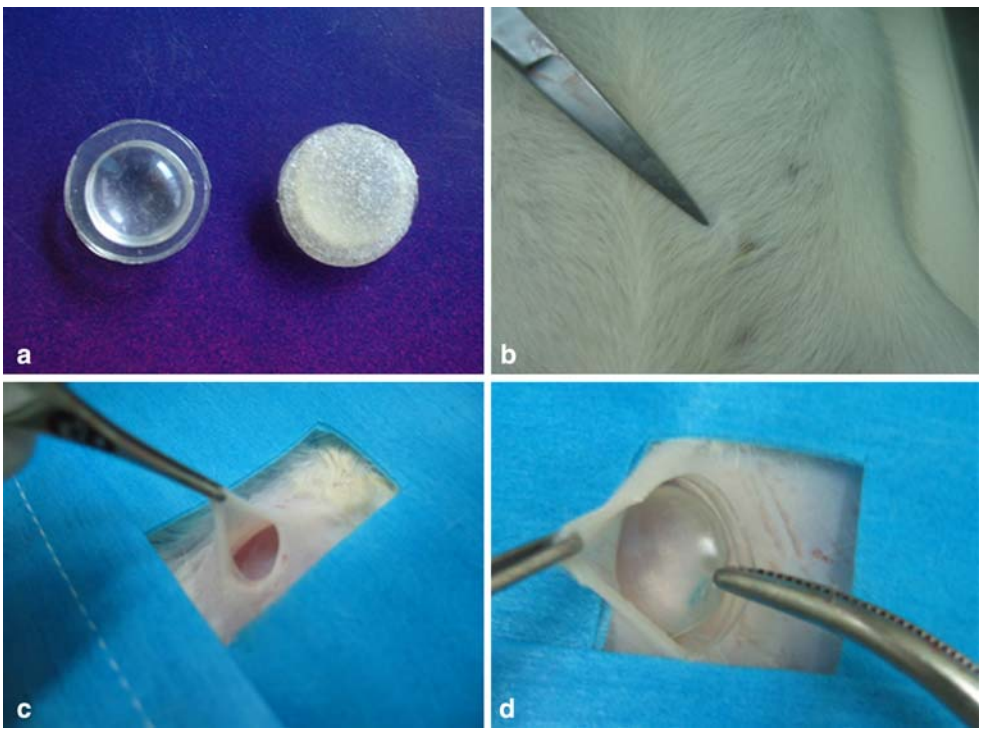

Fig. 1 Silicon breast implant placement. (a) Smooth and textured implants. (b) Mammary gland selection. (c) Subglandular incision. (d) Implant placement

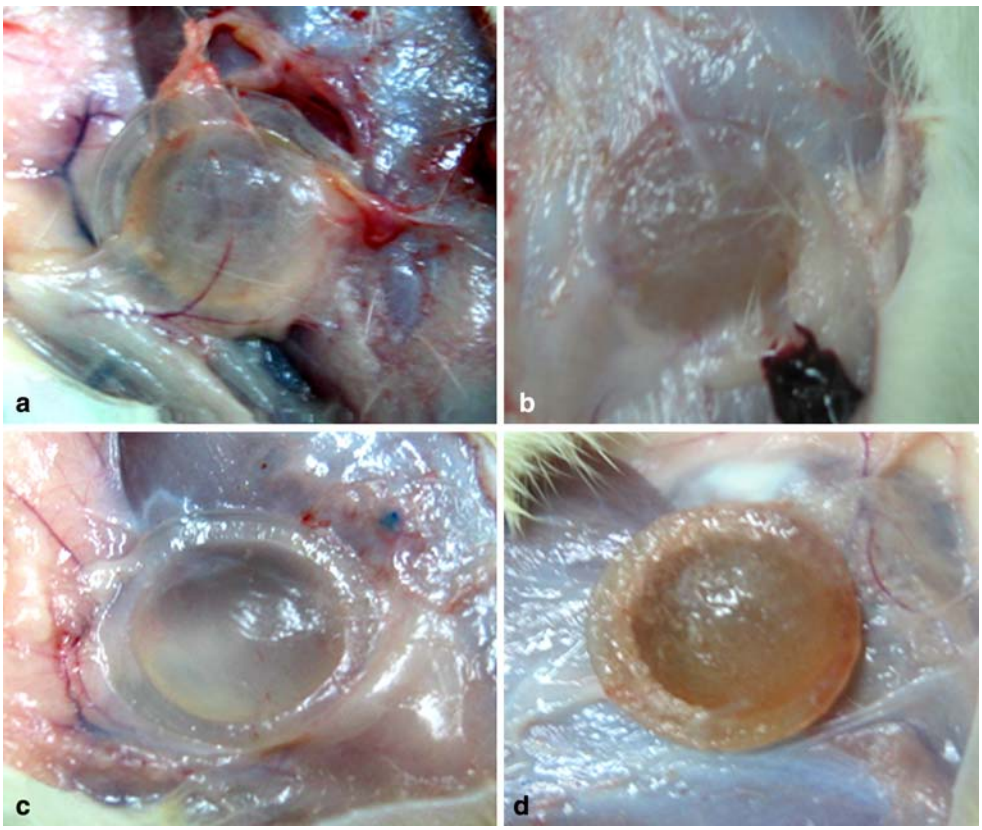

Fig. 2 Macroscopic view of breast implants showing an increase in capsule and opacity in control animals. (a) Control smooth implant. (b) Control textured implant. Treatment with pirfenidone (PFD) reduced capsule thickness around submammary tissue. (c) PFD smooth implant. (d) PFD textured implant

using the Polytron System (Brinkmann, Switzerland) in the presence of Trizol (Invitrogen). Chloroform was added, the aqueous phase was obtained, and the RNA was precipitated with isopropanol at 4°C overnight. The quantity and intactness of RNA were routinely tested by determining the absorbance at 260/280 and the ethidium bromide fluorescence of RNA electrophoresis in 1% formaldehyde-containing agarose gels.

Reverse Transcription

Altogether, 2 μg of RNA extracted from all the samples were added with 240 ng of random primers, 5 m*M* of DTT, 1 m*M* of dNTP mix, 40 U of RNase out inhibitor, and 200 U of RT Superscript III, then incubated for 10 min at 25°C, 60 min at 37°C, and 10 min at 95°C. The samples were stored at –70°C until real-time polymerase chain reaction (PCR) was performed.

Real-Time PCR

Quantitative real-time PCR was performed using a Rotor Gene RG 3000 Sequence Detector (Corbett Research, Sydney Australia) as follows: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 30 s and 60°C for 40 s. The total reaction was designed to occur in 10 μl containing 2 μl of cDNA, 1X of Universal PCR Master Mix (Applied Biosystems), and 1X of final concentration of primers and TaqMan probe from experimental and control genes synthesized by Applied Biosystems (assay on demand). Multiplex PCR real time for collagen αI and TGF-β were performed in duplicate for each animal using glyceraldehyde phosphate dehydrogenase (GAPDH) as a housekeeping gene.

Data analysis was performed according to user bulletin number 2 of Applied Biosystems [35]. Using sequence detection software, we calculated the threshold cycle (Ct) for each reaction, which then was used to quantify the amount of starting template reaction. A difference in Ct values (ΔCt) was calculated for each gene by duplicate. The amount of the target gene normalized to the endogenous gene GAPDH and relative calibrator is given as $2^{-\Delta\Delta Ct}$, where ΔΔCt is $\Delta Ct - \Delta Ct_{GAPDH}$ and ΔCt is $CT_{gene\ target} - Ct_{GAPDH}$.

Statistical Analyses

Results are expressed as mean ± standard deviation. Student's *t* test was used. All *p* values less than 0.05 were considered to indicate a significant difference between groups.

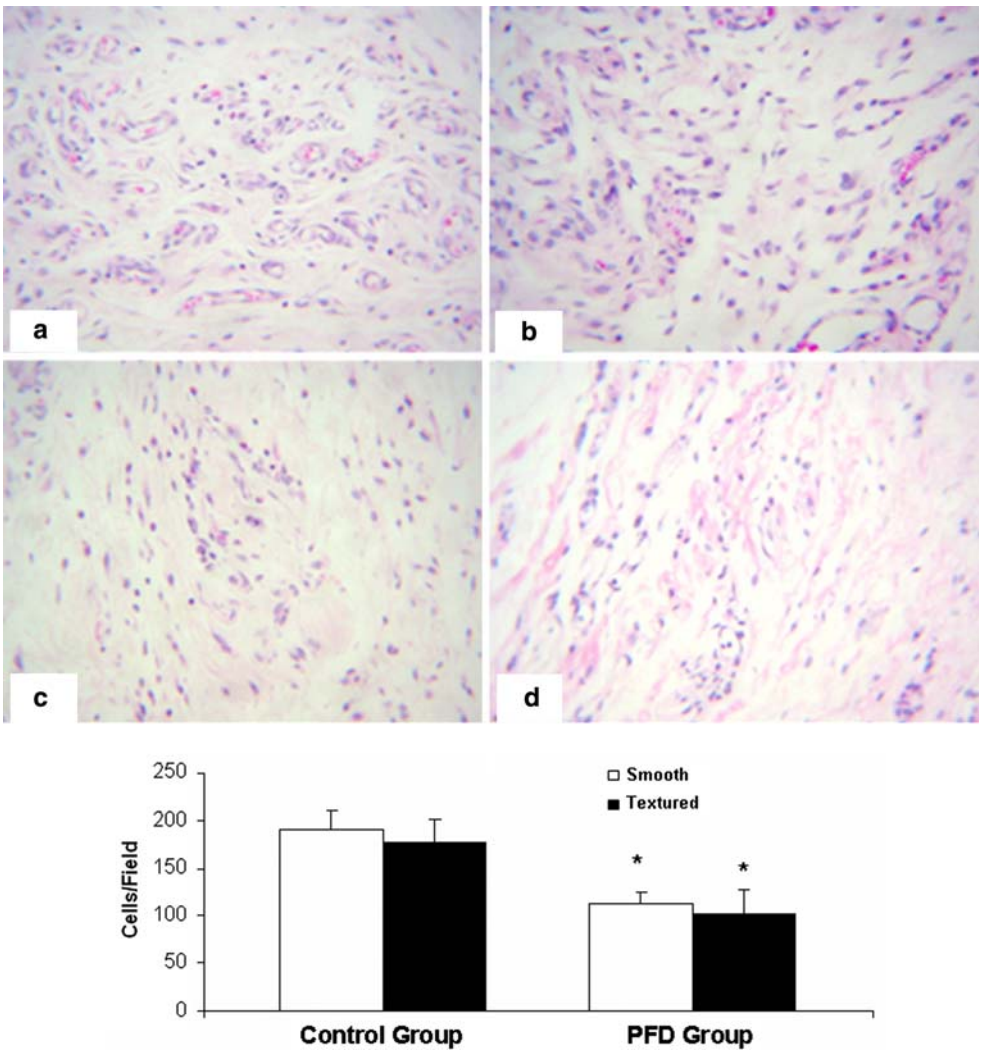

Fig. 3 Hematoxilin-eosin staining after silicone implant placement. The control group presented abundant mononuclear cell infiltration and fibroblast-like cells. In the pirfenidone (PFD) animal group, fewer fibroblast-like cells and diminished recruitment of inflammatory cells were observed. (**a**) Control smooth implant. (**b**) Control textured implant. (**c**) PFD-treated smooth implant. (**d**) PFD-treated textured implant

Table 1 Molecular markers in capsular contracture

Parameters	Control		PFD	
Implant	Smooth	Textured	Smooth	Textured
Inflammatory cells cells/field	191 ± 27	177 ± 31	109 ± 17	97 ± 33
Fibrosis (%)	58.1 ± 3.7	59.8 ± 1.6	28.4 ± 3.6	28.0 ± 4.5
α-SMA positive cells/field	17.6 ± 4.7	19.8 ± 1.9	9.6 ± 1.8	8.9 ± 1.1
TGF-β expression	1.0 ± 0.16		0.38 ± 0.18	
Collagen 1	1.0 ± 0.15		0.14 ± 0.09	

PFD, pirfenidone; α-SMA, smooth muscle actin-α; TGF-β, transforming growth factor-β

Values are mean ± SEM for 10 rats. Textured or smooth implants were implanted as described in the Methods section. Determinations of each parameter were performed according to the methodology described in the Methods section. $p < 0.05$ (significantly different from control condition)

Results

In this study. submammary silicone breast implants were placed in female Wistar rats to evaluate the effect of PFD has on contracture and fibrosis development after 8 weeks (Fig 1). It is important to mention that no animal treated with PFD presented adverse effects such as diarrhea or weight loss. After 8 weeks, the animals presented with an augmented capsule around the implant, with an increase in the opacity on adjacent tissues, making it difficult to visualize the morphology of the implant surface. An exacerbated amount of extracellular matrix also was evident. We did not observe differences between textured and smooth implants (Fig. 2a and b). The PFD treatment caused a reduction in capsule thickness around submammary tissue without evidence of a real capsule and with a clear decrease in the amount of extracellular matrix, a fact confirmed later with specific staining.

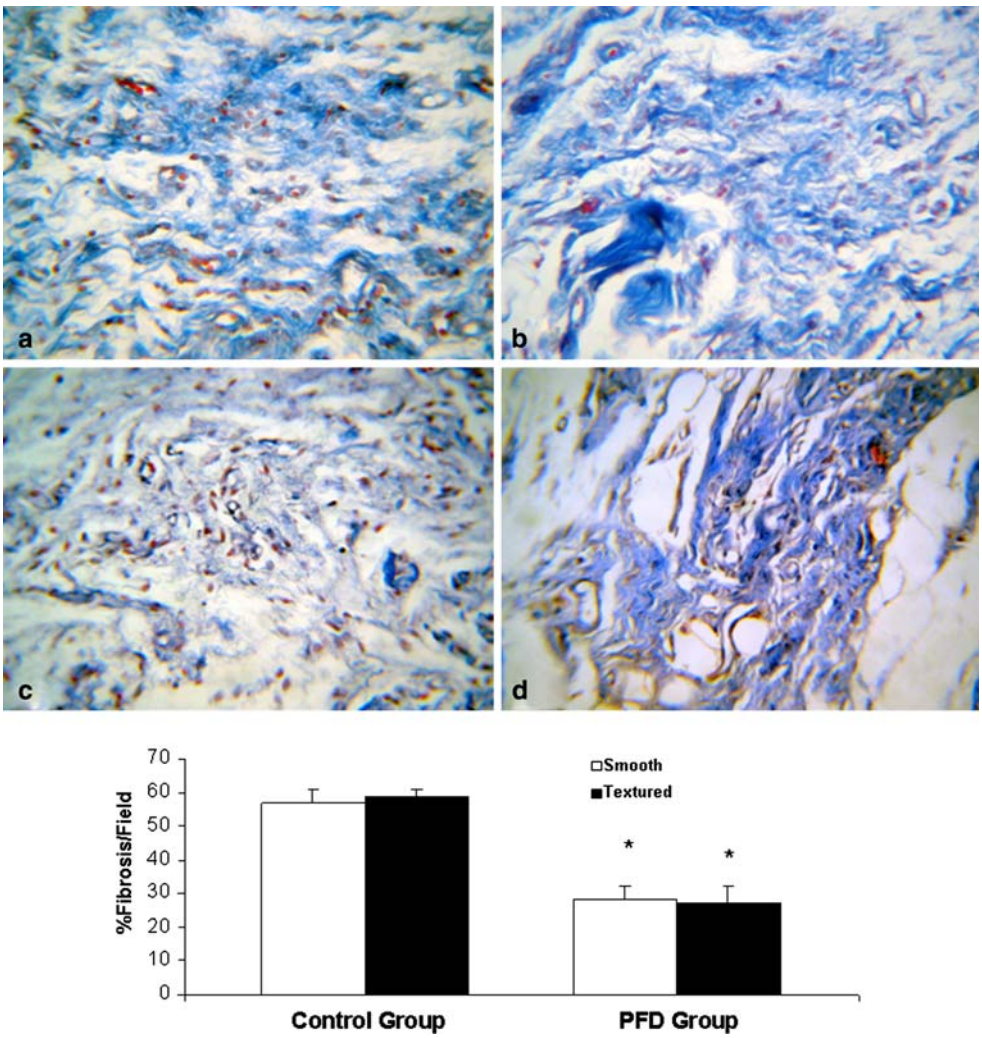

Fig. 4 Masson's staining of mammary tissue from control and pirfenidone (PFD)-treated animals. The total content of collagen in the tissue of the PFD group was 50% less than in the control group with saline. Nonetheless, in the PFD group, a significant difference was observed between smooth and textured implants. (**a**) Control smooth implant. (**b**) Control textured implant. (**c**) PFD-treated smooth implant. (**d**) PFD-treated textured implant

These downregulated events were noticeable to the same extent in rats with both textured and smooth implants (Fig. 2c and d). By using hematoxylin-eosin staining, we could demonstrate that PFD-treated animal groups presented lower fibroblast- like cell proliferation and reduced recruitment of inflammatory cells. In contrast, the control group showed abundant mononuclear cell infiltration, larger fibroblast-like cell proliferation, and the presence of neovascularization. Total cell numbers per microscopic field were considerably reduced when the animals were treated with PFD (n = 10; $p < 0.05$; Fig. 3a–d; Table 1). To detect the degree of capsular fibrosis, tissue was stained with Masson's staining, which showed 50% fewer extracellular matrix proteins by morphometric analysis in the PFD group than in the control group without PFD. Irregular, tight, thick bundles of extracellular matrix can be observed in Fig. 4a and b. On the other hand, Fig. 4c and d clearly shows more lax and loose extracellular matrix proteins, correlating with the lower number of fibroblastic cells shown in Fig. 3d and Table 1.

We did not observe differences in extracellular matrix content when we compared smooth and textured implants after PFD treatment (n = 10; $p < 0.05$). We looked for fibroblast activation by means of immunohistochemistry analysis using anti-α-SMA antibodies, and quantitative data were gathered from multiple stained tissue sections. As can be deduced from Table 1, we found approximately 45% fewer fibroblasts displaying an activated phenotype in PFD-treated animals than in nontreated animals (n = 10; $p < 0.05$) (Fig. 5). These results suggest that PFD induced its antifibrotic effect (among other pathways) via inhibition of fibroblast activation.

We also conducted a series of experiments based on real-time PCR to determine the effect of PFD on the expression of key genes involved in fibrogenesis. Compared with the control group, TGF-β gene expression in PFD-treated rats decreased significantly, by 85% ($p < 0.05$). Collagen 1 gene expression decreased 60% in the PFD-treated animals compared with the untreated animals (n = 10; $p < 0.05$; Fig. 6).

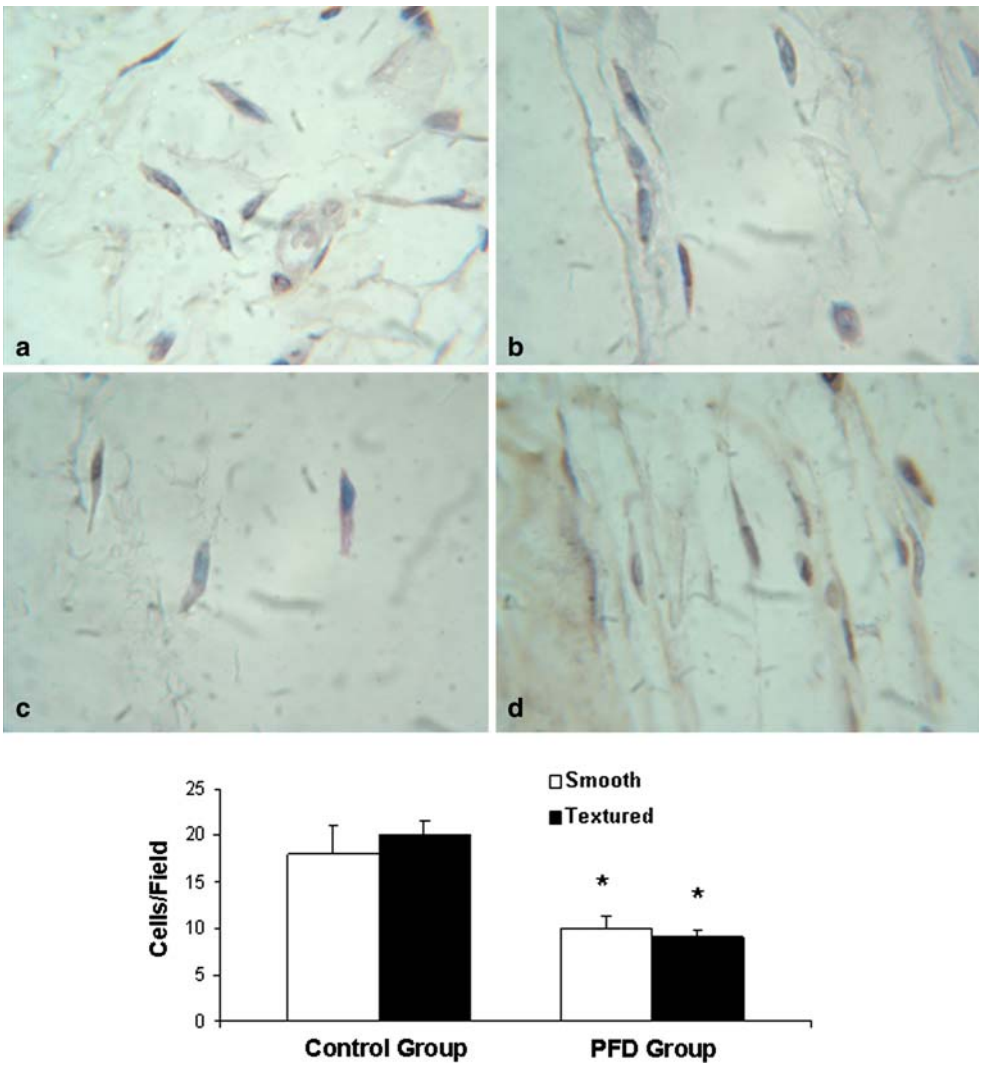

Fig. 5 (**A**) Immunohistochemistry analysis with anti-α -smooth muscle actin (anti-α-SMA) antibody on multiple stained tissue sections. (**a**) Control smooth implant. (**b**) Control textured implant. (**c**) Pirfenidone (PFD)-treated smooth implant. (**d**) PFD-treated textured implant. (**B**) Quantitative analysis of tissue section from panel **A**

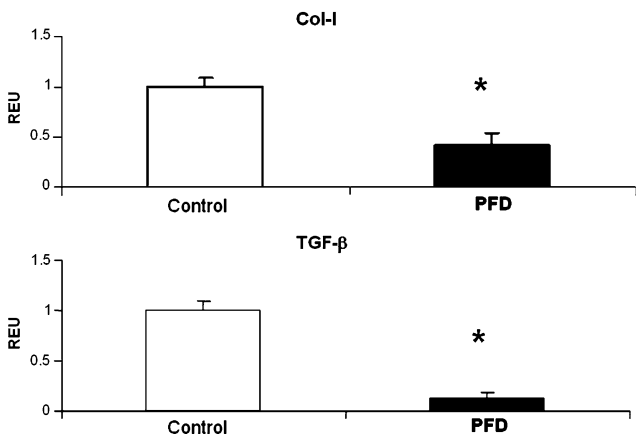

Fig. 6 (**A**) Real-time polymerase chain reaction (PCR) for transforming growth factor-β (TGF-β) in the control and pirfenidone (PFD) animal groups. White bars: control group. Black bars: PFD group ($p < 0.05$). (**B**) Real-time PCR for collagen 1 in the control and PFD animal groups ($p < 0.05$)

Discussion

Breast implantation surgery is one of the most desired aesthetic surgical procedures. Although this type of surgery is increasing in use and safety of performance, the presence of related adverse events continue to be evident. The adverse effects most observed after breast implants surgery include inflammation, capsule contracture, and fibrosis. Different materials to diminish these undesirable effects have been tested.

In this study, we provide evidence concerning the fibrogenic process using two different textures of implants, smooth and textured, with 8 weeks of placement in a rat model. Histologic analysis did not show any intragroup difference for any analyzed parameter. Both the treatment and control groups received smooth and textured implants.

Our results are in agreement with Fagrell et al. [36], who compared capsule contracture between smooth and

textured implants in 20 healthy women. The study showed no significant difference in contracture between smooth and textured implants, although the majority of the patients preferred the smooth implants.

Although new materials for breast implants are being introduced continuously, the risk of adverse effects continues. Several drugs are being used to diminish postsurgery complications. In this context, PFD, a new drug with antiinflammatory and antifibrotic properties was used in this study for an analysis of its effect on contracture and inflammation after breast implantation. With the administration of PFD immediately after mammary implantation and continuing for 8 weeks, we were able to decrease inflammation, contracture, and the thickness of the capsule compared with the control group. In addition, PFD reduced the expression of profibrogenic agents such as TGF-β ?and collagen by 85% and 60%, respectively. It is important to mention that no animal treated with PFD presented adverse effects such as diarrhea or weight loss.

These results are in agreement with the effect of PFD on other fibrotic diseases reported by us and others, in which PFD reduced the fibrosis index by 70% and inhibited TGF-β and collagen expression [28, 29, 37]. Musters et al. [38] observed mammary stromal cell proliferation in a TGF-β1−treated mammary gland 4 and 22 h after pellet implantation suggesting the effect of TGF-β? on ?proliferation of collagen-producing cells. Immunohistochemistry techniques using the anti-α-SMA antibody allowed detection of lower fibroblast proliferation with PFD administration, suggesting that its antifibrotic effect is mediated via inhibition of fibroblast proliferation/activation.

Our results show an antifibrotic and antiinflammatory effect of PFD on surgery implants similar to that reported previously by our group [28, 29] and others [38] for several fibrogenic diseases, demonstrating its high potential as a preventive drug in mammary implants. Besides, PFD treatment provoked minor adverse events, as shown in a previous study by our group, in which patients with established advanced liver fibrosis were treated with PFD at 1,200 mg/day during 12 months. Only 15% of the patients experienced adverse events such as photosensitivity, rash and itching, and gastrointestinal symptoms such as nausea, abdominal discomfort, and diarrhea. After 2 or 3 months of PFD therapy, these reactions disappeared [29]. Our own observations could lead to speculation that a similar oral dose (1,200 mg/day) might be used as a co-adjuvant strategy to prevent undesirable events after breast implantation surgery in humans. We believe a daily intake of this dose for 1 month before breast surgery and a couple of months after the surgical procedure would help to prevent the development of undesirable fibrogenic events. The minor adverse events observed make this drug an attractive alternative for use in healthy women undergoing breast implantation.

Acknowledgment This investigation was supported by Cell Therapy and Technology, S.A. de C.V.

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