The papain local depot impairs the capsule fibrous healing around textured silicone implants in rats.

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***Abstract*— Objective:** To study the tissue repair around the textured mammary implants under the action of papain (PA). **Methods:** Thirty-six Wistar rats were evaluated and randomly distributed into two groups (n = 18): papain (PA) and control (CT). Each group was equally distributed into 3 subgroups (n = 6) and observed on seventh, thirtieth-fifth and ninth pos-operative days. Each animal received a textured implant in the left dorso-axillary region (sham - SH), on were instilled 0.5 mL saline solution 0.9%, and another textured implant on the right dorso-axillary region (papain - PA), on were instilled 0.5 mL of water-soluble solution of papain. The control group (CT) received only textured implant in the left dorso-axillary region with prior instillation of 0.5 mL of saline solution 0.9%. The histological analysis of the 3 subgroups was carried out using picrosirius-red stain and an image analyzing system using the Image Pro Plus™ program to evaluate the thickness and maturation and deposition of collagen fibers. Immunohistochemical evaluation was performed, using micrometric reticules of Weiss (Olympus Labstore™), for myofibroblasts counting only in the 90th day subgroup. **Results:** At 35th and 90th days, the papain group (PA) presented reduction on the fibrous capsule thickness around the implant, in the number of collagen fibers and myofibroblasts, comparing to the control group (CT). **Conclusion:** The papain drug decreased the fibrous capsule formation around the textured silicon implants in rats.

***Keywords*—**Capsular contracture, capsular thickness, inflammation, mammary implant.

I. Introduction

The capsular contracture is the most common adverse effect after breast implant [1]. Fibrotic tissue promotes compression around the implant which distorts and deforms the mass which then compromises the aesthetic result and is associated with painful symptoms [2]-[4]. The development of a fibrotic capsule around foreign material is a physiologic reaction of the organism to protect itself from material it does not recognize [5]. The severity of capsule contracture is directly related to the degree of the local inflammatory reaction [6] and does not depend on the implant surface employed [7]. Although it is not clear the pathogenesis of capsular contracture, this phenomenon seems to be multifactorial [8].

Currently, there is no effective preventive measure for capsule contracture [8]. The conventional treatment may be surgical, by capsulectomy (or capsulotomy) or implant replacement and pharmacological using steroids, anti-leukotrienes, anti-TGF-β, antibiotics or antiinflamatories [2], [3].

Up to now there has been no accurate and reproducible pathologic model for examining capsular contracture [9]. This study assumes that eventual interference by papain (PA) on normal healing could be useful in further studies of a more complex model with induced capsule contracture [9].

Some authors have speculated on the possible modulator action of certain proteolytic enzymes present around the implants in the early stages of healing [10], [11].

Papain is a thiol endopeptidase plant whose activity is similar to the lysosomal cathepsin B enzyme with fibrinolytic and proteolytic action on the normal healing mechanism [12].

It has been suggested that the papain could be helpful when used locally around the implant at the surgical procedure, promoting tissue repair with less fibrotic tissue, thus avoiding the capsule contracture.

The aim of this study was to investigate the papain effects on the fibrous capsule thickness, collagen fibers density and myofibroblasts around textured implants in rats.

II. Methods

The experimental protocol (#1082-06) was approved by the Ethics Committee of the Federal University of São Paulo - Escola Paulista de Medicina (UNIFESP - EPM). The rats were kept according to the guidelines of the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 1996) and according to the ethical principles of the Brazilian College on Animal Experimentation (COBEA).

Were used thirty-six male Wistar rats weighing 250-300g, kept in individual cages at room temperature with photoperiod of 12 hours (light / dark) were used, receiving water and food freely.

Anesthesia was performed by injection of hydrochloride 2-(2,6-xylidine)-5,6-dihydro-4H-1,3-thiazin (Ronpun® - Bayer, Germany) and ketamine hydrochloride (Ketalar® - Parke-Davis, Belgium) in 1:1 ratio, using 1mL.Kg^{-1}. A single dose (60 mg/kg) of cefazolin (Kefazol™ - Eli Lilly do Brasil Ltda -

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São Paulo - Brazil) was given intramuscularly as a prophylaxis of infection.

A textured surface silicone implant was used (pore diameters between 0.05 and 0.25 mm), shell-shaped with two centimeters in diameter and volume of 2 mL of silicone gel (Silimed® Brazil - São Paulo).

Two parallel incisions (1.5 cm) to the left and right of the spine just below the neck were made in eighteen animals. Two pouches were set up at 4 cm from the incision under the *panniculus carnosus* muscle where silicone implants were placed.

Before placing the implant, 0.5 mL of saline solution (sham group - SH) was instilled in the left pouches. The right pouch received a solution of 0.5 mL of 30mg/kg^{-1} papain (USP 27 ® - Papain Tablets for Topical Solution Papaverine - Vermizym, Germany - Formula & Ação Farmácia Ltda. Brazil) (papain group - PA). Other eighteen animals were subjected to only one incision to the left, pouch dissection and instillation of saline solution (0.5 mL), followed by implant insertion (control group - CT) The muscle layer and skin incision were closed with polyamide sutures (Mononylon™, Ethicon, São Paulo, Brazil) (Fig. 1).

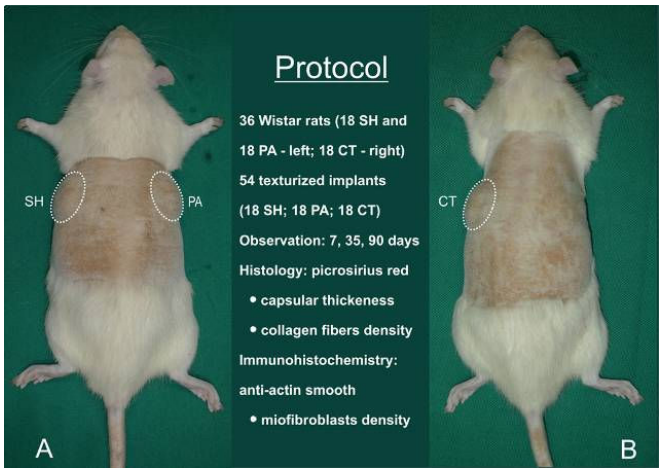

Fig. 1 - *Schematic drawing of the protocol: rat A - two textured implants - sham group (SH) without drugs; papain group (PA) with drugs, rat B - a textured implant - the control group (CT).*

During follow-up, respiratory distress, liquid stools, and refusal of food or water were reported. The wound was carefully monitored mainly for extrusion of the prosthesis as well as for infection and abscess. Once any sign of severe suffering was seen, the veterinarian interrupted the research and the animals were euthanized. Euthanasia was performed on the 7th, 35th and 90th day, under anesthesia, through an intravenous injection of potassium chloride until the cardiac arrest.

The prosthesis and the tissue around were removal was through an incision around the center of the implant (4 cm²) (Fig. 2).

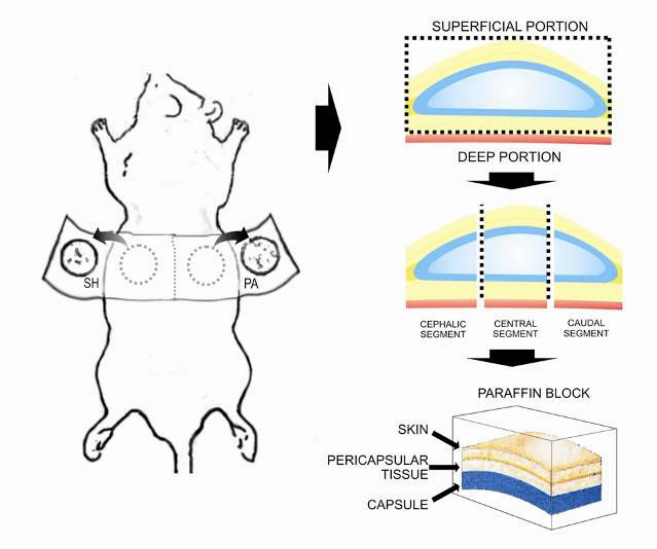

Fig. 2. *Single block with the implant and capsule around it, removed from the rat after 24 hours. The capsule was dissected from the silicone implants and three sections (cranial, medial and caudal) were sent for histological processing.*

In order to evaluate the implant, an incision was made around the center of it (4 cm²). Dissection from the parietal muscular layer to the skin isolated the implant and the per implant tissues in a single block (Fig. 2).The specimens were immersed in 10% buffered formalin. After 24 hours, the capsule around the implant was dissected (Fig. 2), cut into three fragments (cranial, medial and caudal), embedded in paraffin and prepared for histological sections of 5 µm stained with picrosirius red to measure the capsule thickness (using a magnification of 200x) and to calculate the collagen fibers density with polarized light (using a magnification of 400x). The software Image Pro Plus ® was used in both cases.

Other sections were prepared for immunohistochemistry for myofibroblasts counting (Dako Cytomation™ - Biogen Inc™ - Cambrigde, MA - USA), using a micrometric reticule of 100 points (Weiss Olympus® - Labstore®). Muscles and brown vessels were stained and excluded from the percentage of myofibroblasts found in the fibrous capsule.

Statistical analysis was performed using factorial variance tests (one-way and two-way). These were carried out to compare the group's means and test whether there was interaction between times versus treatments. Tukey test (SPSS version 11.0) was employed to identify the "two-way" variation within the same group and it was considered significant when less than 5% ($p \leq 0.05$).

III. Results

Papain was effective to decrease the capsule thickness at the 35 and 90 days when compared with the other two groups (control and sham). These results suggest that the drug had a local action in the healing process (Table I and Figs. 3 and 4).

Table I. Values (Mean ± SD) of the capsule thickness (µm), at 7th, 35th and 90th days of observation in control groups (CT), sham (SH) and papain (PA).

	7 days	35 days	90 days
CT	589.97 (±150.89)	745.08 (±156.37)	705.79 (±81.75)
SH	587.69 (±119.28)	553.33 (±119.2)	611.61 (±135.69)¤
PA	464.53 (±20.65)	397.84 (±92.59)§	443.7 (±55,42)¥

7 days: CT = SH = PA; 35 days: CT > SH > PA§ ($p \leq 0.0094$) ($p \leq 0.006$); 90 days: CT = SH¤ > PA¥ ($p \leq 0.1389$) ($p \leq 0.0123$)

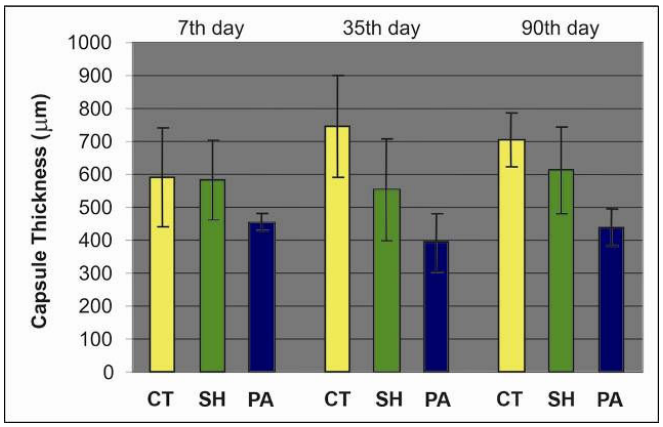

Fig. 3. Values (Mean ± SD) of the capsule thickness (µm), at 7th, 35th and 90th days of observation in control groups (CT), sham (SH) and papain (PA).

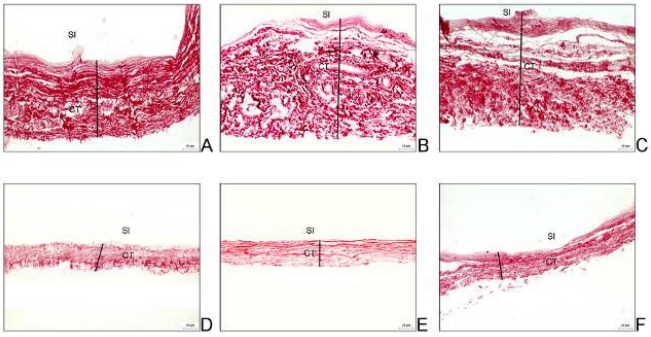

Fig. 4. Photomicrography of the capsule thickness: Control group (CT) at the 7 days - 739.79 µm(A), 35 days 898.62 µm (B) and 90 days 809.34 µm (C); Papain (PA) at 7 days 394.40 µm (D), 35 days 337.45 µm (E) and 90 days 377.14 µm (F). (Picrosirius red - 200X). SI = silicone. FC = fibrous capsule.

Papain was effective to decrease the collagen density at 35 days, but also worked in the sham group (SH), which probably suggests a systemic action in the formation of fibrous collagen in healing. (Table II and Figs. 5 and 6).

Table II. Values (Mean ± SD) of collagen density (µm²) at 7, 35 and 90 days of observation in control groups (CT), sham (SH) and papain (PA).

	7 days	35 days	90 days
CT	8884.38 (±4726.81)	7235.4 (±1061.04)	6964.31 (±1242.69)
SH	6969.93 (±3706.6)	5880.95 (±575.28)	8108.43 (±1127.45)¤
PA	6525.97 (±2175.66)	3470.4 (±90.66)§	3594.11(±531.95)¥

7 days: CT = SH = PA ($p \leq 0.3284$) ($p \leq 0.7997$) ($p \leq 0.2141$); 35 days: CT > SH > PA§ ($p \leq 0.0491$) ($p \leq 0.0001$) ($p \leq 0.0001$); 90 days: CT = SH¤ > PA¥ ($p \leq 0.1239$) ($p \leq 0.0001$) ($p \leq 0.0001$)

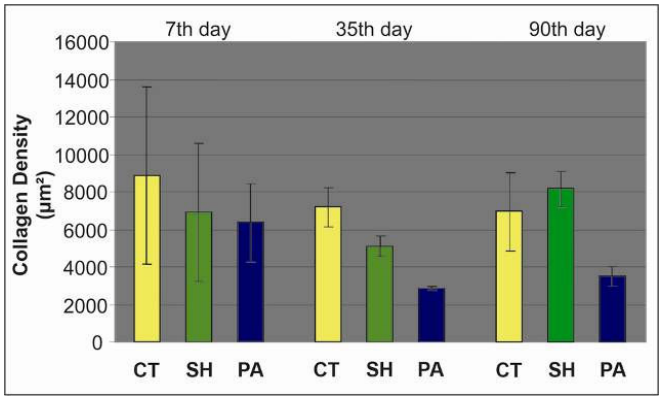

Fig. 5. *Values (Mean ± SD) of collagen density (µm²) at 7, 35 and 90 days of observation in control groups (CT), sham (SH) and papain (PA).*

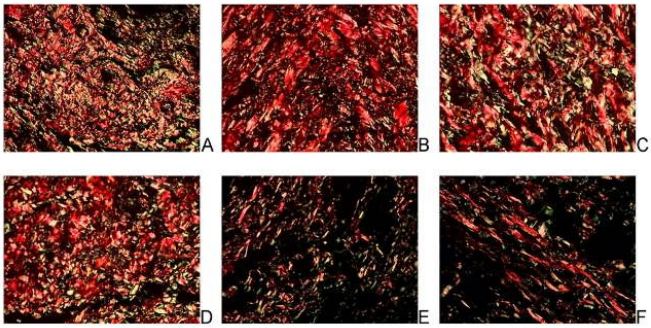

Fig. 6*. Photomicrography of collagen density: control group (CT) to 7th (A) 7681.17 µm², 35th (B) 6318.67 µm² and 90th day of observation (C) 7856.83 µm²; group papain (PA) at 7th (D) 7173.60 µm², 35th (E) 3301.70 µm²;, and 90th (F) 3510.30 µm². The number of collagen fibers is smaller at the 35th day (B). (Picrosirius red, polarized light - 400X). Images captured on screen (Image-Pro Plus ™ software).*

Papain at the 90th day was associated to a lower count of myofibroblasts in the PA group when compared to the control (CT) which could be related to a local effect of the drug.

On the other hand, the number of myofibroblasts was significantly lower in the sham group (SH) which indicates a systemic action (Table III and Figs. 7 and 8).

Table III. Values (Mean ± SD) of myofibroblasts (%) after 90 days of observation in control groups (CT), sham (SH) and papain (PA).

	CT	SH	PA
90 days	32,7 (±13.62)	10,13 (±3.19)§	4,28 (±1.66)¤

90 days: SH§ < CT > PA¤ ($p \leq 0.0001$) ($p \leq 0.0001$) ($p \leq 0.0001$)

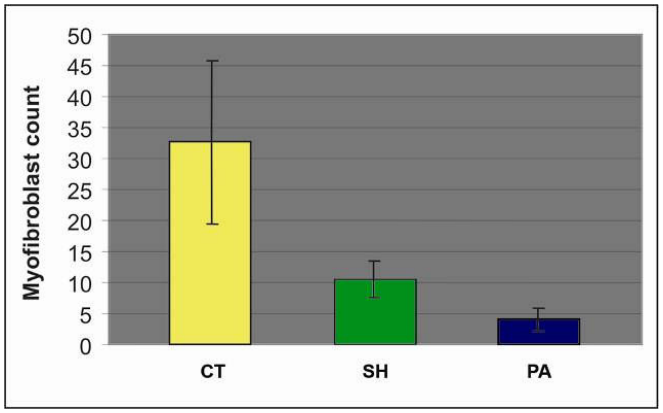

Fig. 7. *Number of myofibroblasts in a hundred fields (Mean ± SD) after 90 days of observation in control groups (CT), sham (SH) and papain (PA).*

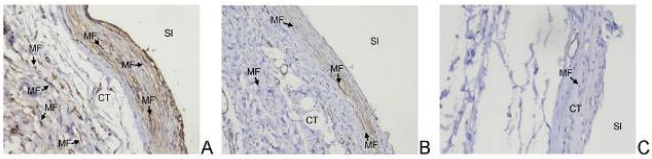

Fig. 8*. Photomicrography of the myofibroblasts counting at 90 days of observation (Immunohistochemistry - 200X). Control group (CT) (A), sham (SH) (B) and papain (PA) (C).*

IV. Discussion

Up to now, all attempts to reduce capsular contracture rate were focused on treating rather than preventing its appearance [4]. The possibility of preventing capsular contracture formation has attracted considerable interest from researcher's worldwide [5].

The papain properties are known to reduce the swelling, or as an anti-inflammatory, antithrombotic and analgesic as well as its proteolytic action [12].

The drug modulates the function of mast cells by reducing the production capacity of cytokines and growth factors, reducing the inflammatory phase and limiting the proliferation of myofibroblasts and collagen deposition. The excess of mast cells produce growth factors, such as TGF-β that will finally promote contracture, scar formation and myofibroblasts proliferation. It seems that the papain main mechanism of action is the regulation of IL-6 cytokines which has a key role in the tissue repair and regeneration [10], [12].

The normal process of the wound healing has a parallel displacement of the wound margin followed by intermittent decrease in the edge number. The peripheral cells flowed into the wound from its side to reduce it approximately keeping it shape as if a water flow makes a land shape of erosion. The area of the wound decrease exponentially [13].

The prolonged and exacerbated tissue reaction has a positive linear correlation with the fibrosis and contraction levels around the implant [6].

Animal models have often been used to study the capsule contracture around implants [2], [3], [8], [14].
Several current methods has been used to quantify the elasticity of the tissues [15] and to calculate by mathematics the architecture of the different cells involved in the inflammation process [16]. Based on several works [2]-[5], [8], [17] that quantified and monitored the behavior of the fibrous capsule, we decided to count collagen fibers, myofibroblasts and measure capsule thickness by utilizing the Image Pro Plus® program.

In an animal model, capsule thickness, collagen fiber density, and myofibroblast counts were investigated to understand the effects of PA in normal healing around textured implants and the possibility of extrapolating the results to abnormal healing that takes place in capsular contracture. Limitations in animal models, such as the absence of capsule contracture and the fundamental differences in the immunohistochemistry and histopathology on periprosthetic physiologic capsules, were considered [9].

Based on the work of Süslü et al. [18] on zafirlukast standard formulation, we initially intended to investigate the parenteral (intraperitoneal, intramuscular, intravenous) and local depot use of papain to determine the median lethal dose (LD-50). During these preliminary tests we found satisfactory results in capsular thickness reduction with the local depot use of papain in the pocket implant. Thus, we proposed the present investigation in which the drug was instilled around the textured implant.

Local depot of substances such as antibiotics [19], [20], antineoplastic agents [4], [21], and steroids [22]-[26] to reduce capsular contracture has been reported. Differences in procedures hinder the ability to form a consensus on the effectiveness of these drugs, and there are no specific reports on the topical route by itself, which we considered a priori as a reliable and feasible one.

The dosage used (30 mg/kg), was based on the preliminary study and proved to be the best one. Higher doses (60 mg/kg) were associated with some implant extrusion.

Capsular contracture may be the result of a progressive response and prolonged tissue repair caused by the presence of the implant [2], [4], [6], [8], [27]. As the lifespan of a rat is about 3 years and a woman's is 85, there is a life proportion of almost 1/30 between them [28]. Therefore, 1–3 months in rats corresponds to 2.5–10 years in humans [28]. Although this might not be an absolute fact, it is enough to estimate the quality of the capsule formed and its stability through time [27]. Some authors [6], [14], [27] reported a relationship between the increases of contracture and the elapsed time. In a pig model, contracture had no significant effect on the capsule's thickness shown on the 180th day (1704 ± 487 μm) and 270th day (1281 ± 636 μm) [18]. Therefore, to study the evolution of capsule formation we used shorter spans: brief (7 days), medium (35 days), and long (90 days) [29].

Our model proved to be trustworthy once the results found in capsular thickness were in accordance to what is reported in the literature. Capsular thickness (μm) of CT (Table I, Fig. 3) on the 7th day (589.97 ± 150.89), 35th day (745.08 ± 156.37), and 90th day (705.79 ± 81.75) were similar to those reported by Bastos et al. [17] using textured implants (range = 450–800 μm) and Karacal et al. [5] (mean = 876.7 μm) using silicone blocks. Using a pig model, Minami et al. [29] demonstrated that the capsule thickness around the textured implant varies from 1.247 ± 606 μm (30 days) up to 921 ± 188 μm (60 days).

Capsule thickness (Table I) and collagen density (Table II) in the acute phase (7th day) did not show significant differences among the three groups (SH, CT, and PA). These results suggest that PA had neither a topical nor a systemic effect on the acute inflammatory response.
In that early stage of inflammation, the PA probably acted on the mast and neutrophils cells and their effects in the fibroblast and collagen matrices appeared in the following stages of the inflammatory response [18].

On the other hand, PA capsule thickness was significantly smaller on the 35th and 90th days compared to that of the CT, and there were no differences between the CT and SH (Table I, Figs. 3 and 4). These results suggest that one dose of PA applied in the pocket had only a topical but effective action in reducing capsule thickness.
Baker's theory [30] on capsule formation is based on the contraction of myofibroblasts and the deposition of collagen around the implant, increasing the contracture even more. Frangou and Kanellaki [4] reported that the reduction in capsule thickness was associated with a decrease in collagen fiber density. Thus, we believe the evaluating collagen density is another way of monitoring capsule formation. We also found parallel results between capsule thickness and collagen density. On the 7th day no significant difference in collagen density was observed among the three groups (SH, CT, and PA). The results suggest that PA was not effective in impairing collagen formation in the acute phase. On the other hand, by the 35th and 90th days collagen density was significantly lower in PA compared to CT which can be attributed to the PA. Nevertheless, we also found a

significant reduction in SH, which might suggest a systemic action.

Desser et al., [10] administrated oral therapy with proteolytic enzymes containing papain in patients with rheumatoid arthritis (n=38), ostheomyelofibrosis (n=7), herpes zoster (n=7), and in seventy-eight healthy volunteers control group and observed statistical decreases in serum levels of TGF-β 1, assessed by enzyme-linked immunosorbent assay (ELISA), in all the patients studied in relation to the control group. It has been demonstrate that proteolytic enzymes reduce TGF-β levels in serum by converting the protease inhibitor α2 macroglobulin (α2 M) from the "slow" form into the "fast" form, whereby the "fast" form binds and inactivates TGF-β irreversible.

TGF-β 1 induces the expression of the proinflammatory cytokine IL-6 [31], and seems also to be a key factor in progression and in immunosuppressant in patients with rheumatoid arthritis [32]. Reduction of elevated TGF-β 1 concentration after enzyme therapy was associated with the beneficial clinical effect of this therapy [33].

The role of α2M in reducing TGF-β has been examined by Tiggelman et al. [34]. These investigators reduced TGF-β-stimulated collagen synthesis in liver myofibroblasts by introducing the "fast" form of α-2M. Activated α2M neutralizes TGF-β, produced by breast-cancer cells, and thereby promotes the activation of NK, LAK and tumor-specific T-cell response [35] by interleukin-2. Reduction of TGF-β overproduction reduces TGF-β synthesis [36]. It has been described by Hall et al. [37] that proteases react with α2M in blood, and convert the "slow" form into the "fast" form. After this reaction, α2M can recognize the receptor LRP on hepatocytes endothelial cells and fibroblasts which is responsible for the rapid plasma clearance of transformed α2M, whereas the "slow" form of α2M shows no affinity for LRP [38]. α2M in the "fast" form (α2M + enzymes) binds TGF-β. TGF-β bound to the "fast" form of α2M cannot bind to its cell receptor and this complex is phagocytized very quickly.

Paczek et al., [39] also concluded that protease administration decreased enhanced transforming growth factor-beta 1 content in isolated glomeruli in an experimental diabetic rats study.

Fibroblasts are cells that mediate a wide variety of responses including wound repair, tissue remodeling and fibrosis. In response to certain types of injury or inflammation these cells undergo certain expressional changes that lead to phenotypical alteration [40]. It have been finding that MK2 (kinase) plays an important role in the development of the myofibroblast phenotype, and implicate the kinase as a target for the treatment of some disease processes [40].

Myofibroblasts are also involved in the physiopathology of contracture so their evaluation is relevant [2]-[5], [8], [17], [21], [30].

Some authors measured the contractile activity of myofibroblasts and demonstrated its relaxation with the use of papaverine (papain) [11], [30].

Gancedo et al. [8] in an experimental study in rats obtained a reduction on capsule thickness, collagen density and myofibroblast count after oral doses of pirfinidone.

Moreira et al. [2], in a rat model obtained the same results by using single pocket delivery dose of zafirlukast.

Bastos et al. [17], in a similar study, showed a reduction on the capsule thickness and on the collagen density, but not on the number of myofibroblasts after peritoneal injection of zafirlukast.

Batra et al. [41], in an experimental study, found less collagen fibers and myofibroblasts around textured implants after the third month due to the presence of certain factors in the transudate that limits the transformation of fibroblasts into myofibroblasts and deposition of collagen fibers.

Wyatt et al. [42], in a histologic study of fibrous capsules in humans, described a natural decrease in the density of collagen fibers around textured implants over time and with the reduction of fibroblasts proliferation due to the presence of fibrinolytic enzymes, which may reduce the local inflammatory process.

Earlier studies of Ajmal et al. [43] with rabbits, with local instillation of 2-mercaptoetane sodium sulphate (Mesna) and Frangou et al., [10] in mice, with mitomycin C, reduced the capsule thickness, the collagen density and the number of fibroblasts.

Karaçal et al., [5] using an experimental model in rats, instilled human amniotic fluid containing hyaluronic acid, in the implant pocket and obtained a reduction on the pressure and on the capsule thickness after six months.

It is believed that, similar to the study of Karaçal et al., papain may offer a tissue repair with more regeneration and less scar formation. As it was hypothesized, papain promoted a significant reduction on the fibrous capsule thickness at 35^{th} and 90^{th} days, when compared to the control group. It also reduced the amount of collagen fibers at 35^{th} and 90^{th} days and the number of myofibroblasts at 90^{th} days of observation, when compared to the control group.

V. Conclusion

The local instillation of a single dose of papain was effective to prevent the formation of fibrous capsule around the textured silicone implants after an evaluation on the capsule thickness, on the collagen fibers density and on the number of myofibroblasts.

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