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Original Article

Fractal features of rabbit dermal wounds treated with platelet-rich plasma and topical rosuvastatin

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Abstract: The healing process comprises a sequence of molecular and cellular events that interact to restore injured tissue. Biomaterials such as platelet-rich plasma (PRP) have been widely used to promote healing by better collagen distribution. Studies have also shown that statin-associated biomaterials may improve endothelial function and increase re-epithelialization. When assessing healing, the analysis of collagen architecture is critical. One form of evaluation used to identify structural changes in the skin is fractal dimension (FD) analysis, which facilitates the characterisation of irregular structures on histologic slides, and quantification of existing alterations by an accurate technique that is independent of the evaluator. The aim of the current study was to assess the feasibility of using rosuvastatin (RSV) alone and combined with autologous PRP to analyze collagen fibers by FD analysis. Skin lesions were experimentally induced in adult male rabbits, and were then treated with either sodium chloride solution (control), RSV gel, autologous PRP gel, or RSV gel and autologous PRP gel combined. Thirty-two biopsies of the lesions were obtained, on days 7, 14 and 17. All treatments were associated with reduced FDs at all three time-points. The FDs of the wounds that were treated with RSV and PRP combined were significantly lower on day 7 ($P < 0.05$), suggesting that the combination may favour the reorganisation of fibres during the initial healing process. The use of FD analysis for collagen evaluation was evidently reliable. More studies are needed, to evaluate collagen and healing associated with RSV and PRP in experimentally induced wounds.

Keywords: Fractal dimension, PRP, rabbits, topical rosuvastatin, wounds

Introduction

The skin is the primary protective barrier of the body, and is critical in defence against foreign bodies and pathogens. Collagen is the main element present in the dermis, where the two main types found are I and III. Specifically, in the dermal matrix type I collagen is the most predominant and type III is present to a lesser extent. In wounds it is the reverse, as type III collagen predominates [1, 2].

Conditions such as burns, surgical wounds and various types of chronic skin ulcers damage the skin and compromise its capacity to function as a barrier, and trigger a natural tissue repair process [3]. Wound healing is a process mediated primarily by platelets, which play a key role in haemostasis, and it consists of inflammation, repair and remodelling of the injured tissue [4].

Biomaterials have been widely used to promote more homogenous healing by better distribution of collagen [3]. Platelet-rich plasma (PRP) is a biomaterial obtained from blood by centrifugation that is rich in growth factors such as platelet-derived growth factor, transforming growth factor beta, and fibroblast growth factor, among others. PRP promotes endothelial and epithelial regeneration and collagen synthesis, and has angiogenic, analgesic and anti-inflammatory properties [5-7].

In addition to the use of PRP in healing processes, studies have shown that statins can have multiple effects including antioxidant, antithrombotic and anti-inflammatory influences, and they have been associated with improved endothelial function [8]. Rosuvastatin (RSV) is considered a new class of statin. It has hypolipidemic effects, and anti-inflammatory action by way of inducing the secretion of cytokines that modulate inflammatory responses, and it reduces the high sensitivity of C-reactive protein-thus it has a beneficial effect

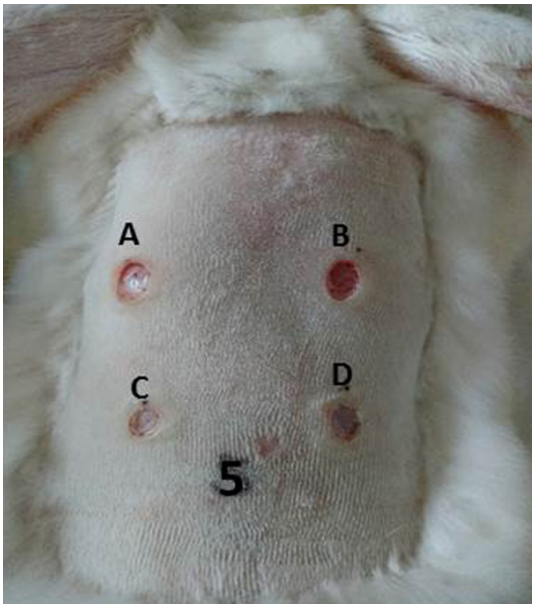


Figure 1. Wound treatment scheme. A. control; B. PRP; C, RSV; D, RSV+PRP.

on excessive immune responses, and can promote inflammatory response resolution [9].

Fractal dimension (FD) analysis is a digital tool that facilitates characterisation of the regularity of an object in different dimensional scales, and the detection of changes in regularity [10]. In human medicine, FD analysis has been used in studies involving neurons, liver carcinomas, and to characterise irregular structures in mounted skin samples [11-15]. Although the results of FD analysis are independent of the evaluator, and thus they are more accurate than those derived from methods that entail a degree of evaluator subjectivity, it is still little used, especially for the analysis of collagen in healing processes.

Thus, the aim of the current study was to investigate the use of RSV alone and RSV combined with autologous PRP in a serial manner to evaluate collagen fibres during wound healing by FD analysis.

Materials and methods

Animals

The animals used were 8 healthy adult male New Zealand white rabbits weighing 3.0±1.0 kg. The rabbits were kept in individual cages at an ambient temperature of 22°C±2°C, and a 12-hour light/dark cycle was utilised. They had access to food and water *ad libitum*. The study was approved by the Ethics and Animal Use Committee of the University of Western São Paulo (protocol number 3478).

Anaesthetic procedure

The rabbits were hand-held during trichotomy of the right and left dorsal regions. They were then anaesthetised by intramuscular (IM) injection with a combination of 2% xylazine hydrochloride (Xilazin® 2%) and zolazepam hydrochloride (Zoletil® 50) at a dose of 15 mg/kg. Thereafter, 0.1 mL of local anaesthetic (2% lidocaine hydrochloride with vasoconstrictor) was applied at the site of each lesion.

Surgical wounds

Skin antisepsis was performed using 70% alcohol, followed by demarcation of the lesions site. An 8 mm punch was used to perform the surgical wound in the dorsal region. The skin fragments were removed using anatomical tweezers, while preserving the musculature [16]. The lesion on the upper left side (lesion A, the control wound) was treated with 0.9% sodium chloride solution. The lesion on the upper right side (lesion B) was treated with autologous PRP in gel form. The lesion on the lower left side (lesion C) was treated with 1.2% RSV gel. The lesion on the lower right side (lesion D) was treated with a gel containing both RSV and autologous PRP (**Figure 1**). Sterile rayon and adhesive dressings (Band Aid®) were then placed on each of the four wounds.

After wound induction, the animals received IM analgesic tramadol hydrochloride at a dose of 0.5 mg/kg twice a day for 3 consecutive days, to minimise initial discomfort. The first dressing change was performed 3 days after wound infliction, and this second dressing remained for the subsequent 4 days. From that point onwards, treatments were administered every 4 days in accordance with the protocol described by Vendramin et al. [17], until day 16 of the experiment. Biopsies were performed on days 0, 7, 14 and 17 for histological analysis.

Skin samples were fixed in 10% buffered formalin solution for 24 hours. Fixed tissue was then embedded in paraffin blocks, and four histological sections from each animal with a

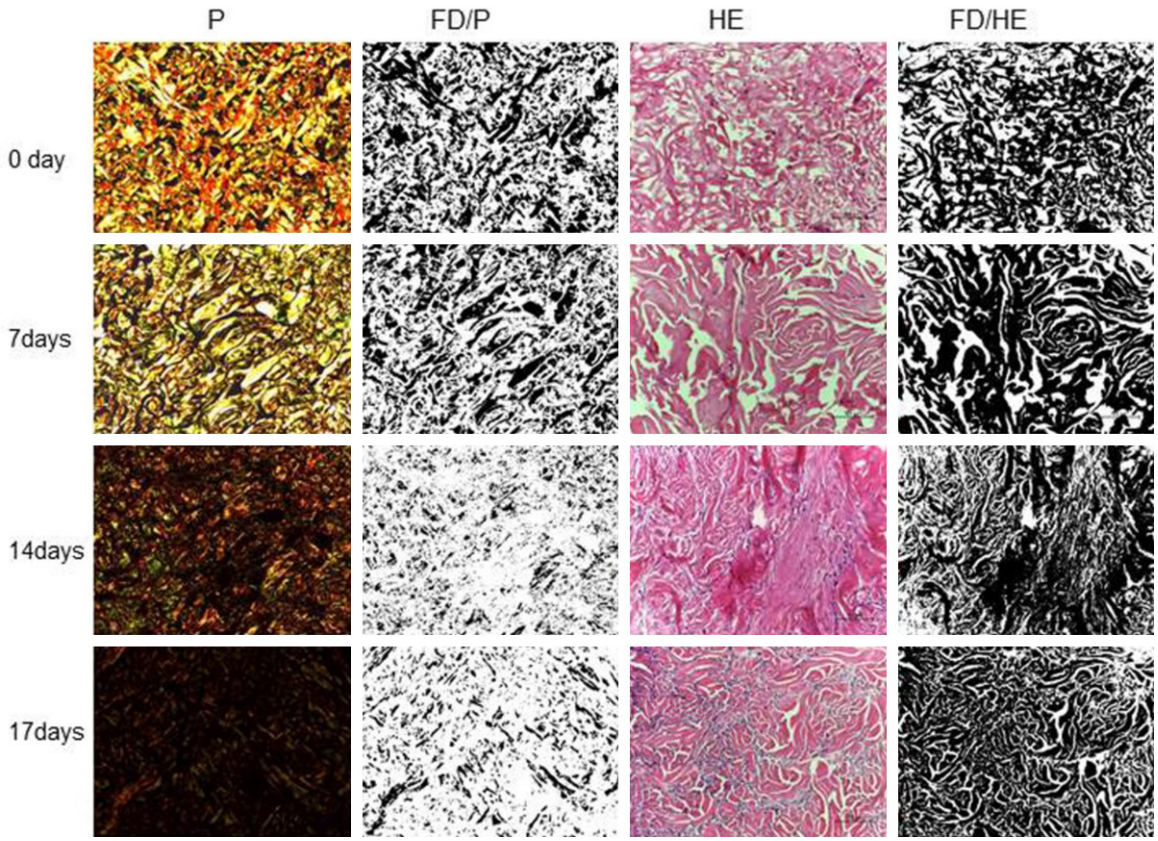


Figure 2. Microscopy and FD analysis of an experimentally induced control wound in a rabbit, at days 0, 7, 14 and 17. P, picrosirius red; FD/P, fractal dimension values of slides stained with picrosirius red; H/E, haematoxylin and eosin; FD/H/E, fractal dimension values of slides stained with haematoxylin and eosin. Magnification 200×.

thickness of 4 μm were stained with haematoxylin-eosin (H/E) solution for collagen evaluation.

Preparation of the RSV

The 1.2% RSV in liquid form was formulated to be PRP incorporated and subsequently all material was gel-transformed with the addition of calcium gluconate. For wound treatment only with RSV gel, 1.2% RSV in the gel form was formulated.

Preparation of platelet-rich plasma (PRP)

After the induction of anaesthesia, 8 mL of blood was collected from the auricular vein using 25G surgical scalp vein needle. The blood was transferred into two vials containing the anticoagulant sodium citrate. From this material an aliquot was taken for automated platelet counting (Sysmex Poch Diff 100iV-Roche automatic counter), then the samples were centrifuged, initially at 200 *g* (Excelsa Baby 206R centrifuge) for 10 minutes to form two fractions. The whole upper fraction corresponding to the plasma plus 200 μL of the lower fraction containing red blood cells was transferred to another tube for further centrifugation at 400 *g* for 10 minutes.

This second step resulted in two distinct fractions, an upper slightly yellowish one known as 'platelet-poor plasma', and a lower reddish one. The platelet-poor plasma was discarded and a new platelet count was conducted on the remainder, to verify that the platelet concentration was greater than six times the initial concentration [17].

Preparation of the PRP gel and the combined PRP + RSV gel

Two tubes were prepared, one for the generation of the PRP gel and the other for the generation of the PRP + RSV gel. In the PRP tube 400 μL of liquid PRP and 100 μL of 10% calcium gluconate were combined. In the PRP + RSV

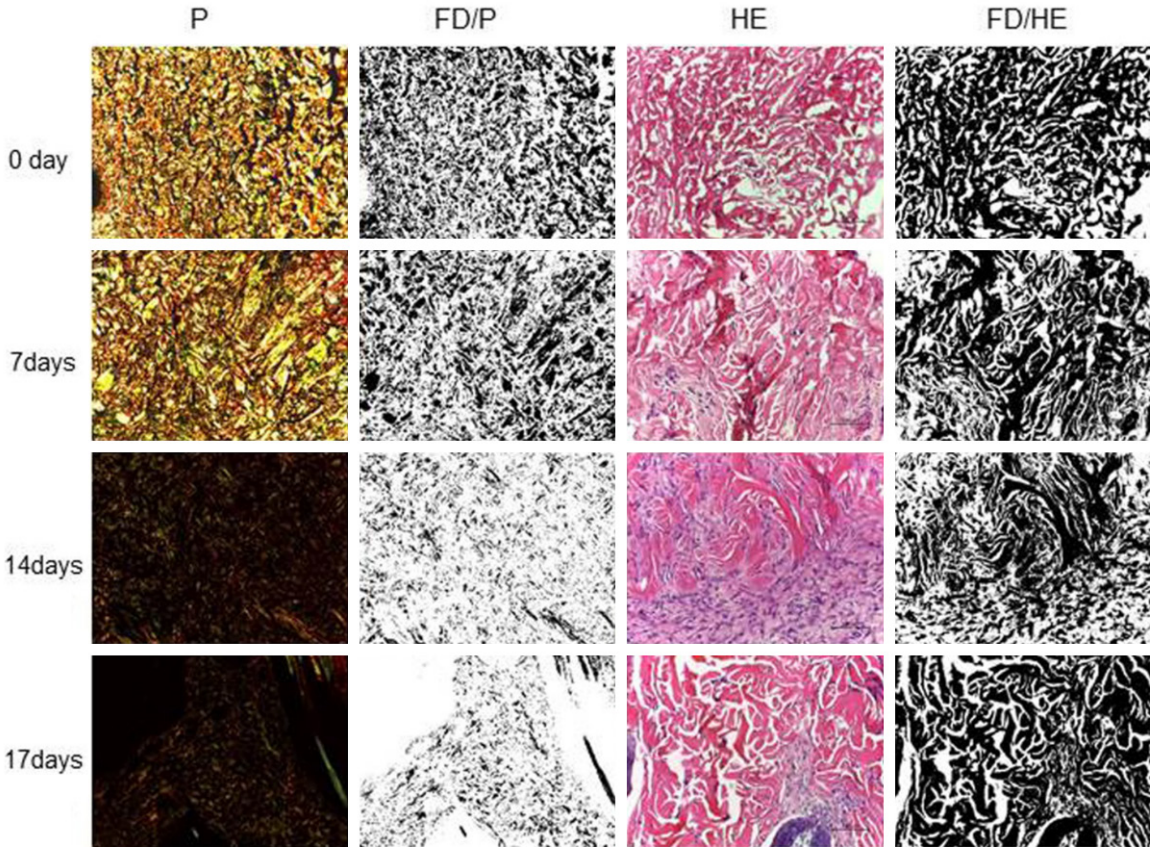
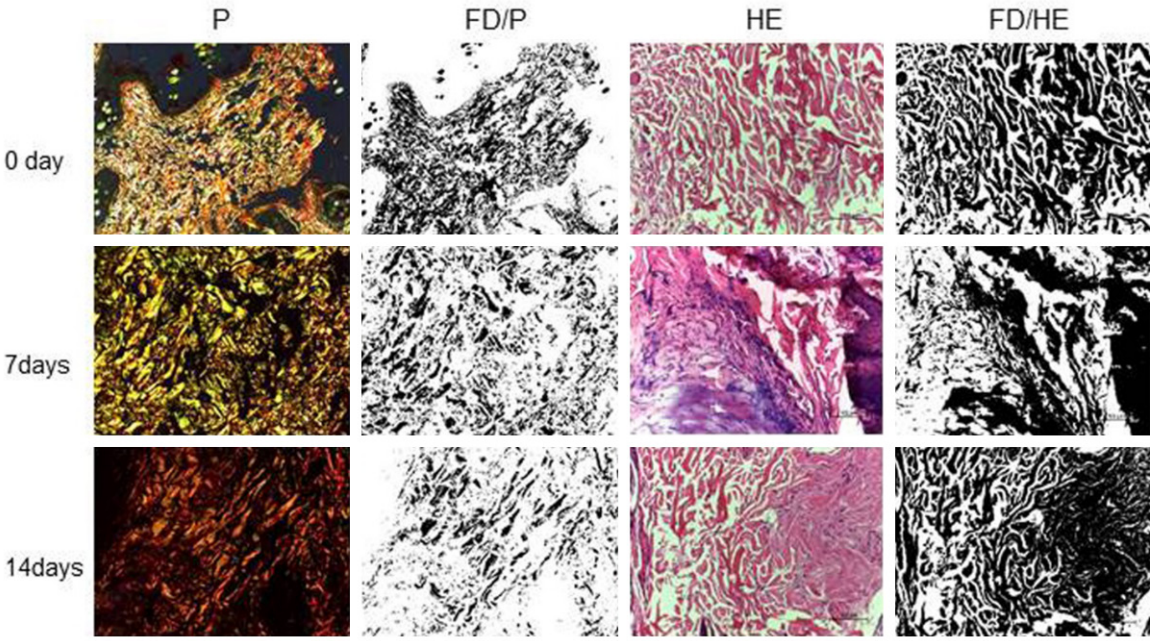


Figure 3. Microscopy and FD analysis of an experimentally induced wound treated with PRP in a rabbit, at days 0, 7, 14 and 17. Legend: P, picrosirius red; FD/P, fractal dimension values of slides stained with picrosirius red; H/E, haematoxylin and eosin; FD/H/E, fractal dimension values of slides stained with haematoxylin and eosin. Magnification 200×.



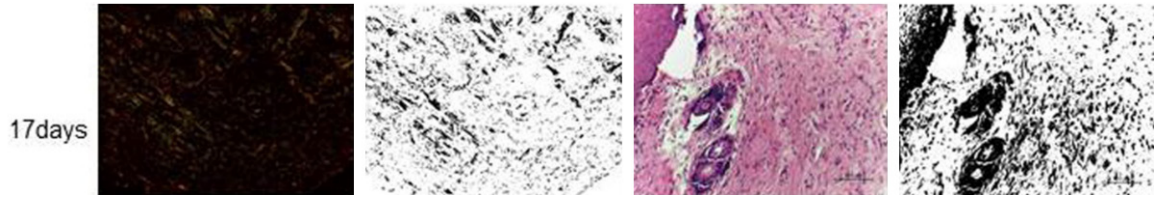


Figure 4. Microscopy and FD analysis of an experimentally induced wound treated with RSV in a rabbit, at days 0, 7, 14 and 17. Legend: P, picrosirius red; FD/P, fractal dimension values of slides stained with picrosirius red; H/E, haematoxylin and eosin; FD/H/E, fractal dimension values of slides stained with haematoxylin and eosin. Magnification 200×.

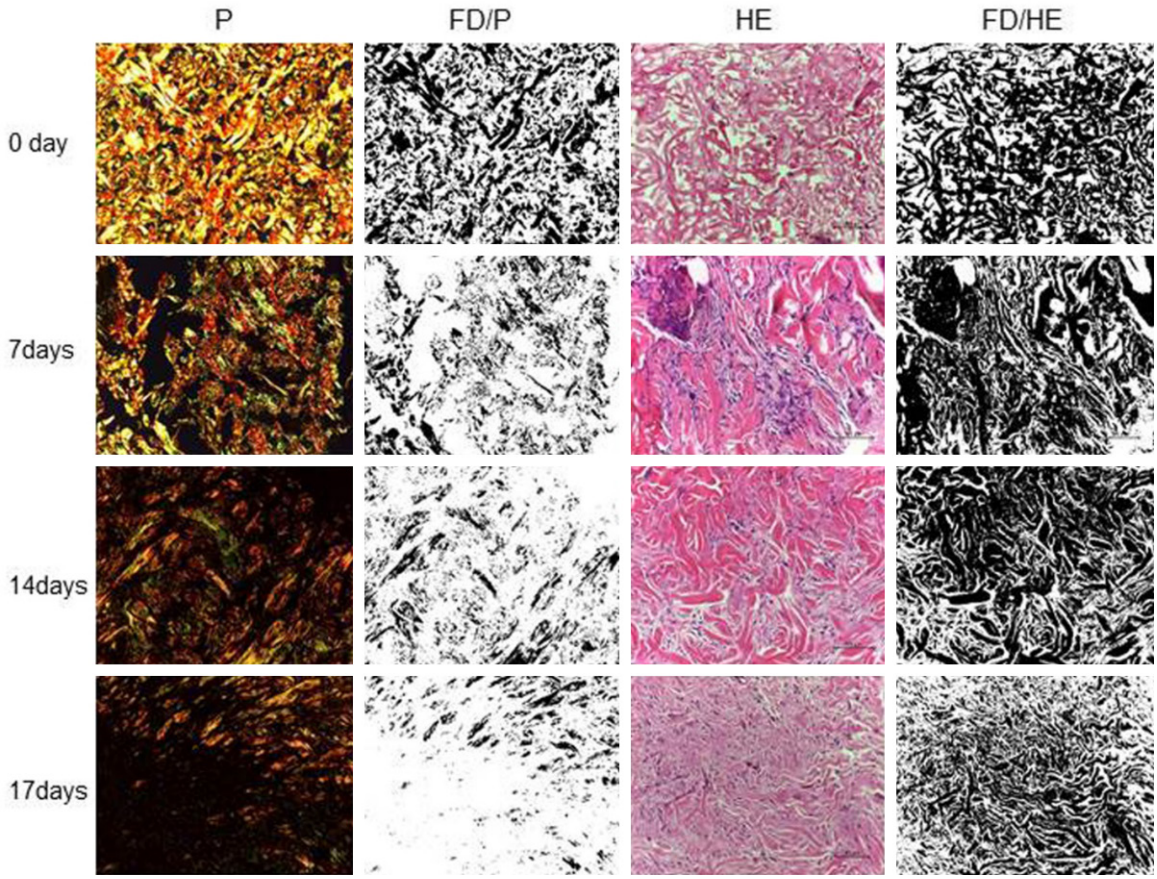


Figure 5. Microscopy and FD analysis of an experimentally induced wound treated with PRP and RSV combined in a rabbit, at days 0, 7, 14 and 17. Legend: P, picrosirius red; FD/P, fractal dimension values of slides stained with picrosirius red; H/E, haematoxylin and eosin; FD/H/E, fractal dimension values of slides stained with haematoxylin and eosin. Magnification 200×.

tube 200 µL of liquid PRP, 200 µL of liquid RSV and 100 µL of 10% calcium gluconate were combined. In both tubes, the final volume of PRP in gel was 0.5 mL.

Microscopy and imaging

To capture images, a polarised light microscope (Leica DM 750, Wetzlar, Germany) coupled to a camera (Leica ICC 50 HD) was used. The images were captured at 200× magnification under the microscope's maximum light intensity setting at a polarisation plane of 90 degrees.

FD analysis

To analyse skin FDs (**Figures 2**-**5**), photographs were binarised for reading and FD values were

Table 1. Mean and standard deviation of the collagen FD values of experimentally induced wounds in rabbits analyzed on days 0, 7, 14, and 17 treated with PRP, RSR, or RSV + PRP, Picrosirius red staining

Moments/Wounds	A	B	C	D
M0	1.73±0.01^{A,a}	1.73±0.01^{A,a}	1.73±0.01^{A,a}	1.73±0.01^{A,a}
M7	1.73±0.03^{A,a}	1.70±0.03^{A,a}	1.66±0.05^{A,b}	1.59±0.07^{B,b}
M14	1.50±0.07^{A,b}	1.45±0.04^{A,b}	1.47±0.05^{A,c}	1.45±0.04^{A,c}
M17	1.41±0.10^{A,b}	1.44±0.07^{A,b}	1.44±0.07^{A,c}	1.44±0.03^{A,c}

^{A,B}Means folowed by different capital letters indicate significant differences between treatments at the same time point. ^{a,b,c}Means followed by different lowercase letters indicate significant differences between time points for the same treatment. *P* value < 0.05. A, control; B, PRP; C, RSV; D, RSV + PRP.

Table 2. Mean and standard deviation of the FD values of experimentally induced wounds in rabbits comparing different days and different treatments, H/E staining

Moments/Wounds	A	B	C	D
M0	1.84±0.03^{A,a}	1.84±0.03^{A,a}	1.84±0.03^{A,a}	1.84±0.03^{A,a}
M7	1.81±0.09^{A,a}	1.85±0.03^{A,a}	1.66±0.05^{A,a}	1.59±0.07^{A,a}
M14	1.81±0.06^{A,a}	1.77±0.10^{A,b}	1.47±0.05^{A,a}	1.45±0.04^{A,a}
M17	1.82±0.05^{A,a}	1.88±0.02^{A,a,c}	1.44±0.07^{A,a}	1.44±0.03^{A,a}

^{A}Means folowed by different capital letters indicate significant differences between treatments at the same time point. ^{a,b,c}Means followed by different lowercase letters indicate significant differences between time points for the same treatment. *P* value < 0.05. A, control; B, PRP; C, RSV; D, RSV + PRP.

estimated by the box-counting method using freely available Image J software (National Institute of Health, USA-NIH; http://rsbweb.nih.gov/ij/). The software considers box-counting in two dimensions, and quantifies pixel distribution in the designated area but does not consider the image's texture. Thus, binarised images and grey level images with the same pixel distributions will have the same FD values. Therefore, the FD analysis of histologic slides was based on relationships between resolution and evaluated scale, and the results could be quantitatively expressed as the FD value of the object, defined as: log Nr/log r^{1} where Nr is the number of equal elements needed to fill the original object, and r is the scale applied to the object. Using this formula, the FD value calculated by the Image J software is always between 0 and 2, and is not affected by different textures.

Statistical analysis

The Shapiro-Wilk test determined that the datasets were not normally distributed, thus non-parametric statistical analyses were used. Control wounds and those of the different treatments were compared by the Kruskal-Wallis test, and within-group differences were assessed using the Student-Newman-Keuls method. All analyses considered P < 0.05 to be statistically significant.

Results

Platelet count

The mean number of platelets at the 17-day time-point was 128.6±28.9/mm^3 and the PRP reached 834.0±110.9/mm^3 exceeding 6 times the initial concentration as recommended by Vendramin et al. [17].

FD analysis

The collagen FD results of slides stained with picrosirius red and H/E are shown in **Tables 1** and **2**, respectively. In the slides stained with picrosirius red, there were significant differences between the control treatment and all of the treatment groups after 1 week, and the treatment group that differed most from the control treatment was the combined PRP + RSV treatment. From day 14 to day 17, there were no significant changes in the results of FD analysis of collagen between the different treatments. In the slides stained with H/E, at day 7 and day 14 there were significant reductions in collagen in wounds that received PRP alone ($P < 0.05$).

In the slides stained with picrosirius red, those derived from wounds treated with PRP + RSV exhibited lower collagen fibres FD values at day 7. In the control wounds there was no significant difference between days 0 and 7, but there were significant differences in the RSV alone group and the PRP + RSV group.

Discussion

Platelets play a central role in wound healing because they release growth factors that directly influence tissue repair, in addition to

cytokines and chemokines involved in the modulation of inflammatory responses [18]. PRP affects the secretion of collagenase, and stimulates the synthesis of collagen types I and III by fibroblasts, which are important structural components of mature scar tissue [19].

In the current study, RSV accelerated the initial wound healing process. A study by Karadeniz et al. [20] demonstrated benefits in lesions in otherwise healthy rats, with improved re-epithelialisation in the statin-treated groups.

Türer et al. [21] analysed the effects of local administration of RSV on calvarial bone defects filled with autogenous grafts in rats, and reported that 1 mg of RSV increased bone regeneration. No studies have evaluated the effects of a combination of RSV and PRP on the healing process. In general, the vast majority of statin studies have been restricted to investigating its lipid-lowering effects [22].

The initial reduction in FD values in the first 7 days after injury suggests that during this period the fibers are just beginning to reorganise; thus some gaps remain, and these gaps result in lower FD values. This observation was most evident in wounds treated with RSV, suggesting that RSV is useful during the first week after injury with regard to the reorganisation of collagen fibres and improved scar quality.

In conclusion, RSV alone and RSV combined with PRP were effective during the first week after injury. However, more studies are needed to evaluate increases in collagen associated with the administration of RSV and PRP in experimental wounds.

Disclosure of conflict of interest

None.

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