Orthopaedics & Traumatology: Surgery & Research (2010) 96, 381—387

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Orthopaedics & Traumatology Surgery & Research

ORIGINAL ARTICLE

Experimental study of tendon healing early phase: Is IGF-1 expression influenced by platelet rich plasma gel?

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Accepted: 1st March 2010

KEYWORDS
Platelet rich plasma;
Tendon healing;
Immunohistochemistry;
Growth factors;
IGF-1

Summary
Background: It is well established that growth factors play a critical role in the healing process of connective tissues. To our knowledge, there are no studies in literature concerning the influence of PRP on growth factors expression.
Hypothesis: The aim of this study was to assess the effect of a single application of platelet rich plasma (PRP) gel in a patellar tendon defect on the spatial and temporal expression of Insulin-like Growth Factor 1 (IGF-1) during tendon healing.
Materials and methods: Twenty-four animals were randomized to receive PRP (PRPFast, Bioteck) in a gel form (PRP group) and 24 to serve as untreated controls (Control group). A defect of 3 mm × 10 mm was surgically created on the tendon under general anaesthetic and in the PRP group, PRP gel was applied to fill the tendon defect whereas no treatment was applied in the control group. Six animals (12 limbs) from each treatment-group were sacrificed after one, two, three and four weeks following treatment. Histological and immunohistochemical staining were performed.
Results: Histology revealed a faster healing process in the tendons of PRP group in comparison with the controls. In the first 2 weeks of healing, IGF-1 was found intracellularly in various type cells, whereas in the last 2 weeks of healing, IGF-1 was detected mainly in tenocytes. Both cytoplasmic and nuclear expressions were present, whereas the larger amounts of immunoexpression were localized in both epitenon and endotenon.

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doi:10.1016/j.otsr.2010.03.010

A superior expression of IGF-1 was seen in PRP group compared with controls ($p < 0.0001$) in both the epitenon and endotenon at each time point except at 4th week of healing where a superior expression of IGF-1 was shown in the endotenon of control group, compared to the PRP group ($p < 0.0001$).
Conclusion: PRP may improve tendon defect healing by overexpression of IGF-1.
Level of evidence: Laboratory control animal study.

Introduction

An injury to the soft connective tissues such as the tendons results to the initiation of a complex wound healing cascade and finally the formation of a scar. It is known that the properties of this scar tissue are inferior when compared to those of normal tissue [1,2]. Therefore, investigators have utilized a variety of tissue engineering techniques in an attempt to improve these inferior properties. One of these techniques is platelet-rich plasma (PRP). PRP provides a pool of concentrated growth factors derived from platelets which are involved in the healing process of tissue defects as well as growth. One of these factors is Insulin Growth Factor 1 (IGF-1) which is a well established factor in the process of tissue healing.

Although there are a few studies which have demonstrated the positive effect of PRP in tendon healing [3], the way that PRP acts is not well documented. We have previously shown that PRP promotes angiogenesis in a patellar tendon defect in rabbits [4]. The aim of the study was to assess the effect on the spatial and temporal expression of Insulin Growth Factor 1 (IGF-1) in the early phase of tendon healing in the same experimental model following application of PRP. Our null hypothesis was that PRP has no influence in the spatial and temporal expression of IGF-1.

Materials and methods

All procedures in the animal experiments were approved by the regional ethical board, while institutional guidelines for the care and treatment of laboratory animals were adhered to. We used 48 skeletally mature New Zealand White rabbits, weighing an average of 3.5 kg. During the surgical procedure, a full thickness window defect of 3×10 mm was created in the mid-part of the patellar tendon. Twenty-four animals were randomized to receive PRP (PRPFast, Bioteck) in a gel form (PRP group) and 24 to serve as untreated controls (Control group). In the PRP group, PRP gel was applied and filled the tendon defect, whereas no treatment was applied in the control group. Six animals (12 limbs) from each treatment-group were sacrificed after one, two, three and four weeks following treatment.

Surgical procedure

The rabbits were anaesthetized with an intramuscular injection of xylazine (Rompun® Injectable, Bayer) at a dosage of 5—7 mg/kg and 0.15 mg of atropine (DEMO S.A.). Ten to 15 min later, ketamine (Imalgene®, Rhone Merieux, France) at a dosage of 12—15 mg/kg was injected intramuscularly. During surgery, supplemental sedation was given administered as required. Local anaesthesia 1 ml of a 2% lidocaine-adrenaline solution (AstraZeneca, UK) was applied at regular intervals at the site of incisions.

The surgical procedure was performed according to the animal model described by Anaguchi et al. [5]. The skin of the right knee was shaved and the operation was performed under aseptic conditions. Following that, a longitudinal skin incision was made on the skin overlying the middle of the patellar tendon. The superficial surrounding fascia was cut longitudinally to expose the patellar tendon. Thereafter, the deep fascia overlying the tendon was opened and a full thickness, 3 mm wide, and 10 mm long tendon substance was excised from the central portion of the patellar tendon with a specially designed knife that had two stainless-steel surgical blades. The 3-mm width of the defect is approximately equal to one-third of the width of the tendon. Four markers were placed at the corners of the defect with 5-0 nylon sutures to identify the location of the resected portion. The PRP gel was then applied and filled the tendon defect. The overlaying fascia was closed with a running suture of 4-0 nylon so that PRP gel applied into the resected portion would not flow out. Skin was closed with clips. The same procedure was performed in the opposite limb and the same procedure was performed in both limbs in the control group, without the application of PRP into the patellar tendon defect. No immobilization was applied after surgery, and the rabbits were allowed unrestricted daily activities in their cages.

PRP preparation

Eight milliliters of blood from an ear vein was collected in a tube, immediately after general anaesthesia. The blood was allowed to stand for 15 min in order to reduce platelets activation during centrifugation. Once centrifugation was complete, the upper half was considered Platelet Poor Plasma (PPP) and was removed by using sterile pipettes. The lower half, the PRP, was retrieved using a pipette by aspirating up to the interphase zone (consisting of blood cells) and was then placed into another glass tube. Two milliliters of PRP was collected for every 8 ml of blood. The PRP was applied in a gel form, manufactured by adding 0.5 ml of procoagulant solution in the tube with the liquid PRP and allowing approximately 15 min for the solution to become a gel.

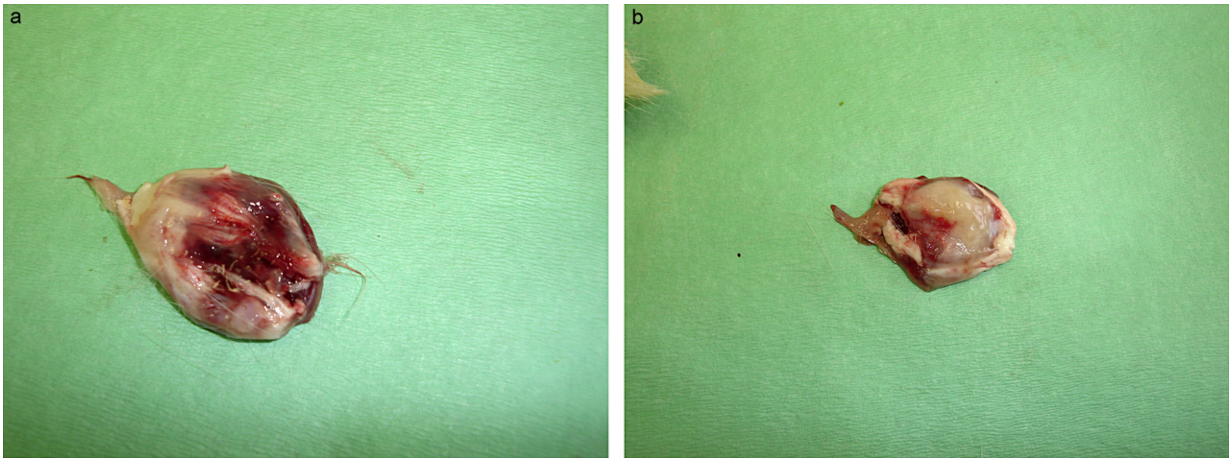

Figure 1 Presentation of a patellar tendon specimen (1st week postoperatively) from (a) control group and (b) PRP group. Macroscopically, a blood clot with a granular tissue is filling the gap in the control group whilst in the PRP group the granular tissue is more prominent.

Sample harvesting

At each time point, the animals were sacrificed with an overdose of intracardiac injection of 10% KCl solution under general anaesthesia. The entire patellar tendon was then removed and dissected free from other tissues (Fig. 1). Then, each tissue was fixed in a 10% buffered formalin solution and cast in a paraffin block. The tendon was sectioned transversely to the longitudinal axis, and stained with hematoxylin and eosin. From each tendon repair site, 12 paraffin sections were made. Of them, three sections were subjected to microscopic examination, while the other nine were immunostained with an anti-IGF-1 primary antibody. All sections were randomly selected and analyzed at both the endotenon as well as epitenon sites by a single pathologist, who was blinded to the treatment groups.

Immunohistochemical staining

Immunohistochemistry was applied on 4 μm thick sections which were obtained from the paraffin blocks and placed on positively charged glass slides. The last ones were left to dry at 37 °C overnight. Deparaffinization was achieved using xylene, followed by rehydration through graded alcohols. For the antigen retrieval, slides were treated in citrate buffered solution 10 mM at pH 6.0 in two cycles of 15 min each. Endogenous peroxidase activity was blocked by immersing the slides in 3% hydrogen peroxide for 20 min at room temperature. Primary antigen, (anti-IGF-1 clone OBT 1090G, AbD Serotec, US), was applied in a dilution 1:100 overnight. A two-step technique (Envision K-5000, Dako Glostrup, Denmark) was used and the bound antibodies were visualized using 3,3′ diaminobenzidine tetrahydrochloride (DAB) as chromogen. Hematoxylin was used for counterstaining. For negative controls, the primary antibody was omitted and replaced by buffered saline serum. For the quantitative analysis, the slides were digitized using a light microscope (Nikon Eclipse 80i, Nikon Corp, Tokyo, Japan) and the images were montaged with the appropriate software (Adobe Photosop CS3®). Then, using semi-automated computerized image analysis with Image ProPlus v5.1 (Media Cybernetics, MD, USA), we evaluated the density of brown diaminobenzidine (DAB) staining, a well-established technique as described in Zafirellis et al. [6]. Values for color density have a range from 0 (black) to 255 (white). Positive and negative control slides were used for the optimal separation of brown and blue stained areas.

Statistical analysis

All results are expressed as mean ± SD. Significant differences among groups were evaluated using the Mann-Whitney U test. A difference of $p < 0.05$ was considered to be statistically significant (SPSS 11.5.0).

Results

Histology

The endotenon had a normal appearance in both groups at all time-points, while the epitenon was thickened from two to 5—6 cell layers by the 4th week time-point. Thickening was more obvious near the repair site. At the 1st and 2nd week time-points, the histological appearance of the repair site was similar in both groups. A blood clot with a granular tissue filled the gap at the 1st week (Fig. 1) with intense mononuclear infiltrates, newly formed vessels and collagen fibers were mixed with plumbed tenocytes which also lost their normal orientation (Fig. 2). At the 2nd week, fibroblast-like cells were obvious and there was inconsistent neoformation of blood vessels with rare presence of collagen fibers and fibrosis. At the 3rd week time-point, there were noticeable differences between the two groups. In the control group the formed tissue remained more immature, with a less compact synthesis absence of tenocyte longitudinal orientation. On the other hand, in PRP group the tissue was denser with fewer less elastic fibers remaining and better tenocyte orientation. Finally, at the 4th week time-point an almost healed tendon with some cellular activity was observed in the control group and a completely healed tendon in PRP

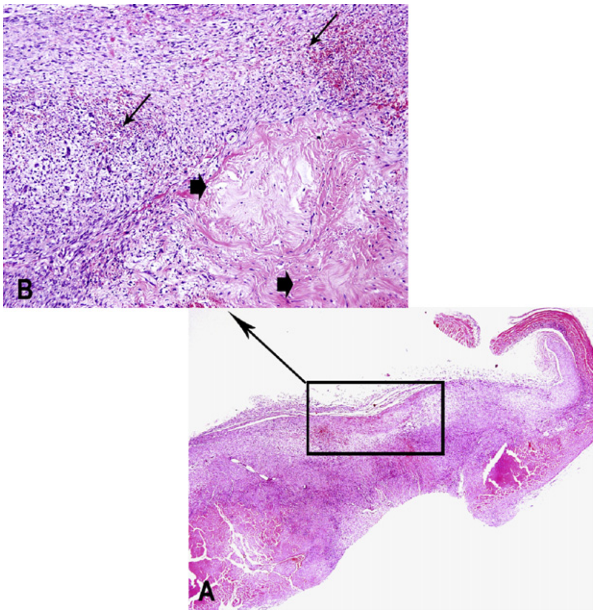

Figure 2 Patellar tendon at 1st week from PRP group (Hematoxylin-Eosin stain). (A) Low power field (20 × original magnification) showing thickening of the epitenon cell layer which fills the wounded site. (B) Under higher magnification (200 ×), a granular tissue is identified (arrows) around the repair site, while the tendon (arrowheads) is disorganized.

group (no clear visible border between repair site and rest of tendon; absent cellular activity). Furthermore, in PRP group the repair tissue which was bridging the gap became more fibrous with increased numbers of more distinct oriented fibroblasts which established a more firm lesion. The number of vessels decreased in both groups indicating the completion of healing process.

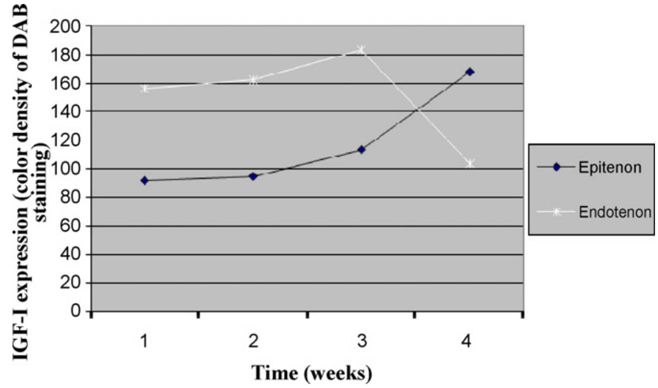

Figure 4 Presentation of IGF-1 expression in the areas of epitenon and endotenon in control group during the healing period. Note that large values of the color density indicate low IGF-1 expression and conversely.

Immunohistochemistry

Spatial expression of IGF-1

In the first 2 weeks of healing, IGF-1 was found mainly in inflammatory cells, endothelial cells, macrophages from the granular tissue which filled the surgical gap and in irregularly shaped tenocytes. In the last 2 weeks of healing, IGF-1 was detected mainly in tenocytes. Both cytoplasmic and nuclear expressions were present, whereas the larger amounts of immunoexpression were localized in both epitenon and endotenon.

Temporal expression of IGF-1

A gradual decrease of IGF-1 was observed during the healing period in both groups. PRP group showed a superior expression of IGF-1 during the first 3 weeks of healing in comparison with the controls, whereas control group showed a superior expression at the 4th week (Fig. 3). Furthermore, in control group during the first 3 weeks of healing a superior expression of IGF-1 was demonstrated in epitenon in comparison with endotenon ($p < 0.0001$), whereas at 4th week a superior

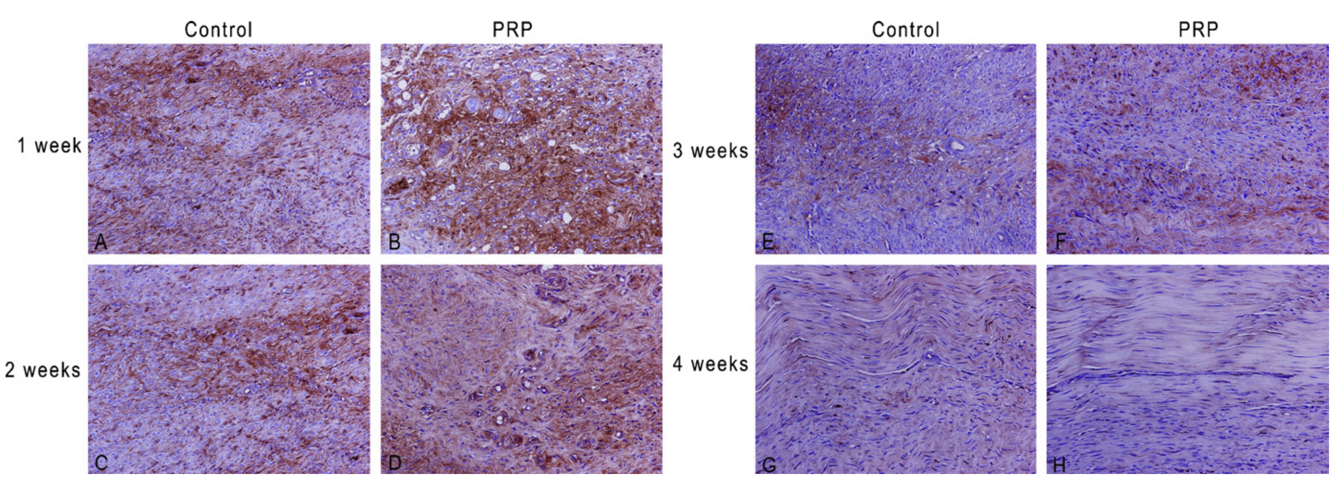

Figure 3 Immunohistochemical detection of IGF-1 in the endotenon, as represented by brown diaminobenzidine staining, 200 × original magnifications. PRP group (right column) expresses larger amounts of IGF-1 at the first 3 weeks compared to control group, while the last one has stronger expression than PRP group at 4th week.

Table 1 Image analysis of color density of the areas of epitenon and endotenon positively stained with IGF-1 in both control and PRP groups. Note that large values of the color density indicate low IGF-1 expression and conversely.

	Control		
Healing period	Epitenon mean color density ± SD	Endotenon mean color density ± SD	p^*
1st week	91.67 ± 10.22	156.39 ± 12.24	< 0.0001
2nd week	94.62 ± 9.42	162.48 ± 12.83	< 0.0001
3rd week	113.45 ± 6.28	183.67 ± 9.75	< 0.0001
4th week	167.92 ± 12.13	103.31 ± 9.25	< 0.0001
	PRP		
Healing period	EpitenonMean color density ± SD	EndotenonMean color density ± SD	p^*
1st week	65.77 ± 6.53	124.32 ± 12.43	< 0.0001
2nd week	69.54 ± 7.54	129.76 ± 7.46	< 0.0001
3rd week	90.33 ± 3.98	151.01 ± 10.71	< 0.0001
4th week	119.76 ± 11.62	184.33 ± 14.12	< 0.0001

*$p < 0.05$ indicates a significant difference between the two groups.

expression was showed in endotenon ($p < 0.0001$) (Table 1, Fig. 4). In PRP group, IGF-1 was significantly increased in the epitenon in comparison with the endotenon in the whole healing period ($p < 0.0001$) (Table 1, Figs. 5 and 6).

In the first 3 weeks of healing, IGF-1 in PRP group was significantly overexpressed in comparison with the controls in both epitenon and endotenon ($p < 0.0001$) (Table 2, Figs. 7 and 8). At 4th week, although IGF-1 in PRP group was significantly underexpressed in endotenon ($p < 0.0001$), there was a persistent significant increase of the growth factor in epitenon in comparison with the control group ($p < 0.0001$) (Table 2, Fig. 7).

Discussion

Our results showed that IGF-1 expression was present during the whole healing process in both PRP and control group. A superior expression of IGF-1 in the epitenon was demonstrated, in comparison with the endotenon in both groups during the first 3 weeks of healing. At the 4th week, PRP group showed a superior expression of IGF-1 in epitenon, in comparison with endotenon. Furthermore, histological evaluation revealed a superior healing process in PRP group compared with the controls.

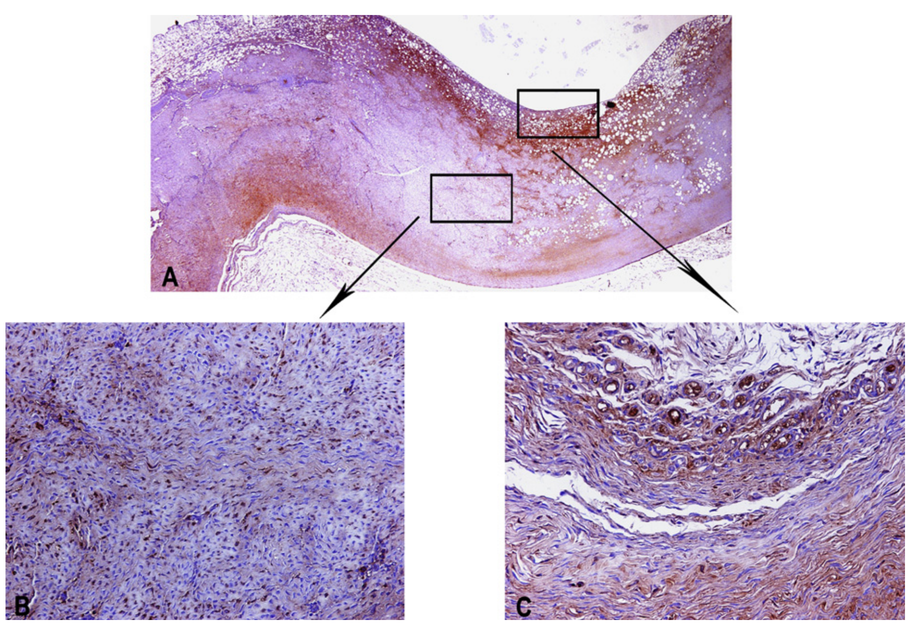

Figure 5 Patellar tendon immunostained for IGF-1 2 weeks after surgery. (A) demonstrates stronger positivity for the antibody in the epitenon cell layer compared to endotenon under 20 × original magnification. (B) A more detailed snapshot from endotenon and (C) from epitenon under 200 × magnification.

Table 2 Comparisons of the expression of IGF-1 between PRP and control group in both the area of epitenon and endotenon. Note that large values of the color density indicate low IGF-1 expression and conversely.

	Epitenon		
Healing period	Control mean color density ± SD	PRP mean color density ± SD	p*
1st week	91.67 ± 10.22	65.77 ± 6.53	< 0.0001
2nd week	94.62 ± 9.42	69.54 ± 7.54	< 0.0001
3rd week	113.45 ± 6.28	90.33 ± 3.98	< 0.0001
4th week	167.92 ± 12.13	119.76 ± 11.62	< 0.0001
	Endotenon		
Healing period	Control mean color density ± SD	PRP mean color density ± SD	p*
1st week	156.39 ± 12.24	124.32 ± 12.43	< 0.0001
2nd week	162.48 ± 12.83	129.76 ± 7.46	< 0.0001
3rd week	183.67 ± 9.75	151.01 ± 10.71	< 0.0001
4th week	103.31 ± 9.25	184.33 ± 14.12	< 0.0001

*$p < 0.05$ indicates a significant difference between the two groups.

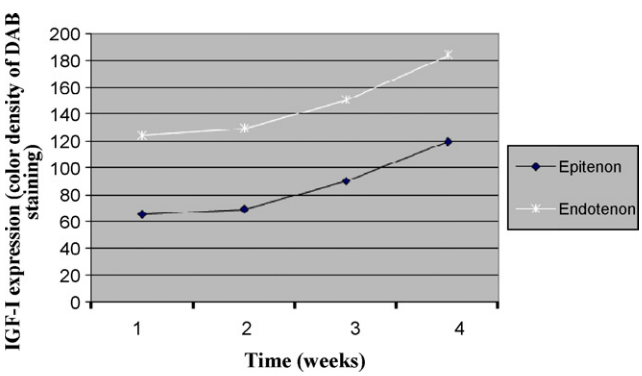

Figure 6 IGF-1 expression in the areas of epitenon and endotenon in PRP group during the healing period. Note that large values of the color density indicate low IGF-1 expression and conversely.

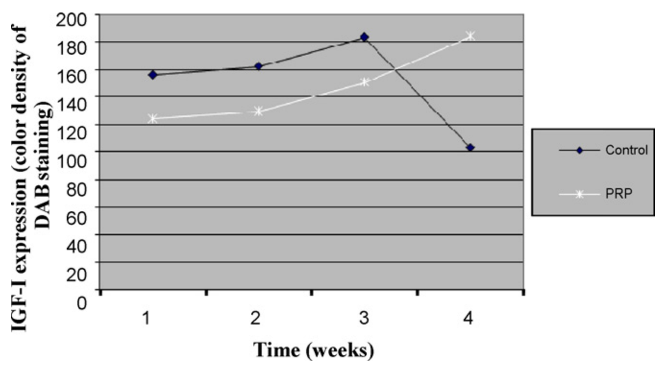

Figure 8 Presentation of the expression of IGF-1 in PRP and control group in the area of endotenon. Note that large values of the color density indicate low IGF-1 expression and conversely.

The immunohistochemical expression of IGF-1 in the whole early phase of healing, the intracellular expression in various cell types, the localization of the growth factor in the epitenon and the repair site, and the gradually decrease of the expression of IGF-1 in both groups, as demonstrated in our study, are in agreement with previous reports [7—9].

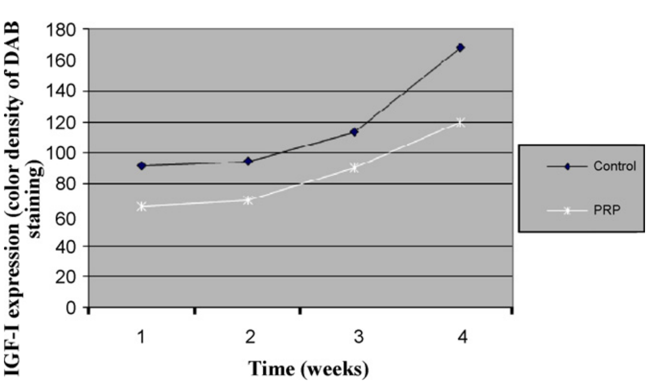

Figure 7 Presentation of the expression of IGF-1 in PRP and control group in the area of epitenon. Note that large values of the color density indicate low IGF-1 expression and conversely.

Furthermore, the superior expression of IGF-1 in the epitenon, in comparison with the endotenon was previously explained by Gelberman et al. They demonstrated that when no sutures were placed in the tendon's cut edges, and no immobilization was applied postoperatively, there was minimal response from the endotenon and a greater initial response from epitenon fibroblasts [10,11].

Another important finding in our study is that at the 4th week, PRP alter the spatial expression of IGF-1, demonstrating a superior expression in epitenon, whereas controls showed a superior expression in endotenon. Sharma and Maffulli suggested that at the initial stage of the healing process, collagen is produced by epitenon cells, whereas endotenon cells synthesized collagen later [12]. It is known that IGF-1 is a stimulator of collagen production [13], and the inversion of this phenomenon in the PRP group suggests a role for PRP in mechanisms of tendon healing. Furthermore, IGF-1 is known to be a potent stimulator of cell proliferation [13]. The change in the IGF-1 expression in the endotenon as observed in our study seems to be in keeping with cellular activity. For example, at the 4th week in the PRP group where no cellularity was histologically found, the IGF-1 expression is lower than the control group where some cellularity is still present suggestive of a degree of healing process. The most likely explanation is that PRP provides a pool of growth factors (including IGF-1) that help expedite the healing process which certainly seems to happen at a faster rate in histological terms. Correlation between IGF-1 expression and cellularity is more difficult to assess in the epitenon as there was a small increase in the number of cell layers in both groups at 4 weeks which indicate cellular activity but difficult to make further assessments.

The positive effect of PRP in tendon healing was previously showed in vitro and in vivo. In a study on humans, tenocytes cultures was demonstrated that PRP stimulates cell proliferation and total collagen production, and slightly increases the expression of matrix-degrading enzymes and endogenous growth factors [14]. On the other hand, Kajikawa et al. showed that locally injected PRP in a rat patellar tendon injury model is useful as an activator of circulation-derived cells for enhancement of the initial tendon healing process [15]. Finally, PRP promotes angiogenesis in the healed tendon [4], whereas various studies demonstrated the positive effect of PRP in the mechanical properties of the healed tendon [3,16—18].

These studies are in accordance with our findings, concluding that PRP enhances and accelerates the tendon healing process. PRP contains a wide variety of growth factors. At the site of an injury, the platelets are activated and release their growth factors and those in turn stimulate the healing process. Platelets normally initiate to a formation of scar. Tendon healing can be regarded as refined from scar formation, and it therefore seems reasonable to assume that the addition of an increased number of platelets might improve the process. Further investigation with possibly longer follow-up of other growth factors contained in PRP, and biomechanical evaluation of the healed tendons under the influence of PRP are necessary in order to understand and establish the way that PRP acts in tendon healing. Another potential clinical application of the results of this study in combination with biomechanical and histological data of other studies [4,18] is the healing and rehabilitation timing of the surgically created patellar tendon defect during graft harvesting for anterior cruciate ligament reconstruction. This would obviously require appropriate clinical trials.

Conflict of interest

None (for all authors).

Acknowledgements

Authors would like to thank Perikles Kakias, Vissaris Kakias and Maria Kemerli for their contribution in tissue preparation, and Bioteck for offering PRPFast.

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