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The Effects of Platelet-Rich-Plasma (PRP) Injection On Ligament Injury

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Background: Soft tissue injuries are becoming a problem especially among active people and athletes, thus doctors are focusing on PRP injection however variety of study results are making the beneficial effects of PRP towards soft tissue healing unclear. The purpose of this study is to clarify the effects of PRP injection on the healing aspect of a ligament injury. **Methods:** The experimental study used European rabbit (*Oryctolagus cuniculus*), which was divided into the control and treated group. The 22 samples, ankle ligaments were injured. The treated group was injected with 4 ml PRP taken autologously. On the 2nd day the samples were examined for hematoma. 4 samples from each group were also examined histopathologically. On the 2nd week, the ligament thickness of the remaining samples from each group was examined ultrasonographically. **Results:** The clinical result showed lower presence of hematoma on the group injected with PRP compared to the control group. The Capillary dilation was less on the treated group compared to the control group. The Inflammatory cells were less on the treated group compared to the control group. The Fibroblasts Cells was less on the treated group compared to the control group. The fibrocytes was more abundant on the treated group compared to the control group. The average of Ligament Thickness was thicker on the treated group compared to the control group. **Conclusion:** The Injection of PRP is beneficial to a ligament injury based on its effect on enhancing healing on the inflammation, regeneration, and remodeling phases.

Keywords: PRP, Inflammation, Healing, Ligament Injury.

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INTRODUCTION

Soft tissue injuries are common among active people especially athletes. The problem is tackled daily by specialists from the Sports Injury department. The time it takes for the tissue to heal is becoming one of the major concerns regarding the injury. The concerns lead doctors to focus on certain methods, one of which is the use of Platelet-Rich-Plasma (PRP). One of the examples is the use of the method to treat tendinosis, which showed promising results[1]. Some of the substances in PRP are said to produce anti-inflammatory effects[2] as well as pro-inflammatory effects[3]. PRP is also claimed to be beneficial in the regeneration as well as the remodeling phase of an injury.

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Remodeling phase, which majorly comprises of collagen III production by fibroblasts, is aided by the presence of extra growth factors in the PRP[4, 5].

Despite the interest towards the method, and apparent widespread use, there is a lack of high-level evidence regarding randomized clinical trials assessing the efficacy of PRP in treating ligament and tendon injuries[6]. Some studies have demonstrated that increased cellularity during early wound healing may have a positive impact on ligament and tendon[7]; however, other studies have found no such relationship[8]. This creates a problem in which physicians are unsure whether the treatment of PRP is justified.

The varieties of study results are making the beneficial effects of PRP towards soft tissue healing quite unclear[9].

Ankle injury is the most common musculoskeletal injury[10]. Therefore, we would like to focus the soft tissue injury to an ankle ligament injury as tendon and ligament injuries are ones of the most common health problems affecting the adult population[11].

The purpose of this study is to clarify the effects of PRP through an experimental study on rabbits by comparing the soft tissue healing on several groups with a variety of treatments after purposely injuring the rabbit.

MATERIALS AND METHODS

This is a True Experimental Study, performed to prove the effects of Platelet-Rich-Plasma (PRP). The study uses the animal *Oryctolagus cuniculus.* They were purchased from the Tropical Disease Center of Airlangga University, Surabaya. The rabbits were adapted to their cages for 2 weeks before the study starts. The population consists of male domestic rabbits (Oryctolagus cuniculus) aging 4-6 months, each weighing 2500-4500 grams; appear healthy without any presence of disease or abnormality. The rabbits will be kept in the Institute of Tropical Disease of the University of Airlangga, Surabaya.

All of the rabbits will be conditioned well based on the environment that they are living in as well as the food and water. With the minimum size of 16 samples, the researcher chose 32 samples for both the control and treatment groups. Rabbits were chosen because the animals are suitable to represent the condition of soft tissue injury on humans as mammalian models. Other advantages are the fact that the animals are: docile and non-aggressive, easily bread, cheaper, and having short vital cycles. Due to the death of 10 samples during the adaptation, 22 samples are used and divided into control and treated group. First, all of the samples are injured by spraining the ankle. This was performed by over endorotation, inversing, and plantar flexion. Before the procedure was performed all of the samples were injected with 3 ml of lidocaine as a local anesthesia then, the treated group was injected with 4 ml PRP taken from 10 ml of whole blood.

On the 2nd day, all of the samples of the group were examined clinically through the presence of hematoma. Afterwards, 4 samples from each group were sacrificed to be examined through a histopathological examination using Hematoxylin and Eosin staining with 100 times magnification to examine the inflammatory phase through the parameter of: amount of inflammatory cells, and capillary dilation, as well as to examine the regeneration and remodeling phase through examining the amount of fibroblasts and fibrocytes. Afterwards, the 8 samples were euthanized by using ether.

On the 14th day, all of the remaining samples' ligament thicknesses were examined using ultrasonography. The data was taken in the form of percentage compared to a healthy and intact contralateral ankle ligament.

The days of each examination process were chosen earlier than the healing phase should take place naturally based on the theory of healing to actually see the effects of PRP without the possibility of the physiological process of healing taking place regardless of the action of PRP.

The comparison between two groups of the positive and negative results of hematoma were analyzed using chi square, whereas the numerical data of inflammatory cells, capillary dilation, fibroblasts, fibrocytes, and ligament thickness were analyzed using paired sample t-test. All of the analyzes were processed using IBM the IBM SPSS 22 with the degree of validity of 95%.

RESULT

The description of both treatment and control group can be seen on table 5.1 and table 5.2 and for further analysis and the p-value it can be seen on table 5.3.

Table 5.1. Description of control group of *Oryctolagus cuniculus*

	Hematoma (+/-)	Capillary Dilation (per Field of View)	Inflammatory Cells (per Field of View)	Fibroblast cells (per Field of View)	Fibrocytes (per Field of View)	Ligament Thickness (millimeters-percentage)
N	8	4	4	4	4	4
X	2	2	71.5	9.75	27	22.5
SD	0	0	5.196	1.708	3.916	22.825

Table 5.2. Description of treatment group of *Oryctolagus cuniculus*

	Hematoma (+/-)	Capillary Dilation (per Field of View)	Inflammatory Cells (per Field of View)	Fibroblast cells (per Field of View)	Fibrocytes (per Field of View)	Ligament Thickness (millimeters-percentage)
N	14	4	4	4	4	10
X	1.21	1	19.75	6.75	36	58.71
SD	0.426	0	2.754	1.258	1.414	21.83

As seen from table 5.1 and table 5.2 the result shows a statistically higher amount of hematoma of the group samples, which wasn't given a PRP injection, compared to the group, which was given the injection with a significant p-value, which can be seen on table 5.3. As seen from table 5.1 and table 5.2 the result shows a statistically higher amount of capillary dilation of the group sample, which wasn't given a PRP injection, compared to the group, which was given the injection with a significant p-value, which can be seen on table 5.3.

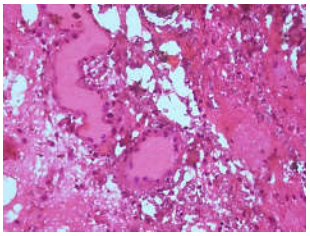

Figure 5.1
An abundance of dilated capillaries on the tissue taken from the control sample

As seen from table 5.1 and table 5.2 the result shows a statistically higher amount of inflammatory cells of the group sample, which wasn't given a PRP injection compared to the group, which was given the injection with a significant p-value, which can be seen on table 5.3.

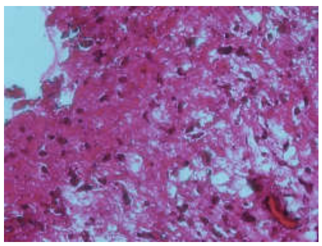

Figure 5.2
An abundance of inflammatory cells on the tissue taken from the control sample

As seen from table 5.1 and table 5.2 the result shows a statistically higher amount of fibroblasts of the group sample, which wasn't given a PRP injection, compared to the group, which was given the injection with a significant p-value, which can be seen on table 5.3.

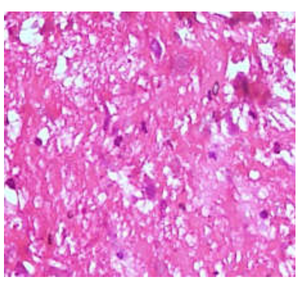

Figure 5.3
A number of fibroblasts on the tissue taken from the control sample

As seen from table 5.1 and table 5.2 the result shows a statistically lower amount of fibrocytes of the group sample, which wasn't given a PRP injection compared to the group, which was given the injection with a significant p-value, which can be seen on table 5.3.

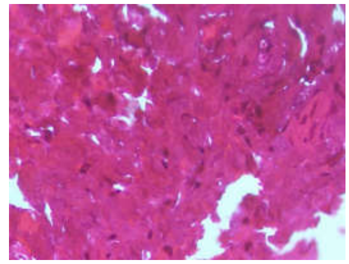

Figure 5.4
A number of fibrocytes on the tissue taken from the treated sample

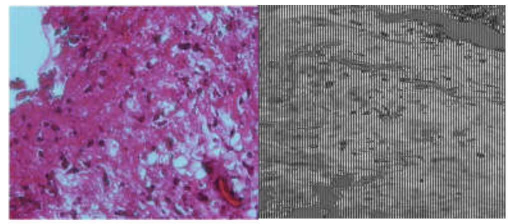

Figure 5.5
Comparison of overall inflammatory process: Control group (above) compared to Treated group (below)

As seen from table 5.1 and table 5.2 the result shows a statistically lower ligament thickness of the group sample, which wasn't given a PRP injection compared to the group which was given the injection with a significant p-value, which can be seen on table 5.2.

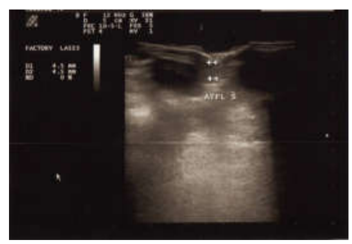

Figure 5.7
No hypoechoic area on the Anterior Tibiofibular Ligament (ATFL) of the rabbit's ankle, ligament is 100% intact
[taken from one of treated samples]

Figure 5.8
0.96 mm hypoechoic area on the Anterior Tibiofibular Ligament (ATFL) of the rabbit's ankle from the total ligament of 3.7 mm, ligament is 74.1 % intact [taken from one of treated samples]

Figure 5.9
0.85 mm hypoechoic area on the Anterior Tibiofibular Ligament (ATFL) of the rabbit's ankle from the total ligament of 2.9 mm, ligament is 70 % intact [taken from one of treated samples]

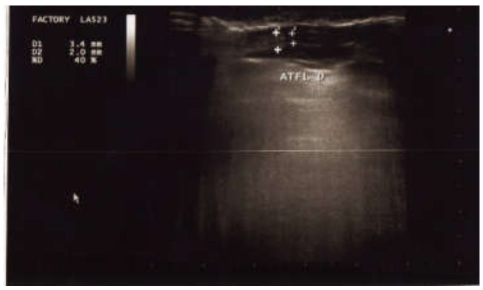

Figure 5.10
2 mm hypoechoic area on the Anterior Tibiofibular Ligament (ATFL) of the rabbit's ankle from the total ligament of 3.4 mm, ligament is 41.2 % intact [taken from one of treated samples]

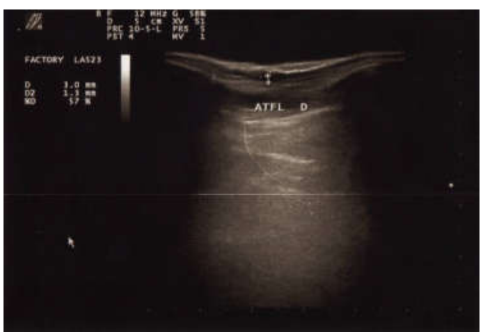

Figure 5.11
1.3 mm hypoechoic area on the Anterior Tibiofibular Ligament (ATFL) of the rabbit's ankle from the total ligament of 3 mm, ligament is 57 % intact [taken from one of control samples]

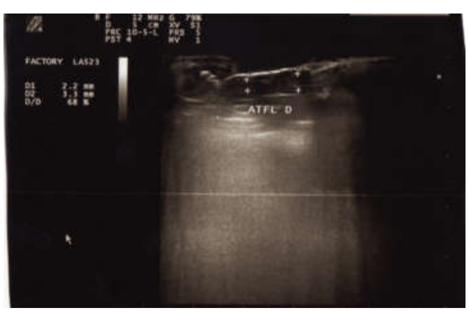

Figure 5.12
Total tear of Anterior Tibiofibular Ligament (ATFL) of the rabbit's ankle, ligament is 0% intact [taken from one of control samples]

Table 5.3
Both treatment and control group of Oryctolagus cuniculus Analysis and p-value

Variable	NaCl 0.9 %	PRP 4 ml	p-value
Hematoma (+/-)	2	1.2 ± 0.4	0.001
Capillary Dilation (amount per Field of View)	2	1	0.029
Inflammatory Cells (amount per Field of View)	71.5 ± 5.2	19.8 ± 2.8	<0.001
Fibroblast Cells (amount per Field of View)	9.8 ± 1.7	6.8 ± 1.3	0.003
Fibrocytes (amount per Field of View)	27 ± 3.9	36 ± 1.4	0.005
Ligament Thickness (millimeters-percentage)	22.5 ± 22.8	58.7 ± 21.8	0.017

DISCUSSIONS

Effect of Platelet Rich Plasma on Inflammation

The result, when compared between the treatment group and the control, as seen previously from the result pages, is proven to be significant with the p value of $p<0.05$. Upon Injury towards the ligament, the first phase of healing to take place was the inflammation phase. The phase usually last for 24 to 72 hours, even 2 weeks for some pathological cases[12]. The examination was performed at the 2nd day to examine whether the process has already taken place or not.

Theoretically, during the examination, the ligament tissue will still be in the inflammation phase. However, PRP contains both pro-inflammatory and anti-inflammatory mediators[3].

The pro-inflammatory mediators will initiate and support the process through several ways, for example neutrophil attraction by IL-8[13].

The anti-inflammatory mediators, on the other hand, limit the process to prevent the phase from extending by reducing the activity of NF-κB, an example of one of many mechanisms[2]. Theoretically, the treatment group, which was injected with PRP, would have had a shorter, more successful inflammatory phase compared to the control group. In order to examine the phase, the histopathological examinations were focused on the amount of neutrophils as inflammatory cells, and the abundance of dilated vessels in the area. The clinical examination is based on one of the cardinal signs of inflammation, which is swelling or hematoma.

Bradykinins contribute to the prolonged vascular permeability. Cytokines like prostaglandins are also produced; they will be released with any breach of the cell membrane integrity. The permeability leads to extravasation that causes edema[14]. Based on the result, it can be seen that all 8 subjects of the control group are positive with hematoma, whereas the treatment group which consists of 14 subjects; 3 were presented with hematoma at the 2nd day while the rest didn't show any signs of swelling. This shows that those injected with PRP have a shorter period of swelling compared to the ones not injected.

The injected group was also shown to have less dilated capillaries compared to the control group, which could be seen in the result. The result shows that all 4 subjects of the injected group have normal capillaries overall compared to the 4 subjects of the control group, which have dilated capillaries. This shows that the process is still taking place, the dilated capillaries occur as a response of injury.

The cells around the area would release histamine, which would cause a brief vasodilation in the non-injured vessels. This would create a combination of whole blood exudate and serous transudate, which is responsible for the red, swollen, hot, and painful environment. If there are blood vessels that are also cut during the injury, they will pour whole blood to the wounded area. The non-injured vessels will dilate in response to the signals produced by the injured tissue[12]. Certain prostaglandins could extend the period of long-term vascular vasodilation[14]. The vasodilation on the control group shows that prostaglandins are still heavily produced in the area.

The amount of inflammatory cells, in this case neutrophils, is the most representative of showing whether or not a tissue is still in the phase of inflammation. This is due to the fact that neutrophils are the first cells to respond to an injury[14]. The treatment group shows 19.8 ± 2.8 neutrophils for each field of view, whereas the control group shows 71.5 ± 5.2 neutrophils for each field of view. This shows that most of the subjects of the group which was injected with PRP show that the inflammation phase is no longer taking place compared to the control group which show that most subjects are still heavily undergoing the process.

Effect of Platelet Rich Plasma on Regeneration and Remodeling

The result, when compared between the treatment group and the control, as seen previously from the result pages, is proven to be significant with the p value of $p<0.05$. Theoretically, after the inflammation phase, regeneration and repair phase will take place followed by the remodeling phase (Hardy, 1989). As mentioned in the literature review, the process is stimulated by certain growth factors like PDGF, TGF-β, and VEGF[15]. PRP works as a stimulation of this phase as it contains these substances[16].

The process of regeneration could last up to 3 to 6 weeks with the end result of repaired tissue wound. The ankle ligaments of the subjects from both groups were examined using ultrasonography at the 2nd week to see if there's a difference in the rate of healing. Theoretically, in the 2nd week, the process will still be at its earliest stage. However, the result from the subjects of the treatment group shows that most of the subjects displayed a positive sign of healing shown by the thickness of the ligament with a mean of 58.7 ± 21.8 mm. This is far better compared to the control group, which has a 22.5 ± 22.8 mm ligament thickness. This finding is consistent with the fact that the growth factors delivered by PRP are heavily involved in the repair of ligament injury[17, 18].

Combining the result with the fact fibroblast proliferation and collagen production is of the upmost importance during this phase, it can be said that PRP is indeed beneficial as it can increase both cell production and cellular component by enhancing fibroblast proliferation and collagen production, therefore strengthen healed ligament[17].

Effect of Platelet-Derived-Growth-Factor (PDGF) as a part of PRP

PDGF has several mechanisms of action like chemotaxis, cell proliferation, angiogenesis, ECM deposition, and remodeling[19]. It also stimulates the action of fibroblasts. The work of this substance can be seen on the experiment from the fact that the regeneration and repair phase of the injury injected with PRP was performed faster than the ones without the injection, evident by the large presence of fibrocytes, which shows that the major action of fibroblasts have already taken place.

Effect of TGF-β as a part of PRP

The action of this growth factor consists of stimulating proliferation as well as differentiation in cells, more specifically, angiogenesis; stimulating blood flow to injured areas; and stimulating other growth factors to be released[20]. The work of this particular substance can be seen by the enhanced inflammation phase of the injury injected with PRP compared to the injury that wasn't injected.

Effect of Fibroblast-Growth-Factor (FGF) as a part of PRP

FGF is a protein that stimulates the process of proliferation and differentiation. It is stimulating the process of migration and replication of fibroblasts as well as regulates the release of collagen from fibroblasts by macrophages[21]. The action of the protein can be seen from the enhanced regeneration and repair process on the treatment group compared to the control group.

Effect of Insulin-like Growth Factor (IGF) as a part of PRP

The two types of IGF, IGF-1 and IGF-2, act differently on stimulating the healing process. Their mechanisms of action are similar but rather different, IGF-2 doesn't rely as much on growth hormone secretion like IGF-1 but it's not as effective as IGF-1. They stimulate proliferation, apoptosis, cellular differentiation, and cell migration. IGF-1 also stimulates the synthesis of proteoglycan and collagen I by the fibroblasts[1, 6]. The actions of both of these growth factors can be seen with the improved work of fibroblasts on the treated group.

Effect of Vascular Endothelial Growth Factor (VEGF) as a part of PRP

The inflammation process of the treated group was done faster than the control group because of the earlier improved vascularization of the area of injury injected with PRP. The result is indeed consistent with the fact that the growth factor stimulates vasculogenesis and angiogenesis.

Effect of Epidermal Growth Factor (EGF) as a part of PRP

The boosted 2 healing phases of the treated group is also consistent with the existence of EGF as a part of PRP. Epidermal Growth Factor (EGF) stimulates cell growth, proliferation, and differentiation. EGF has some implications in fibroblast function and formation of granulation tissue[22].

Effect of Platelet Rich Plasma on Interleukin-8 (IL-8) as a part of PRP

The inflammation phase of the treated group is indeed faster to resolve compared to the control group. One of the major growth factors to be responsible is IL-8. Interleukin-8 (IL-8) is produced by a number of cells, such as macrophages, epithelial cells, and endothelial cells[23].

IL-8 functions as a chemotaxis towards neutrophils and certain other granulocytes to attract them to the injury site. It also stimulates the process of phagocytosis as well as angiogenesis[13].

Result Differences Among Studies

Previous study results show a variety of differences, in which some studies claim that it is not as effective as one might think and other studies claim that it does have a major impact on healing[9]. Based on the result of this study, PRP does have a positive effect towards soft tissue healing. Not all currently marketed PRP devices are equal; some do not concentrate viably active platelets in sufficient numbers to produce a healing enhancement. As mentioned previously, some method of PRP extraction ended up damaging the platelet content, which explains the criticisms regarding the efficacy of PRP.

In addition, some PRP studies that use animal models that have a blood volume that is too small to produce PRP. These studies have used donor blood due to that reason. This method resulted in a homologous preparation, not autologous; which is the true PRP. The use of donor animal blood platelets gives an overt immune reaction and leads to false-negative results that may falsely be ascribed to PRP[24]. The positive result of this study may be linked to the fact that this study used a standardized method of PRP extraction and homologous blood sample from the subject.

CONCLUSION AND SUGGESTIONS

From this research, it can be concluded that the injection of PRP is beneficial to a ligament injury on the healing phases of inflammation, regeneration, and remodeling, which can be seen from the fact that hematoma and inflammation phase examined histopathologically that resolved quicker on the treated group compared to the control group as well as the thicker ligament of the group injected with PRP.

For further study, the author of this study suggests that the restoration of function to the ligament is also examined to further prove the effectiveness of PRP at the remodeling stage of healing. In relation to the first suggestion, a collagen type examination would provide better result to see whether the thick ligament consists mostly of fibrotic or functional ligament tissue. In order to assess the function of the ligament, tensile strength measurement is also needed. The effectiveness of PRP based on its ratio and concentration should also be evaluated.

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