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Original Article

A histopathological and clinical assessment on the effect of microneedling on the autologous platelet-rich plasma in the experimental wound healing model

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Abstract

Aim: Platelet Rich Plasma (PRP) is a method used in dentistry and aesthetic applications in order to accelerate tissue healing. The purpose of this study is to assess the effect of PRP on accelerating wound healing and the contribution of microneedling application, i.e. multiple injection during PRP, and the volume effect of the PRP material to healing.

Materials and Methods: In the study, 16 5-month-old male white New Zealand rabbits with an average weight of 2.7-3.0 kg were used. The four wounds were made on the back of each rabbit, and PRP, Normal saline(NS), microneedling(MN), and Sham procedures applied. Eight rabbits were sacrificed by taking their tissues for histological assessment at the end of 14 days. Epithelization, VEGF expression, angiogenesis, inflammatory cell, fibroblast, reticular fiber, and collagen parameters were evaluated. Eight rabbits were followed up for macroscopic healing.

Result: The PRP procedure decreased the healing duration and wound area compared to other procedures($p<0.05$). The NS procedure decreased the healing duration compared to the Sham procedure and provided a significant decrease in wound area compared to the MN and Sham procedures($p<0.05$). Although the MN procedure decreased the wound area compared the Sham procedure, this difference was not statistically significant($p>0.05$). PRP procedure increased staining scores and densities of epithelization, VEGF expression, angiogenesis compared to Sham procedure($p<0.05$). PRP procedure increased staining score of collagen compared to Sham and NS procedures and staining density of collagen compared to other procedures($p<0.05$). MN procedure decreased staining density of inflammatory cell compared to Sham procedure($p<0.05$).

Conclusion: The evaluation of simultaneous microneedling and PRP procedure on the 14th day of wound healing did not provide more positive synergistic effects on healing. In order to reduce the volume effect of the PRP amount given, the amount of PRP applied at each point should be as low as possible.

Keywords: Microneedling; PRP; proliferation phase; wound healing

INTRODUCTION

Numerous growth factors, proteins, and cytokines play an active role in wound healing (1,2). These structures are effective in all the phases of wound healing. Many cell and growth factors such as fibroblast, mesenchymal stem cell, and inflammatory cell as well as cytokines and protein infiltration occur in the wound. New capillary vessels and structures are formed and proliferated. A metabolically rich environment that also supports repair develops. Necrotic and damaged tissues are eliminated in the proliferation phase and new tissues similar to the tissue environment of the said area develop again. In the remodeling phase, new developed tissue is reshaped and rearranged. Also, cell density and vascularity decrease and collagen fibers are reshaped. As a result, mesenchymal cell proliferation, extracellular matrix formation, and scar formation occur (3).

Local treatments are more efficient in case of vascular problems in the tissues during wound healing, ageing, and failure to provide blood flow and perfusion due to damages. Topical ointments, oils, growth factors, stem cell, and microneedling are also used for local effect in the wound edge such as autologous platelet rich plasma (PRP) in order to accelerate the healing process (4-6). Also, PRP and microneedling are mostly used together even in clinical applications.

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New areas of application are developed by learning the contribution of the growth factors, cytokines and numerous protein release of thrombocytes to healing (7). It is reported that obtaining PRP both strengthens the existing healthy tissue in aesthetic applications and accelerates healing in chronic wounds and graft applications. (2,3,7,8). Platelets include powerful growth and platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-ß), adipose-derived stem cells, dermal fibroblasts, and epidermal growth factor (EGF) Autologous platelet rich plasma (PRP) includes a higher concentration of platelets than blood in the same amount. (7,9). The use of PRP or PDGF accelerates healing in many chronic and non-healing wounds such as diabetes and pressure ulcers. For this purpose, plastic and aesthetic surgery, maxillofacial surgery, dentistry, cardiovascular surgery, orthopedics, and dermatology are widely used (9-11).

Microneedling enables to restore the transepithelial potentials and electrotaxis of epithelial skin cells and initiate the collagen synthesis (4,12). At the wound edge, epithelialization slows down and microneedling around wound skin may stimulate epithelial migration and angiogenesis for new capillaries. Also, microneedling leads to the multiple cutaneous injuries breaks the old collagen structures in the scars, and stimulates migration and proliferation of fibroblasts, Thus, a new collagen production is provided (4). Microneedling gives a clear advantage because of regenerating skin and reducing scars (13). Electrical Stimulation is used in the treatment of decubitus ulcers and wounds of the lower extremity caused by vascular failure and diabetes that have not responded to standard wound treatment in the United States (14).

During autologous PRP applications, numerous microneedling applications are also done to the said site and the volume effect of PRP in the intercellular area develops. The aim of the present study is to investigate the contribution of microneedling applied at the wound edges during the injection of autologous PRP in experimental wound model and the volume effect of PRP to wound healing.

MATERIALS and METHODS

Model Selection

All the study procedures used in this study were approved by the Animal Research Local Ethics Committee of our institution (dated 24/06/2015 and numbered 39). 16 male New Zealand white rabbits aged 5 months and having an average weight of 2.7-3.0 kg were used. Rabbits were placed in a separate cage. All of them were kept in environmentally controlled conditions at 23 ± 2°C with appropriate humidity and a 12-h light cycle. The rats were given food and water ad libitum and were randomly assigned to 4 experimental groups including 16 animals in each.

Experimental Design

The four distant wounds were made on the back of each rabbit. Thus, each rabbit was included 4 study groups: PRP, Normal Saline (NS), Microneedling (MN), and Sham groups. The same procedure was applied to all rabbits. Eight rabbits were sacrificed by taking their tissues for histological assessment at the end of 14 days. Eight rabbits were followed up for macroscopic healing and calculating the area of wound contraction.

1. PRP procedure

3cc blood was collected from the rabbit's ear vein and PRP was obtained. The edges of the wound in the left upper quadrant were injected intradermally its PRP with a 30G needle every other day. PRP was intradermally applied to the wound edges by leaving a 1mm distance.

2. Normal saline (NS) procedure

The same amount of saline (0.9% physiological saline solution) as the amount of PRP obtained with 30G needles was injected to the edges of the wound on the right upper quadrant every other day.

3. Microneedling (MN) procedure

In the lower left quadrant, edges of the wound were microneedled to the same depth with 30G needles as in the PRP application, which was regularly obtained every other day.

4. Sham procedure: In the lower right quadrant, no injection was administered to edges of the wound.

Surgical Procedure

Each rabbit was anesthetized with an intramuscular injection of 5-10 mg/kg xylazine (Rompun, Bayer, Turkey) and 30-35 mg/kg ketamine HCL (Alfamine®, Ege-Vet, Turkey) and allowed to breathe spontaneously. Before the surgical procedure, the rabbits were stabilized in the appropriate position on the operation table. We shaved backs of the rabbits using an electric razor and prepared them using a surgical scrub with 10% povidone iodine solution. All the surgical procedures were performed by the same surgeon (N.K.O.) and using aseptic technique and powderless gloves were used in the surgery. Intramuscular analgesic drug (4 mg/kg Carprofen) was administered.

After the rabbits were anesthetized and prepared, four circular areas having a 20-mm diameter were drawn on the dorsal region at equidistant from the midline, and its boundaries were determined with a ruler and pencil. Four circular skin defects having a 20-mm diameter and full-thickness were created by removing skin from the back of the animals to the panniculus carnosus at the base of the floor. Hemostasis was done in the wounds.

Preparation of Autologous Platelet Rich Plasma

Three milliliters of fresh blood taken from the ear veins of rabbits under aseptic conditions was injected slowly into the anticoagulant tube containing sodium citrate (10:1). 0.5 ml blood was taken into a separate tube and platelet count (489.000/µl) was measured with complete blood panel (Mindray BC-6800). The blood was centrifuged at 160 G for 10 min. The components of blood were

separated into 2 sections: 1 supernatant that was clear and constituted PRP and the other section containing erythrocytes and leukocytes. The supernatant was again centrifuged at 300 G for 10 min to form a pellet of rabbit platelets. Complete blood counts (Mindray BC-6800) were found to reach approximately three times of the number of platelets (1463.000/µl). (10:1) Calcium chloride was added for platelet activation before the injection. Platelet count was not calculated in other applications because sufficient PRP was provided. The same procedure was repeated in PRP applications and the PRPs obtained with this method were used in the defects.

Macroscopic Assessment of Healing and the Area of Wounds Follow-Up

After the wound was created in all groups, dressings were performed every day by soaking with saline. Every other days, the wound was measured and its area and percentage of contraction were calculated. Wound sizes were drawn on a transparent acetate on the days 0, 3, 6, 9, 12, 15, 18, and 24. Corel Draw X5 and Golden Software Didger 3 programs were used to calculate the wound areas. Transparency drawings were transferred to digital media on a 1/1 scale through a browser. The JPG files were drawn in a DXF file format in Corel Draw X5, a vector drawing program, with each wound drawn in a closed curve. These files were opened in Didger 3 software used for geological digitization and the area of each closed curve was calculated in mm². The percentage of wound contraction was calculated with following formula: the current wound area × 100/first-day wound area. Besides, the macroscopic healing time of all groups was determined in days. Photographs of each wound were taken using a camera in suitable mode with the same distance at an angle of 90° to the plane of the wound.

Histology and Immunohistochemistry

On day 14, eight rabbits were sacrificed by intraperitoneally administering 10 mg/kg thiopental sodium solution. For histological assessment, the 20-mm diameter tissue containing the wound was carefully removed until the tissue of the panniculus carnosus. Full-thickness biopsy samples extended from the outside edge to the center of the treated area. All tissue samples were fixed in 10% neutral formalin for 24 h at room temperature. After fixation, wound samples were dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin. The sections were cut at 3 µm using a microtome (Thermo Microm HM 340E; Thermo Fisher, Waltham, MA), mounted on glass slides, dewaxed, rehydrated through graded alcohols, and stained with hematoxylin and eosin (H & E), Silver Stain, Azan trichrome, and anti-vascular endothelial growth factor (VEGF) (sc7269; Santa Cruz Biotechnology, Dallas, TX). The images were captured using a light microscope (Olympus BX51) and scanning electron microscope (SEM) (Tescan Mira3 XMU (Brno, Czechia)). Wound healing for each group was evaluated using the scoring system for epidermal and dermal regeneration (Table 1). Assesment were semiquantitatively using a visual scoring method with grades ranging from 0 to 3 (0=no cell, fiber or no epithelization; 1=few cell, fiber or initiation epithelization; 2=medium number of cell, moderate fibers or epithelization covering; 3=spreading and many cell, numerous fibers or epithelization covering; 4=very common cell, excessive fiber, or epithelization covering). In the proportional evaluation, the density of the cells was evaluated. (0 = no cell, fiber or no epithelization; up to 30% = few cell, fiber or initiation epithelization; 30% to 50% = medium number of cell, moderate fibers or epithelization covering; 50% to 80% = spreading and many cell, numerous fibers or epithelization covering; 80% to 100% = very common cell, excessive fiber, or epithelization covering) (15-17).

Table 1. Histopathological scoring (Score:0,1,2,3,4) and grading (Dansity: %) of wound healing

Epithelization	İnflamatory cell density (InfCD)	Fibroblast cell	Reticular fibers	Collagen fibers	Anjiogenesis	VEGF immünolocalization
0: no epithelization	**0**: no inflamatory cells	**0**: no fibroblast cell	**0**: no fiber	**0**: no fiber	**0**: very few new vessels	**0**: slight immünolocalization
1:initiation epithelization, %30	**1**: focal and few cells, %30	**1**: few fibroblast cells, %30	**1**: few fibers, %30	**1**: few fibers, %30	**1**: few new vessels, %30	**1**: few immünolocalization, %30
2:medium epithelization covering, %30-50	**2**:medium number of cell,%30-50	**2**:medium number of cell, %30-50	**2**:moderate fibers, %30-50	**2**:moderate fibers, %30-50	**2**:moderate new vessels, %30-50	**2**:moderate immünolocalization, %30-50
3:spreading epithelization covering, %50-80	**3**:spreading and many cell, %50-80	**3**: many fibroblast cell, %50-80	**3**:numerous fibers, %50-80	**3**:numerous fibers, %50-80	**3**: lots of new blood vessels, %50-80	**3**: numerous immünolocalization, %50-80
4: very common epithelization covering, %80-100	**4**: very common cell, %80-100	**4**: very common cell, %80-100	**4**: excessive fiber, %80-100	**4**: excessive fiber, %80-100	**4**: many new blood vessels, %80-100	**4**: very extensive immünolocalization, %80-100

Statistical Analysis

The data analysis was performed using IBM SPSS Statistics for Windows Version 24.0. (IBM Corp., Armonk, New York, USA). The data are presented as mean ± SEM with median and range (n=8 for each group) and analyzed with Kruskal-Wallis test with post-hoc Dunn's test. Statistical significance between the procedures was analyzed via ANOVA or repeated measures ANOVA test followed by Tukey post hoc test. Values for $p \leq 0.05$ were considered as statistically significant.

RESULTS

The practice of the study had no adverse effects on the surrounding tissue or wound healing in any rabbit. No infection, irritation, erythema, hematoma or any complication were observed in the healing process in all groups. In order to determine the healing duration with eight rabbits sacrificed for the immunohistochemical examination, no significant difference was found between the areas measured on the same study days (days 0, 3, 6, 9, and 12) only between the eight followed rabbits (p>0.05).

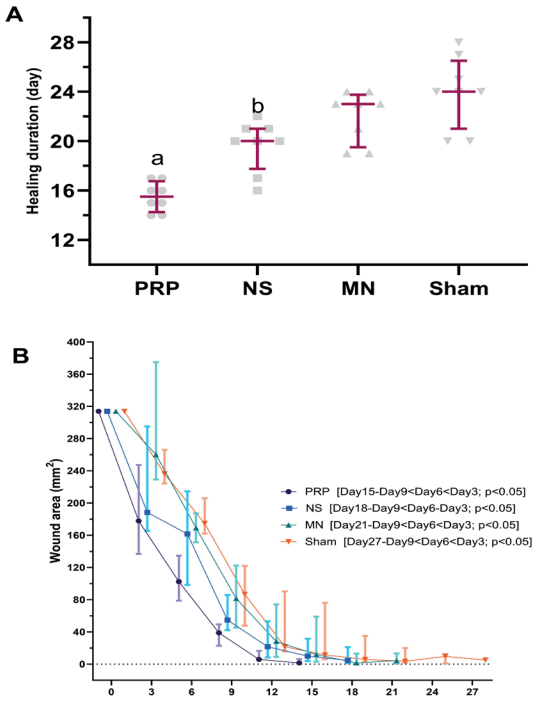

Figure 1. The datas are show comparison of the statistical of the groups. A; Healing duration of rabbits undergoing all procedures. aSignificantly different when compared to other procedures (p<0.05). bSignificantly different when compared to Sham procedure (p<0.05). B; Wound area of rabbits undergoing all procedures from day 0 to day 27. Overall, the PRP procedure decreased the wound area compared to the other procedures (p<0.05). The NS procedure provided a significant decrease in wound area compared to the MN and Sham procedures (p<0.05). Although the MN procedure decreased the wound area compared the Sham procedure, this difference was not statistically significant (p>0.05). Regarding the measurement days, the decreasing effects of all procedures on wound area were, ranked in order of success according to statistical significance; NS, normal saline was applied; MN, microneedling was applied; and Sham, sham procedure was applied

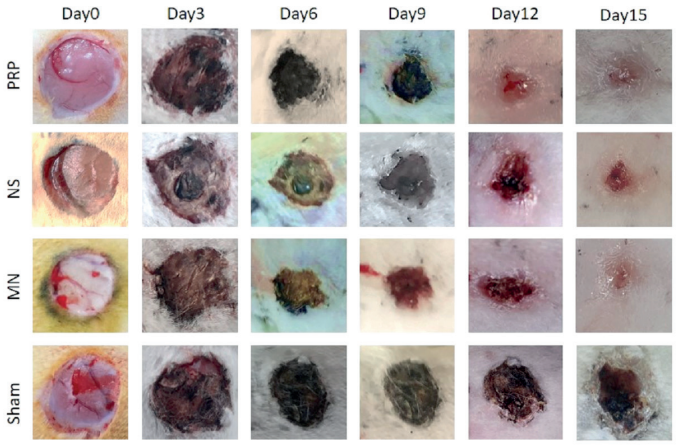

Figure 2. Representative images of wounds obtained from all the groups on days 0, 3, 6, 9, 12, and 15

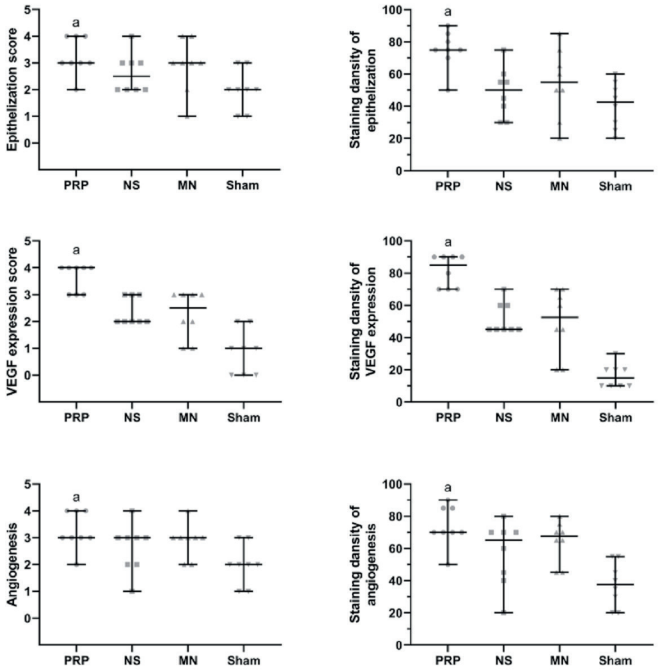

Figure 3. Staining scores and densities of epithelization, VEGF expression, angiogenesis assessed in wound biopsy of rabbits undergoing all procedures. aPRP procedure significantly increased staining scores and densities of epithelization, VEGF expression, angiogenesis compared to Sham procedure (p<0.05). PRP, platelet rich plasma was applied; NS, normal saline was applied; MN, microneedling was applied; and Sham, sham procedure was applied

In this study, the effects of PRP, NS, and MN procedures on wound healing in a rabbit model were investigated. In this study, healing duration and wound area were used for macroscopic assessment. Immunohistochemical evaluation was performed by evaluating epithelialization, VEGF expression, angiogenesis, inflammatory cell, fibroblast, reticular fiber and collagen of healing wound tissue. Figure 1A shows the healing duration of the study groups. The PRP procedure significantly decreased the healing duration compared to the NS, MN, and Sham procedures (p<0.05). The NS procedure significantly decreased the healing duration compared to the Sham procedure (p<0.05). There was no significant decrease in

the healing duration of MN procedure with NS and sham procedures (p>0.05). Figure 1B shows the wound areas of the study groups from day 0 to day 27. Overall, the PRP procedure significantly decreased the wound area compared to other procedures (p<0.05), and following the PRP procedure, the NS procedure provided a significant decrease in wound area compared to the MN and Sham procedures (p<0.05). Although the MN procedure decreased the wound area compared the Sham procedure, this difference was not statistically significant (p>0.05).

Figure 2 shows the images of wounds obtained from all the groups on days 0, 3, 6, 9, 12, and 15. The area of each wound was measured on all these days.

Figure 3 displays the staining scores and densities of epithelization, VEGF expression, and angiogenesis assessed in wound biopsies. PRP procedure significantly increased staining scores and densities of epithelization, VEGF expression, angiogenesis compared to Sham procedure (p<0.05). No significant difference was found among the other procedures in terms of these parameters (p>0.05).

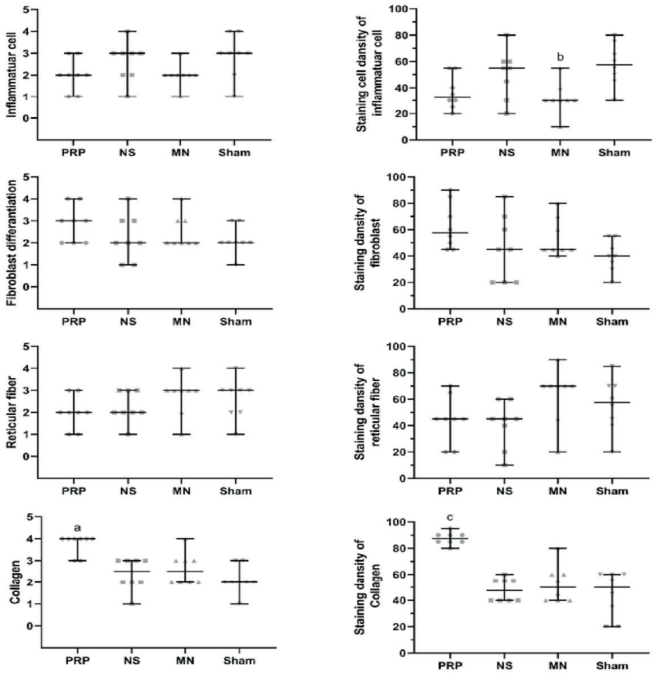

Figure 4. Staining scores and densities of inflammatory cell, fibroblast, reticular fiber, and collagen assessed in wound biopsy of rabbits undergoing all procedures. aPRP procedure significantly increased staining score of collagen compared to Sham and NS procedures (p<0.05). bMN procedure significantly increased staining density of inflammatory cell compared to Sham procedure (p<0.05). cPRP procedure significantly increased staining density of collagen compared to other procedures (p<0.05)

Figure 4 shows the staining scores and densities of inflammatory cell, fibroblast, reticular fiber, and collagen assessed in wound biopsy of rabbits undergoing PRP, NS, MN, and Sham procedures. PRP procedure significantly increased staining score of collagen compared to Sham and NS procedures (p<0.05). MN procedure significantly increased staining density of inflammatory cell compared to Sham procedure (p<0.05). PRP procedure significantly increased staining density of collagen compared to MN, Sham, and NS procedures (p<0.05). No significant difference was found among the other procedures in terms of these parameters (p>0.05).

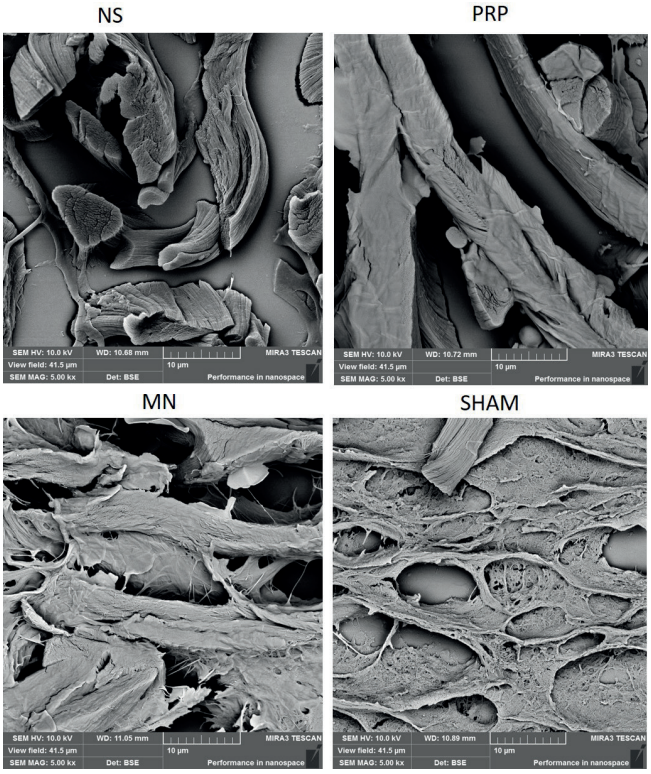

Figure 5. In the PRP procedure, the reticular fiber density decreased and instead, regular and thick collagen fibers were located. In the SEM images, the collagen alignment and thickness in the MN procedure were quite good. In the NS procedure, normal saline was given additionally in order to create a volume effect along with the MN procedure; in other words, the effect of the MN procedure on collagen was expected in this procedure but it did not happen. In the SEM images, collagen alignment in NS procedure was more dispersed

In the SEM images, the important difference is that in histological assessment, regular and thicker collagen fibers are located in the PRP procedure (Figure 5). The collagen alignment and thickness in the MN procedure were quite good in the SEM images taken and it was seen that collagen alignment of NS procedure was more dispersed (Figure 5).

DISCUSSION

When the healing durations of the wounds to which PRP, NS, MN, and sham procedures were applied, were assessed in the present study, healing in the PRP procedure was quite faster than other procedures, and the reduction in the wound areas was more every passing day. There was a significant healing in NS procedure when compared to the sham procedure. In NS procedure, significant rapid reduction was present in the wound sites when compared to the MN and Sham procedures. The reduction in the wound sites in MN procedure was similar to the NS procedure

and no statistically significant difference was observed even though it was quicker than the Sham procedure. According to the immunohistochemical assessment in the healed wound tissue, the epithelization, VEGF expression, angiogenesis staining scores, and densities of the PRP procedure increased when compared to the sham procedure. Also, collagen staining density increased more than all the other procedures and collagen staining score increased more than NS and sham procedures. Staining density of inflammatory cell increased more in the MN procedure than the Sham procedure. The results obtained from the present study were assessed with those reported in the literature.

Assessment of the immunohistochemical status of the healing wound is important in terms of the treatment management and healing follow-up (18). Histological assessment should include main factors such as inflammation, angiogenesis, wound contraction, epithelization, fibroplasia, and remodelling phases (19). In the present study, especially the proliferation and remodelling phases were taken into consideration. There are a limited number of studies assessing the cellular effects in many aspects. We used epithelialization, VEGF expression, angiogenesis, inflammatory cell, fibroblast, reticular fiber and collagen structures in the wound tissue that healed in histological assessment. The wound was also macroscopically followed p in terms of the day of healing and the reduction in the wound site. The present study is worthy because it evaluates both the macroscopic follow-up and numerous cellular changes immunohistochemically. Also, it assesses the cellular effects of PRP on wound healing and the contribution of other procedures to this effect.

In medicine, PRP is used to treat wounds, burns, musculoskeletal system diseases, dermatological diseases, dentistry, and various clinical cases such as post-surgery tissue healing (12,20-22). There are numerous studies regarding the subdermal, intradermal, intralesional uses and effects of PRP, and its use as a gel on the wound in order to accelerate wound healing. In almost all of these studies, it is highlighted that it accelerates healing, increases flap viability, collagen sequence and infiltration on the wound, and has positive effects (23-26).

Similar to PRP, microneedling is one of the locally applied methods. It is used to smooth the skin with the scar and to treat fine wrinkles. It encourages the repair of irregularly aligned collagen on the reticular dermis and induces collagen production (27,28). The purpose in microneedling is to produce thousands of micro needle lesions from epidermis to the papillary dermis and to initiate the normal wound healing process that results in type III and I collagen synthesis. As a result, collagen tightening and alignment occur naturally (4,27,28). The studies conducted on stria have shown that it increases the elastin density (28).

Today, PRP and MN procedures are used separately or together both in aesthetic procedures and in wound and scar treatment in experimental and clinical applications (9,27,20). The success in these applications definitely depend on both the microtraumas in MN procedure and the positive cellular effect of PRP on the wound healing. In the present study, it was observed that the PRP procedure applied group clinically showed faster healing than the other groups as a result of histological assessment and follow-up. However, healing is also quite good in the microneedle procedure. In the studies, it is specified that the expression of the matrix metalloproteins controlling the collagen fiber formation and scar fibrillation is taken under control via the microneedling method, and accordingly, it has rapidly contributed to wound healing via collagen modulation (4). In the present study, there was no statistically significant difference between the MN procedure and other procedures in terms of collagen modulation in the tissue. However, the collagen alignment and thickness in the MN procedure were quite good in the SEM images taken (Figure 5). Accordingly, in the present study, differently from the MN procedure, it was expected to have the collagen score be better with the synergistic effect in the PRP procedure. This is because microneedling was also performed in the PRP procedure. In the PRP and NS procedures, a little PRP and normal saline were given in addition to the MN procedure. In the SEM images, it was seen that collagen alignment of NS procedure was more dispersed. NS procedure has a positive effect on the collagen thickness however, it could not provide a regular collagen alignment (Figure 5).

In the studies on the use of PRP in wound healing, it is specified that it accelerates epithelization, and contributes to wound healing together with the release of the factors required for cell activation, the development of granulation tissue, and acceleration of angiogenesis (29-31). The results of the present study also support the cellular treatment intervention in order to accelerate surgical wound healing in an experimental wound model. The data obtained can be interpreted as the positive effect for wound healing in PRP procedure is provided by the cellular and protein richness of PRP.

Fibroblasts and numerous growth factors releasing from the fibroblasts have important roles in wound healing and these factors increase in the healing process and contribute to the wound healing (32). It is reported that PRP application increases fibroblast migration and proliferation (33). In the present study, fibroblast proliferation increased slightly in all the groups. However, this increase did not reach to a statistical significance among the study procedures. In the tissues, reticular fibers are the structures showing continuity with the collagen fibers and they form a large fibrillary network together. They provide structural support for the cellular components (34). In the present study, the effect of the procedures on wound healing of the reticular fibers could not be detected in the assessment performed on the 14th day of wound healing.

There are studies reporting that PRP reduces inflammatory cell infiltration and accelerates healing in wound healing (31,35). In the current study, no significant increase or decrease could be obtained in the inflammatory cell score or density in the assessment performed on the 14th day of wound healing in the PRP procedure. It is reported that inflammatory response and inflammatory cell infiltration of microneedling reduced the inflammatory cytokines (4,36). In the microneedling procedure of the study, as there was no significant difference in the staining score of the inflammatory cell from other procedures, there was a significant decrease in the staining density of the inflammatory cell.

The study has some limitations. Immunohistochemical assessment was only performed in the proliferation phase of wound healing. A study including the assessment of cellular changes on different days would be more worthy. In addition to the semiquantitative scoring, providing a quantitative scoring provides to obtain data that support each other more. Working with multiple groups by increasing the number of needling in the MN procedure could enable the assessment of the synergistic effect.

In the present study, it was determined that the evaluation of simultaneous microneedling and PRP application on the 14th day of wound healing did not provide more positive synergistic effects on healing. According to the results of NS procedure, the amount of PRP applied at each point should be as low as possible in order to reduce the volume effect of PRP.

CONCLUSION

The present study has the characteristics of being a preliminary study to cause new studies regarding the investigation of the amount given during PRP application and the number of needling that will contribute to wound healing or clinical success. In clinical applications, it is considered that the number of needlings used in microneedling application should be changed according to the purpose of application (scar, wrinkle, wound healing, etc.) and the amount given during the PRP application should be as little and frequent as possible.

****The preliminary study of the present study was presented as an oral presentation at V. International multidisciplinary congress of Eurasia, 2018, Barcelona.*

Competing interests: The authors declare that they have no competing interest.

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Ethical approval: Sivas Cumhuriyet University Animal Research Local Ethics Committee (dated 24/06/2015 and numbered 39).

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