Utilization of Digital Imaging Analysis in Evaluation of the Effect of Autologous Bone Marrow and Platelets Rich Fibrin in Primary Wound Healing Pattern in Rats

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ABSTRACT

Background and Purpose: Recent studies suggested that the pattern of wound healing is responsible for scarring. In this experimental study we tried to evaluate the pattern of primary wound healing in incisional wounds after applying autologous unprocessed bone marrow and platelet rich fibrin. This evaluation was done by examination of the wounds during healing process macroscopically, microscopically and by digital imaging analysis.

Methods: We compared 4 groups of rats with the same incisional wounds. Group (A) was the positive control, Group (B) was injected with platelet rich fibrin, Group (C) was injected with bone marrow and Group (D) was injected by both.

Results: Adding autologous unprocessed bone marrow with or without platelet rich fibrin improve the time of healing, scar appearance and consistency, microscopic inflammatory response, fibrosis area and depth of fibrosis. Epithelial thickness, collagen deposition and remodeling were similar to the normal healing process.

Conclusion: Digital imaging analysis is a technology that may documents objectively the improvement in wound healing after adding bone marrow and/or platelet rich fibrin. But, there will be much to do to investigate and document these effects on biological and biochemical basis on a larger sample size.

Key Words: Digital imaging analysis – Bone marrow injection – Mesenchymal stem cells – Platelet rich fibrin – Scar prevention – Wound healing.

INTRODUCTION

Normal skin contains bone marrow-derived cells that are involved in host defense and inflammatory processes, including wound healing. Hematopoietic and mesenchymal stem cells are mobilized from the bone marrow into circulation after tissue injury. These cells will be hosted at the site of injury. After that they regulate the migration and proliferation of epithelial and dermal cells during the inflammatory phase [1-3]. Stem cells derived from the bone marrow could home to injured tissues and act on wound repair and tissues regeneration [4-6]. Adult bone marrow derived hematopoietic stem cells are the precursor to all blood cells, “fibrocytes” and “endothelial progenitor cells” [7,10]. Stem cells have been shown to home in the site of injury improving healing process [11-15].

Platelets have many functions beyond that of simple hemostasis. They secrete growth factors which are responsible for increasing cell divisions, collagen production, attracting other cells, starting vascular growth, and initiating cell differentiation [16-21]. Platelet-Rich-Fibrin (PRF) can hold these growth factors in a fibrin network. So, a gradual sustained release can accelerate and improve wound healing over a period of time. PRF has found a place in the regenerative field owing to its advantages over PRP [22,23]. Lundquist R and colleagues reported that the platelet counts of PRF and PRP are similar but with low cost and great ease of the procedure [24-26]. Also fibrin has been shown to be an excellent provisional scaffold providing a conductive surface for cell attachment, adhesion, and migration during the initial phase of the healing process [16].

In this preliminary pilot experimental study we tried to evaluate the pattern of primary wound healing in incisional wounds after applying autologous unprocessed bone marrow and platelet rich fibrin. We hypothesized that when we enrich the wound with bone derivative cells and growth factors that found in the platelet rich fibrin (which may act also as scaffold); the healing will be much better with minimal inflammatory responses and minimal scaring.
METHODOLOGY

The study was conducted in Suez Canal University and in Pathology Department of the National Research Center of Egypt from October 2014 to June 2015. This was experimental, controlled and descriptive clinical trial done on twenty Sprague-Dawley male rats. We followed our national institution's guidelines in experimental studies. Rats weighed between 300 to 400 grams. They were divided into 4 groups. Each group consists of 5 rats (4 active participant + 1 spare animal if a wound get infected or an animal died). The animals were anaesthetized by intra peritoneal thiopental injections. Hair was removed with a depilatory cream.

Group A (active control): All wound models in the study were incisional wounds with primary closure [27]. 2cm incision was done at the back; deep to incise the cutaneous maximus muscle of each rat then sutured by polyglactin 910 Suture (Vicryl Rapide™) (subcuticular technique to direct the healing process to be by primary intension) as shown in Fig. (1).

Group B: The same as Group A. Before surgery a 3ml whole blood were drawn by retro-orbital approach then immediately centrifuged (3000 RPM for 10 minutes) [28]. The resulted PRF was taken and placed in the central part of the wound before final closure. This is shown in Fig. (2).

Group C: The same as Group A. Then a heparinized 20cc syringe with 18G needle was introduced into the right femur of the animal percutaneously. About 0.5ml of bone marrow was suctioned [29]. The suctioned BM was then injected to the edges of the central part of the wound (after closure) of each animal. As shown in Fig. (3).

Group D: The same maneuvers of Groups A, B & C were done to each animal of this group.

Post operatively all animals received one dose of antibiotic intra muscularly. Local antibiotic cream were applied twice daily for 1 week. Fortunately, no infected wounds or dead animals experienced during the period of the study.

Assessment of wound healing pattern:

Wound excisional biopsies (whole length of the wounds) were taken from each group (one animal each time) at the intervals 2, 4, 6 and 8 weeks. Only the central parts of the specimen were examined histologically. The wounds were photographed before excision to evaluate the wound grossly. Each time the slides were stained by Hematoxylin and eosin stain, and Masson’s trichrome stain.

The evaluation of the pattern of healing depended on: Gross pictures and Microscopic pictures. Computerized image analysis was done regarding: inflammatory reactions, epithelization, collagen, fibrosis, and presence or absence of skin appendages. Measurements were taken using LIECA QUIN DM3000 LEDimage analysis system, founded in Pathology Department of the National Research Center of Egypt. Each slide compared with the normal skin (passive control).

RESULTS

The results here were not in sequence manner. Each finding represents a specific finding for one specific animal in a specific time (as a preliminary data for a pilot study).

1- Gross picture:

By photographing the site of the injury before taking the biopsies and by comparing them subjectively, we found that:

The healing time and healing pattern were much better in Groups C and D than Groups A and B. Also Group B was slightly better than Group A. During biopsy taking we found that the tissues were much bulky and tough in Groups A and B. Scars were more obvious in Groups A and B than in Groups C and D. This is presented in Fig. (4).

2- Depth of fibrosis:

By measuring the distance between the epithelium and the deepest part of fibrosis in all biopsies we found that; all groups were near each other in the depth of fibrosis after 2 weeks of injury. But with the end of the 8th week, this depth of fibrosis decreased in all groups except in Group A which became the deepest. The least depth of fibrosis was in Group D. Those results are presented in Figs. (5-8) and (Table 1).

3- Extent of fibrosis area:

We shaded the area of fibrosis in all slides and measured them by imaging analysis. We found that; after 2 weeks of injury there were variable degrees of fibrosis on all groups. In the 4th week all groups decreased in the area of fibrosis to be near each other. By the end of the 8th week the results varied. The wider area of fibrosis was in Group A and the narrower area of fibrosis was in Group D then Groups C and B. Those results are presented in Figs. (5-8) and (Table 1).

4- Epithelial thickness:

Epithelium got thickened in all groups after 2 weeks. This thickening decreased in all groups
with passing of time. After 8 weeks the epithelial thickness became near each other. By comparing this finding with the epithelium thickness of the normal skin, we found that after 8 week of injury the epithelium became slightly thicker than the original pattern. Those results are presented in Figs. (7-9) and (Table 1).

5- Inflammatory cell count:
Marked inflammatory response was recorded on Group A. This was noticed in the first 3 animals of this group at 2, 4 and 6 weeks. This reaction reduced after 8 weeks to be near the results of other groups. These results are presented in Fig. (10) and (Table 1).

6- Pattern of collagen density:
We compared the amount of collagen density in all slides with the collagen in the normal skin. After 2 weeks, the collagen increased in all groups. This density decreased in all groups after 4 weeks to be increased again after 6 weeks; also in all groups. By the end of 8 weeks the collagen density decreased and became near the value founded in normal skin. It was slightly more in Group A and slightly less in Groups B, C and D. Those results are presented in Figs. (11-13) and (Table 1).

7- Pattern of skin appendages and microscopic fat of bone marrow cells:
After careful examination of all slides prepared from skin tissue obtained from the 4 groups of rats, no microscopic evidence of residual bone marrow elements was seen. Complete continuous epithelization was restored in all groups. The skin adnexal distribution was regained efficiently in groups received BM and combined BM & PRF more than the control group and PRF only group. This can be shown in Fig. (7) and (Table 1).

<table>
<thead>
<tr>
<th>Table (1): Represents the multiple variants regarding the groups through the period of the study.</th>
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<tbody>
<tr>
<td>Fibrosis depth</td>
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<td>Normal skin</td>
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* "Letter" = group of rats.
* "First number" = number of the rat in its group.
* "Second number" = number of weeks after surgery (time of excisional biopsy).
* Depth of fibrosis at the power 4x from the surface epithelium till the deepest point of fibrous tissue. (Measuring unit was micrometer).
* Area of fibrosis by drawing the area of fibroblastic proliferation and collagen deposition at power 4x. (Measuring unit was micrometer square).
* Measuring the epithelial thickness over the incision, 10 measurements then taking the median, at the power of 20x. (Measuring unit was micrometer).
* Inflammatory cell numbers were measured at 40x in 5 different areas of dense inflammation (frame area = 16638.6 micrometer square). Then the total numbers were taken.
* Collagen density was measured on the Masson stained slides using (measure grey) application at the power 40x at different 5 areas (frame area = 16638.6 micrometer square) then taking the median.
Fig. (1): (A) Incision and (B) Suturing.

Fig. (2): (A, B) Blood sample withdrawal, (C, B) Gross and microscopic picture of platelet rich fibrin, (E, F) Handling and positioning of platelet rich fibrin.
Fig. (3): (A) Bone marrow withdrawal from the femur of the rat, (B) Microscopic picture of the harvested bone marrow, (C) Injection of the bone marrow immediately after closure of the wound.

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<td><strong>Group D</strong> (PRF + BM)</td>
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Fig. (4): Comparison between the four groups regarding gross pictures of the scars in the different times of biopsy.
Fig. (5): Comparison between the four groups regarding fibrosis depth.

Fig. (6): Comparison between the four groups regarding fibrosis area.

Normal skin biopsy (without injury)

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<td><strong>Group D</strong> (PRF + BM)</td>
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Fig. (7): Comparison between the four groups regarding the different times of biopsy (stained by Hematoxylin and eosin stain-power 4x).
Fig. (8): Comparison between the four groups regarding the different times of biopsy (stained by Hematoxylin and eosin stain-power 4x) (with shadow).

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>2 weeks</th>
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<td>B (PRF)</td>
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<td>C (BM)</td>
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<td>D (PRF + BM)</td>
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Fig. (9): Comparison between the four groups regarding epithelial thickness.

Fig. (10): Comparison between the four groups regarding inflammatory cell count.
**Fig. (11):** Comparison between the four groups regarding collagen density.

**Fig. (12):** Comparison between the four groups regarding the different times of biopsy (stained by Masson’s trichrome stain-power 40x).
DISCUSSION

Wound healing has three principal phases: inflammatory, proliferative, and remodeling. Platelet-Derived Growth Factor (PDGF) is released initially by platelets in the inflammatory phase during the formation of the initial thrombus. These growth factors attract, recruit, and activate additional macrophages. Transforming Growth Factor Beta (TGF-B) is released by macrophages and platelets stimulating collagen formation [30,31]. Adding autologous bone marrow in animal wound models accelerate wound healing and may reduce scar formation [31,32]. Also bone marrow-derived mononuclear cells increase lower limb perfusion and promote foot ulcer healing in diabetics [34]. After wound healing, some stem cells remain at the site of trauma and others go back to the bone marrow [35]. On the other hand, Joanne E and colleagues, conclude that bone marrow-derived endothelial progenitor cells do not contribute significantly to new vessels during incisional wound healing [36]. Cellular therapeutics try to enhance tissues repair and reduce inflammations, so scaring can be [6,37].

PRP gel is very useful for the treatment not only of chronic or non-healing wounds, but also can be very effective even in acute trauma wounds. This is due the release of many of active growth factors [38,39]. Platelet-Rich Fibrin (PRF) also used to promote tissue healing. It is easier in preparation and application than Platelet-Rich Plasma (PRP). It has low cost, and lack of biochemical modifications (no anticoagulant or thrombin is required) [22,40].

Many studies tried to combined cells from bone marrow and platelet rich plasma to enhance healing. Ravari and colleagues reported that a combination of bone marrow stem cells, platelet-derived wound
healing factors, fibrin glue and bone marrow-impregnated collagen matrix can improve the management of diabetic foot ulceration [41]. Heffner and colleagues, concluded that addition of bone marrow derived mesenchymal stromal cells and platelet rich plasma on a collagen matrix may reduce incisional hernia formation in rats [42]. Also, Dong and colleagues stated that bone marrow aspirate concentrate and platelet rich plasma helped to improve bone healing in distraction osteogenesis of the tibia in human [43]. Combination of MSCs and PRP has many advantages. It is safe with minimal side effect because both MSCs and PRP are autologous, nontoxic, less invasive and have no or limited immunogenicity [44].

So, we tried in this study to bring “artificially” all factors needed for optimal wound healing to the site of injury. These factors include bone marrow mesenchymal stem cells, bone marrow hematopoietic stem cells, paracrine factors of both bone marrow and platelets, and fibrin network. The results were matching most of literatures, but the point here that we tried to compare the results in 4 groups subjectively. We analyzed the outcome of wound healing grossly and microscopically. We reported in the results that the wound healing was much better in both time and quality when we add the bone marrow and/or platelet rich fibrin.

**Inflammatory response and fibrosis:**

Scarring is believed to be secondary to prolonged inflammation [45]. A phenomenon that appears to be intrinsic to fetal skin, wound heals without scarring. The presence of stem cells in the fetal wound may be an important mechanism resulting in scarless repair [46].

In this study; we found that in normal wound healing process there was increased inflammatory response in 2, 4 and 6 weeks after injury in comparison with other groups. These may be the main cause of increased depth and areas of fibrosis in this group of rats. After 8 weeks the inflammatory cells reduced but the fibrosis still remarkable. When we added bone marrow with or without platelet rich fibrin, the inflammatory cells were less all over the period of the study. We found that the fibrosis also became less in the depth and area of fibrosis in these groups.

**Epithelial thickness:**

A number of growth factors and cytokines stimulate re-epithelialization [47], until contact inhibition happens. They continue to proliferate and migrate across the surface of a wound until they contact each other [30]. Recent studies have shown that cells derived from the bone marrow contribute fibroblast-shaped cells and other cells such as keratinocyte phenotype [48-49].

We planned in our study that the wounds-in all groups-healed with primary intention by subcuticular absorbable sutures to reduce the tissue gap and having the best epithelization. So; by analyzing the histological slides in our study, we found that there were increase in epithelial thickness in all groups after 2 weeks. This thickness decreased gradually with time to be near the epithelial thickness of the normal skin after 8 weeks in all groups. This finding strongly suggests that the appearance of scar is mainly due to the amount of fibrosis below the epithelium and not due to the epithelial thickness.

**Collagen in wound healing:**

Type I collagen in the dermis is about 80% to 90% of skin collagen [30]. Hematopoietic and mesenchymal cells are responsible for producing collagen types I and III for repairing dermis. Therefore, collagen type III is considered to be important for wound healing [11]. During remodeling, type III collagen is replaced by type I collagen to restore the normal dermal collagen. Collagen production continues for 6 weeks and then followed by collagen degradation [30].

Unfortunately, we did not define the type of collagen in our study, but we think that the amount of collagen may match this phenomenon. We found that after 2 weeks of injury, the collagen density increased in the 4 groups (may be due deposition of type III) in early stages of wound healing. Then the density decreased in the 4th week to become increased again in the 6th week (may be due replacing type III by type I) in remodeling process. At the end of the 8th week, the collagen density in all groups was near the value of normal skin. Addition of bone marrow with or without platelet rich fibrin did not interfere much with the amount of collagen production or remodeling in our study.

**Skin regeneration:**

Haihong and colleagues reported that; labeled mesenchymal stem cells were noted in hair follicles, sebaceous glands, blood vessels, and dermis in full-thickness wounds [38]. Bone marrow mesenchymal stem cells are significantly involved in the regeneration of functional hair follicles. Bone marrow mesenchymal stem cells in the wound enhance the proliferation of keratinocytes and increase the number of regenerating appendage-like structures [11]. In the past few years, several research groups have explored the feasibility of
using platelet-rich matrices as tissue engineering scaffold [21].

We tried in our study to regenerate the skin scaffold with platelet rich fibrin. We think that it played a role especially in combination with bone marrow. We noticed that the skin appendages were preserved in group that received this combination. We cannot assure if it is true regeneration or it is preservation of original appendages due to minimal fibrosis.

Conclusion:

Adding heparinized autologous unprocessed bone marrow and/or autologous platelet rich fibrin; may improve the pattern of incisional wounds healing. This improvement can be documented objectively by using the digital imaging analysis. This preliminary work suggests that there were differences in the time of healing, scar appearance and consistency, microscopic inflammatory response, fibrosis area and depth of fibrosis. Epithelial thickness, collagen deposition and remodeling were similar to the normal healing process. To illustrate significant statistical differences we need a larger sampling and longer follow-up periods. There will be much to do to investigate and document these effects on biological and biochemical basis.

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