HISTOPATHOLOGICAL STUDY OF PLATELETE RICH PLASMA FOR TREATMENT OF INDUCED CUTANEOUS ULCER IN DIABETIC RABBITS INDUCED BY STREPTOZOTOCIN

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ABSTRACT

The present study investigates the effect of platelets rich plasma (PRP) on the healing of skin ulcers in rabbits in which diabetes mellitus (DM) was induced using Streptozotocin. The study was applied on 12 adult rabbits of both sexes with a mean weight of 1000-1500 g. They were divided randomly into two equal groups (six rabbits per group). DM was induced in two groups by injected Streptozotosin 65 mg / kg B.W IV. Ulcers were made in 3 cm length and 2 cm width in all rabbits in the gluteal region under the general Anesthesia by mixture of xylazine hydrochloride and ketamine hydrochloride in sterile conditions. The first group was used as control; the second group was treated with platelets rich plasma on the site of ulcers.The first group in the 7 days, showed large ulcers and pus; in the 14 days, were ulcers continued with thickened epidermal. Second group, in the 7days, showed skin ulcers with dermal thickening and a beginning of skin formation. In the 14th days, treated group showed small ulcer remaining with dermal fibrosis.ELISA results have been shown the effect of platelets rich plasma on tumor necrosis factor alpha (TNFα) concentration in blood after 10 daysof skin lesions inducing. The natural concentration of TNFα is 6.4 pg/ml in range (4.2-7.9) pg/ml. The first group had a concentration below 5.3 pg/ml, due to the DM. The second group was highly concentrated in 10.4 pg/ml. The results of the histopathological and ELISA confirmed that there are a marked healing and elevating TNFα concentration in the treated group.

INTRODUCTION

Platelets rich plasma (PRP) is an autologous concentration of platelets in concentrated plasma, which is extensively used to support tissue healing and reduce wound healing time.PRP is abundance of growth factors (GF)and has both mitogenic and chemotactic properties on various cell types, present in a wound, such as; fibroblasts, keratinocytes and epithelial cells (1). It stimulates local production of growth factors and enhances wound closure and improves angiogenesis and re-epithelialization. Also, it has been reported that PRP may suppresses cytokines release and limit inflammation, interacting with macrophages to improve tissue healing and regeneration (2).

Platelets are formed from megakaryocytes and synthesized in bone marrow by pinching off pieces of the cytoplasma. Platelets are small discoid blood cells. The average platelet countsare ranging from 1.5–3.0 × 10^{-5}/ML of the circulating blood; with an average half-life of about 7 days. Preparation of Platelet Rich Plasma (PRP) involves obtaining several milliliters of blood and special systems for enrichment of the sample. The price of this procedure strongly varies (3). Platelets secrete variety of growth factors after activation and degranulation which acting as messengers to regulate the various processes. The role of growth factors in wound healing has been well described in acute wound healing.Growth factors are stored in the form of α-granules in platelets and when platelets are activated, they in turn release multiplicity of growth factors. After the formation of platelet coagulum, the activated platelets are interspersed among the fibrin strands forming a matrix within the clot, which helps keeping the growth factors within the mesh. Eventually They diffuse out into the surrounding tissue. Growth factors act locally to recruit undifferentiated cells to the injury site by chemo-attraction, also, stimulate mitosis in the undifferentiated cells. Stem cells are attracted to areas of high concentration of growth factors.Cellular movements occur by forming attachments to the matrix/scaffold. Growth factors attach to stem cell receptors, thereby activating genes which control cell division. They also attach to cell receptors and control the genetic expression of stem cells via modulations of signal transduction pathways of secondary proteins, resulting in cellular division and differentiation. Mitosis occurs via a signal transduction pathway through the tyrosinase kinase located on the cell membrane. Receptor activation leads

to the activation of secondary messenger proteins, which enter the cell nucleus and influence the expression of the genes responsible for triggering mitosis, angiogenesis, and macrophage activation (4).

Platelet derived growth factor (PDGF) is first growth factor that was identified. It has been synthesized through recombinant DNA technology and has been used topically for treating chronic diabetic ulcers (5). Other identifiable growth factors include transforming growth factor (TGF-β), which has β1 and β2 as isomers, platelet derived angiogenesis factor; platelet derived epidermal growth factor, fibroblast growth factor, keratinocyte growth factor, insulin like growth factor; interleukin-1, vascular endothelial growth factor, epidermal growth factor, osteocalcin, osteonectin, fibrinogen, and fibronectin. Other groups of growth factors are collectively called the adhesive proteins. These include fibrinogen, fibronectin, and thrombospondin. They are known to participate in thrombus formation and some mitogenic action (6). On the other hand, a variety of active substances at high concentrations are released and enhance healing in acute and chronic wounds through stimulation of cellular migration and proliferation (7).

Diabetesmellitus is induced by Streptozotocin (STZ, 2-deoxy-2- ({[methyl (nitroso) amino] carbonyl} amino)-β-D- glucopyranose) is a combination of α- and β-stereoisomers (appear as a pale yellow or off-white crystalline powder and very soluble in water), ketones, and lower alcohols which is slightly soluble in polar organic solvents (8). STZ is a naturally occurring compound, synthesized by the bacterium Streptomyces chromogenes, that shows broad spectrum antibacterial properties and is used to induce both insulin-dependent and non-insulin-dependent diabetes mellitus (9).

MATERIALS and METHODS

Twelve healthy adult domestic rabbits (*Lepus cuniculus*) with body weight ranged (1000g-1500g) were brought from local markets. Rabbits were kept to an adaptation period for one month in the animal house of Veterinary Medicine Collage / Basra University. The experimental animals were kept in individual cages, provided with ration composed fodder which is consist of dry bread, lettuce and tap water. All of the rabbits were given a prophylaxis drug against coccidiosis Amprollium (1g/L of

drinking water). Animals were maintained in air-condition quarters(24C°) under standard husbandry condition with alternate 12 hours' light/ dark (10).

The experimental design included 12 rabbits were induced of diabetes mellitus by using Streptozotocinwhich induced ulcer in gluteal region of rabbits.Rabbits thendivided into two groups as; first group is a control which remained without treatment. Thesecond group was treated with platelet rich plasma (PRP).

Preparation of Sodium Citrate Buffer:

The preparation of 1 molarsodium citrate buffer were done by mix 2.1 grams of citric acid and 2.94 grams of sodium citrate in 50ml of distilled water. Then, we add NaOH to adjusted pH to 4.5 finally the volume was completed to 100ml (11).

Preparation of STZ for IV Injection:

Depend on the weight of animals and the dose of STZ (65mg/kg I.V.), the suitable amount of STZ was dissolved in citrate buffer freshly. STZ was prepared freshly (20 minute before injection) and container was covered with aluminum foil to protect the buffer from light exposure (12).

Induction of Diabetes

Diabetes mellitus was induced in the overnight fasted rabbits by a single I.V. injection of STZ at a dose of 65mg/kg of body weight.STZ was dissolved in citrate buffer (pH 4.5) and freshly prepared before injection. After five hours from STZ injected, water contain 5% glucose was giveninstead of drinking water to overcome the high insulin released to all rabbits injected with STZ. Hyperglycemia in rabbits followed up for five days.Using AccuChek Active meter. Rabbits have been reached to 300 mg/dl of blood glucose considered diabetic(13).

PRPpreparation(includedapproximately)6 mL whole blood were withdrawn into centrifuge tubes containing EDTA. The blood was centrifuged in a standard laboratory centrifuge machine at 1.500 rpm for 5 min. Subsequently, yellow plasma (containing buffy coat with platelets and leukocytes) was taken up into a second centrifugation at 3.500 rpm for 10 min. Plasma was performed to combine the platelets into a single pellet. The plasma supernatant, which was platelet-poor plasma (PPP) and contained relatively few cells was removed. The pellet of platelets, termed

the buffy coat/plasma fraction (PRP), was re-suspended in 1 mL residual plasma and used for the platelet gel(14). PRP was stored at room temperature in a conventional shaker until use. Diabetic ulcers were dressed by prp in the first day.

Enzyme-linked immunosorbent assay ELISA (Elabscience/China) was used to detectedtumor necrosis factor alpha (TNF-α) in tenth day of induced of skin lesions.

Histological Process:

Specimens werecollected from the wound area including a part of the skin edge on the 7thand 14th days postoperative. The specimens were preserved in 10% formalin solution for slide preparation. Dehydration of specimens were doneby upgrading alcohol from 70% to absolute alcohol 100% for two hours in each concentration.The clearing process occurred byusing xylene for ½ hours for each solution; thenembedding (Blocking) with paraffin wax. Paraffin is allowed to solidify around and within the tissue for keeping intact all parts of the tissue when sections are cut. After the samples were embedded, it were put in the oven (58C°). Then, it poured in a block of paraffin.The specimen was cutting using microtome and staining withordinary H&Estaining (15).

RESULTS

Serum TNF-α levels in control group was (5.3 pg/mL), however TNF-αserum concentration in platelet rich plasma (PRP) group was elevated to (10.4 pg/mL). (Fig. 1)

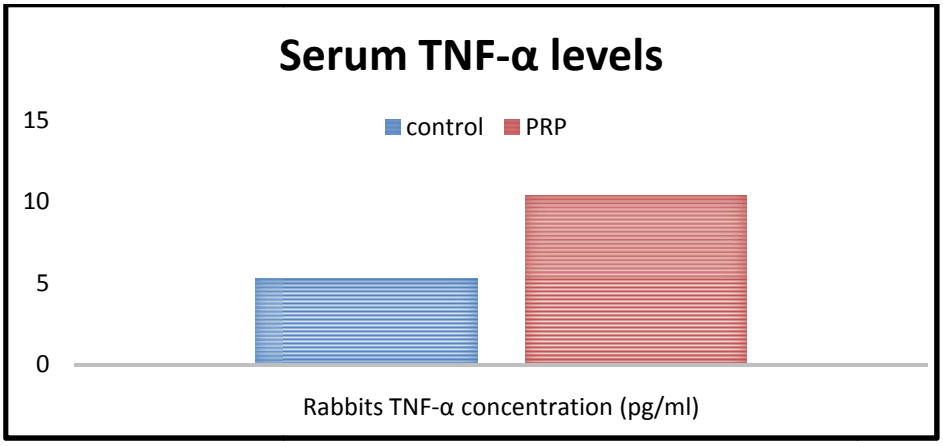


Figure (2). Circulating levels of TNF-alpha

Histopathogical Examination

After experimental period scarified the animals had a thin outer portion the epidermis, which is the keratinised stratified squamous epithelium of skin. control group (this group has induced the diabetes mellitus and diabetic ulcers) In the 7th day of induction of the diabetic ulcers is a very complex and accompanied with Large ulcer left with infiltration of the inflammatory cells, thickened epidermal, dermal fibrosis, scab forming, no hair follicles, sebaceous glands and abscess figures (2 and 3).In the 14th day of induction of the diabetic ulcers, rabbits showed ulcers with thickened epidermal, dermal fibrosis, no hair follicles and sebaceous gland figures (4 and 5).

The diabetic ulcers of rabbits which treated with platelet rich plasmashowed clear revealed histopathological changes. In the 7th day of induction of the diabetic ulcers, animal showed ulcers included thickening in epidermis, scabbing formationand not yet epidermal covering above complete healing with epidermal formation figures (6,7,8).In the 14th day of induction of the diabetic ulcers, the platelet rich plasma treated group show Small ulcer, large scab, moderate thickening of epidermal and moderate dermal fibrosis figure (9).

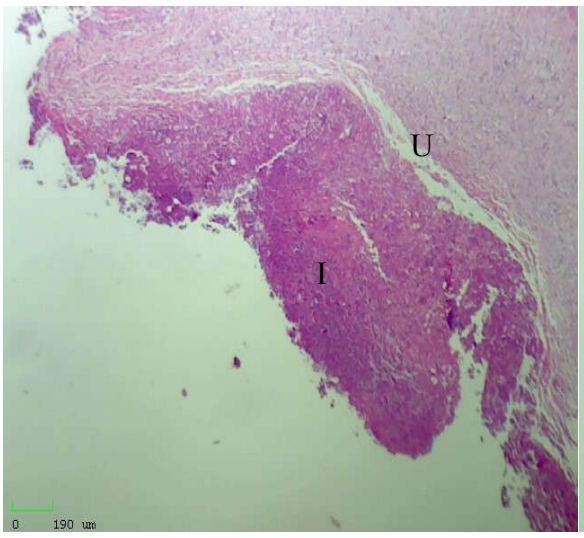


Figure (2) Diabetic control group (7th day). Large ulcer (U) left with infiltration of the inflammatory cells (I). 4X H&E

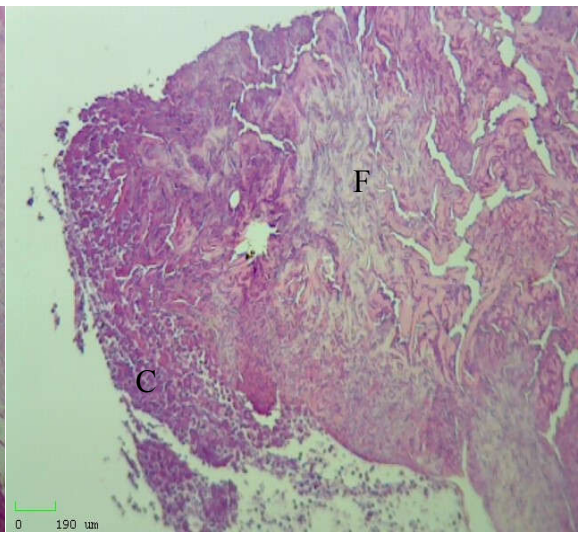
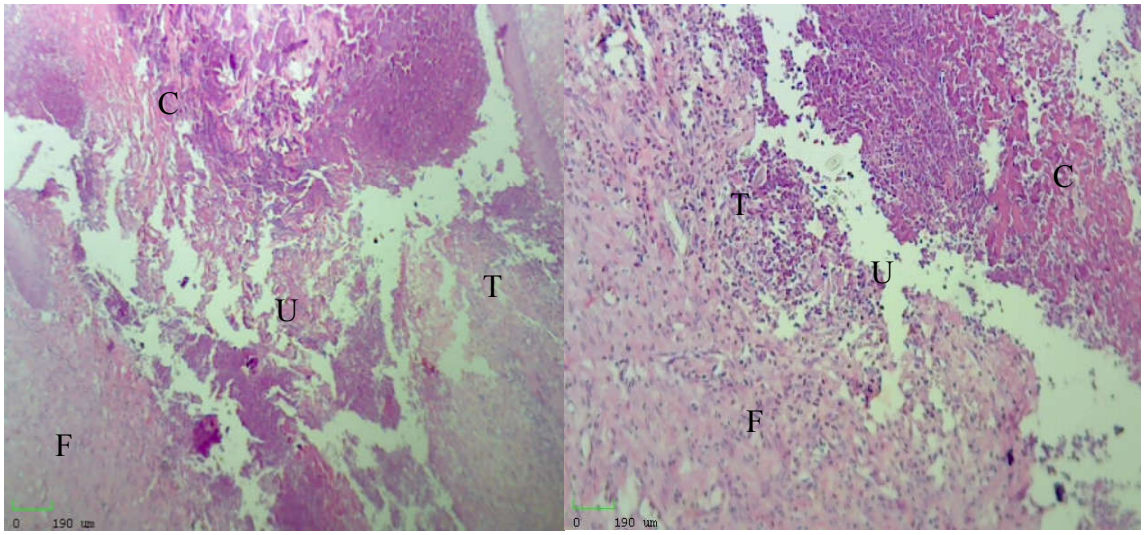


Figure (3) Diabetic control group (7th day). Thickened epidermal, dermal fibrosis (F), scab (C) forming and no hair follicles and sebaceous glands. 10X



). Ulcer (U) with Thickened epidermal (T), dermal fibrosis (F), scab (C) forming and no hair follicles and sebaceous glands. 10X

day). Ulcer (U) with Thickened epidermal (T), dermal fibrosis (F), scab (C) forming and no hair follicles and sebaceous glands. 10X H&E

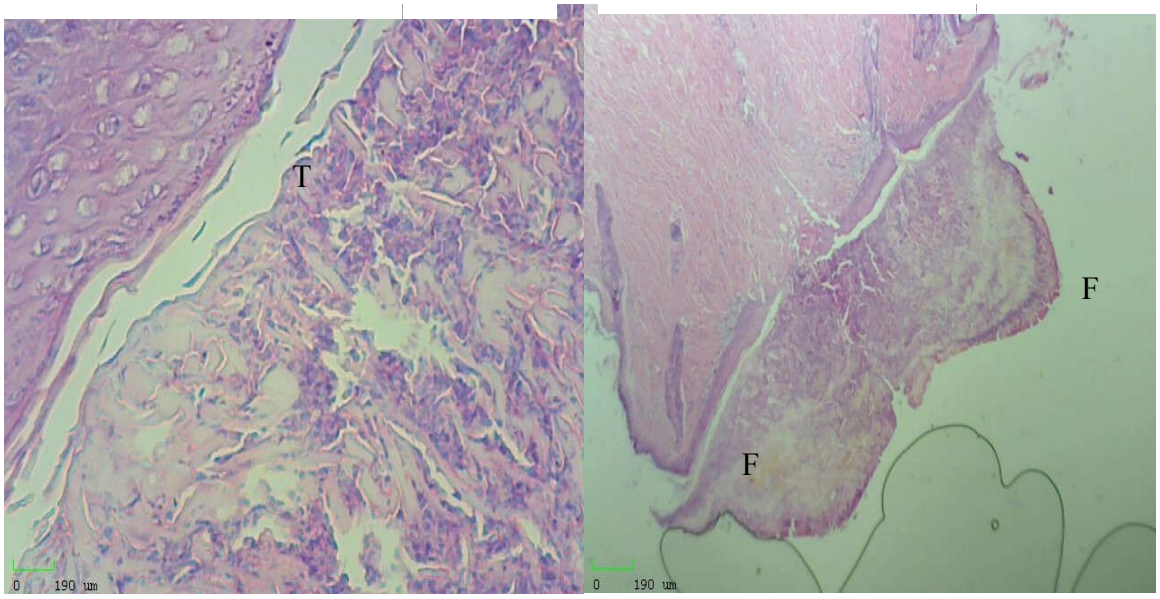


Figure (6) Platelet rich plasma (prp) treated group (7th day). Ulcer with thickening (T) in epidermis and scabbing formation. 4X H&E

Figure (7) Platelet rich plasma (prp) treated group (7th day). Ulcer consist of fibrous tissue (F) and not yet epidermal covering above complete healing with epidermal formation. 40X H&E

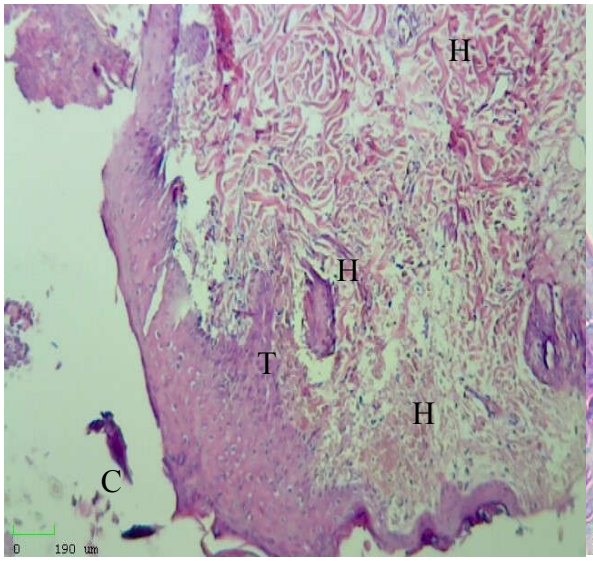


Figure (8) Platelet rich plasma (prp) treated group (7th day). Thickened(T) epidermal, Scab (C) formation and hair(H) formation.40X H&E

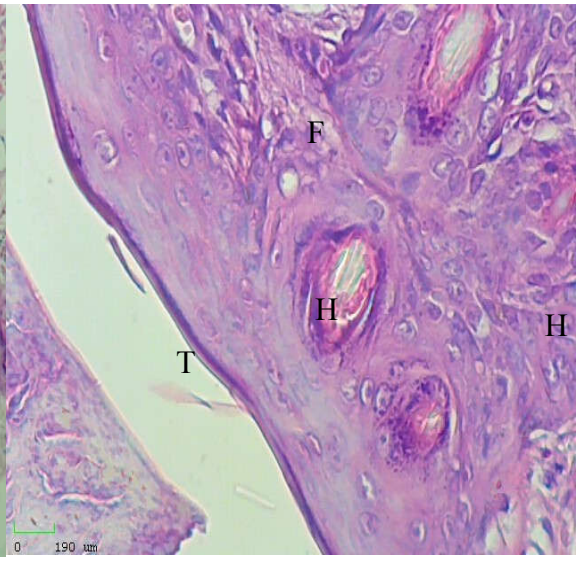


Figure (9) Platelet rich plasma (prp) treated group (14th day). Thickening (T) of epidermal, fibrosis (F) in derm and few hair follicles(H) formation. 10X H&E

DISCUSSION

This study demonstrates the immunological and histological findings after 7th ,14th of treatment by the platelet rich plasma to stimulate the healing process after the ulcerinducingin the diabetic rabbits.

The results showed that under high-glucose microenvironment, there was less vascular regeneration of skin ulcers compared to low-glucose microenvironment (16,17). Many scientists have employed different methods to support ulcer healing (18),therefore this project used PRP to treat ulcers in diabetic rabbits.

ELISA

Serum TNF-α level was measured by enzyme-linked immunosorbent assay (ELISA) technique. TNF-a, is secreted by invading macrophages and monocytes after injury in 2 to 5 days (19). The TNF induces extracellular migration and tube formation in the absence of proangiogenic factors, suggesting that TNF can directly activate signalling pathways for epithelial cell migration (20).Therefore,serum TNF-α level in the control group(5.3 pg/mL) and theserum TNF-α levels in platelet rich plasma (PRP) group(10.4 pg/mL)

Histological Findings

Diabetic control

In the 7th day, the control group showed large ulcers, infiltration of the inflammatory cells and dermal fibrosis. In the 14th day, the control group showed dermal fibrosis, no hair follicles and sebaceous glands formation these group healed by second intention this observation agreed with (21).

PRP group

The application of PRP seems to have a positive effect in the healing process of chronic ulcers, accelerating the rate of wound healing, decreasing local pain and the possibility of infection (22). Experimental studies usually present satisfactory results of PRP application; which conduct an experiment in equine lower limb wounds applying PRP onto ulcers. Histological results showed improvement of the wound healing with a presence of organised fibroblasts and better orientation of collagen fibres (23).

In the7th day,prp group showed ulcers with dermal fibrosis. This result is similar to Yazawa; who used PRP in rabbit ear ulcers and concluded that from the seventh post-treatment day, ulcers treated with PRP presented an increased progress in epithelialisation and limited appearance of granulation tissue with seven days treatment (24). Clinical studies on the healing progress of chronic ulcers using PRP equally present encouraging results (25).

In 14th day, the prp group showed dermal fibrosis and few hair follicles formation.The results are consistent by (26), who found the PRP caused formation of fibrous tissue by the end of two weeks which causing faster healing of diabetic ulcers. The role of PRP as a local covering is required to provide the growth factors locally at the wound area. This role is suggested to be beneficial because diabetic ulcers are deficient in growth factors. This work result agrees with (27).

دراسة نسيجية مرضية للبلازما الغنية بالصفائح الدموية في علاج القرح الجلدية المحدثة في الأرانب المصابة بمرض السكري المستحدث بالستربتوزوتوسين

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الخلاصة

أجريت الدراسة الحالية للتحقق من مدى تاثير البلازما الغنية بالصفائح الدموية على ألتئام القرح الجلدية المحدثة في الأرانب المستحدث فيها داء السكر تجريبيا بأستخدام عقار الستربتوزوتوسين. أجريت الدراسة على 12أرنبابالغةمنكلاالجنسين معدل اوزانها (١٠٠٠-١٥٠٠) غم وقد قسمت عشوائيا الى مجموعتينستة أرانبلكل مجموعة تم أستحداث السكري فيها حيث حقنت بعقار الستربتوزوتوسين (٦٥) ملغم/كلغم داخل وريد الأذنلاستحداث السكري وبعدها تم عمل قروح جلدية بطول ٣ سموعرض ٢ سم جرحفيجميعالأرانبفيالمنطقةالكفليةتحتتأثير خليطزيلازين هيدروكلوريدوهيدروكلوريدالكيتامين في ظروف معقمة.

كلا المجموعتين استحدث فيها السكري والقرح الجلديةالمجموعة الاولى تمثل مجموعة السيطرة،المجموعة الثانيةتم علاجها بالبلازما الغنية بالصفائح الدموية على موقع القرح.

وأظهرتالدراسة في المجموعة الأولى في اليوم السابع وجود قروح كبيرة متبقية وتكون قيح أما في اليوم الرابع عشر استمرت القروح مع تثخنات في البشرة، المجموعة الثانيةفي اليوم السابع ظهرت القروح الجلدية مع تثخن وبداية تكوين البشرة أما في اليوم الرابع عشر فوجدت بقايا قروح صغيرة مع حدوث تليف في البشرة.

نتائج الاليزا اظهرت تاثير البلازما الغنية بالصفائح الدموية على ارتفاع تركيز عامل التنخر الورمي (TNFα: Tumor Necrosis Factor) في الدم في اليوم العاشر من احداث الجروح والقرح الجلدية. TNFα يعتبر من السايتوكينات التي تنتج بواسطة الخلايا البلعمية والوحيدات خلال الألتهاب ويكون مسؤولا عن تحفيز الخلايا المولدة الليفية وافراز عوامل النمو وتنظيم عمل الخلايا البلعمية. التركيز الطبيعي لل TNFα هو 6.4 pg/ml معدل pg/ml(٤.٢-٧.٩) ففي المجموعة الاولى كان تركيزه أقل من المعدل الطبيعي وهو ٥.٣ نظرا لأستمرار القروح. المجموعة الثانية كان تركيزه عاليا فيها ١٠.٤pg/mL. نتائج الأليزا اكدت بأن هنالك أرتفاع في تركيز TNFα في المجموعة المعالجة بشكل واضح.

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