RESEARCH PAPER

Omegaven Improves Skin Morphometric Indices in Diabetic Rat Model Wound Healing

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Abstract The global prevalence of diabetes is estimated to be 200 million people, and it is likely to increase to 333 million people by 2025. Different cells, are known to participate in three main phases of wound healing. Omega-3 fatty acids influence cytokines and growth factors which affects the presence of inflammatory cells in wound area as well, but how this event specifically influences the role of fibroblasts, macrophages and angiogenesis in wound healing is not obviously understood. In this experimental study seventy male Wistar rats after induction of diabetes type-one by streptozotocin (STZ) (55 mg/kg) were divided into two groups, Experimental group receiving omegaven intraperitoneally and control group which underwent the injection of mineral oil. Streptozotocin was used for the induction of diabetes type 1. Diabetic male wistar rats were scarified at 1, 3, 5, 7 and 15 days after the excision was made. To estimate orphometric indices, histological sections were provided by stereological methods. It was found that wound area significantly decreased on day 7 in experimental group by omega-3 fatty acids. The number of fibroblasts increased significantly on days 5 and 7 in the experimental group. The number of neovascular significantly decreased on day 7 in the experimental group. This study implied that it seems omegaven is able to improve morphometric indices during wound healing and make healing faster.

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Introduction

The incidence of diabetes mellitus is increasing tremendously world-wide. Complications arising from diabetes

have become serious public health issues. Research on human and animal models has identified a large number of changes associated with diabetes at the molecular level in delayed wound healing.¹ Macrophages can be found throughout the body and in different organs, in skin and the lamina propria of epithelial tissues as well. Most of times they may be seen around the vessels, lamina propria

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appendages and skin^{2,3}. Tissue macrophages are embedded in extracellular matrix, express receptors for collagens, elastin, proteoglycans, and reticular/fibrillar constituents, including bound cytokines and in turn generate potent extracellular proteinases and anti-proteinase inhibitors to modulate matrix, cellular and plasma protein functions.⁴

Fibroblast is central to the processes of extracellular matrix deposition and remodeling. It functions in different ways, as a synthetic cell, it deposits a collagen-rich matrix, and as a signaling cell, it secretes the growth factors important for cell-cell communication during repairing process.^{5,6} A neuropathic foot ulcer is a severe complication of diabetes that can result in a more severe complication; a lower extremity amputation. According to an earlier study about 7% of people with diabetic neuropathic foot ulcer had an amputation and in the past 10 years there had been a remarkable increase in the number of minor as compared to major amputations.⁷ Previous studies have suggested that possible etiologies for impaired wound healing in diabetes are accompanied with decreasing growth factors such as keratinocyte growth factor (KGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) production and excess protease activity.^{8,9} Omega-3 (eicosapentaenoic and docosahexaenoic) acids are essential for humans. The important member of these families (EPA and DHA) has various effects on immune system and inflammatory reactions,¹⁰ Their functions are changing membrane fluidity, the regulation of transcription of genes, protein acylation and making less changes in cytokines and interleukins.¹¹ Omega-3 fatty acids are able to affect macrophages, fibroblasts and even epidermal regeneration as well.¹² Since there haven't yet been any stereological-based study to determine the relationship between omega-3 fatty acids and these above mentioned elements in diabetic wound, the current study is carried out to determine this relationship.

Materials and Methods

This experimental study was approved by the Medical Ethical Committee at Arak University of Medical Sciences, Arak, Iran (protocol no.92-148-1). Seventy male Wistar rats weighed (200–250 g) were obtained from Pasteur Institute of Iran and after the induction of diabetes type-one by streptozotocin (STZ) (55 mg/kg), rats were divided into two groups, the experimental group (DB, OMV) receiving omegaven intraperitoneally at a dose of 1 mL once a day per rat¹³ and the control one (DB, MiO) which underwent the injection of mineral oil, 1 mL once a day per rat. The animals were maintained individually in separate cages with free access to food and water in a room with controlled humidity and temperature (22–24 °C) on a 12-h light/dark cycle.

Induction of Type1 Diabetes Mellitus

In the present study all 70 rats (experimental and control) were randomly chosen and after 12-h fast, each rat underwent

single intraperitoneal injection of (STZ) (Zanosar Pharmacia & Upjohn Co., Kalamazoo, MI49001, USA) at a dose of 55 mg/kg body weight in distilled water (DW).¹⁴ Seven days after (STZ) injection, blood glucose measurements (Bionime, Rightest GM310, Bionime Corporation, Switzerland) were taken from tail blood.¹⁵ Type 1 diabetes was defined for each animal if blood glucose levels were consistently above 300mg/100 ml one week after the injection. During this period diabetic rats (DB) showed clinical signs of diabetes mellitus such as polyuria, polyphagia and weight loss.¹⁶ After a monthly consistent hyperglycemia, animals were considered eligible for the remainder of the study. During the study blood glucose levels for all rats were measured every three days.

Wounding Model

To provide excisional wound (day0) each animal was anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg). The back of all rats were shaved and a full-thickness excisional wound (20 mm × 20 mm; Fig. 1) was made to the level of the panniculus carnosus muscle.¹⁷ Wounds were not sutured or covered, but were allowed to be healed by secondary intention. Wounds were inflicted in the same manner for all rats (Fig. 1A, B).

Omega-3 Administration

In experimental group (n = 35) omegaven (Fluka/Sigma-Aldrich®, St. Louis, MO) was given intraperitoneally at a dose of 1 mL per rat (100 mL omegaven contains 2.82 g of EPA and 3.09 g of DHA) once a day.¹⁸ Administration started intraperitoneally at a dose of 1 mL once a day from 10 days before starting the study and continued until the end of each analysis.

Macroscopic Assessment

Body weight and blood glucose levels were measured on the day of surgery (day0) and then every three days after the surgery until the end of each analysis. To measure changes of the wound area for all the experimental and control groups, photographic method¹⁹ was used and therefore all wounds were photographed (Yashica CMH150 Full HD 1080P Digital Camcorder) in similar pattern (the similar distance from camera to the wound, the similar magnification of camera, the similar magnification of photos in windows photo viewer) then the photographs were conformed to a simple meshwork built-up 225 squares designed to evaluate wounds surface (Fig. 2A, B). Real dimension of each square is 5 × 5 mm. According to stereological rules, only if left and lower flanks and the plus symbol of any square of the grid are conformed to the wound then this square can be considered.¹⁶

The total number of counted squares could be used in SPSS software to compare wound areas in all groups.

Figure 1 A, B. The Pattern of the excisional wound (20 mm × 20 mm) on the back of the rats.

Counting the number of each square conforming wound area makes it possible to compare total wound area in each specimen, these numbers are analyzed by SPSS software. The photograph of scabs in healing wound area in day 7, in experimental and control groups, Fig. 3A and B. Comparing the extent of scabs in Fig. 3 A and B.

Microscopic Analysis

On days 1,3,5,7 and 15 rats were sacrificed by intraperitoneal overdose of pentobarbital. The complete area of the wound consisting the excised area adjacent to normal skin was removed. The excised tissues were fixed in formalin (pH = 6.9) and embedded in paraffin. Sectioning was performed according to stereological methods.^{16,19} Stereological methods were precise techniques used to obtain information from three-dimensional structures on the basis of the observation of two-dimensional sections. In this study, 15 × 2 (6 μm thickness) sections from the healing wounds of each rat were obtained. The sections were stained with H&E, observed and evaluated by stereological methods. 1050 random fields of all specimens of each group (n = 7) (8400 microscopic field, totally 7.43 mm² for all groups) were photographed and a 10 × 100 objective lens (NIKON-Eclipse E2000-Videocamera DS-Fi1, Japan) was applied, all photos were used to count cells by

stereological methods (Fig. 4). The quantitative evaluation of any tissue elements were based on counts obtained in vertical sections.²⁰

Statistical Analysis

For all experiments SPSS (IBM) version16 (Kolmogorov Smirnov, Leven’s test, student’s t-test for independent samples and One-way Anova) for the statistical analysis of the macroscopic and microscopic tests was used, p-values ≤ 0.05 were considered statistically significant. Data were expressed as mean and standard deviation (SD).

Results

Macroscopic Analysis

Weight and blood glucose level of all animals were measured every three days. In both the experimental and control groups, weight and blood glucose levels changed from day to day, but these changes were not significant (p > 0.05). All 5 diabetic rats died during the first week following (STZ) injection and were replaced by new animals. The measurement of wound area by photographic

Figure 2 A, B. The Measurement of the wound area. The Photograph of wound area (Fig. 2. A) is conformed to the grid meshwork including 225 squares (5 × 5 mm) designed to evaluate wound surface (Fig. 2. B).

Figure 3 A&B. Day 7, as it is clear in figures, the extent of scab formed in the experimental group in fig. A. is less than that in the control group in fig. B. the photographs are taken in same focus, distance and magnification.

method cleared that although wound area decreased on days 5 and 7 in the experimental group this reduction was significant only in the experimental group on day7, (Fig. 5).

Microscopic analysis

According to microscopic figures as they are shown in Fig. 6A&B. in Fig. 6 A the wound is healed faster and there is a small scar in the wound area (arrows) but in Fig. 6B. It is clear that there is a great area between the wound edges (arrows) which is filled with scar.

Based on the microscopic assessment, the administration of omegaven fatty acids caused the reduction of neo-vasculars on days 3,5 and7 as well, but this reduction was significant only on day7 ($p = 0.00$); Figs. 7,8,9.

Also there was a significant increase in the number of fibroblasts on day5, 7 ($p = 0.00$); Figs. 7, 8. There was a remarkable reduction in macrophages only on day 7 ($p > 0.05$); Fig. 9. Comparing the experimental and control groups we found a significant reduction of epithelium thickness on day7 ($p = 0.00$); Fig. 9.

Discussion

Many earlier studies have indicated deleterious effects of diabetes mellitus on the wound healing. Growing evidence has shown a reduction in collagen organization, diminished blood supply, increased blood viscosity, defects in leukocyte function, unbalanced production of growth factors, cytokines, proteases, delays in inflammatory cell infiltration and finally conversion from acute to chronic inflammation.^{21,22} Recent studies conducted within the field of diabetes have revealed significant delays in the wound healing and biomechanical parameters of skin after wounding in comparison to healthy rats.²³ Previous studies indicates that EPA and DHA may alter membrane fluidity Act as arachidonic acid antagonist, and inhibit the production of inflammatory eicosanoids, adhesion molecules and

cytokines.²⁴ Measuring the wound area and comparing DBOMV and DBMIO groups, the current study demonstrated that the experimental group showed a significant reduction of the wound area on day 7. It was found that eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids induced faster wound closure comparing to the control group. Definitely wound contraction played a key role in defining the dynamics of wound closure. In all full thickness wounds specially in the excisions, contraction was known to peak between 5–15 days after wounding and continues even later than the wound reepithelialization.²⁵ Fibroblasts and myofibroblasts are clearly among the most important and abundant cells in the wound area. In physio-

Figure 4 Stereological technique to obtain information regarding three-dimensional structures based upon the observation of the two-dimensional sections. In this figure, a sample photograph of a random histologic section is conformed randomly with a special grid for ease in distinguishing and counting the tissue elements. This operation has been done for all figures.

Figure 5 Comparing of the macroscopic evaluation of wound area between experimental and control groups. There was a remarkable difference between the experimental and control group in the wound area only on day seven ($p = 0.000$).

logical conditions these cells are responsible for tissue homeostasis and tissue contractile, they move within the collagen matrix and reorganize the surrounding collagen fibrils at the periphery of the collagen lattice first, and the mechanism for contraction is based upon cell-cell contractions.²⁶ By the way in tissue injuries, fibroblasts will be activated and differentiated into myofibroblasts, which generate large contractions and actively produce extracellular matrix ECM proteins to facilitate wound closure.

Fibroblasts and myofibroblasts are responsible for conducting traction and contractile forces respectively to promote wound contraction.²⁷ Wound environment contains high molecular weight proteins that promotes fibroblast functions essential for the healing process,²⁷ e.g. TGF-beta released from platelets and inflammatory cells that enhances wound contraction,^{28,29} stromelysin-1 which is crucial metalloproteinase for the organization of a multi-cellular actin network which promotes wound contraction.³⁰ Some of these cytokines are on opposite side for example: IL-1- β ³¹ and IL-10 Which impede wound healing,³² Tumor necrosis factor TNF- α and interferon IFN- γ

which all these cytokines inhibit the contraction, it seems that IL-1 beta is the most powerful among them.³³ Based on previous studies omegaven can inhibit the production of some of these inflammatory cytokines such as IL-1, IL-6, and TNF- α , that eventually leads to increasing the ability of wound contraction³⁴ or may decreases the number of cells that are able to secrete these cytokines.³⁵ Some studies indicate that diabetes causes the increasing blood viscosity that leads into serious problems with tissue microcirculation and also causes hidden ischemia in tissues which has been noted to be the source of diabetic retinopathy, defects and delay in wound healing and even quantitative reduction in biomechanical indices.^{36,37} It is clear that omega-3 fatty acids decrease blood viscosity, platelet and fibrinogen aggregation. In no clinical trial, there has been any criteria of increasing blood loss due to the ingestion of omega 3 fatty acids.³⁸ Plenty of studies have revealed therapeutic effect of omega 3 fatty acids on ischemia.^{38,39} It is also clear that omega-3 fatty acids may act as a modulator or an accelerator in fibroblasts proliferation and collagen synthesis during the wound healing.^{12,40} Among fatty acids, omega-3 polyunsaturated fatty acids possess the most potent immunomodulatory or anti-inflammatory activities, and among them, EPA and DHA are more biologically potent.

Some of the effects are acting upon intracellular signaling pathways, transcription factor activity and gene expression. According to previous studies omega-3 fatty acids are able to reduce Plasma macrophage chemoattractant protein 1 MCP-1 that may lead into reducing macrophages.⁴¹ These results are on the basis of invivo and invitro studies. In present study, the reduction of macrophages may be related to the reduction of MCP-1 by EPA and DHA. Many studies suggested that Omega-3 fatty acids are able to increase the amount of capillaries in adipose tissue, the same mechanism may cause increasing capillaries in wound area.⁴² Although on days 3 and 5 after wounding,

Figure 6 A&B. The microscopic photographs of wound areas and adjacent normal skin on day15. In fig. A in the experimental group, the edges of wound are closed together. In Fig. 6B. The edges of wound are yet far from each other and there is a great area composing connective tissue and fibroblasts between edges.

Figure 7 The statistical analysis of histological quantification of different elements in the wound area and adjacent normal skin three days after surgery in experimental group (DB,OMV) and in control group (DB,MIN), (Mean $\pm$ SD based on stereological methods).

neovasculars started to increase, but it seems after that it begins to reduce significantly because of the regulatory effect of omega-3 fatty acids on angiogenic factors on day 7. In control group the number of neovasculars increases from day to day during the study.

Conclusion

Based on results of this study it seems that Omegaven, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are able to improve morphometric indices during the wound healing and cause healing faster.

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Figure 8 The statistical analysis of histological quantification of different elements in the wound area and adjacent normal skin five days after the surgery, (Mean $\pm$ SD based on stereological methods).

Figure 9 The Statistical analysis of stereological quantification of different elements in the wound area and adjacent normal skin, on the seventh day after the surgery, (Mean $\pm$ SD based on stereological methods).

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