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RESEARCH ARTICLE

Histological Evaluation of Effect of Thymosin Beta 4 on Wound Healing of Skin

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ABSTRACT

Skin wound healing is a normal biological process, achieved through four precisely and highly programmed phases: hemostasis, inflammation, proliferation, and remodeling. For a wound to heal successfully, Thymosin beta 4 (Tβ4) is a highly conserved, naturally occurring, water-soluble regenerative peptide that has biological activities of promoting angiogenesis, anti-inflammation, anti-apoptosis, and anti-fibrosis and has many important functions in wound repair. This study aims to histologically examine the effect of systemic Thymosin beta 4 treatment with regard to skin wound healing in rats. A total number of 36 Western Albino rats with body weight (250–350 g) and aging from (1–2 months) were divided into two main groups each group was subdivided into 3 subgroups (6 rats in each) according to the healing period (1, 3 and 7 days). Full full-thickness wound of a circular area 2 mm in depth was made on the dorsum of each rat with a sterile biopsy punch of 5 mm diameter. The present study shows the role of Tβ4 in increasing wound contraction, inflammatory cell, and blood vessel proliferation at certain days of wound healing. Exogenous Tβ4 has a bioactive role in wound healing when administrated systemically as it accelerates the healing process at certain phases.

Keywords: Histology, skin wound healing, Thymosin beta 4.

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1. Introduction

Skin wound healing is a natural physiological route that occurs in four exact and highly regulated stages: hemostasis, inflammation, proliferation, and remodeling. For a wound to heal properly, all four stages must occur in the correct succession and interval. It is not a simple process involving highly organized crosstalk between cells, growth factors, inflammatory cytokines, and vascular system [1], [2].

Hemostasis is characterized by vasoconstriction and blood clotting to prevent blood loss alongside providing a scaffold for cell migration and inflammation occurs directly upon injury from day 4 to day 6, during wound formation collagen is exposed instigating activation of the clotting cascade (extrinsic and intrinsic pathway) starting the inflammatory process a biological response to harmful stimuli or pathogen [3] After an injury occurs; the cell membrane releases thromboxane A2 and Prostaglandin 2-α, the clot that forms are composed of platelets, collagen, fibronectin, and thrombin; these factors release growth factors and cytokines such as FGF, TGF-, TNF, KGF, and FDGF which encourage thrombosis, angiogenesis, and reepithelialization. In turn, the fibroblasts deposit new collagen and glycosaminoglycans. These proteoglycans create the interior of the wound, helping to stabilize it and function as scaffolding [4], [5].

Neutrophils are an integral part of the inflammatory phase that secrete signals that amplify inflammatory response at the early stages of healing and also produce antimicrobial substances and proteases that help killing pathogens clearing the wounding location of bacteria, non-viable tissue, and cellular debris [6]. When blood monocytes reach the wound site, they develop into tissue macrophages continue phagocytosis release many chemokines, growth factors, and cytokines which stimulate cell proliferation molecules, recruiting fibroblasts, endothelial cells to support collagen formation, repair the damaged blood vessels and synthesis of extracellular matrix (ECM) along with keratinocytes [7], [8]. By the entry of these cells, the proliferative phase then starts 2–10 days after tissue injury including re-epithelization, neoangiogenesis, peripheral nerve regeneration, granulation tissue formation, a highly vascularized but poorly differentiated soft tissue, and collagen deposition these are the principal steps in building portion of wound healing [9],

[10]. Net collagen production will continue for at least 4–5 weeks following injury. Initially, the collagen deposited is thinner than collagen in intact skin and orientated parallel to the surface.

The remodeling phase, the last phase begins from day 21 after injury lasts a few months and can continue for up to one year or more. Granulation tissue maturation includes a gradual decline in vascular components, the number of proteoglycans and glycosaminoglycans (GAGs), and the water associated with the (GAGs) will decrease. Changes in collagen type, quantity, and structure improve tensile strength [11]. Initially, large amounts of type III collagen were generated, but they were replaced by type I collagen, the dominant fibrillar collagen in the skin, due to the action of matrix metalloproteinases (MMPs), which increased the tensile strength of the scar [12].

Based on their isoelectric point differences, the thymosin family is classified into three groups: α, β, and γ thymosin. Thymosin β (Tβ) has an isoelectric point of 5.0–7.0 [13].

β-thymosins are N-terminally acetylated peptides with a molecular mass of roughly 5 kDa and 40–44 amino acid residues [14]. Thymosin β4, the earliest member of the family, is biochemically separate from and the most numerous and physiologically active member of the thymosins family, present in all tissues and most types of cells except red blood cells [15], [16].

Thymosin beta 4 promotes blood vessel formation, anti-apoptosis, anti-inflammation, and anti-fibrosis via influencing chemokines, cytokines, and specific proteases, as well as up-regulating gene expression and matrix molecules. It has been reported to enhance the healing of different wounds, such as diabetic ulcers, burns, and pressure ulcers [17]. After an injury, platelets, macrophages, and many other cell types produce thymosin beta 4, which protects cells and tissues from further damage. It also reduces the quantity of myofibroblasts in wounds, resulting in less scar formation and fibrosis [15]. Tβ4 logically considered as an adjunct therapy to antibiotics to prevent and accelerate wound healing [18]. This study aims to histologically evaluate the effect of systemic Thymosin beta 4 treatment with regard to skin wound healing in rats.

2. Materials and Methods

2.1. Animals

A total number of 36 Western Albino rats with body weight (250–350 gm) and from (1–2 months) in age were used in the present study in randomized clinical trial design. The rats were kept under standard laboratory conditions. The rats were randomly divided into two main groups. Each group was subdivided into 3 subgroups according to the healing period (1, 3 and 7 days) each group contained 6 rats. On the dorsum of each animal, a full-thickness wound of 2 mm depth was created using a biopsy punch of 5 mm diameter which was sterilized previously with ethanol alcohol 70% [19] Fig. 1.

1. Experimental group (n = 18 rats) received an Intraperitoneal injection of Thymosin βeta 4 (60 µg in 300 µl) [20] on the day of wounding and every other day thereafter for 7 days.

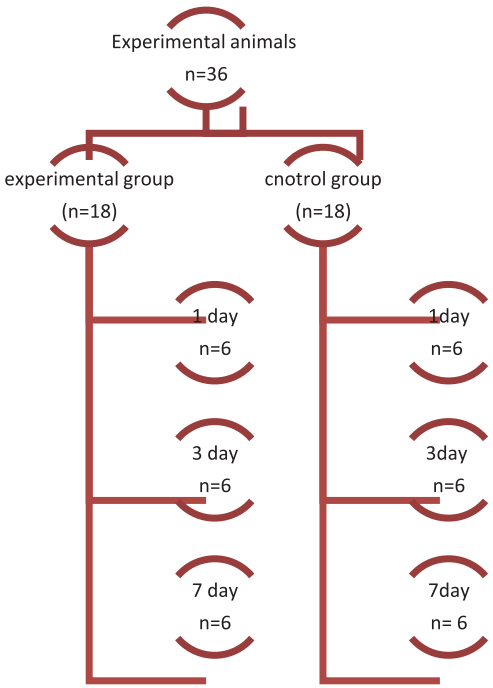

Fig. 1. The diagram represents the study design for these experimental animals and their division into two main groups, each of which is later divided into three groups, with 6 animals per day.

2. Control group (n = 18 rats) received identical amounts of saline intraperitoneal on the same injection schedule.

This study was approved by the Animal Care and Research Committee of Al Mustansiriya University (Approval: coded as MUOPA 1), which was conducted under the Laboratory Animals Guide Care.

2.2. Surgical Procedure

Each rat has been weighed to determine the amount of anesthetic necessary. The general anesthesia was induced by Intramuscular injection of Xylazine 2% (Germany) (0.4 mg/kg B.W) plus Ketamine HCL 50 mg (40 mg/kg B.W). Hair of skin in this region was shaved at first using a hair clipper followed by hair removal lotion and a plastic spatula [21], [22]. The skin was disinfected with 70% ethyl alcohol as a disinfectant. One circular wound was made at the dorsal skin with a biopsy punch 5 mm in diameter. At the end of each healing period (1, 3 and 7 days), all experimental rats were sacrificed they were anesthetized by general anesthesia using the same procedure mentioned previously before the sacrifice procedure. After sacrifice, wounds are removed with punch biopsy larger than used in wounding to permit even removal of intact skin around the wound margin [21].

2.3. Histological Slides Preparation

All tissue specimens were first fixed immediately in freshly prepared 10% neutral formalin then processed in a routine paraffin embedding medium then the sample had serial sections using a microtome so 5 µm thickness sections were mounted on clean glass slides for routine Haematoxylin and Eosin staining (H&E) after deparaffinization. All studied samples (experimental and control groups) were examined for histopathological examination.

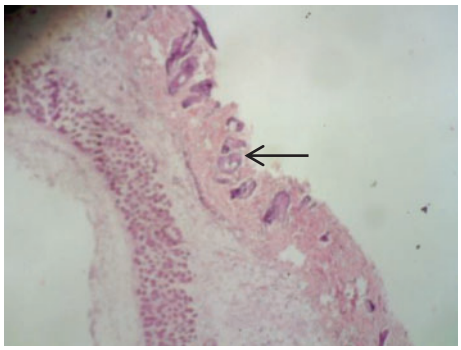

Fig. 2. Histological findings for 3-day duration of wound healing in experimental group show necrotic area (black arrow) ×10.

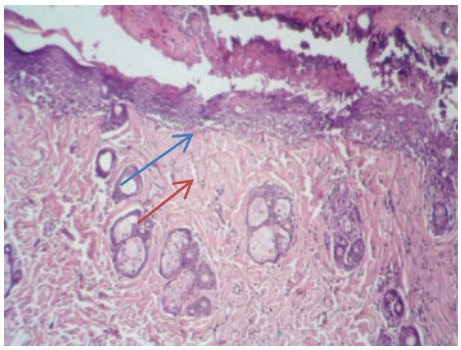

Fig. 3. Histological findings for 3-day duration of wound healing in the control group show new epithelium (blue arrow), collagen fiber (red arrow) ×10.

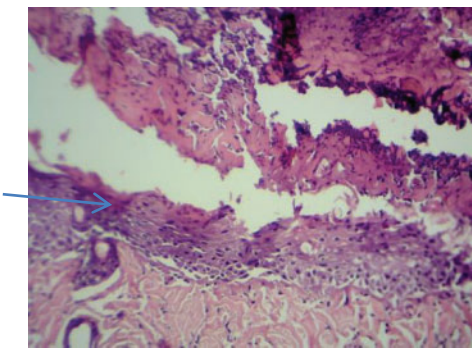

Fig. 4. 3-day duration in control group ×20. New epithelium (blue arrow).

2.4. *Histological Analysis*

A light microscope was used to examine all stained sections (OlympusBX51, Tokyo, Japan) and photographed with a digital camera. For analysis of inflammatory cells, each sample was examined over at least five microscopic areas under power ×40. The inflammatory cell number of each field was counted and the mean number of examined cells was reported [23]. To determine the epithelial thickness, measure the distance between the outermost layer of keratin to the innermost basal layer of the epidermis at the wound margins. The average of the two measurements was obtained using the Image J computer program [24]. For the blood vessel analysis, the numerical density of blood vessels was settled in 45 μm^2 in three fields. The mean number of blood vessels was recorded [25].

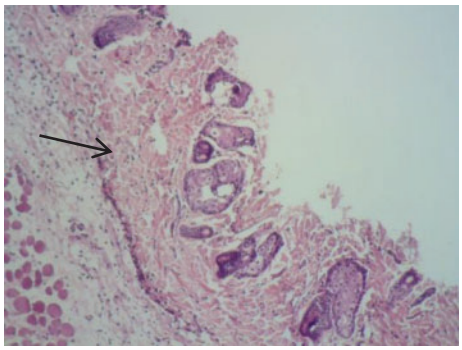

Fig. 5. Experimental group, 3-day duration H&E ×20. New epithelium (black arrow).

2.5. *Statistical Analyses*

SPSS software version 24.0 (Statistical Package for the Social Sciences) was used for statistical analysis. After establishing the normal distribution of the data using the paired sample t-test, ANOVA test was used to examine treatment efficacy and significance. The descriptive statistics of mean, standard deviation (SD), and a p 0.05 show a significant difference.

3. Results

When evaluating wound healing in the experimental group on day 3 notice the presence of necrotic tissue in some areas (Fig. 2). In other areas, new epithelial tissue begins to form in both control Figs. 3, 4 and experimental groups Fig. 5. The assessment of wound contraction was measured in mm for the healing periods for the studied groups. Table I shows the data measured at different healing periods for wound contraction. On day 7, a statistical difference was established between the control and experimental groups whereas non-significant differences were

TABLE I: Descriptive Statistics and Paired Sample T-test of Wound Contraction in All Groups Throughout Each Healing Period

Days	Groups	N	Descriptive statistics				Paired sample t-test	
			Minimum	Maximum	Mean	SD	p-value	Sig.
Day 1	Control	6	3.8	5.30	4.55	1.061	0.69	NS
	Exp.	6	2.84	4.78	3.81	1.372		
Day 3	Control	6	3.60	5.99	4.795	1.69	0.63	NS
	Exp.	6	2.70	4.90	3.8	1.556		
Day 7	Control	6	1.39	7.48	4.435	4.306	0.034	S
	Exp.	6	2.98	3.97	3.475	0.7		

Note: *$p < 0.05$ Significant. **$p > 0.05$ Non-significant.

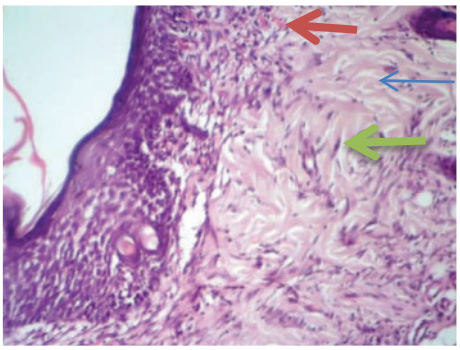

Fig. 6. 7-day duration in experimental group (H&E) magnification power ×20. Collagen fiber (blue arrow). Fibroblast (green arrow). Blood vessel (red arrow).

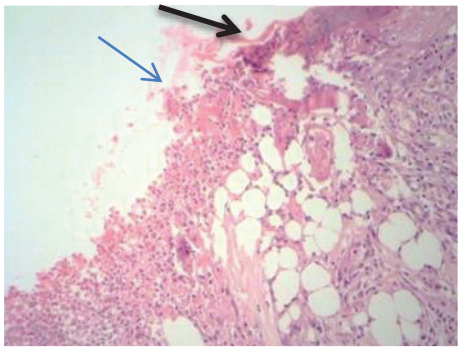

Fig. 7. 7-day duration in control group (H&E) magnification power ×20. Epithelial discontinuity (black arrow). Remanent of necrotic tissue (blue arrow).

TABLE II: ANOVA Test of Wound Contraction in All Groups Throughout Each Healing Period

Group	F-test	p-value
Control	3.765	0.046
EXP	4.56	0.039

Note: *p < 0.05 Significant.

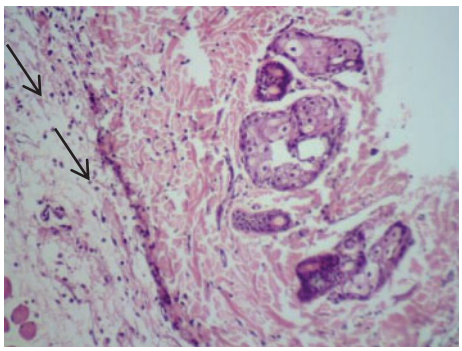

Fig. 8. 3-day duration experimental group ×40. Inflammatory cells (black arrow).

recorded on days 1 and 3 in both groups (Figs. 6 and 7). According to the ANOVA test, the wound contraction showed significant differences among healing periods for both control and experimental groups as shown in Table II.

Regarding the inflammatory cell, the greatest mean value recorded for the control group was on day 3 and the lowest value was on day 1, whereas the highest mean value recorded for the experimental group was on day 3 and the lowest value was on day 7 (Fig. 8). On day 1, there was a high statistically significant difference between the control and experimental groups and on day 7 significant difference was recorded. The ANOVA test shows a highly significant difference between all healing periods in the control group and a significant difference in regarding experimental group (Tables III and IV).

Concerning the epithelial thickness, all data was shown in Table V, which means values increase with time. For all groups investigated across different periods, the greatest mean values were obtained on day 7 and the lowest on day 1, with a statistical difference between the control and experimental groups on day 7 (Figs. 6 and 7). In ANOVA test significant differences among all healing periods in the experimental group (Table VI).

Table VII shows the blood vessel count, with the experimental group having the highest mean value on day three. On day 1, the control and experimental groups had the lowest mean values. There was a statistically significant difference between the control and experimental groups on days 3 and 7. According to ANOVA test Table VIII shows significant differences among all healing periods in control and experimental groups (Fig. 9).

4. Discussion

Tβ4 is a multifunctional regenerative protein found in nearly every cell type and bodily fluids such as wound fluid promote cell migration, stem cell recruitment, and differentiation, This study found that the experimental group had more wound contraction than the control group after 7 days; demonstrate that systemic Tβ4 promotes wound closure by increasing in migration of keratinocyte from the wound edges cover defect area reducing microbial infection by this it promotes dermal wound repair in normal and aged animals Furthermore, it reduces the number of myofibroblasts in wounds, resulting in scar elimination and future fibrosis [26], [27].

The findings of the present study indicate the lowest mean value in experimental groups in all studied healing periods; According to the fact that Thymosin beta 4 increases collagen synthesis and the deposited collagen is well organized within the newly deposited connective tissue matrix [28].

Sosne *et al*. proposed that secreted laminin-5 (a recognized migratory factor) stabilizes migrating keratinocytes to construct the new basement membrane zone during skin wound reepithelialization and $T\beta_4$ increases the expression of laminin-5 (LM-5) [29]. We suggest the systemic treatment of thymosin beta 4 increases wound contraction and re-epithelialization. It is a crucial part of wound healing since mean epithelial thickness values increased over time for all studied experimental groups in different durations.

When injured and when the barrier breaks down, monocytes, neutrophils, and macrophages are attracted to the area of injury [30], [31]. Afterwards, keratinocytes become activated. This is accomplished by the expression of numerous cytokines and growth factors. Keratinocytes with the activated phenotypethis migratory, hyperproliferative cells that generate and release extracellular matrix components and signaling polypeptides. At the same time, their cytoskeleton is changed by the creation of certain keratin proteins that are required for re-epithelialization [32].

TABLE III: Descriptive Statistics and Paired Sample T-test of Inflammatory Cell Count in Each Healing Period for All Groups

Days	Groups	N	Descriptive statistics				Paired sample t-test	
			Minimum	Maximum	Mean	SD	p-value	Sig.
Day 1	Control	6	4.15	88.50	46.325	59.64	0.00	HS
	Exp.	6	30.58	86.69	58.635	39.68		
Day 3	Control	6	58.20	88.95	73.575	21.74	0.076	NS
	Exp.	6	48.20	97.98	73.09	35.2		
Day 7	Control	6	43.67	93.56	68.615	35.28	0.036	S
	Exp.	6	12.98	78.45	45.715	46.29		

Note: *p < 0.05 Significant. **p > 0.05 Non significant. ***p < 0.0001 High significance.

TABLE IV: ANOVA Test of Inflammatory Cells Count for All Groups in Each Healing Period

Group	F-test	p-value
Control	6.715	0.000
EXP	5.562	0.028

Note: *p < 0.05 Significant. ***p < 0.001 High significant.

TABLE V: Descriptive Statistics and Paired Sample T-test of Epithelial Thickness in (μm) for All Groups in Each Healing Period

Days	Groups	N	Descriptive statistics				Paired sample t-test	
			Minimum	Maximum	Mean	SD	p-value	Sig.
Day 1	Control	6	0.11	0.17	0.14	0.042	0.098	NS
	Exp.	6	0.18	0.39	0.285	0.148		
Day 3	Control	6	0.37	0.78	0.575	0.29	0.112	NS
	Exp.	6	0.41	0.85	0.63	0.311		
Day 7	Control	6	0.90	1. 88	1.39	0.693	0.049	S
	Exp.	6	0.88	1.45	1.165	0.403		

Note: *p < 0.05 Significant. **p > 0.05 Non significant.

TABLE VI: ANOVA Test of Epithelial Thickness in (μm) for All Groups in Each Healing Period

Group	F-test	p-value
Control	2.05	0.058
EXP	2.89	0.048

*p < 0.05 Significant. **p > 0.05 Non significant.

TABLE VII: Descriptive Statistics and Paired Sample T-test of Blood Vessels Count in Each Healing Period for All Groups

Days		N	Descriptive statistics				Paired sample t-test	
			Minimum	Maximum	Mean	SD	p-value	Sig.
Day 1	Control	6	0.60	1.35	0.975	0.53	0.231	NS
	Exp.	6	1.25	1.98	1.615	0.516		
Day 3	Control	6	1.43	2.65	2.04	0.863	0.049	S
	Exp.	6	1.15	4.97	3.06	2.701		
Day 7	Control	6	0.70	1.60	1.15	0.636	0.048	S
	Exp.	6	1.09	3.44	2.265	1.662		

Note: *p < 0.05 Significant. **p > 0.05 Non significant.

TABLE VIII: ANOVA Test of Blood Vessels Count for All Groups in Each Healing Period

Group	F-test	p-value
Control	2.193	0.048
EXP	5.89	0.008

Note: *p < 0.05 Significant.

Regarding the inflammatory cells, our results show a significant difference in the control group in comparison to the experimental group in all healing periods confirmed by other studies that Tβ4 reduces levels of many

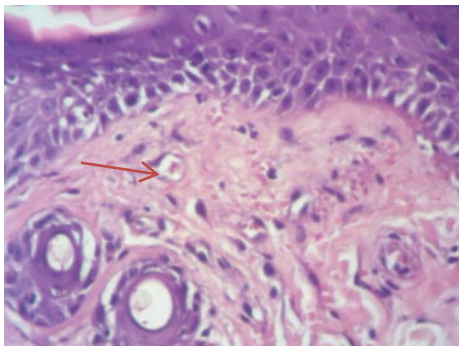

Fig. 9. Histological findings of 3-day duration in control group ×40. Blood vessels (red arrow).

inflammatory cytokines such as TNF-α and chemokines in many tissues, including the eye, heart, and skin also decreases inflammatory cell infiltration and decreases reactive oxygen species by increasing antioxidant enzymes and decreasing pro-apoptotic and pro-inflammatory genes [33], [34]. Tβ4 is also included in angiogenesis, endothelial cell migration ($T\beta_4$ considered an angiogenic small molecule), and thrombosis. It is released by platelets after activation and contributes to aggregation. The quantity of Tβ4 increases at injury locations suggesting a crucial role of this biopeptide in wound healing [35]. Our observation of blood vessel count showed a statistically significant difference between the control and experimental group on days 3 and 7; The primary intracellular G-actin sequestering peptide ($T\beta_4$) not only adds to evidence for thymosin Beta 4 activities. It also promotes angiogenesis by raising the rate of endothelial cell adhesion and spreading on matrix components, as well as stimulating the migration of human umbilical vein endothelial cells [36]. It up-regulates VEGF expression forming new blood vessels in the wounded tissues. In summary, Thymosin beta 4 treatment improves wound healing by enhancing different parameters of the repair process including wound closure, and epithelial formation in agreement with Noori *et al*. along with promoting angiogenesis and inducing inflammatory response [37].

5. Conclusion

Recent research has shown the possible applicability of Tβ4 and its underlying mechanisms. This study shows the histological study of the effect of systemic application of Tβ4 in skin wound healing of rats at the recommended dose. The main purpose of Thymosin beta 4 as a very well-tolerated peptide is to promote quick wound healing by promoting cell migration to the site of the injury, new blood vessel formation, decreased inflammation, and reduced scar tissue and adhesions.

Conflict of Interest

Authors declare that there is no conflict of interest.

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