Original Article

Role of biomarkers in determination of Wound Age: Histopathological, Immunohistochemical and molecular Study

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ABSTRACT

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Background: Wound age determination is a critical aspect of forensic investigations, offering valuable insights into the timing of traumatic events. Biomarkers, including VEGF, α-SMA, TNF-α, and TGF-β1 have shown promise in aiding wound age determination. The primary objective aimed to determine the appropriateness of VEGF, α-SMA, TNF-α, and TGF-β1 biomarkers for wound age determination in rats. We conducted histopathological, immunohistochemical, and molecular assessments to investigate changes in these markers over different post-wounding time intervals. ***Methods:*** Forty-eight healthy adult male albino rats were separated into 8 equal groups. based on the post-wounding time interval. Histopathological examinations, immunohistochemical staining, and gene expression analyses were performed on skin samples to assess the statement of transforming growth factor beta (TGF-β1), tumour necrosis factor-alpha (TNF-α), alpha-smooth muscle actin (α-SMA), and vascular endothelial growth factor (VEGF). Data were analysed statistically to determine the significance of changes over time. ***Results:*** The histopathological analysis revealed distinct changes in skin tissue over time, ranging from ulceration to reepithelialization and tissue regeneration. Immunohistochemical examination demonstrated varying levels of VEGF and α-SMA expression, with marked increases in later post-wounding periods. Molecular analysis indicated a significant upregulation of TNF-α and TGF-

β1 expression, reaching peak levels around seven days post-wounding. ***Conclusions:*** The use of VEGF, α-SMA, TNF-α, and TGF-β1 as potential biomarkers for wound age determination. The expression patterns of these markers in the rat skin suggest their suitability for forensic applications.

Keywords: Wound Age Determination; VEGF; α-SMA; TNF-α; TGF-β1; Histopathology; Immunohistochemistry; Gene Expression.

I. INTRODUCTION:

The morphologic functional property of the wound is described as the interruption of the tissue structure's continuity (Khalaf et al., 2019).

Inflammation, granulation tissue creation, and remodelling are the three stages of skin wound healing, which are thought to be a complex and well-organized biological response involving a large number of regulating chemicals for instance, growth factors and cytokines (Saba et al., 2019).

Though it can be difficult in forensic medicine, estimating a wound's age can help reconstruct crime scenes and help apprehend suspects (Elzahed et al., 2020). Nowadays, one of the main criteria used in forensic examinations is the age of the wound (Pakiş, 2016).

Advancements in forensic techniques have made it possible to check many markers at once and evaluate the wound at both the molecular and cellular levels (Li et al., 2020). Numerous techniques have been developed for estimating the age of the wound, comprising reverse transcription polymerase chain reaction, immunohistochemical staining, and regular histological inspection (RT-PCR) (Khalaf et al., 2019).

Immunohistochemistry is the preferred technique in forensic pathology due to its simplicity and dependability when applied to formalin-fixed paraffin-embedded tissue. Unlike most methods, this one allows the material of interest to be located inside the substructures of the tissue or cells (Elzahed et al., 2020).

The use of RNA for forensic reasons has been made possible by developments in molecular biology (Saba et al., 2019). Early-stage wound ageing can be accurately determined by measuring the mRNA levels of wound-healing factors and inflammatory cytokines. In a sample that has been kept for many months or perhaps years, RNA has been found (Zhang et al., 2015).

Biological materials and molecular pathology methods have emerged as

helpful indicators for estimating the wounds age (Akbaba et al., 2014). Vascular endothelial growth factor (VEGF) can be utilised as a marker to determine the age of a wound because it is a crucial angiogenic factor in the development of new granulation tissue during wound healing (Khalaf et al., 2019). The closing and healing of wound tissue is known to be largely dependent on myofibroblasts, which have contractile properties and aid in wound contraction through alpha-smooth muscle actin (α-SMA), a protein involved in numerous cellular processes such as cell motility, division, and the production of contractile force (Wang et al., 2016).

Tumor necrosis factor-alpha (TNF-α) is a multipurpose proinflammatory cytokine that triggers signalling pathways related to cellular differentiation, apoptosis, inflammation, and cell survival. It can cause some tumour cells to die and is mostly secreted by mast cells, fibroblasts, lymphoid cells, and macrophages (Peyron et al., 2021).

There are 33 members in the transforming growth factor beta (TGF-β) superfamily. Mammals have predominantly TGF-β1, β2, and β3 isoforms; however, TGF-β1 is predominant in the healing of cutaneous wounds. Platelets, fibroblasts, keratinocytes, and macrophages all make them (Borena et al., 2015).

Given that wound-age estimate is a complex and multifaceted subject, using a mix of multiple parameters may help to lower estimation errors (Birincioğlu et al., 2016).

Thus, this work's objective was to determine the significance of biomarkers in determination of wound age at different times in rats by histopathological examination, immunohistochemical examinations using some tissue markers (VEGF, α-SMA) and gene expressions in rat skin using tissue markers (TNF-α, TGF-β1). Also, to assess the following markers' suitability for use in forensic cases when they are needed to determine the age of a wound.

II. MATERIAL AND METHODS:

I- Chemicals:

The immunostaining markers, VEGF and α-SMA, were acquired from Abclonal Technology and AM Biomedical Company in Egypt. Diethyl ether and 40% formalin were purchased from Al-Gomhoria Pharmaceutical Company in Egypt.

The HERAPLUS SYBR® Green qPCR Kit was procured from (Willowfort, UK).

II- Animals:

The research involved 48 healthy adult male albino rats, weighing between 150 and 200 grams, which were sourced from the animal house at the Faculty of Veterinary Medicine, Benha University. These rats were accommodated in appropriately ventilated wire mesh cages, had access to water and a standard diet, and were subjected to a 12:12 hours light/dark cycle with room temperatures maintained between 22 - 24oC. The rats were acclimatized to their environment for two weeks before the experiment's commencement to guarantee their general health .

Exclusion criteria:

Animals with prior illnesses, injuries, or infected wounds, characterized by redness, edema, or the formation of a pyogenic membrane, were excluded from the study.

III- Wound model (induction of wound):

Wound induction involved anaesthetizing the rats with diethyl ether inhalation, shaving the dorsal back area to create a 2 x 2 cm bare region according to Khalaf et al. (2019), marking wound margins, and making a 2 cm full-thickness incision extending to the adipose tissue. The wounds were left untreated for 14 days. Each rat was individually housed, provided with food and water, and subsequently sacrificed through cervical decapitation after being anesthetized with diethyl ether at intervals of 12 hours, 1, 3, 5-, 7-, 10-, and 14-days post-wounding.

IV- Animal grouping:

The rats were split up at random into eight equal groups, each consisting of six rats:

Control group (GI): Rats without any injuries, **Group II**: Rats sacrificed 12 hours after wound infliction, **Group III**: Rats sacrificed 1 day after wound infliction, **Group IV**: Rats sacrificed 3 days after wound infliction, **Group V**: Rats sacrificed 5 days after wound infliction, **Group VI**: Rats sacrificed 7 days after wound infliction, **Group VII**: Rats sacrificed 10 days after wound infliction and **Group VIII**: Rats sacrificed 14 days after wound infliction.

Ethical consideration:

The investigation was conducted in compliance with the guidelines for the use and care of laboratory animals (Clark et al., 1997**).** The Benha

University Faculty of Medicine's Ethical Committee gave its approval to the study design. So, the minimum estimated number of animals to offer valid results was used.

V- Tissue collection:

At the euthanisation time, the injured skin was removed, leaving an unbroken 1-cm border and stored in glass Teflon for RNA extraction and RT-PCR gene expression, while others were maintained in 10% neutral buffered formalin with a volume ten times that of the tissues for histological and immunohistochemical analysis. The skin specimens of six unwounded rats (control group) were also looked at.

VI- Histopathological study:

Samples of the complete thickness of the injured skin were gathered and fixed in 10% neutral buffered formalin right away. Paraffin part was completed (5 micrometre) and stained with hematoxylin and eosin (H&E) as a routine method according to Perry et al. (2016) and examined by light microscope to assess the diagnosis and to evaluate various histopathological features and inflammatory response.

VII-Immunohistochemical study:

In the immunohistochemical study, the Strept-avidin-biotin peroxidase complex method, as described by Buchwalow and Böcker (2010) and Duraiyan et al. (2012) was used to evaluate VEGF and α-SMA expression in skin sections.

The immunohistochemical staining process involves several steps. First, Sections measuring 5 µm were cut from blocks that had paraffin embedded in formalin and then deparaffinized in xylene, followed by rehydration in 95% ethyl alcohol. After rinsing in distilled water, parts were subjected to endogenous peroxidase blocking using 3% hydrogen peroxide in methanol.

Retrieval of microwave antigens was done in citrate buffer (pH 6) for 15 minutes, and slides were washed in phosphate buffer saline (PBS). To block non-specific staining, normal goat serum was applied to cover tissue sections, and then slides were exposed to primary antibodies against VEGF or α-SMA (Abcam) at 1:400 dilutions overnight at 4°C. Subsequently, secondary biotinylated antibodies were applied, followed by streptavidin enzyme label. Color development was achieved by using Diamino-Benzedine (DAB) chromagen, counterstaining with Mayer's hematoxylin, dehydrating in alcohol, clearing in

xylene, and covering the slides with Canada balsam.

Morphometric study:

The interactive measuring menu of image analyzer (LeciaQwin 500 image analyzer computer system, England) in anatomy department, faculty of medicine, Menoufia University, was used to measure the intensity of VEGF and α-SMA immunostaining in immunostained sections. These measurements were obtained by total magnification ×200 from 5 non-overlapped sections *(Zhuang et al., 2013).*

Interpretation of the immunohistochemical staining results:

Vascular endothelial growth factor (VEGF) staining was considered positive when detected as brownish nuclear staining and alpha smooth muscle actin (α-SMA) staining was considered positive when detected as brownish cytoplasmic staining.

VIII- Two step RNA relative quantitation using real-time PCR (qRT-PCR) (Wacker and Godard, 2005):

I. RNA Isolation and Purification:

The process began with sample homogenization, where 1 ml of GENEZOLTM Reagent was added to 50-100 mg of tissue sample, followed by incubation and transfer to a new RNase-free tube. Phase separation involved the addition of chloroform, centrifugation, and transfer of the aqueous phase. RNA precipitation included the addition of isopropanol, incubation, centrifugation, and removal of the supernatant. After being air-dried and cleaned with ethanol, RNA was resuspended in water devoid of RNase.

II. Reverse Transcription:

To convert RNA into cDNA, 10 µl of purified tissue extract was combined with random primer and incubated at 70°C for 3-5 minutes. Buffer, water, dNTPs, and Reverse Transcriptase enzyme were added, followed by incubation at 45°C for 1 hour.

III. Quantitative Polymerase Chain Reaction (qRT-PCR):

Sequence-specific primer pairs were employed in real-time PCR using the generated cDNA as detailed in **(Table 1)**. The qRT-PCR was completed utilizing the HERAPLUS SYBR® Green qPCR Kit. The PCR cycles involved initial denaturation at 95°C, denaturation at 95°C, annealing at 54-66°C, elongation at 72°C, and a final elongation at 72°C.

Table (1): Primer sequence of TGF-β1, TNF-α and housekeeping genes, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

TGF-β1	TNF-α	GAPDH
Forward primer sequence 5□-CAGTAAATGTATGGGGTCGCAG.	Forward primer sequence 5-CCCAGGCAGTCAGA TCATCTTC-3	Forward primer sequence 5-ATGGAAATCCCATCACCATCTT-3′
Reverse primer sequence 5□-GGTGTCAGTGGGAGGAGGG.	Reverse primer sequence 5-AGCTGCCCCTCAGCTTGA-3.	Reverse primer sequence 5-CGCCCCACTTGATTTTGG-3′

Calculation of the results:

To calculate the results, the target gene expression was determined for both the wound groups and the control group. The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which remains relatively stable, was used as a reference gene. The fold change or relative quantity (RQ) was computed using the 2 to the power of minus delta-delta CT (2-ΔΔCT) method, as proposed by Livak and Schmittgen (2001). This method is widely employed for quantitative real-time polymerase chain reaction (qPCR) data analysis, utilizing the threshold cycle (CT) to determine the relative expression of a gene in the target and reference samples by using the reference gene as a normalizer.

The delta CT was obtained by subtracting the CT value of the target gene from that of the reference gene (ΔCT = CT (target gene) − CT (reference gene)). The delta-delta CT, representing the difference between the wound and control tissue (ΔΔCT = ΔCT (target sample) − ΔCT (reference sample)), was then calculated. The ultimate outcome is displayed as the fold change in the target gene's expression in the target sample compared to the reference sample, normalized against the reference gene.

Statistical analysis:

Data processing and data collection for statistical analysis were done with SPSS (Statistical Package for Social Science, version 28). The normality of distribution was assessed with the Shapiro test, assuming normality at P>0.05. Data were summarized as mean ± standard deviation (SD). The variance, which is calculated by dividing the total squared deviations from the mean by the number of observations minus one, is equal to the positive square root of the standard deviation (SD). To evaluate the statistical significance of differences between groups and the

control group, One Way ANOVA tests were conducted, followed by post hoc LSD tests for between-group comparisons. The Pearson correlation coefficient was employed to assess the linear association between two quantitative variables. A P value greater than 0.05 was considered nonsignificant, P values below 0.05 were considered significant, while those below 0.01 were considered highly significant. These values indicate the likelihood that the observed differences were not the result of chance (Kothari, 2004, Prakash et al., 2005).

III. RESULTS:

This study utilized 48 healthy adult male albino rats weighing 150 to 200 g, sourced from the animal house at Benha University's Faculty of Veterinary Medicine. The research aimed to assess wound age determination in rats through histopathological examination, immunohistochemical analysis with tissue markers (VEGF, α-SMA), and gene expression profiling (TNF-α, TGF-β1) in rat skin. The rats were separated into eight groups, including a control group and various wound groups sacrificed at different time intervals. All rats survived the study without wound infections or complications during the healing process.

I-Histopathological results:

Microscopic examination of intact skin tissue sections from all animals of control group (group I) exhibited normal histological structure of the epidermis and dermis layers of the skin. The epidermis consisted of stratified squamous epithelium formed of multiple layers of cells as stratum basalis, stratum spinosum and stratum corneum, and the dermis formed of 2 layers: the stratum papillarosum, the topmost layer (papillary layer), The stratum reticularosum is the thicker and deeper layer of the dermis (reticular layer). Sebaceous gland, sweet gland, hair. **(Figure 1 A and B).**

At the same time, the histopathological examination of skin tissue sections of all animals in group II (skin samples were taken at 12-hour post wounding) showed ulceration of the epidermis and disturbance of dermis structure and disarrangement of the fibers, lost areas are also seen **(Figure 1 C and D).**

At one day post wounding (group III), histopathological examination of skin tissue sections of all animals in this group showed degeneration of dermis and inflammatory cell infiltration,

degeneration of the glands and blood vessel congestion are also seen **(Figure 1 E and F).**

At three days post wounding (group IV), histopathological examination of skin tissue sections of all animals in this group showed reepithelialization of the epidermis, fibroblast appear with flat nucleus, monocellular infiltration and granulation tissue formation **(Figure 1 G and H).**

At five days post wounding (group V), histopathological examination of skin tissue sections of all animals in this group showed reepithelialization of the epidermis, fibroblast appear with flat nucleus, severe monocellular infiltration and granulation tissue formation and neovascularization. The dermis showed a cut section of the shaft of the hair follicle **(Figure 1 I and j).**

At seven days post wounding (group VI), histopathological examination of skin tissue sections of all animals in this group showed marked reepithelialization of the epidermis, neovascularization, the dermis showed proliferation of the fibroblasts and well-organized fibers and fibroblast appear with flat nucleus for collagen fibers deposition, severe monocellular infiltration and granulation tissue formation **(Figure 1 K and L).**

At ten days post wounding (group VII), histopathological examination of skin tissue sections of all animals in this group showed marked reepithelialization of the epidermis, neovascularization, severe monocellular infiltration and granulation tissue formation and proliferation of fibroblast. Fibroblasts appear with flat nucleus for collagen fibers deposition and well-organized fibers (**Figure 1 M and N).**

At 14 days post wounding (group VIII), histopathological examination of skin tissue sections of all animals in this group showed the epidermis covered with a layer of keratin, new hair follicle and multiple glands **(Figure 1 O and P).**

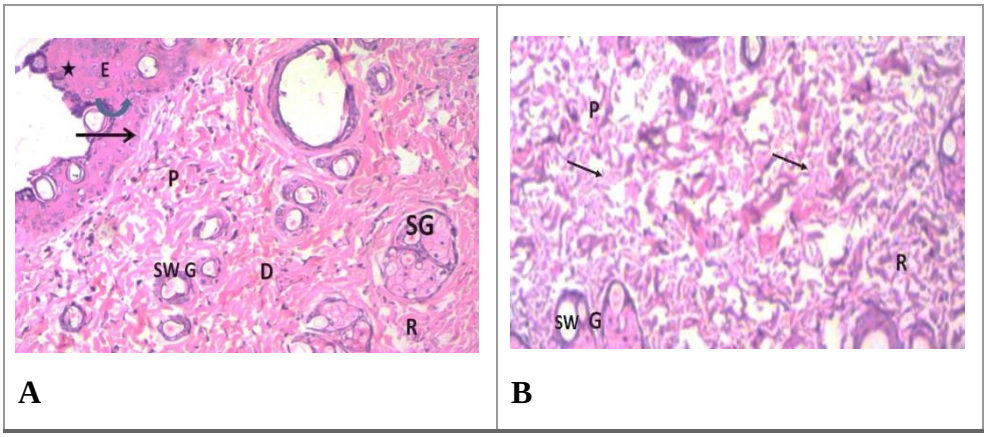


Figure 1(control group I): A: Photomicrograph of a section of the skin of control group showing two layers, epidermis (E) consisted of multiple layers of cells as stratum basalis (arrow), stratum spinosum (curved arrow) and stratum corneum (star), and dermis (D) formed of papillary layer (P) and reticular layer (R). Notice sebaceous gland (S G) and sweet gland (SW G) also seen (H&E ×100). B: Photomicrograph of a section of the skin of the control group showing the dermis formed of papillary layer (P) and reticular layer (R). Sweet glands (SW G) andfibers (arrow) also seen (H&E ×100).

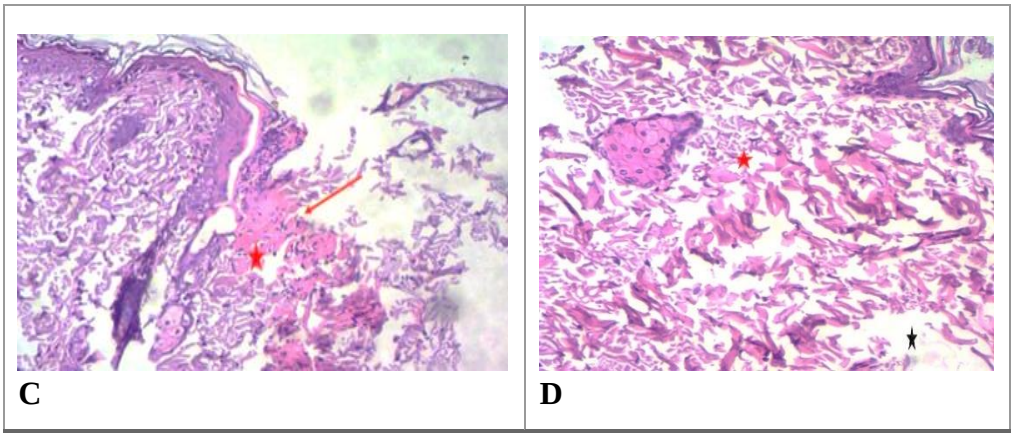
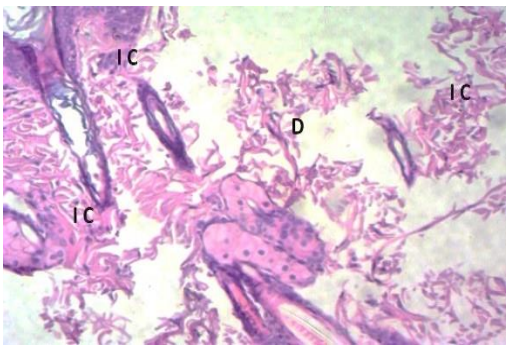


Figure 1 (group II) : Photomicrographs of skin sections 12 hours post-wounding. C: showing epidermal ulceration (red arrow) and dermal disturbance(red star) . D: showing dermal disturbance , fiber disarrangement (red star) , and lost areas(dark star) (H&E ×100).



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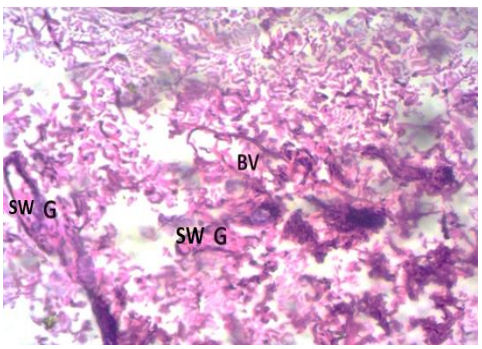

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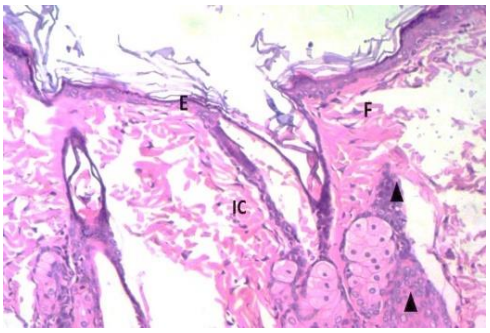
Figure 1 (group III) : Photomicrographs of skin sections One day post-wounding. E: depicting dermal degeneration(D) and inflammatory cell infiltration(IC) . F: exhibiting degeneration of sweat glands(SW G) and blood vessel congestion(BV) (H&E ×100).



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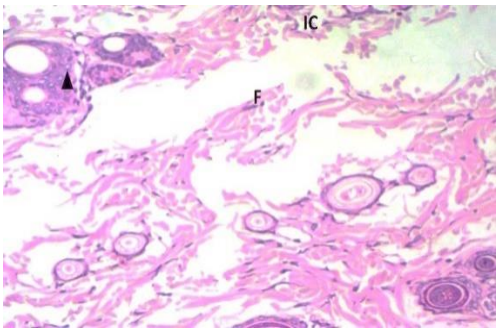

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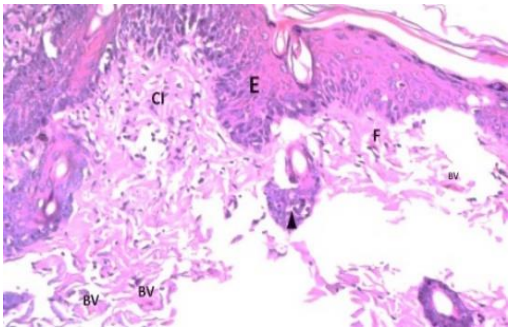
Figure 1 (group IV) : Photomicrographs of skin sections Three days post-wounding. G: with reepithelialization (E) , fibroblast appearance (F) , and granulation tissue(arrow head) , monocellular infilteration (IC) . H: displaying dermal fibroblasts(F) , monocellular infiltration(IC) , and granulation tissue (arrow head) (H&E ×100).



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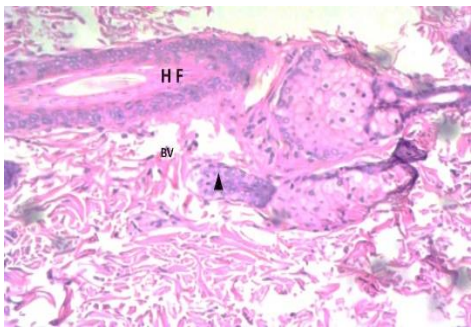

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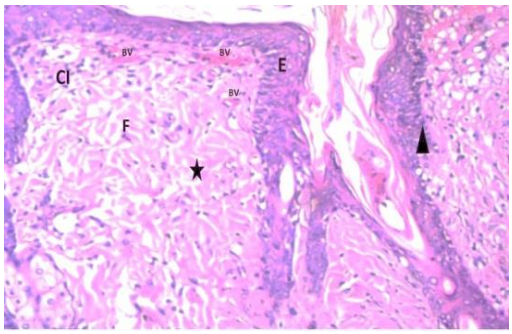
Figure 1(group V): Photomicrographs of skin sections Five days post-wounding. I: featuring reepithelialization (E) , fibroblasts (F) , severe monocellular infiltration (CI) ,

and granulation tissue (arrow head) . J: showcasing dermal hair follicle (HF) , granulation tissue (arrow head), and neovascularization (BV). (H&E ×100).



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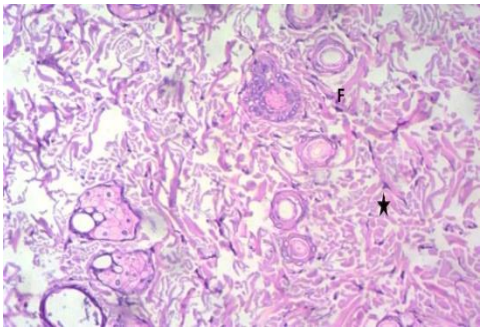

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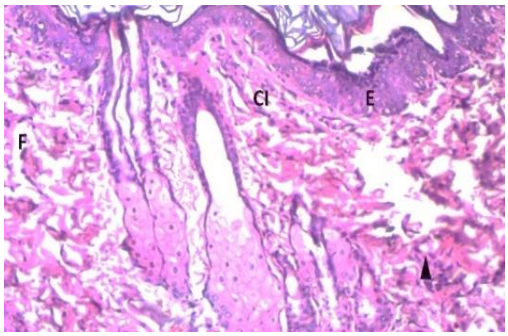
Figure 1(group VI): Photomicrographs of skin sections Seven days post-wounding. K: revealing marked reepithelialization (E) , neovascularization (BV) , fibroblasts (F) , severe monocellular infiltration (CI) and granulation tissue formation (arrow head) and well organized fibers (star). L: showing the dermis with proliferation of the fibroblasts (F) and well organized fibers (star) (H&E ×100).



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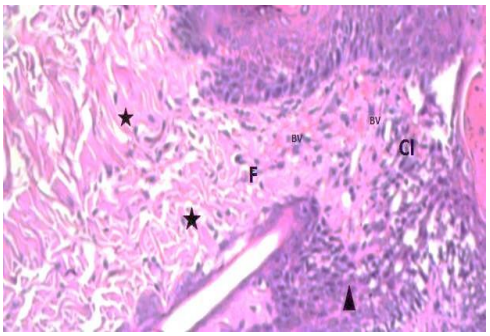

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Figure 1(group VII): Photomicrographs of skin sections Ten days post-wounding. M: demonstrating reepithelialization (E), monocellular infiltration (CI) , granulation tissue (arrow head) , and fibroblast proliferation (F). N: showing neovascularization (BV) fibroblast appear with flat nucleus (F) for collagen fibers deposition, severe monocellular infiltration (CI) and granulation tissue formation (arrow head) and well organized fibers (star) (H&E ×100).

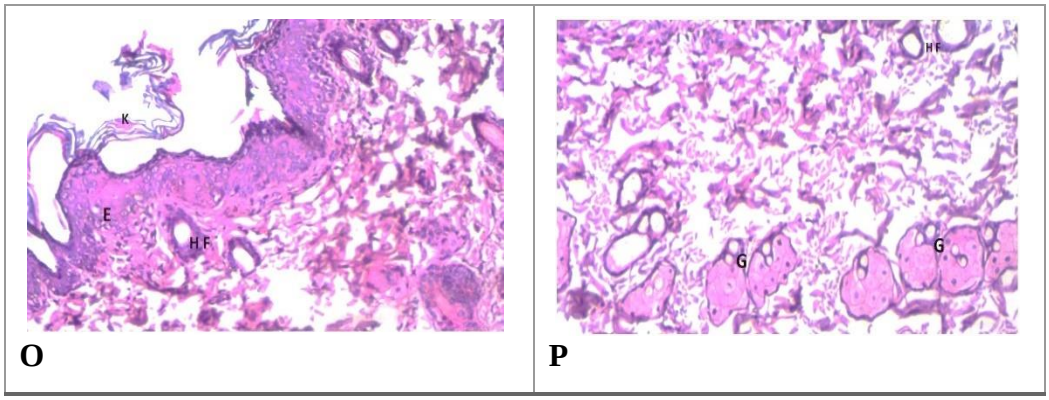

Figure 1(group VIII): Photomicrographs of skin sections 14 days post-wounding. O: showing the epidermis (E) covered with a layer of keratin (K), new hair follicle (HF). P: showing new hair follicle (HF) and multiple glands (G) (H&E ×100).

II-Immunohistochemical results:

A) Immunohistochemical study of Vascular endothelial growth factor(VEGF) in wound groups:

Immunohistochemical examination of all skin sections in control group (intact skin), group II (skin samples were taken at 12h after inducing the wound) and group III (skin samples were taken one day after inducing the wound) showed mild VEGF expression as seen in **(Figure 2 A, B and C.**

Whereas immunohistochemical examination of all skin sections in group IV (skin samples were taken three days after inducing the wound), group V (skin samples were taken five days after inducing the wound) and group VI (skin samples were taken seven days after inducing the wound) showed moderate VEGF expression as seen in **(Figure 2 D, E and F).**

As well, immunohistochemical examination of all skin sections in group VII (skin samples were taken ten days after inducing the wound) and group VIII (skin samples were taken 14 days after inducing the wound) showed severe VEGF expression as seen in **(Figure 2 G and H).**

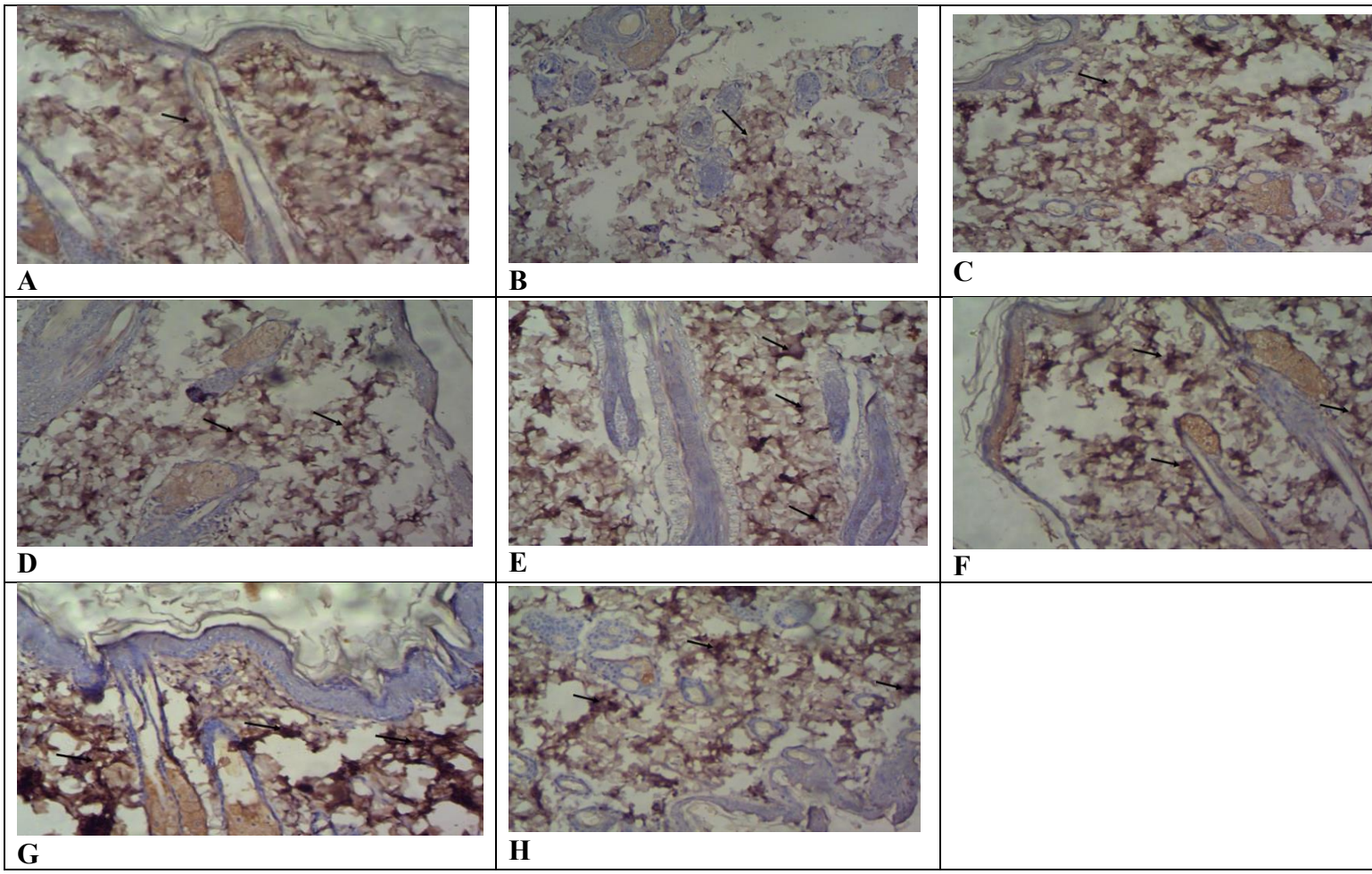

Figure 2: Immunohistochemical stained section for VEGF in the skin showing: A; control group is showing mild VEGF expression (Strept –ABC ×200). B; group II (skin samples were taken at 12h after inducing the wound) is showing mild VEGF expression (Strept –ABC ×200). C; group III (skin samples were taken one day after inducing the wound) is showing mild VEGF expression (Strept –ABC ×200). D; group IV (skin samples were taken three days after inducing the wound) is showing moderate VEGF expression (Strept –ABC ×200). E; group V (skin samples were taken five days after inducing the wound) is showing moderate VEGF expression (Strept –ABC ×200). F; group VI (skin samples were taken seven days after inducing the wound) is showing moderate VEGF expression (Strept –ABC ×200). G; group VII (skin samples were taken ten days after inducing the wound) is showing severe VEGF expression (Strept –ABC ×200). H; group VIII (skin samples were taken 14 days after inducing the wound) is showing severe VEGF expression (Strept –ABC ×200).

B) Immunohistochemical study of Alpha-smooth muscle actin (α-SMA) in wounds groups:

Immunohistochemical examination of all skin sections in control group (intact skin), group II (skin samples

were taken at 12h after inducing the wound) and group III (skin samples were taken one day after inducing the wound) showed mild α-SMA expression as seen in **(Figure 3 A, B and C).**

Whereas immunohistochemical examination of all skin sections in group IV (skin samples were taken three days after inducing the wound), group V (skin samples were taken five days after inducing the wound) and group VI (skin samples were taken seven days after inducing the wound) showed moderate α-SMA expression as seen in **(Figure 3 D, E and F).**

As well, immunohistochemical examination of all skin sections in group VII (skin samples were taken ten days after inducing the wound) and group VIII (skin samples were taken 14 days after inducing the wound) showed severe α-SMA expression as seen in **(Figure 3 G and H).**

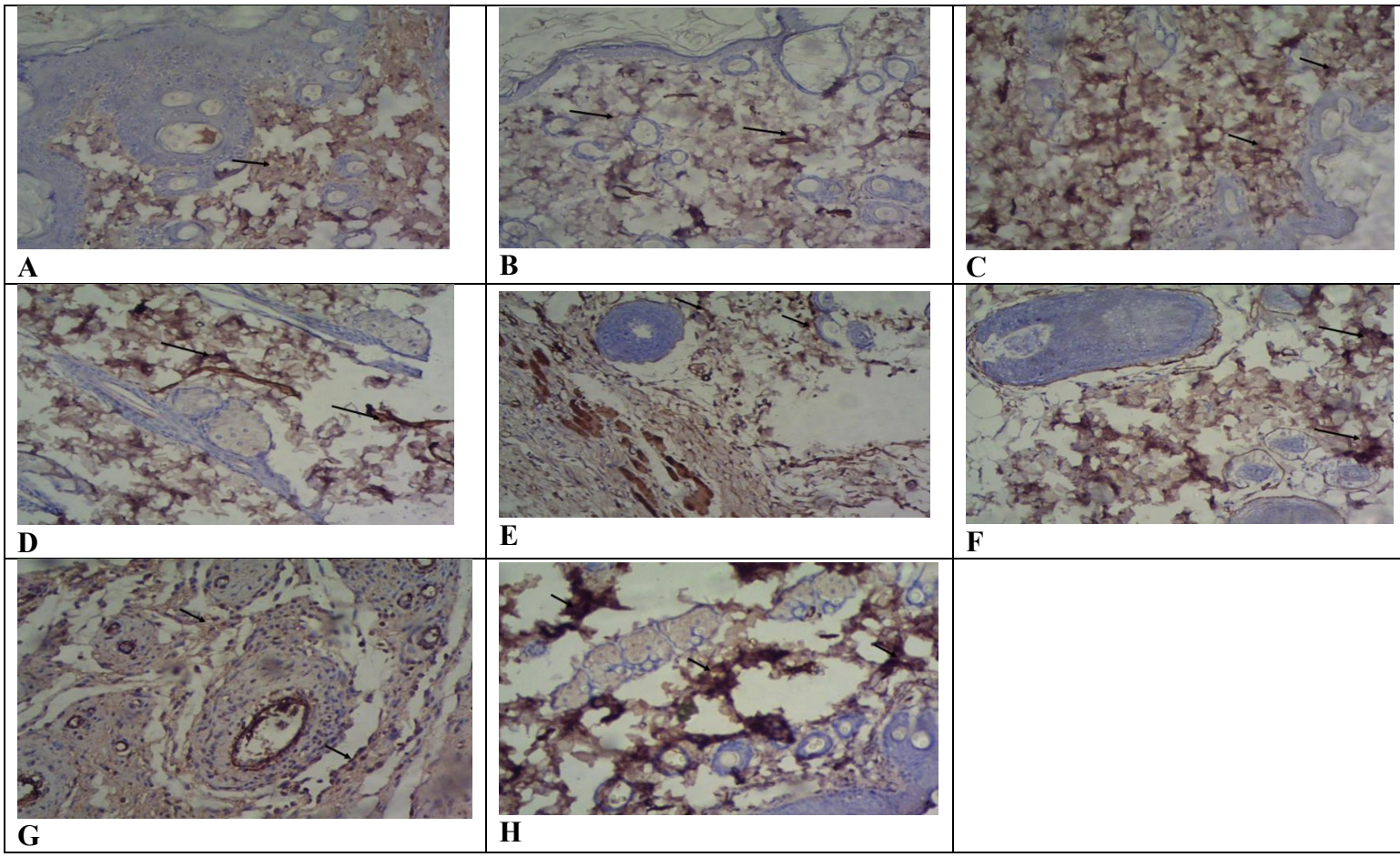

Figure 3: Immunohistochemical stained section for α-SMA in the skin showing: A; control group is showing mild α-SMA expression (Strept –ABC ×200). B; group II (skin samples were taken at 12h after inducing the wound) is showing mild α-SMA expression (Strept –ABC ×200). C; group III (skin samples were taken one day after inducing the wound) is showing mild α-SMA expression (Strept –ABC ×200). D; group IV (skin samples were taken three days after inducing the wound) is showing moderate α-SMA expression (Strept –ABC ×200). E; group V (skin samples were taken five days after inducing the wound) is showing moderate α- SMA expression (Strept –ABC ×200). F; group VI (skin samples were taken seven days after inducing the wound) is showing moderate α-SMA expression (Strept –ABC ×200). G; group VII (skin samples were taken ten days after inducing the wound) is showing severe α- SMA expression (Strept –ABC ×200). H; group VIII (skin samples were taken 14 days after inducing the wound) is showing severe α- SMA expression (Strept –ABC ×200).

A- Differences between the studied groups regarding α-SMA morphometry:

There was a highly significant ($p < 0.001$) increase in α-SMA in group II as compared to control group as showed in **(Table 2).**

Also there was a highly significant (p< 0.001) increase in α-SMA in each group of groups: (III, IV, V, VI, VII and VIII) as compared to control group and the preceding group of each of these groups as showed in **(Table 2).**

Table (2): Comparison among different groups regarding α-SMA morphometry.

Groups	α-smooth muscle actin
Control group I Normal healthy rats	87.31± 5.80
Group II (12 hours after wound infliction)	100.75±7.13*
Group III (1 day after wound infliction)	129.00±11.48*¥
Group IV (3 days after wound infliction)	249.70±37.92*¥
Group V (5 days after wound infliction)	331.32±34.57*¥
Group VI (7 days after wound infliction)	557.23 ± 56.9*¥
Group VII (10 days after wound infliction)	750.51±21.17*¥
Group VIII (14 days after wound infliction)	851.50±86.35*¥
One -Way ANOVA test	312.1
P value	<.001(HS)

Number or rats of each group: 6 rats, Data were represented as mean ± SD, SD: standard deviation, * Highly significant difference with control group at p value <.001, ¥ Highly significant difference with the preceding group at p value <.001.

B- Differences between the studied groups regarding VEGF morphometry:

There was a highly significant (p< 0.001) increase in VEGF in group II as compared to control group as showed in **(Table 3).**

Also there was a highly significant (p< 0.001) increase in VEGF in each group of groups: (III, IV, V, VI, VII and VIII) as compared to control group and the preceding group of each of these groups as showed in **(Table 3).**

Table (3): Comparison among different groups regarding VEGF morphometry.

Groups	VEGF
Control group I Normal healthy rats	158.12± 23.01
Group II (12 hours after wound infliction)	229.76± 11.60*
Group III (1 day after wound infliction)	302.9± 28.27*¥
Group IV (3 days after wound infliction)	398.88± 39.04*¥
Group V (5 days after wound infliction)	496.99± 7.72*¥
Group VI (7 days after wound infliction)	594.74± 32.81*¥
Group VII (10 days after wound infliction)	742.93± 59.76*¥
Group VIII (14 days after wound infliction)	911.97±78.23*¥
One -Way ANOVA test	234.9
P value	<.001(HS)

Number or rats of each group: 6 rats, Data were represented as mean ± SD, SD: standard deviation, * Highly significant difference with control group at p value <.001, ¥ Highly significant difference with the preceding group at p value <.001.

Correlation between VEGF and α-SMA morphometry with the time of wound and Simple linear regression for time of wound:

Figure 4 and 5 showed positive correlation (significant increase) between VEGF and SMA morphometry and time of wound. Also, simple linear regression showed that VEGF and SMA markers are highly significant predictors of wound time (p <.001).

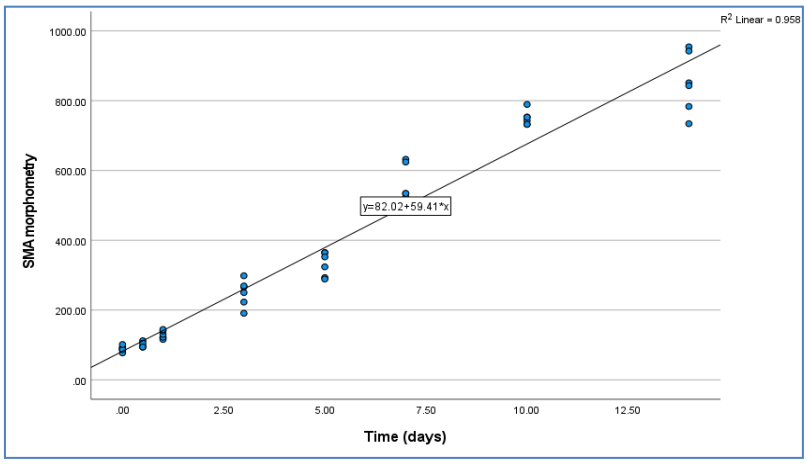

Figure 4: Scatter graph showing significant positive correlation between SMA morphometry and time of wound, the regression coefficient (R2) and equation are also shown.

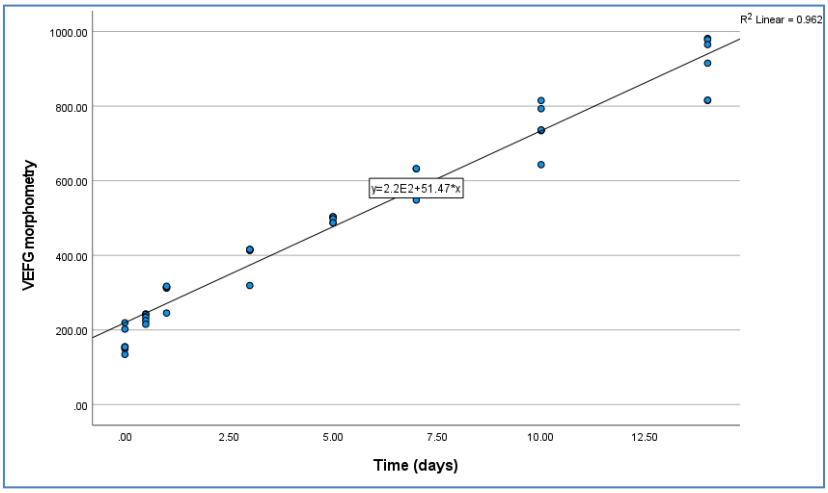

Figure 5: Scatter graph showing significant positive correlation between VEGF morphometry and time of wound, the regression coefficient (R2) and equation are also shown.

III-The mRNA expression of tumor necrosing factor alpha (TNF-α) and transforming growth factor (TGF-β1) genes results:

The present study showed a highly significant increase in expression of both TNF-α and TGF-β1 in group II, III, IV, V and reached to the peak in group VI then a sharp downregulation was detected in the group VII, VIII as shown in **(tables 4 and 5).**

A- Differences between the studied groups regarding relative quantity (R Q) of mRNA expression of TGF-β1 marker:

Table (4) showed a highly significant ($p< 0.001$) increase in TGF-β1 in group II as compared to control group,

a highly significant (p< 0.001) increase in TGF-β1 in group III as compared to control group and group II, a highly significant (p< 0.001) increase in TGF-β1 in group IV as compared to control group and group III, a highly significant (p< 0.001) increase in TGF-β1 in group V as compared to control group and group IV, a highly significant (p< 0.001) increase in TGF-β1 in group VI as compared to control group and group V, a highly significant (p< 0.001) increase in TGF-β1 in group VII as compared to control group but a highly significant (p< 0.001) decrease in TGF-β1 in group VII as compared to group VI and a highly significant (p< 0.001) increase in TGF-β1 in group VIII as compared to control group and group VII.

Table (4): Differences between the studied groups regarding relative quantity (R Q) of mRNA expression of TGF-β1 marker.

Study groups	TGF-β1 marker (RQ) (Mean ±SD)
Control Group I	1.0± 0.0
Group II	*3.625±0.343
Group III	*¥ 4.501±0.182
Group IV	*¥ 5.733±0.145
Group V	*¥ 8.539±0.246
Group VI	*¥ 86.313±0.164
Group VII	*¥ 1.393±0.257
Group VIII	*¥ 2.556±0.282
One -Way ANOVA test	1008
P value	<.001(HS)

*Highly significant difference with control group (p <.001), ¥ Highly significant difference with the preceding group (p <.001), Number or rats of each group: 6 rats, TGFβ1: transforming growth factor beta.

B- Differences between the studied groups regarding relative quantity (R Q) of mRNA expression of TNF-α marker:

Table (5) showed a highly significant (p< 0.001) increase in TNF-α in group II as compared to control group, a highly significant (p< 0.001) increase in TNF-α in group III as compared to

control group and group II, a highly significant (p< 0.001) increase in TNF-α in group IV as compared to control group and group III, a highly significant (p< 0.001) increase in TNF-α in group V as compared to control group and group IV, a highly significant (p< 0.001) increase in TNF-α in group VI as compared to control group and group V, a highly significant (p< 0.001) increase in TNF-α in group VII as compared to control group but a highly significant (p< 0.001) decrease in TNF-α in group VII as compared to group VI and a highly significant (p< 0.001) increase in TNF-α in group VIII as compared to control group and a significant (p <.05) increase as compared to group VII.

Table (5): Differences between the studied groups regarding relative quantity (R Q) of mRNA expression of TNF-α marker

Study groups	TNF-α marker(RQ) (Mean ±SD)
Control Group I	1.0± 0.0
Group II	* 3.363±0.132
Group III	*¥ 3.498±0.140
Group IV	*¥ 14.508±0.282
Group V	*¥ 31.289±0.166
Group VI	*¥ 37.086±0.0
Group VII	*¥ 2.133±0.079
Group VIII	*Ɛ 2.294±0.02
One -Way ANOVA test	663
P value	<.001(HS)

*Highly significant difference with control group, ¥ Highly significant difference with the preceding group, Ɛ significant difference with the preceding group, Number or rats of each group: 6 rats, TNF-α: Tumor necrosis factor-alpha.

IV. DISCUSSION

The calculation of the age of wounds is a crucial aspect of forensic medicine, with medico-legal implications. Recent advances in forensic techniques have allowed for a deeper understanding of the wound's vitality and the amount of time that has passed after the injury through cellular and molecular analysis, and simultaneous assessment of multiple markers. Skin-wound healing involves three phases: inflammation, proliferation, and maturation (Khalaf et al., 2019).

This study, conducted on 48 healthy adult male albino rats, aimed to evaluate the role of biomarkers in determining wound age using histopathological examination, immunohistochemical analysis of

tissue markers (VEGF, α-SMA), and gene expression profiling (TNF-α, TGF-β1). The rats were divided into different groups and sacrificed at various time intervals following wounding. All rats survived the study without wound infections or complications during the healing process, underscoring the potential practical applicability of these markers for forensic wound age determination.

In this study, skin wound histopathological examination revealed the following timeline of changes: At 12 hours: Epidermal ulceration and dermal structure disturbance, at 1st day: Degeneration of dermis, gland disruption, mild edema, and vessel congestion, at 1st, 3rd, 5th, 7th, and 10th days: Mononuclear cell infiltration, at 3rd and 5th days: Reepithelialization, fibroblast proliferation, and granulation tissue formation, at 7th and 10th days: Collagen tissue development.

Khalaf et al. (2019) reported similar findings at 1st day. Sharma et al. (2010) and Fouad et al. (2019) found neutrophils dominant in wounds less than 12 hours old, with a shift to lymphocytes and macrophages between 12-24 hours. Macrophages peaked from 1st to 3rd day.

Oehmichen (2004) observed macrophages appearing from 3 to 7 hours, peaking at 1-2 days. Unmesh and Rema (2012) found maximum lymphocyte concentration after 48 hours, followed by increasing macrophages from the fourth day. Yeh et al. (2010) noted macrophages at 3rd day or later in human skin wounds.

Reports on macrophage appearance vary from 2 to 24 hours, with methodological and observer factors influencing timing (Dettmeyer, 2018). When wounds heal, neutrophils and macrophages release growth factors and cytokines (Ellis et al., 2018).

Inflammatory cell infiltration timing varies in studies, but Wang et al. (2016) found it within 1-10 days. Sharma et al. (2010) observed mononuclear infiltration at 24 hours. Khalaf et al. (2019) and Dimaio et al. (2001) reported granulation tissue by 5-7 days. Fibroblasts and collagen formation were seen from 3rd day (Wang et al., 2016) and 3rd-14th day (Khalaf et al., 2019, Vinay et al., 2017). Accurate wound dating may require immunohistochemical methods (Fouad et al., 2019). For determining the age of wounds, immunohistochemical analysis is now the only method utilised (Khalaf et al., 2019).

In this study, α-SMA and VEGF showed mild expression at 12 hours, 1st day, moderate expression at 3rd, 5th, and 7th days, and severe expression at 10th and 14th days.

Khalaf et al. (2019) found similar patterns for α-SMA and VEGF, with an increase from 5th to 7th days. Wang et al. (2016) noted VEGF upregulation from 1st to 10th day.

VEGF plays a key role in angiogenesis during wound healing (Khalaf et al., 2019) *and* (Wang et al., 2016). Myofibroblasts and macrophages are involved in VEGF secretion during skin wound healing (Khalaf et al., 2019). Myofibroblast α-SMA expression increases around the 6th day (Putra et al., 2020). Gao et al. (2009) observed α-SMA elevation on the 3rd day after injury, marking myofibroblast transition.

In general, myofibroblasts express α-SMA in granulation tissue about one week after wound formation (Putra et al., 2020).

In this study, mRNA expression of TNF-α and TGF-β1 increased from 12 hours to 7th day, peaking at 7th day, then sharply declined at 10th and 14th days.

Khalaf et al. (2019) reported a similar pattern for TGF-β1 mRNA expression, increasing until the 7th day and declining thereafter. Kuninaka et al. (2022) noted TGF-β1 upregulation peaking at the 6th day.

TNF-α mRNA expressions increased from 12 hours to 7th day, peaking at 7th day, then declining, as also found by (Wang et al., 2016). Grellner and Madea (2007) observed TNF-α expression within 15 minutes of injury, maintaining up to 90 minutes. Elzahed et al. (2020) suggested TNF-α for wound age estimation within 2 hours.

The combination of these markers may provide a more accurate calculation of the wound age.

V. CONCLUSIONS

The study highlights the time-dependent expression patterns of (VEGF), (α-SMA), (TNF-α), and (TGF-β1) in skin wounds, emphasizing the critical importance of determining wound age, particularly in the long term, within the field of forensic medicine. It can be concluded that using of these markers together gives a true account of the wound age estimation.

VI. RECOMMENDATIONS

Spotting the light on the results of this research, it could be recommended to use vascular endothelial growth factor (VEGF), alpha-smooth muscle actin

(α-SMA) tumor necrosing factor alpha (TNF-α) and transforming growth factor (TGF-β1) as a useful tools for the estimation of wound age in forensic practice. Also, It is recommended to use such research methods on human samples to support the use of these markers as objective methods for wound dating determination.

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Conflict of interest:

The authors declared no conflicts of interest.

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دور الدلالات الحيوية في تحديد عمر الجرح: دراسة هستوباثولوجية وهستوكيميائية مناعية وجزيئية

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الخلفية: يعد تحديد عمر الجرح جانبًا مهمًا لتحقيقات الطب الشرعي، حيث يقدم رؤى قيمة حول توقيت الأحداث المؤلمة. أظهرت المؤشرات الحيوية، بما في ذلك عامل نمو بطانة الأوعية الدموية، وأكتين العضلات الملساء ألفا، وعامل نخر الورم ألفا، وعامل النمو المتحول بيتا 1، نتائج واعدة في المساعدة في تحديد عمر الجرح. **كان الهدف الأساسي هو** تقييم مدى ملاءمة عامل نمو بطانة الأوعية الدموية، وأكتين العضلات الملساء ألفا، وعامل نخر الورم ألفا، والمؤشرات الحيوية لعامل النمو المتحول بيتا 1 لتحديد عمر الجرح في الفئران. أجرينا تقييمات التشريح المرضي، والكيميائية المناعية، والجزيئية للتحقيق في التغيرات في هذه العلامات على مدى فترات زمنية مختلفة بعد الجرح.

الطريقة: تم تقسيم ثمانية وأربعين من ذكور الجرذان البيضاء البالغة الأصحاء إلى ثماني فئات على أساس الفاصل الزمني بعد الإصابة. تم إجراء فحوصات نسيجية مرضية، وفحص كيميائي مناعي، وتحليلات التعبير الجيني على عينات من الجلد لتقييم بيان عامل نمو بطانة الأوعية الدموية، وأكتين العضلات الملساء ألفا، وعامل نخر الورم ألفا، وعامل النمو المتحول بيتا 1. وتم تحليل البيانات إحصائيًا لتحديد أهمية التغيرات مع مرور الوقت.

النتائج: كشف التحليل النسيجي المرضي عن تغيرات واضحة في أنسجة الجلد مع مرور الوقت، تتراوح من التقرح إلى إعادة الظهارة وتجديد الأنسجة. أظهر الفحص المناعي الكيميائي مستويات متفاوتة من عامل نمو بطانة الأوعية الدموية وتعبير الأكتين في العضلات الملساء ألفا، مع زيادات ملحوظة في فترات ما بعد الإصابة اللاحقة. أشار التحليل الجزيئي إلى زيادة كبيرة في تنظيم تعبير عامل نخر الورم ألفا وعامل النمو المتحول بيتا 1، حيث وصل إلى مستويات الذروة بعد حوالي سبعة أيام من الإصابة.

الاستنتاجات: النتائج التي توصلنا إليها تدعم فائدة عامل نمو بطانة الأوعية الدموية، وأكتين العضلات الملساء ألفا، وعامل نخر الورم ألفا، وعامل النمو المتحول بيتا 1 كمؤشرات حيوية محتملة لتحديد عمر الجرح. تشير أنماط التعبير عن هذه العلامات في جلد الفئران إلى مدى ملاءمتها لتطبيقات الطب الشرعي.

التوصيات: بعد إلقاء الضوء على نتائج هذا البحث، يمكن التوصية باستخدام عامل نمو بطانة الأوعية الدموية (VEGF)، وأكتين العضلات الملساء ألفا (α-SMA) عن طريق الفحوصات الكيميائية المناعية وعامل نخر الورم ألفا (TNF-α)، تحويل عامل النمو (TGF-β1) عن طريق تفاعل البوليميراز المتسلسل للنسخ العكسي (RT-PCR) كأدوات مفيدة لتقدير عمر الجرح في ممارسة الطب الشرعي.وايضا يوصى بشدة بتطبيق طرق البحث هذه على عينات بشرية لدعم استخدام هذه العلامات كطرق موضوعية لتحديد تاريخ الجرح.