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Research Article
The Regenerated Capacity of Curcumin in the Migration of Epidermal Stem Cells Promotes Skin Wound Healing in a Wistar Rat

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Abstract

Background and Objective: Turmeric contains a polyphenol called curcumin, which is similar to stem cells in that it can renew identical cells and preserve proliferation and multipotency. Curcumin's anti-inflammatory and stemness properties as well as regenerative benefits, were investigated in this study. **Materials and Methods:** Thirty Wistar rats were randomly assigned into five groups (n = 6), negative-control group, treated with normal saline only, curcumin-treated I group, applied topically with curcumin for 7 days, curcumin-treated II group for 14 days, curcumin-treated III group for 21 days and curcumin-treated IV group for 28 days. Wound healing was assessed on days 7, 14, 21 and 28 when skin-wounded tissue were prepared and stained with H&E, Masson's trichrome special stain and CD34 marker for immunohistochemistry (IHC) utilization. **Results:** The curcumin decreased granulation tissue intensity vs. the control group on days 7 and day 14. Curcumin significantly reduced the mean of angiogenesis vs. the negative-control group. However, curcumin significantly improves epidermal thickening vs. the negative-control group (score 1 vs. 2, $p<0.05$), respectively at days 21 and 28. Curcumin improves the collagen fibre, on day 21 as fascicle vs. mixed when compared to the negative-control group. While, on days 21 and 28 the mature collagen was profoundly deposited vs. moderately in the curcumin vs. control groups. **Conclusion:** we conclude that curcumin significantly accelerated the healing course via modulation in the amount, maturation and arrangement of collagen fibre. Curcumin had a putative regenerative property upon upregulation of CD34 as epidermal stem cell marker expression in epidermis and hair follicles.

Key words: CD34, collagen fibre, curcumin, epidermis thickness, follicular stem cells, masson's trichrome stain

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Injuries, particularly chronic injuries are incapacitating aspects that delay a patient's healing process and significantly reduce the patient's standard of living[1]. Only 1-3% of the medications registered in the Western pharmacopoeia are useful for skin-related diseases, such as wounds, according to a prior investigation. One-third of herbal treatments, on the other hand, can be utilized efficiently for this objective[2]. As a result, work on generating novel medications from natural ingredients capable of treating wounds is currently in progress to improve therapeutic benefits and speed up the wound-healing stage[3]. Any interruption of the skin layers that alters its basic structure and function is referred to as a wound[2]. In wound repair, which consists of a chain of dynamic and well-orchestrated biological processes, a succession of sequential phases characterize the regeneration and growth of wounded tissue. Coagulation, inflammation, proliferation and remodeling are some of the processes that take place in the body[4,5]. When this finely tuned process fails, a persistent wound develops. After four weeks of adequate therapy, a chronic wound does not heal in the predicted stages, does not heal within three months or stays 40-50% unhealed[6].

In the extracellular matrix (ECM) of skin and connective tissues, collagen is the most prevalent structural protein. Collagen-based scaffolds are a promising candidate for tissue regeneration due to their biocompatibility, biodegradability and low protein binding[7,8]. Furthermore, it has been shown to successfully heal many soft and hard tissues and is fundamentally comparable to the native ECM[9]. Collagen-based scaffolds can absorb wound exudates and keep the wound site hydrated, which promotes cell migration and skin regeneration[10].

Because there are so many different types of stem cells in the skin and its appendages, they have a tremendous capacity for regeneration (SCs). These SCs maintain skin homeostasis and control skin damage in physiological settings. Epidermal Stem Cells (EPSCs) are of special interest among these SCs because of their quantity and accessibility. The EPSCs are also easier to collect than embryonic stem cells, which are similar to adipose-derived stem cells, which have been widely used in regenerative medicine and clinical trials[11]. Research on EPSCs for potential regeneration strategies to get around the constraints of conventional therapeutic procedures has been going on since the 1970s. Numerous successful EPSC-based methods to speed up wound healing or restore permanently lost skin have made their way into the clinic[12].

Plants with a long history of ethnomedicinal usage have been shown to have significant therapeutic potential for a variety of maladies and disorders[13,14]. Various potential plant species, on the other hand, require a thorough scientific examination to determine their traditional usage[15]. Curcuma aromatic, a medicinal plant growing most abundantly in India, Bangladesh, China, Thailand, Cambodia, Malaysia, Indonesia and the Philippines, is one such plant with ethnomedicinal properties. Most tropical locations of Africa, America and the Pacific Ocean Islands have grown it[16]. Curcumin is a yellow-coloured natural phenol found in turmeric. Antimicrobial, antioxidant, anticancer, anti-inflammatory and wound-healing effects are all well-known. Curcumin appears to have changed the expression of a variety of growth factors, enzymes, MMPs and interleukins when applied topically, resulting in quicker healing[17,18].

The purpose of this study was to assess the possible usage of topical curcumin's biological activity on the wound healing process and its relationship to regeneration capability in a rat model.

MATERIALS AND METHODS

Study area: This study was conducted in December, 2021-March, 2022, The housing and experiment were done at the Animal House of the Veterinary Teaching Hospital at the University of Sulaimani's College of Veterinary Medicine. While the H&E with IHC processing was done at the Histopathology Lab of Shoresh Hospital/Sulaimnai Province/Iraq.

Animal model and experimental design: Thirty Wistar rats (200-250 g), aged 4-6 weeks, were procured from the Animal House of the Veterinary Teaching Hospital at the University of Sulaimani's College of Veterinary Medicine. They were kept in a 12/12 hrs light/dark cycle at 22±2°C, with unlimited access to water and food. This work was approved by the Local Ethical Committee for Animal Experimentation, College of Veterinary Medicine, University of Sulaimnai (permission 030591, 2 December, 2021) based on CONCEA (National Animal Experiment Control Council) ethical norms for animal experimentation.

Rats were given intraperitoneal xylazine/ketamine (1:1) anaesthesia (ketamine 50 and xylazine 5 mg kg^{-1}) and then rounded full-thickness incisions were made on the backs of each animal (2 cm under the inter-scapular area).

The animals were randomly assigned into five groups (n = 6), negative-control group, treated with normal saline only, curcumin-treated I group, applied topically with curcumin for 7 days, curcumin-treated II group, applied topically with curcumin for 14 days, curcumin-treated III group, applied topically with curcumin for 21 days and

curcumin-treated IV group, applied topically with curcumin for 28 days. The ointment of curcumin was chosen for wound healing activity evaluation and prepared by mixing 5 g curcumin+ 5 g vaseline+ 1 cc glycerin, each rat was treated with curcumin ointment on the area of the wound throughout the experiment time according to groups using day interval.

Sample collection: Each group's animals were euthanized in a 1:2 of Xylazine and Ketamine after 7, 14, 21 and 28 days. The injured tissues were surgically removed after death certification and treated for at least 24 hrs in 10% neutral buffer formalin. The tissue sections were prepared and stained with hematoxylin and eosin. Then 3 sections of 4 µm thickness were prepared for H&E, Masson's trichrome special stain and CD34 marker for immunohistochemistry (IHC).

Assessment of wound closure or retraction rates (WCR): The animals were positioned on a flexible platform and the wounds were photographed using a digital camera (Nikon D50) at a standard distance of 2 cm from the horizontal base immediately after completing the experimental surgical procedures as well as after each euthanasia time. To examine the wounded areas, the photos were processed using morphometric software (Image J analyzer, Japan) (on days 0, 7, 14, 21 and 28). An equation was used to calculate wound closure rates:

$$WCR = \frac{iWA - fWA}{iWA} \times 100$$

where, WCR is represents the wound closure rate, iWA represents the initial wounded area (day 0) and fWA represents the final wounded area (at days 0, 7, 14, 21 and 28). A clinical evaluation was also undertaken as well as a descriptive analysis of the macroscopic (visible) aspects of the wounds using the following criteria: hyperemia, edema and suppuration[19].

Histo-morphometric analysis of wound healing: Each paraffin-embedded specimen was cut into 4 mm thick histological slices and stained with H&E and Masson's trichrome stain. A descriptive-analytical study for different parameters was done as seen in Table 1. The tissue sections were examined and photographed (AmScope™, Japan) under a light microscope.

Table 1: Assessment of different histological structures for wound healing scores[20]

Histological parameters	Scoring system		Days of healing	Histological staining
Density of granulation tissue	Profound	1		
	Moderate	2		
	Mild	3		
	Absent	4	7 and 14th	H&E stained
Pattern of granulation	Vascular granulation	1		
	Fibro-vascular granulation	2		
	Fibro granulation	4	7 and 14th	H&E stained
Mean of angiogenesis	Profound	1		
	Moderate	2		
	Mild	3		
	Absent	4	7	H&E stained
Epidermal thickness	Profound	1		
	Moderate	2		
	Mild	4	21st and 28th	H&E stained
Pattern of collagen fibre	Reticular	1		
	Mixed	2		
	Fascicle	3	21st and 28th	
Collagen fibre orientation	Vertical	1		
	Mixed	2		
	Horizontal	3	21st and 28th	
Amount of early collagen	Profound	1		
	Moderate	2		
	Minimal	3		
	Absent	4	14th	
Amount of mature collagen	Profound	1		
	Moderate	2		
	Minimal	4	21st and 28th	

Assessment of the granulation tissue intensity and epidermis thickness: The granulation tissue intensity and epidermis thickness were photomicrographed from each histological section at power (100X) at 5 different points using image analyzer software (AmScope™, Japan) after being calibrated. The granulation tissue intensity was measured on day 7 with day 14 and then the mean was taken and used for the statistical analysis. Additionally, the pattern of granulation tissue was also assessed in Table 1. Each histological section's epidermis thickness was photomicrographed. The average thickness of the skin epithelial lining was calculated by measuring the top-down epidermal thickness in five different places (thickest points).

Assessment of collagen fibre: For assessing the morphological pattern of collagen fibres on days 14, 21 and 28. Three histological sections for each duration had been stained by Masson's trichrome and analyzed under light microscopy (Leica, Germany). The morphological features and architectural arrangement of the fibres were also observed (Table 1). Each histological segment was photomicrographed in ten fields (400X) and the percentage of the area containing collagen fibres was calculated using the software Image analyzer (AmScope™, Japan).

The total healing score in each case was calculated by adding the scores of individual criteria, healing status was graded as poor (9-14), fair (15-20), good (21-25) and excellent (26-29).

Immunohistochemical analysis: For immunohistochemistry evaluation, serial sections of 4 µm thickness were taken on days 21 and 28, tissues were soaked in paraffin wax. In xylene, paraffin-embedded slides were dewaxed and hydrated. Sections were heated for 20 min in a microwave oven in a 10 mM sodium citrate buffer (pH 6.0) and then cooled in de-ionized water. Endogenous peroxidase activity was reduced by incubating the slices in 3% hydrogen peroxide for 5 min. The CD34 rabbit polyclonal antibody was treated overnight at 4°C with primary antibodies (1:100, DAKO, Denmark). As indicated by the manufacturer, staining was detected using biotin-labelled anti-rabbit secondary antibodies and streptavidin coupled to horseradish peroxidase (DAKO Cytomation, USA). Diaminobenzidine was used to see the reaction products (Sigma-Aldrich Co., St., Louis, MO, USA). Hematoxylin counterstained, dehydrated as per usual method and coated with cover slips.

The CD34 was used to stain the nuclei. Computer-assisted image analysis software was used to examine slices under a microscope (Motic, Japan) (Am scope version 2.5 software, Japan). Section of 100 µm with 400X magnification was taken from each skin segment and studied under a microscope, depending on the size of the area. Mean values were calculated using CD34 expression.

No staining or 0 for 5%, 1 for 5-25%, 2 for 25-50%, 3 for 50-75% and 4 for >75% positive staining were used to determine the amount of positively stained epithelial cells in IHC labelling of CD34. The CD34 staining intensity was rated as faint (+1), moderate (+2) or strong (+3). The positive reactivity extent and level of staining intensity were multiplied to provide a total staining score ranging from 0-12[21].

Statistical analysis: The SPSS Statistical Software was used for all statistical analysis (SPSS for Windows, version 22.0). The data was presented in Mean+SE format. The Shapiro-Wilk test was used to determine if the data were normal or not. A repeated measures model ANOVA (SPSS version 24.0) (by IBM, USA) was used to compare control and curcumin on animal groups, with the LSD test used for the subsequent comparisons. Differences were considered significant when $p<0.05$.

RESULTS

Impact of curcumin on wound retraction at different time-points: The rate of wound retraction was measured using Am scope software on day 0, 7, 14, 21 and day 28 after the creation of the wound. The rate of contraction of the wound was significantly higher in the curcumin groups vs. the control positive group, particularly after day 14 of the treatment. Overall, the wounds, which had been treated with curcumin were completely closed ($p<0.05$) vs. the control positive group (Fig. 1 and Table 2).

Effect of topical curcumin on granulation tissue intensity and angiogenesis: The curcumin decreased granulation tissue intensity vs. the control group on day 7, while, the pattern was more progressed (fibrovascular vs. vascular) in curcumin vs. control in which the score (2 vs. 1), respectively, at day 14 the

Table 2: Mean±SEM of wound retraction in control positive and curcumin-treated groups

Time-points	Negative control	Curcumin-treatments
Day 0	00.00±00.00	00.00±00.00
Day 7	23.00±3.93	36.50±3.93**
Day 14	41.50±4.33	57.66±4.33***
Day 21	54.00±2.30	68.33±2.30***
Day 28	65.16±2.16	75.33±2.16***

Values are Mean±SEM, each dot represents a mouse. A two-way ANOVA test was used for the statistical analysis and ***$p<0.05$

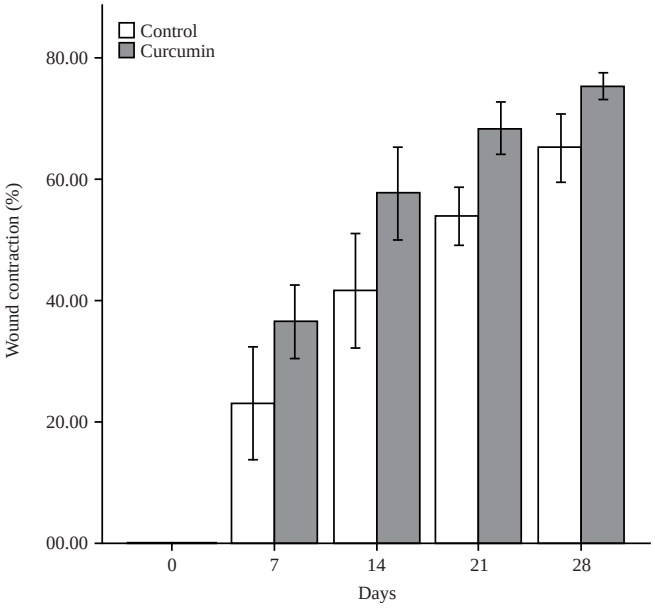

Fig. 1: Assessment of wound retraction at diverse time points between the control positive and curcumin-treated groups

Table 3: Mean±SE for granulation tissue intensity, angiogenesis and epidermal thickening in different groups

Parameters	Negative control	Curcumin-treatments	Total scores
Granulation tissue day 7	150.46±17.80	128.96±14.76	3
Granulation tissue day 14	141.91±8.94	125.24±7.58	4
Angiogenesis day 7	50.00±17.41	21.10±3.32	3
Epidermal thickening day 21	21.77±3.38	26.87±14.76	3
Epidermal thickening day 28	19.86±2.73	21.23±1.55	3

Table 4: Histomorphic evaluation of collagen fibre in control and curcumin groups

Parameters	Negative control	Curcumin-treatments	Total scores
Early collagen fibre day 14	40.48±5.85	54.80±7.79	3
Early collagen fibre day 21	63.10±5.38	122.21±15.29	3
Early collagen fibre day 28	70.91±6.14	125.67±12.58	3

rate of granulation tissue was still high in the control group vs. curcumin group but both of them had a fibrovascular pattern (score 2 vs. 2) as in Fig. 2a-d. Curcumin significantly reduced the mean of angiogenesis vs. the control group (score 2 vs. 1, $p<0.01$) in the curcumin vs. control. However, curcumin significantly improves epidermal thickening vs. the control group (score 1 vs. 2, $p<0.01$) respectively on days 21 and 28 (Fig. 3a-c and Table 3).

Impact of curcumin on collagen in the healing process: Curcumin improves the collagen fibre organization that was found in Fig. 4a-b, on day 21 as fascicle vs. mixed (score 3 vs. 2) in curcumin (Fig. 4b) in comparison to the control group (Fig. 4a), while on day 28 both groups showed organization in the collagen fibre as fascicle (score 3). At 21 days the collagen fibre oriented as horizontal vs. mixed in (control vs. curcumin) groups correspondingly as in Fig. 4, while at day 28 both groups showed horizontal arrangement (score 3). The curcumin enhanced early collagen fibre deposition at day 14 profoundly vs. moderately in the control group (score 1 vs. 2) respectively. On days 21 and 28 the mature collagen profoundly deposited vs. moderately in the curcumin vs. control groups, (score 1 vs. 2) individually.

Curcumin enhanced healing and recorded excellent degree vs. good degree in the control group (score 26 vs. 22), respectively, for all parameters mentioned in Table 4.

Role of curcumin in skin regeneration via regulation of CD34 expression: The expression of CD34 found as brownish granule by the membrane pattern in keratinocytes in the stratum spinosum and hair follicle (HFs) was first observed on day 14th then on the 21st and 28th in both groups (control and curcumin-treated) groups, demonstrated that CD34 positivity in the in follicular epithelial cells were stronger in

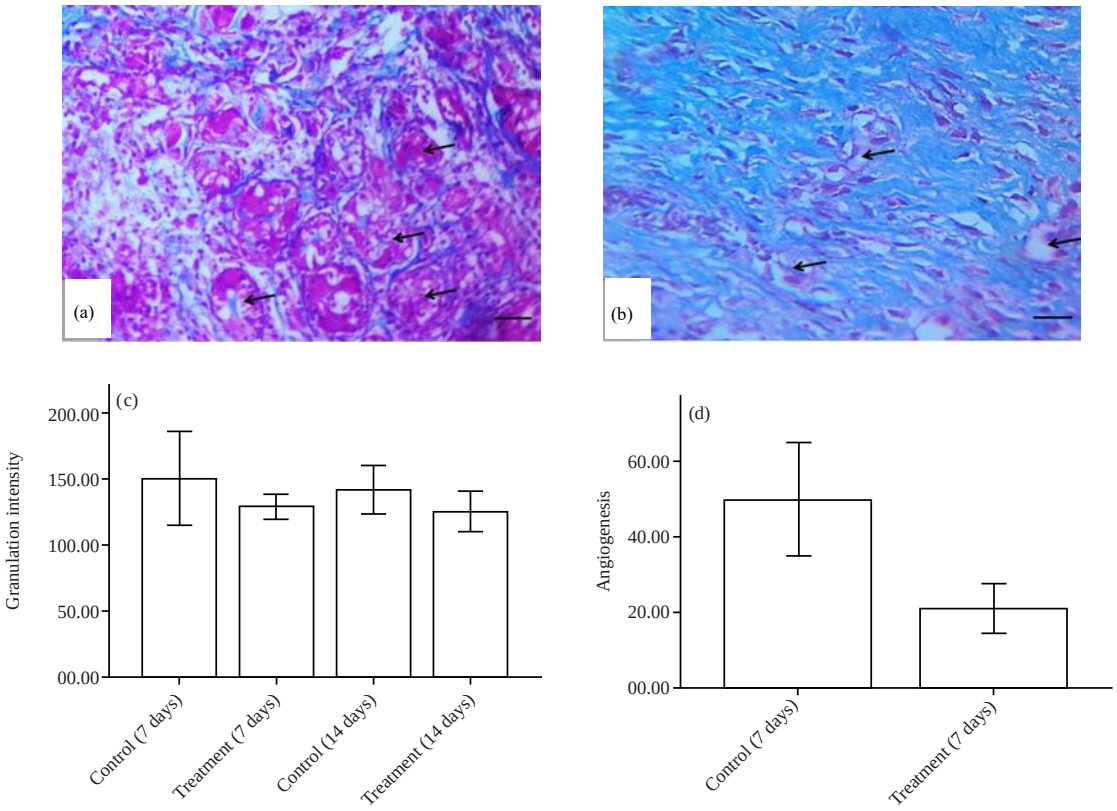

Fig. 2(a-d): Granulation tissue and angiogenesis on days 7 and 14 showed, (a) Vascular granulation tissue in the control group (Black arrow), (b) Fibrovascular granulation tissue in the treatment group, (Masson's trichrome stain, scale bar 20) (Black arrow), (c) Bar chart showed raising granulation tissue intensity in control vs. treatment groups on days 7 and 14, x-axis: Experiment groups and (d) Bar chart showed a significant increase in angiogenesis level in the control group vs. treatment group on days 7

x-axis experiment groups

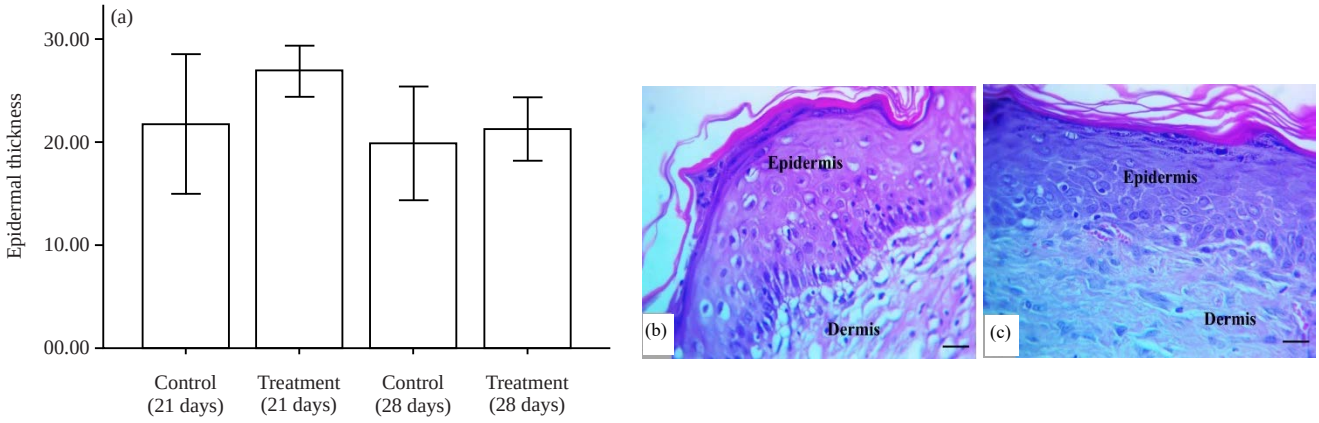

Fig. 3(a-c): Epidermal thickening in control and treatment group, (a) Bar chart showed improvement in epidermal thickening in curcumin vs. control group on days 21 and 28, x-axis: Experiment groups, (b) Organized skin histological structure in the control group and (c) Well organized in curcumin group at day 28

(H&E stain, scale bar 20)

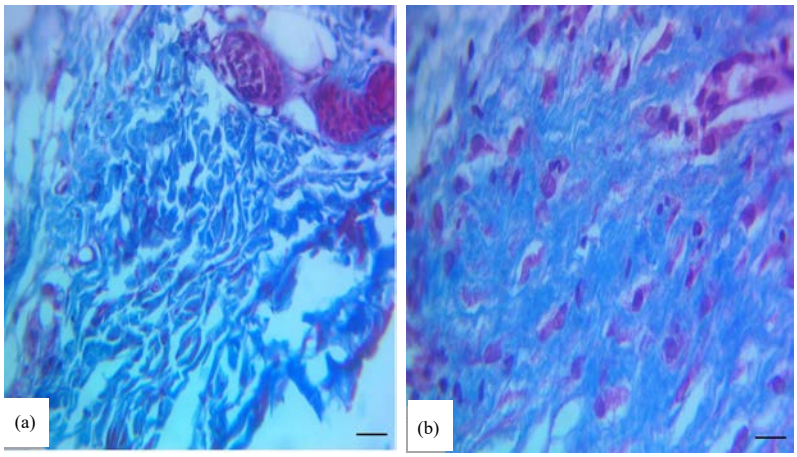

Fig. 4(a-b): Organization, orientation and intensity of collagen fibre at day 21, (a) Collagen fibre organized and oriented in the mixed pattern by a moderate degree in the control group and (b) Collagen fibre organized as fascicle and oriented horizontally to a profound degree in the curcumin group
Masson's trichrome stain, scale bar 20

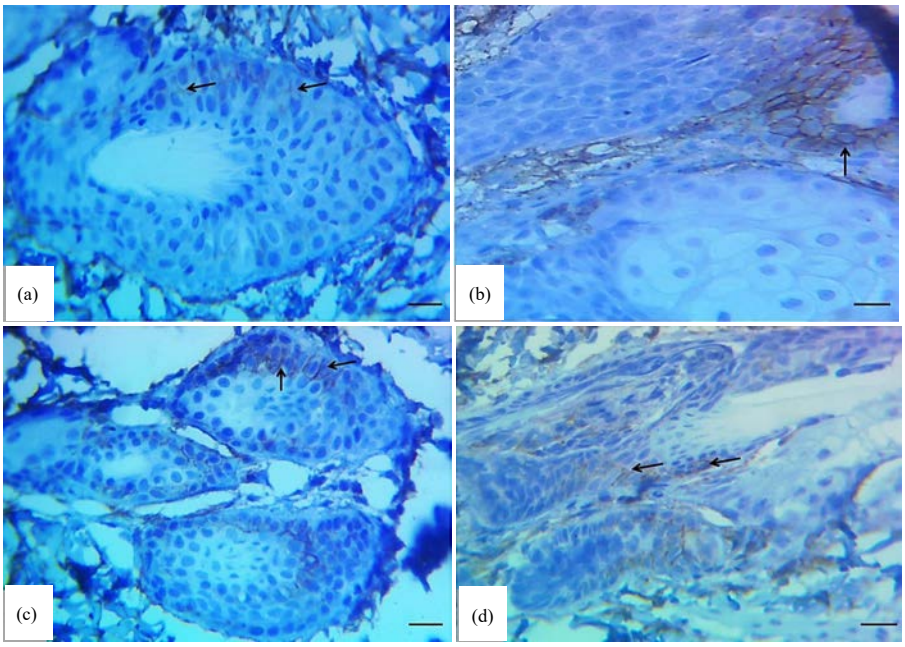

Fig. 5(a-d): Membranous CD34 expression in the hair follicle or follicular epithelial cells on days 14th, 21st and 28th, (a) Weak-localized expression (sum score 2) in mice of the control negative group on day 14th (Black arrow), (b) Strong-diffuse expression in the follicular epithelial cells in curcumin-treated groups (sum score 6) on day 14th (Black arrow) and (c) Moderate-localized expression of CD34 in hair follicles (sum score 4) in mice of the control negative group on day 28th (Black arrow) and (d) Moderate-diffuse expression in the follicular epithelial cells in curcumin-treated groups (sum score 6) on day 28th (Black arrow)

curcumin-treated wounds than in the control wounds at 14th, 21th and 28th days for example in control groups showed weak-localized expression (sum score 2) in mice of the control negative group vs. curcumin-treated groups (sum score 6) with strong-diffuse expression at day 14th (Fig. 5a-b). While, at days 21th and 28th, moderate-localized expression of CD34 in hair follicles (sum score 4) were seen in mice of the control negative group vs. moderate-diffuse expression

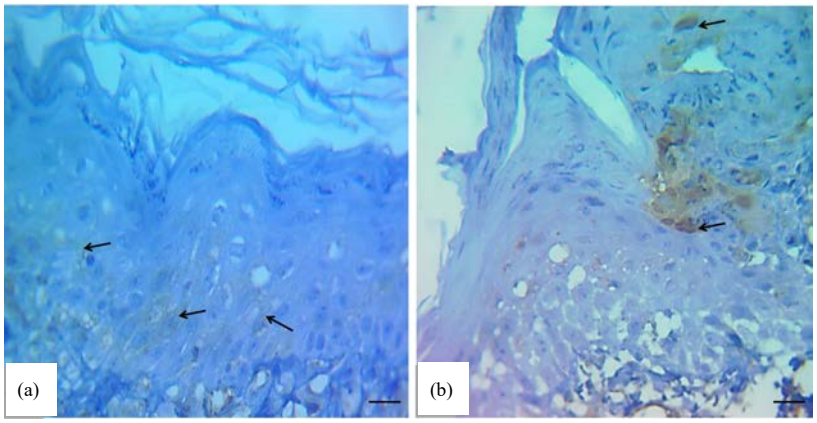

Fig. 6(a-b): Membranous CD34 expression in the keratinocytes of epidermis on day 28th, (a) Weak-localized expression (sum score 1) in the keratinocytes in stratum spinosum in mice of the control negative group on day 28th (Black arrow) and (b) Strong-localized expression in the epidermis epithelial cells in curcumin-treated groups (sum score 3) on day 28th (Black arrow)

in the follicular epithelial cells in curcumin-treated groups (sum score 6) on day 28th (Fig. 5c-d).

The expression of CD34 in keratinocytes of stratum spinosum in the epidermis revealed high expression of CD34 in the curcumin-treated group by moderate-strong localized positive immune CD34 (score 3) vs. localized-weak expression (score 1) in the control group (Fig. 6a-b).

DISCUSSION

For cutaneous healing, the alliance of various distinctive tissues and cell populations is required for this sophisticated process that involves inflammation, fibroplasia, neovascularization, wound contraction and resurfacing of the wound defect with epithelium the cooperation played a critical role in the wound healing process[22].

Curcumin has a long history of use in medicine[23], owing to its anti-neoplastic[24], anti-ageing[25] and qualities to heal wounds[26].

In the current study the facilitated wound contraction, which was observed in the curcumin-treated wounds, indicated a positive effect of the curcumin treatments on the process of wound healing more specifically on days 21st and 28th. The process of wound contraction seems to be related to the tensile strengths that originated from the deposited fibroblasts, myofibroblasts and collagen fibrils[27]. Curcumin-treated wounds appear to have a higher number of myofibroblasts, which could explain why they contract so quickly[28].

When compared to control groups, the results showed that curcumin could facilitate wound regeneration with a better level of integrity while reducing inflammation, following are our findings[29,30], Curcumin is thought to reduce inflammation by reducing the production of Tumour Necrosis Factor (TNF-) and interleukin-1 (IL-1) cytokines, according to prior research[31,32]. Curcumin was also found to inhibit PI3K and pAKT while increasing IB expression on days 4 and 10 after injury[33]. Curcumin has also been studied for its biofunctional properties, specifically its anti-inflammatory, antioxidant, radical scavenger and antibacterial properties, all of which play important roles in wound healing. Furthermore, it can stimulate the generation of growth factors that are important in wound healing[29]. Furthermore, earlier research on curcumin pretreatment in excisional skin wound healing indicated that curcumin may have a role in wound healing[34]. Another theory was that curcumin, by reducing inflammation, helps damaged tissue to begin the final stages of healing, such as proliferation, sooner[35].

On day 7, topical treatment of curcumin resulted in a significant reduction in neoangiogenesis as well as a decrease in granulation tissue. Sidhu *et al.*[36], in agreement with current findings[36], discover decreased angiogenesis in the curcumin-treated groups on day 7 after injury but Kant *et al.*[31], was discovered that using 0.3% curcumin in pluronic F-127 gel regularly can increase vascular density in the wound region.

On day 21, both groups had completed re-epithelialization but only the curcumin group had a well-organized skin compartment. Curcumin has been proven to have a substantial effect on wound closure speed in previous research[37] and curcumin treatment was shown to decrease the production of ROS and increase cellular proliferation in accelerating wound healing[38].

Collagen is an essential element of connective tissues, which provide structural support, stability and an environment for regenerating tissue and it also aids wound healing. Fibroblasts create collagen, which helps the wound gain tensile strength during wound healing[39].

Curcumin's beneficial effect on wound healing was evident in the current investigation as evidenced by a rise of wounds that healed faster via the increase in collagen content and improving the maturation, organization and orientation of collagen fibre vs. the control group, there is an almost 3-fold increase in the earlier maturation of collagen fibres in curcumin-treated wounds vs. control group, curcumin significantly improved the mending qualities as evidenced by broad deposition of well-organized collagen fibrils and collagen synthesis, which was consistent with our findings[40,41]. Collagen concentration per unit area and thus tissue tensile strength improves when newly generated collagens are deposited at the wound site[38].

This was well consistent with[42], curcumin may have enhanced host stem/progenitor cell recruitment into implanted films and expedited wound healing, according to researchers[43].

Certain nutraceuticals could act as stem cell mobilizers in rodent models[44] and curcuminoids could contribute to increasing SC mobilization[45]. Curcumin stimulated migration-related transcription factors, which improved cell migration[46]. As a scavenger, curcumin can protect stem cells against oxidative stress, free radicals and I/R, resulting in antiaging actions[47].

Dermal fibroblasts stimulate local paracrine growth factor stimulation of stem cells in the hair bulge[48]. Current data revealed the improvement of healing in the curcumin group vs. control group on the 14th, 21st and 28th throughout the experiment by enhancing the expression of CD34 which are epidermal stem cells that localized in hair follicles from day 14 in the exciting area at the side of the wounded area that effect in accelerating healing at the day of 21 and 28, CD34 was found within the hair follicle structures, indicating the origin of epithelial cells and the skin basal cell nucleus, which was similar to earlier research[49]. Keratinocyte stem cells in skin appendages are nearly entirely responsible for eventual re-epithelialization in severe dermal wounds. Multipotent SCs are found in HFs and are activated at the start of a new hair cycle and after wounding to provide cells for HF regeneration and epidermal healing[50].

It has become clear that improving the topical use of curcumin by using a high dose and decreasing sensitivity in a few people through modifying its formulation by joining or adding nanoparticles is necessary to ensure that curcumin has the greatest therapeutic effects on skin wounds. Also, further studies utilizing IHC and FISH are necessary for understanding proliferative and regenerative Wnt pathways that include epidermal stem cells for documenting the further impact of curcumin as a stem cell enhancer product.

CONCLUSION

Curcumin significantly accelerated the healing course via modulation in the amount, maturation and arrangement of collagen fibre. Furthermore, curcumin has regenerative properties on skin repair upon upregulation of CD34 expression in epidermis and hair follicles.

SIGNIFICANCE STATEMENT

In this study, we confirmed the role of curcumin treatment in promoting Epidermal Stem Cells (ESCs) proliferation and providing a pro-regenerative microenvironment, which has a significant impact on the effectiveness of collagen fibre regeneration and accelerates wound healing within a short period and without any side effects. Full-thickness skin wound healing revealed hopeful consequences in this study but more research is required to clarify the signalling pathway of this effect.

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