EFFECT OF THYMOQUINONE ON WOUND HEALING IN ALLOXAN-INDUCED DIABETIC RATS

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ABSTRACT

Objective: *Nigella sativa* and its active constituent thymoquinone (TQ) have been extensively documented for its pharmacological values, but its application in wound healing in particular in a diabetic wound healing model is less documented.

Methods: In our study, alloxan-induced diabetic rats were used as a chronic delayed wound model and topical administration of TQ 10% w/v were used to assess the role and function of TQ in wound healing through wound contraction and histological analysis.

Results: Although statistically insignificant, we found out that TQ accelerated wound healing in post-wounding day 3 (inflammatory phase), whereas aggressively decelerating wound healing in post-wounding day 7 (proliferation phase). In addition, our histological analyses of wound granulation tissues at post-wounding day 14 substantiate our claim by showing that TQ treatment had delayed wound healing progression of the diabetic rats.

Conclusions: Our study shows that TQ accelerates wound healing during the inflammatory phase; however, decelerate rapidly during the proliferation phase. We speculate the acceleration of wound healing during the inflammatory phase was due to its well-documented antioxidant, anti-inflammatory, and antimicrobial properties while its deceleration of wound healing during the proliferation phase was due to its well-documented antiangiogenic effect.

Keywords: Wound healing, Thymoquinone, *Nigella sativa*, Diabetes mellitus.

INTRODUCTION

Naturally, cutaneous wound heals spontaneously and does not necessitate any medical aid [1]. It is a continual, overlapping but independent event occurring in four described phases mainly hemostasis, inflammation, proliferation (early granulation), and remodeling (late granulation) with their own standard time frame.

Chronic non-healing ulceration is commonly associated with diabetes mellitus affecting approximately 2-4% of patients [2-4]. Ulceration is as a result of collective tissue breakdown manifested by impaired healing [4,5]. This is primarily associated with a series of macrovascular and microvascular alteration that leads to many systemic complications [4,6]. These systemic complications will result in delayed wound healing that may eventually lead to limb amputation [4,7]. Non-traumatic lower limb amputation is one of the worst complications of diabetes. Treatment is naturally difficult due to predominance of polymicrobial with multidrug resistance and capability of biofilm formation [8].

Most modern medicines today are derived from the study of medicinal plants. Plants have been phytochemically screened and tested for the past 210 years [9]. It is appealing to consider effective phytochemicals such as to treat against bacterial infection and at the same time, improve inflammatory and angiogenic functions in wound healing.

Thymoquinone (TQ) (2-methyl-5-isopropyl-1,4-benzoquinone) was first phytochemically screened and isolated by El-Dakhakhny [10]. As of yet, *in vivo* model has yet been employed to evaluate wound healing ability of *Nigella sativa* and its active constituent TQ. The nearest preclinical trial of such was using a scratch assay of fibroblast migration using *N. sativa* seed extract described recently by Ab Rahman *et al.* [11] TQ strong antioxidant-associated capacities and strong antimicrobial property, all suggested that TQ is a desirable wound healing alternative [12]. Due to TQ high pharmacological value and the severity of chronic non-healing complication, it is imperative to explore the medicinal activity of TQ in wound healing therapy. Hence, our present study was conducted to investigate to determine the effect of TQ in the wound healing progression of alloxan-induced diabetic rats.

METHODS

Animal

Male Wistar rats aged 10-12 weeks were used in all experiments. Approval for work and procedures with animals has been granted by the UBD University Research Ethics Committee. Rats were kept under optimal controlled humidity on a 12 hrs light/dark circle. Feeding pellets and sufficient access to clean water were provided.

Diabetes induction

Animals were fasted overnight with access to water *ad libitum*. Alloxan monohydrate (Sigma Chemicals, USA) was freshly dissolved in 0.9% saline solution (w/v). Animals were administered with a single dose of alloxan administration at 120 mg/kg through the intraperitoneal route.

Blood glucose levels were measured at 36 hrs and 72 hrs after alloxan administration to confirm hyperglycemia and before the experimental conduct. During the course of the experiment, blood glucose levels and body weight of rats were recorded at day 0, day 7, and day 14 to ensure successful diabetes induction. Blood glucose parameter was as previously described [13], rats with blood glucose readings of ≥300 mg/dL were used for the study.

Wound excision

Rats were anesthetized using ether fume inhalation method as previously described [14]. Excisional wounds were made as previously described with slight modifications [15]. Dorsal skin was shaved using electric razor and cleaned using repeated application of ethanol. An excision full thickness of approximately 5 mm was made to the size of approximately 225 mm^2 (15×15 mm) using sterilized

surgical equipment. Wounds were cleaned with repeated flushing of 0.9% w/v saline solution and dried with cleaned gauze. The first topical application was then administered to the wound with their own assigned treatments. Wounds were left exposed uncovered and monitored daily.

Groupings and treatments

Diabetic rats were grouped into two groups at 5 animals per group. Control vehicle only group was administered topically with white petroleum jelly (Vaseline® Jelly) while treated group was administered topically with 10% of TQ (TQ powder; Sigma Chemicals, USA) in petroleum jelly (w/v). TQ was administered on alternate days for 14 days.

Wound contraction

Photographs of wounds of all the rats were taken and respective wound area measurements were determined using Image J 1.48v software (National Institute Health, USA). The percentage of wound contractions were calculated using formula as previously described [15]: Rate of wound contraction = [(original wound area-open area on final day)/ original wound area] ×100%.

Histological observation

At post-wounding day 14, animals were euthanized using carbon dioxide asphyxiation, and the wound granulated tissue was harvested and fixed in 3.7% neutral buffered formaldehyde for at least 36 hrs for histological analysis. Tissues were then processed and embedded in paraffin wax. Serial sections of 10 μm thick were cut and stained with hemotoxylin for histological investigation. Images were visualized and captured under digital microscope Olympus ix73 with the aid of cellsens software. Observation was in accordance to relative expectation of size, shape, and color of cells in hemotoxylin and eosin staining [16].

Statistical analysis

Statistical significance was determined using one-way ANOVA, followed by Tukey's HSD *post hoc* test. Data were represented as the mean ± SEM, and the difference was considered significant at $p < 0.05$.

RESULTS

Effect of blood glucose and body weight in alloxan treatment

Alloxan-induced rats which were confirmed hyperglycemic were used in the experiment. From day 0 of experimental study, rats showed that there were no significant trends in their blood glucose levels and body weight measurements at weekly intervals (Table 1). The rats demonstrated no fluctuations in blood glucose levels and body weights during the course of the experiment.

Gross observation of wound contraction

Macroscopic evaluation of wound healing progression in Fig. 1a characterized the early formation and shedding of scabs as well as the size of the wound site through wound contraction measurement. As wound healing progress, there are noticeable differences at post-wounding day 7 and 14 between the experimental groups. Evidently, TQ is shown to decelerate wound healing after 7 days of treatment and is further decelerated at post-wounding day 14. Although insignificant, average percentage of wound contraction in Fig. 1b supports the wound healing deceleration in TQ treatment. In addition, there is noticeable increase in wound contraction at post-wounding day 3 in effect of TQ treatment.

Histological observation of wound granulation at post-wounding day 14

Delayed wound healing model as expected in diabetes-induced rats, both groups have shown unorganized collagen depositions (Fig. 2). In addition, hematoxylin staining shows visible well-developed capillary systems in both experimental groups. Furthermore, both groups show the infiltration of inflammatory cells and fibroblasts. This indicates both are in the active proliferation phase.

However, there are noticeable morphological differences in cell infiltration levels, suggesting they are not in the same timeline phase with one another (Fig. 2). In the control group, histological assessment shows relatively low infiltration of inflammatory cells in comparison to TQ-treated group. The reduction of inflammatory cells in control group indicates wound healing is faster than the wound healing in TQ-treated group. Furthermore, high infiltration of fibroblast in control group shows that wound healing is actively progressing, exiting the proliferation phase. Low infiltration of fibroblast in TQ-treated group indicates the beginning timeline of fibroblast recruitment, thus a little behind from exiting the proliferation phase.

DISCUSSION

Our study primarily focuses on the effect of TQ in treating delayed chronic wound healing as a result of metabolic impairment of diabetes. Our results have shown possible selective effect posed on different wound healing cascades. Wound healing is a dynamic and complex system; thus, repair processes are in stages but overlapping cascades [17]. During these cascade events, wound permeation differs

Table 1: Average blood glucose levels and body weights

Group	Blood glucose (mg/dL)			Body weight (g)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Control	512±45	495±43	539±34	207±9	207±9	207±9
TQ	513±46	516±50	518±34	184±10	184±10	184±10

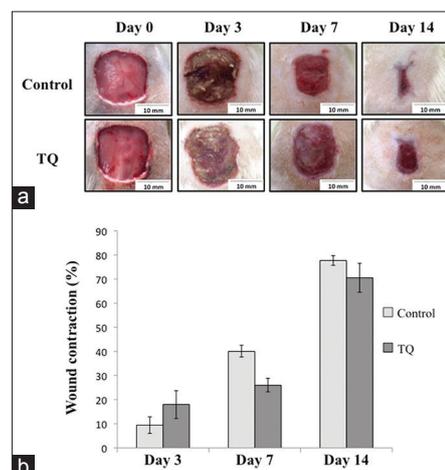Data are presented as mean±SEM (n=5 per group)

Fig. 1: (a) Wound healing progression in diabetic models with vehicle (control) and TQ treatments at given post-wounding time points, (b) average percentage of wound contractions at post-wounding day 3, 7, and 14; data are presented as mean ± SEM (n=5 for each group)
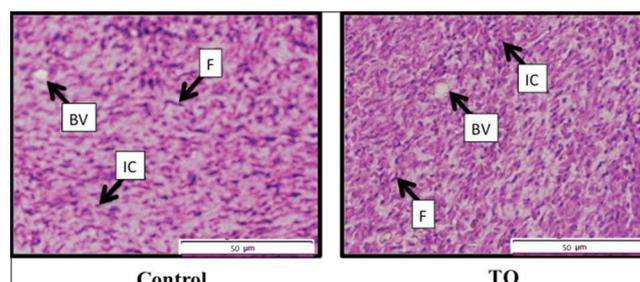

Fig. 2: Histological section of wound granulation tissues of control and TQ-treated groups on post-wounding day 14 (arrows: BV: Blood vessel; F: Fibroblast, IC: Inflammatory cell)

greatly due to physiological changes and transition from one phase to another. For instance, the behavior of drug for vasculature uptake is higher in a vasodilated environment compare to vasoconstricted environment [18].

Our result shows that for the early wound healing cascades, TQ has been effective in accelerating wound healing. During this early phase, an initial acute inflammatory response is onset to combat harmful stimuli [19]. This usually lasted for 3 days, and is simulated as our post-wounding day 3 in wound model. This acute inflammatory response is predominantly a non-specific immune response against infection and injury [20]. Neutrophils and macrophage infiltrations are important in the acute inflammatory phase of wound healing as these cells remove debris and kill bacteria through phagocytosis [21,22]. Moreover, this cascade is important to prepare the wound bed for subsequent recruitment and proliferation of cells; however, excessive inflammatory response will impair tissue granulation processes such as angiogenesis [17,23].

A study has shown that dysfunctional proinflammatory cellular function in hyperglycemic microenvironment hinders suboptimal interaction of reepithelialization process [24]. This shows that hyperglycemic metabolic impairment affects wound healing ability. Chronic wound healing in diabetic patients usually manifests badly in diabetic ulcer, and this occurs as a consequence of excess reactive oxygen species (ROS) and dysfunctional inflammatory function [21,25]. It is usually characterized by continual influx of neutrophils that release cytotoxic enzymes and inflammatory mediators that cause damage to surrounding tissue [26]. This response leads to a progressive shift in the type of cells present in the area of inflammation; thus, simultaneously destruct and heal tissue concurrently [19]. These, in turn, produce an imbalance of ROS status and lead to oxidative stress [26].

TQ has been documented as having antioxidant and anti-inflammatory effects effective for inflammatory diseases [20]. A study of metabolic impaired rats treated with curcumin, which also possessed antioxidant and anti-inflammatory effects show the acceleration of wound healing by relieving the excessive oxidative stress and minimize cytotoxic degradative environment in diabetic wound healing model [26]. In addition, improved wound morphology was observed from a different study, indicating a prominent role of the cell infiltrations and antioxidant activity [27]. TQ is also well known for its antimicrobial effect and thus effective in minimizing local inflammatory response and infection. A study of wound healing in burn model shows that these inflammatory and antimicrobial effects are responsible for accelerated wound healing capability of TQ [12]. However, more thorough investigation is needed to determine the role of TQ during the early phase of the wound healing (post-wounding day 3).

Alternatively, TQ is widely used and deemed effective in combating cancer and tumor, as opposed to the direction we want to achieve, for wound healing therapy. Early prognosis indicates that the disruption of angiogenic function in cancer cell is thought to be selective, affecting only the cancer cells, but not the normal cell [28,29]. This predicted selective effect of TQ was based on study showing toxicity in normal cells at the minimal level [30]. This is probably due to the quiescent nature of wound vascularization [29]. However, anticancer therapy using antiangiogenic medication shows interuption in wound healing processes as its cutaneous side effects [19].

Our study demonstrated a possible cutaneous effect from TQ treatment during the active angiogenesis process in our wound healing model at post-wounding days 7 and 14. During the early granulation phase, concurrent phases display the transitioning of wound healing event from inflammatory phase to proliferative phase indicated by the reduction of inflammatory cells. Myofibroblasts and microvessels are more predominant in wound healing, with peak at 7 days and progressively reduce overtime [31]. The granulation phase started when angiogenesis begin to occur, establishing a new vascular network [29].

This organization of vascular system provides necessary oxygen and nutrient supplements for wound healing repair [22]. Angiogenesis induction in endothelial cells is mediated by angiogenic factors such vascular endothelial growth factor (VEGF) [29]. These factors induce cell migration and proliferation of endothelial cells and thus direct capillary tube morphogenesis and vascular remodeling [29].

Inadequate angiogenesis independently leads to delay wound healing, and this is not so uncommon to people with diabetes [29,32]. Hyperglycemia induces pathological changes in endothelial function resulting to abnormal angiogenesis [33]. The local metabolic environment is impaired by diabetes manifesting macro- and micro-vascular dysfunction that contributes to delayed wound healing [23]. The inhibition of endothelial response to injury may manifest occurrence of wound dehiscence, bleeding, and wound infection [29]. It is worth noting that impaired wound healing in diabetes is not only just characterized by a decrease in angiogenic function but also in reduction of endothelial progenitor cell recruitment [5]. Saito *et al.* had discovered that the subsets of endothelial progenitors are altered in diabetic rats, resulting in impaired endothelial vascular differentiation [34].

As angiogenesis function is disrupted, wound healing becomes chronically inflamed. Chronic inflammation hinders progression to subsequent granulation phase due to imbalance ROS and oxidative stress in wound [21]. This causes lipid peroxidation of organelles and cellular membranes, responsible to disrupt the function of fibroblast and endothelial cells [35]. In addition, lipid peroxidation is suspected to impair VEGF expression for normal angiogenesis process [35]. Antioxidant agents are effective to combat excessive ROS activity and thus minimize lipid peroxidation [22,25]. TQ has shown to improve endothelial function by reducing oxidative stress [36]. However, its antiangiogenic effect is probably stronger in decelerating wound healing.

To the best of our knowledge, the pathway of TQ in decelerating wound healing progression still remains unknown. Nevertheless, our histopathological analysis on post-wounding day 14 has indicated that wound healing is decelerated in TQ-treated group. However, more follow-up study is needed, and we suggest the utilization of oxidative stress and vascular motif to potentiate better investigation series.

CONCLUSION

Our data show that TQ accelerate wound healing in diabetic model during the inflammatory phase, this is possibly due to its well-known antioxidant, anti-inflammatory, and antimicrobial properties. However, TQ shifts its function, as wound healing is severely decelerated during granulation phase. We speculate the deceleration of wound healing is attributed due to TQ's antiangiogenic property.

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