Total Particulate Matter and Wound Healing: An *in vivo* Study with Histological Insights[1]

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Objectives Wound healing in the skin is a multifarious orchestration of cellular processes and cigarette smoking may be a cause for delayed wound healing. The aim of this study was to investigate the plausible association between exposures of cigarette total particulate matter (TPM) and wound healing. **Methods** An *in vivo* wound healing model of mice was established for determination of assorted events of wound healing, dermal matrix regeneration, re-epithelialization, and neovascularization. A total of 72 adult mice, separated in eight groups, were exposed to TPM for 12 days. **Results** A highly considerable diminution in wound closure ($P<0.001$) was pragmatic among all TPM-treated mice from day 6 to day 8 post-wounding. Histological investigations unveiled a noteworthy impede in the outcome of re-epithelialization, dermal matrix regeneration and maturation of collagen bundles among all TPM-exposed wounds. Delayed commencement of neovascularization was pragmatic among all TPM-treated mice, on day 12 post wounding. Abbot curve, angular spectrum, and other different parameters of 3D surface behavior of wounds revealed a very highly significant reduction ($P<0.001$) in angiogenesis on days 6 and 8 post-wounding, which points that application of TPM instigates extensive delay in trigging the progression of angiogenesis, resulting in delayed onset of wound healing. **Conclusion** Our annotations validate the damaging effects of TPM on wound healing and excessive use of TPM may lead to the production of chronic wounds and oral ulcers.

Key words: Cigarette; Total particulate matter; Wound healing; Re-epithelialization; Angiogenesis

INTRODUCTION

Cigarette smoking, at present era, is the most widespread leisure interest among different generation groups and culture[1]. In general community, this is going to portray as a status symbol without knowing its destructive effects. Generally, cigarette smoking is adopted for relaxation or habitual purposes[2]. The harmful effects of smoking on health are numareous, depending on the amount and way of use (smoked, snuffed or chewed) leading towards even death[3]. Likewise, Department for Disease Control and Prevention of the United States has nominated tobacco use as "the single most important preventable risk to human health in developed countries and an important cause of premature death worldwide"[4]. Individuals who smoke get involved in many health hazards regarding cardiovascular diseases, insulin resistance, elevated heart rates, hypercholesterolemia, *etc*.[5]

Wound healing is the natural process of regenerating dermal and epidermal tissue in body[6]. During wound healing, a set of complex biochemical events take place in a closely orchestrated cascade to repair the damage[7]. These events overlap in time and may be artificially categorized into separate steps: the inflammatory, proliferative, and remodeling phases. In the inflammatory phase, bacteria and debris are phagocytized and removed, and different factors are released, which cause the migration and division of cells involved in the proliferative phase[6]. The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction[8].

Cigarette smoke is an intricate mixture of countless toxic substances. There are principally

[1]This work was supported by the grant from Post Doctor Program, Chonbuk National University (2008).

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0895-3988/2009
CN 11-2816/Q

three categories of blend engender during smoking: mainstream cigarette smoke (inhaled by the smoker), side stream cigarette smoke (inhaled by non-smokers in places where smoking is allowed), and total particulate matter (TPM) of cigarette smoke, which is the sticky particles comprised of thousands of chemicals fashioned by burning tobacco. It is the particulate component of tobacco smoke without nicotine and water[9]. According to the constituents of cigarette, there are various references that designate the association between cigarette smoke and impaired wound healing[10]. Nicotine is a vasoconstrictor that reduces dietetic blood flow to the skin, resulting in tissue ischemia and impaired healing of injured tissue[11]. Cigarette smoking also boosts platelet adhesiveness, raising the risk of thrombotic micro vascular occlusion and tissue ischemia[12]. Additionally, proliferation of red blood cells, fibroblasts, and macrophages is reduced by smoking[13]. Carbon monoxide diminishes oxygen transportation and metabolism, whereas hydrogen cyanide hampers the enzyme systems required for oxidative metabolism and oxygen transport at the cellular level[14]. Thus, several toxic constituents of cigarette smoke propose prospective mechanisms by which smoking may destabilize expeditious wound repair. The abridged capability of wound repair is of particular concern in patients undergoing plastic or reconstructive surgery[15].

In our preceding investigations, we have elucidated different detrimental effects of mainstream cigarette smoke solutions (MSCSS) and side stream cigarette smoke solutions (SSCSS) on wound healing[16]. In extension to our earlier studies, the current experiment was premeditated to monitor different toxic effects of *in vivo* application of TPM from commercially available cigarettes on wound healing. This *in vivo* model gave us a thorough determination of different events of wound healing, dermal matrix regeneration, re-epithelialization and neovascularization, in a programmed and time dependent manner with a profound view of wound healing in comparision with the control group.

MATERIALS AND METHODS

Animals

A total of 72 BALB/c mice, at the age of 4-5 weeks, were acquired from University of Veterinary and Animal Sciences, Lahore. All the animals were kept under the same conditions of temperature with free access to normal tap water and commercial feed. At the start of experiment, all the animals were weighed and divided into 8 groups, 9 in each group (Table 1).

TABLE 1

Experimental Groups with Respective Treatments of Total Particulate Matters (TPM) from Six Commercial Cigarettes

Group	Mice (*n*)	Treatment
A	9	TPM-1
B	9	TPM-2
C	9	TPM-3
D	9	TPM-4
E	9	TPM-5
F	9	TPM-6
G (+ive Control)	9	Petroleum Jelly
H (-ive Control)	9	NA

Preparation and Administration of Total Cigarette Particulate Matter from Commercial Cigarettes

A market survey was carried out to select the most extensively smoked commercial cigarettes, which were then used to prepare TPM. TPM was prepared immediately before use essentially as previously described[17]. In brief, TPM was collected onto the Cambridge filter pad, then placed in 10 mL dimethylsulfoxide (DMSO) and filtered through 0.22 μm pore filters. The pH of the TPM solutions was adjusted to 7.0. Moreover, 10% TPM solutions, prepared in petroleum jelly, were topically applied to a 3 mm wound area on the dorsum of all mice in treatment groups daily for 12 days.

Wounding and Preparation of Wound Tissue

Mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (10 mg/kg) and xylazine (1.5 mg/kg). For the management of pain and suffering of animals, buprenorphine (an opioid analgesic) was given subcutaneously at a dose rate of 0.04 mg/kg postoperatively, which produces analgesia up to 12 hours. Moreover, buprenorphine injection was also repeated before each manipulation. The hairs on the dorsum of mice were removed by depilatory cream and dorsum was subsequently wiped with 70% (v/v) ethanol. A wound was created as previously described[18]. Briefly, a 3 mm-diameter excision was created on the dorsum of mice with a sterile biopsy punch. Wounds were left undressed and mice were housed separately after wounding.

Macroscopic Evaluation of Wounds

During the 12-day experiment, the wound closure activities were macroscopically recorded by photography. All wounds were visualized using a high-resolution Lebeca cam (PanWest, China) built-in CMOS image sensor (320,000 Pixel)

supporting high quality VGA (640×480) resolution with 24 bit RGB color. The cam lens made up of 5 glasses contributes to picture improvement over regular lenses. The magnification was made adjustable by manual placement of a camera from the object. To ensure an objective 3D measurement of wound healing, serial images with respective x, y and z dimensions were recorded at 30 frames/s using a camera shutter speed of 1/2 000 s.

Image Processing System to Quantify Wound Closure

Computer elaboration and image processing were performed with an image probing system (IPS) using a 32-bit color quality under Windows XP environment. A great deal of information regarding IPS (a combination of SPIP software and Adobe photoshop 6.0) has already been explained elsewhere[19-20]. The display screen (IBM-LG, Korea) supported with SVGA graphics (NVIDIA GeForce 2 MX 100/200) and adjusted to resolution 800×600 pixels, provides an optimal optical representation. After image acquisition, all images were imported to scan probing image processing software (IBM-Denmark) that works on specific algorithm[21]. Respective x, y, and z dimensions of each image were loaded to determine different parameters for wound healing quantification. 3D surface roughness, one of the main parameters in 3D image analysis to evaluate the cellular activities, was measured for precise quantification of wound healing. Other different imperative parameters (abbott curve, angular spectrum, Sci) of 3D evaluation of micrometer and/or nanometer scale neovascularization were also quantified for holistic wound healing quantification.

Abbott curve (graphical presentation of the height of blood vessels at wound site) is a vital parameter used to quantify overall vascular activities at a particular area. Angular spectrum (graphical presentation of the angular distribution of blood vessels at wound site) is a graphical representation of the progression of cellular activities at wound sites, while Sci is the ratio of the void volume of the unit sampling area at the core zone over the root mean square deviation. This vital parameter of 3D quantitation is used for determination of fluid retention at that surface and a larger Sci value designates better neovascularization at that surface[22].

Histological Examination of Wound Healing

For in-depth investigating of wound healing at different intervals, three mice from each group were sacrificed on days 4, 8, and 12 post-wounding. The complete wound, including the scab and 3 mm of the epithelial margins, was excised at each time point, fixed in a 10% formaldehyde solution and processed for histological examination. These tissues were then embedded in paraffin wax, cut into 3-μm thick sections, mounted on glass slides and stained with haematoxylin and eosin (H&E) for routine light microscopy. Histological sections were also digitized with a spot camera and different parameters of wound healing were quantified.

Statistical Analysis

Statistical analysis was carried out using SPSS 13 software (SPSS Inc. Chicago, Illinois 60606, USA). All data were expressed as $\bar{x} \pm s$. Groups of data were compared with analysis of variance (ANOVA) followed by Student's t test. $P<0.05$ was considered statistically significant[23].

RESULTS

Cigarette Total Particulate Matter Significantly Disrupted Wound Contraction

To explicate the function of TPM in wound contraction, the wound sites were scrutinized by macroscopic examination of wound photographs. On day 2 post-wounding, the wound sites in all TPM-treated groups were analogous in size and manifestation to those of the groups G and H. The wound margins were straightforwardly predictable by an abrupt interruption in the epithelial and dermal permanence. Different arrangements of wound contraction ranging from moderate to incomplete wound contraction were pragmatic among different groups from day 5 to day 12 post exposure (Fig. 1). A highly significant diminution ($P<0.001$) in wound contraction of all TPM-treated groups was observed from day 6 to day 12 post exposure (Fig. 6a), which symbolizes that TPM significantly adjourns the events of wound contraction resulting in delayed wound healing.

Delayed Re-epithelialization among Cigarette Total Particulate Matter Treated Groups

The re-epithelialization progression subsequent to wounding was scrutinized by microscopic assessment. Different extents of re-epithelialization were observed among all TPM-treated groups, demonstrating more obvious impede outcomes (Fig. 2a) than in control groups, thus illustrating comprehensive delay in re-epithelialization of wound sites on day 12 post-wounding. Histological (H & E) investigations of TPM-treated wounds on different days post-wounding

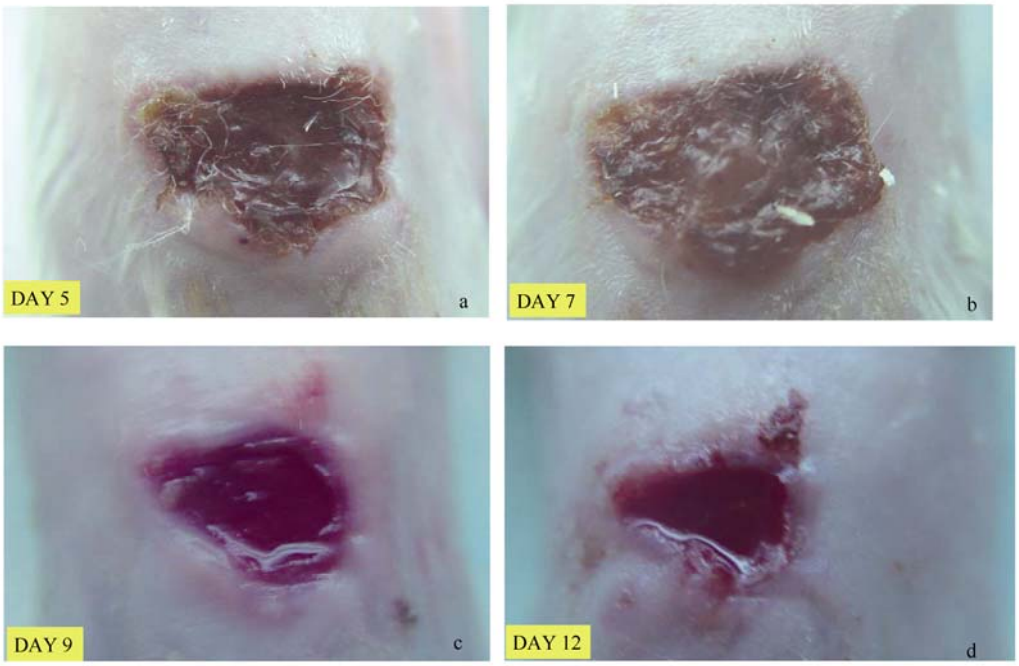

FIG. 1. Macroscopy showing effects of TPM from cigarettes on wound contraction. Comparison of skin wound healing in TPM-treated mice on different days post wounding represent a significant delay in wound contraction of mice after treatment with TPM.

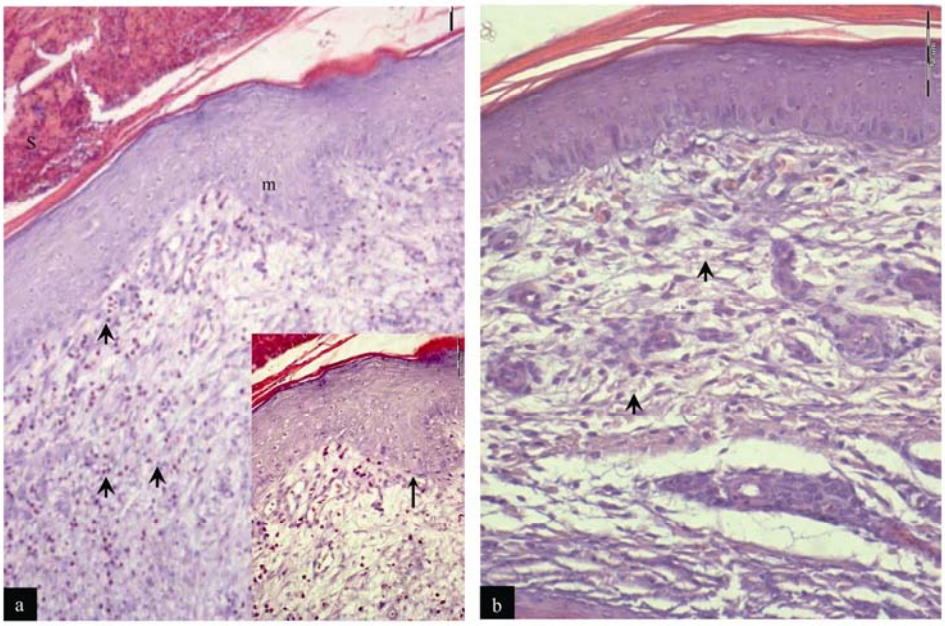

FIG. 2. Histological micrographs elaborating delayed re-epithelialization and dermal matrix regeneration on day 12 post-wounding in mice after treatment with TPM. Histological analysis of TPM-treated wounds revealed a disorganized migratory tongue (m, inset) of epidermal cells penetrating into the wound bed covered by scab (S) in close contact with the provisional matrix (a). Moreover, several inflammatory cells could also be seen (arrowheads). Deterioration of variable intensities in collagen bundles with several inflammatory cell (arrowheads) was observed in all TPM-treated groups (b).

revealed an asymmetrical development of neoepidermis and neodermis towards the wound axis after wounding. A scab (S) enclosed the wound in close contact with the provisional matrix (Fig. 2a). An immature development in both neoepidermis and neodermis was observed among all TPM-treated wounds with defective re-epithelialization and a thin granulation layer. On day 12 post-wounding, agitatedly activated migratory tongue (m) of epidermal cells infiltrating the wound bed of TPM-treated groups was observed under the scab. The forward branch of the tongue (tongue tip) was composed of a monolayer, progressively pursued by disorganized multilayered epithelium (Fig. 2a, inset) and then a bilayer epithelium towards the wound boundaries (tongue tail). Additionally, inflammatory cells (Fig. 2a, arrowheads) were nonetheless markedly evident on day 12 post-wounding, representing delayed wound healing. On day 12 post-wounding, a moderately straight tongue composed of multilayered epithelium and exclusively enclosed wounds by stratified neoepithelium were observed in the control group. Histological analysis thus revealed a nearly healed morphology of the control wounds, which was consistent with the wound closure observations above.

Cigarette Total Particulate Matter Disrupted the Normal Process of Dermal Matrix Regeneration

The composition of the dermal matrix underwent transformations from an original fibrin clot to a granulation tissue and a collagenous dermis. All the wounds, on day 8 post-wounding, were plugged by variable levels of provisional matrix containing blood vessels and invading cells consistent with inflammatory elements. On day 12 post-wounding, delayed granulation tissues with asymmetrical intensities were recognized in all TPM-treated wounds, nevertheless underprivileged with collagen activities (Fig. 2b). On day 12 post-wounding, the connective tissues from the control group illustrated that an advanced intensity of the collagen bundles acquired an ‘‘angel curl shaped structure’’, while deteriorations of variable intensities in collagen bundles were observed in all TPM-treated groups (Fig. 2b).

Delayed Trigging of Neovascularization in TPM-treated Wounds

In the control group, a noteworthy (P<0.001) increase in the height of Abbott curve was observed on day 6 post-wounding, while a substantial retardation in the height of Abbott curve on days 8, 10, and 12 post-wounding showed inclusive revitalization of wounds and quiescent neovascularization. On days 6 and 8 post-wounding, highly significant dwindle in the height of Abbott curve was observed in all TPM-treated groups (Figs. 3a, b), while a significant increase in the height of Abbot curve was established during days 10 and 12 post wounding (Figs. 3c, d), imitating that application of TPM can instigate extensive impediment in trigging the process of angiogenesis resulting in delayed commencement of wound healing (Fig. 6b).

Blood vessels in non-treated wounds were more profoundly observed on days 6 (P<0.05) and 8 (P<0.001) post-wounding with homogeneous dissemination covering the utmost region of wound (Fig. 6c). A significant decline in the spread and distribution of the angular spectrum of control wounds at the later stages of wound healing, advocateed the customary physiological development of vascular inactivity after wound healing (Fig. 6c). Extensive retardations in the angular spectrum with a dejected and irregular distribution of blood vessels were pragmatic in all TPM-treated groups on day 6 post-wounding (Fig. 4a), whereas a significant increase (P<0.001) in the angular spectrum was observed on days 10 and 12 post-wounding (Figs. 4c, d, 6c).

IPS elucidated an ascending escalation in surface roughness of the control wounds from day 6 to day 10 post-wounding, while least activities were observed at the wound site of mice on day 12 post-wounding. A significant decrease in surface roughness of the variable intensities was observed in all TPM-treated groups on days 6 and 8 post-wounding (Figs. 5a, b), which inflicted an impede neovascularization. A significant increase (P<0.05) in the wound surface roughness was observed on days 10 and 12 post-wounding (Figs. 5c, d). A considerable reduction (P<0.001) in Sci magnitude was observed in all TPM-treated groups on days 6 and 8 post-wounding, which led to a significant augmentation of different intensities in Sci values for all TPM-treated groups on day 12 post-wounding (Fig. 6d).

Histological micrographs of wounds from control samples on day 12 post wounding, revealed quiescent vascularization in close proximity to the endodermal margin of skin, while mesodermal vicinity of wound was filled with significantly modified extracellular matrix. On day 12 post wounding, all the wounds treated with TPM showed significantly ample neovascularization (arrowheads) in the mesodermal area where extracellular matrix was exclusively shattered (Fig. 7). Likewise, inflammatory cells were also prominently observed in the whole mesodermal area, which corresponded to belated wound healing.

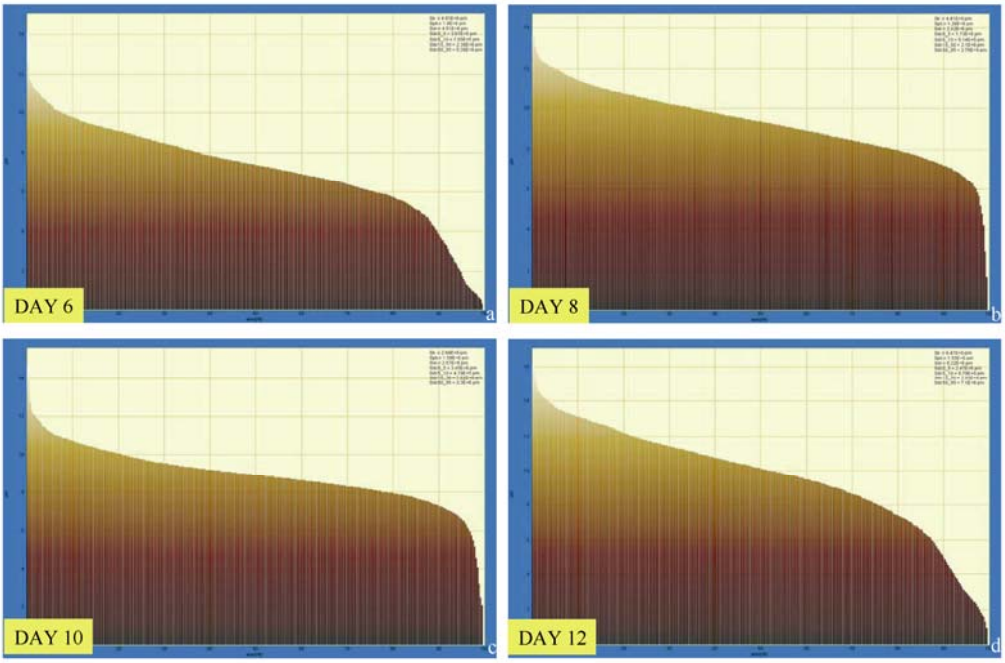

FIG. 3. Outline of the height of Abbott curve for TPM-treated wounds on different days post-wounding. Impede was noted in the height of Abbott curve during early days (a) with a gradual increase in the consecutive days (b, c, d), revealing delayed onset of angiogenesis in TPM-treated groups.

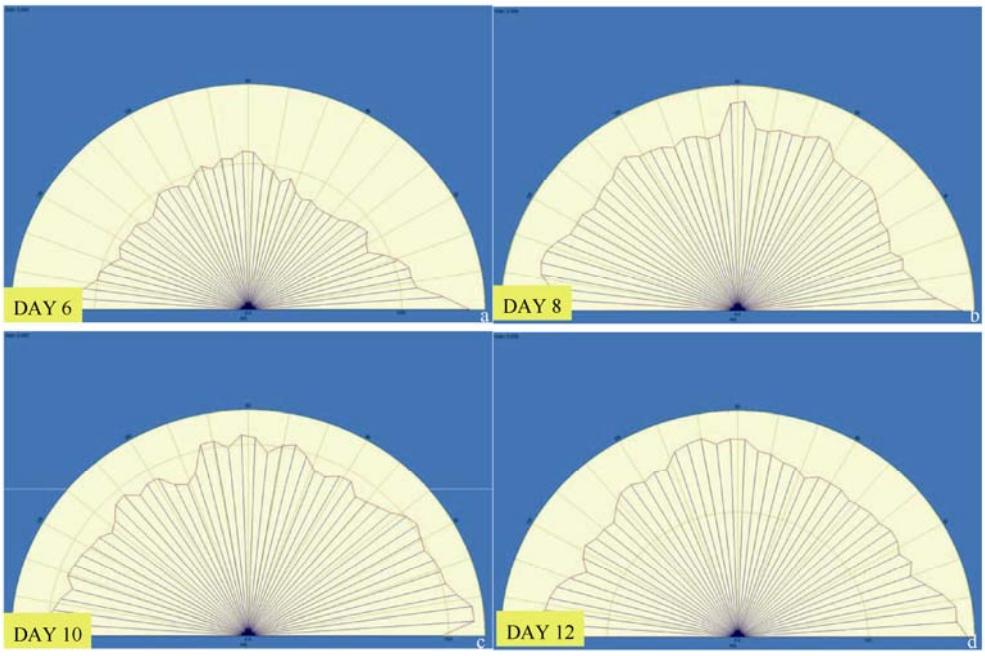

FIG. 4. Delayed and altered distribution of blood vessels on different days post-wounding among TMP-treated groups. Dejected and irregularly distributed blood vessels were observed in all TPM-treated groups on days 6 and 8 post-wounding (a, b), while significant activation of angiogenesis was observed in TPM-treated groups (c, d) showing retarded angiogenic process.

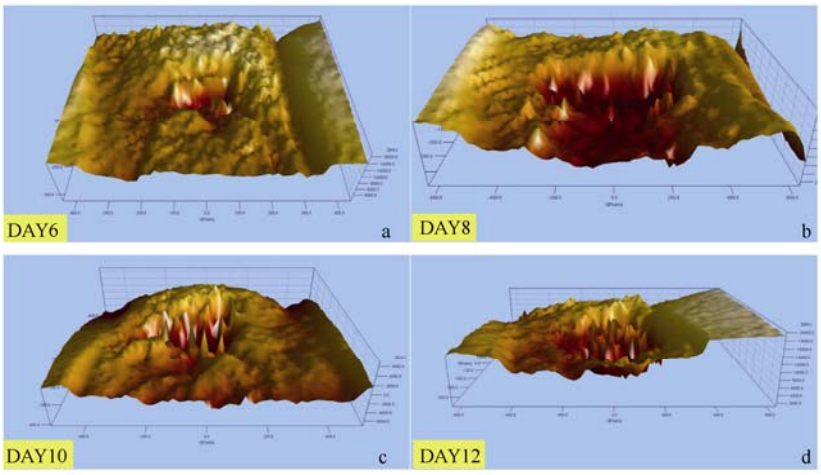

FIG. 5. Scan probing images highlighting delayed wound healing activities at different days post-wounding among TPM-treated groups. An early halt (a, b) and late progression (c, d) in the surface roughness of TPM-treated groups imposed retarded neovascularization.

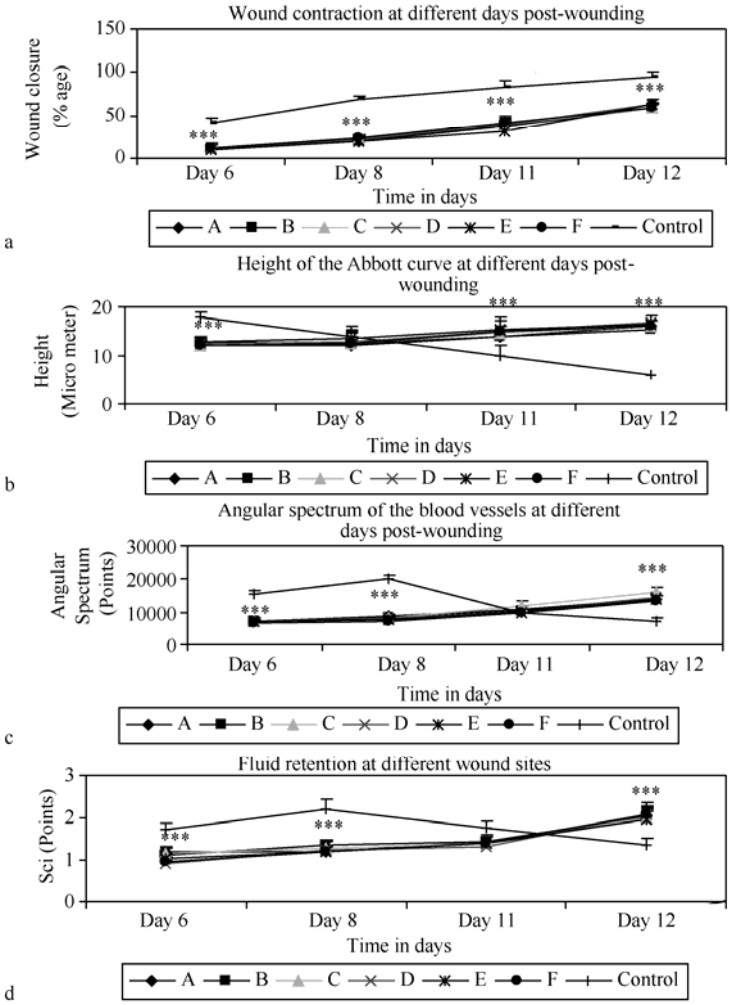

FIG. 6. Significant decrease in wound contraction (a), height of Abbott curve (b), angular distribution (c), and fluid retention at wound sites (d) in TPM-treated groups. (***P<0.001).

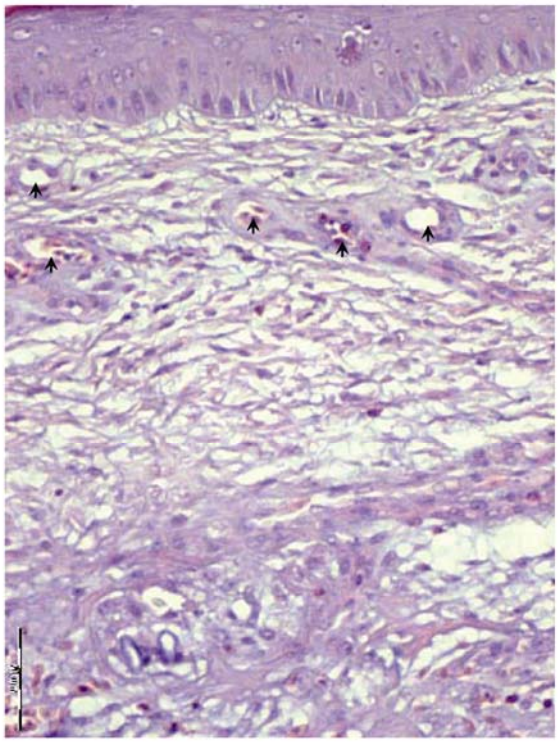

FIG. 7. Histological micrographs of wounds after treatment with TPM on day 12 post-wounding. Significant neovascularization was observed near the marginal area of wounds representing belated wound healing in TPM-treated groups.

DISCUSSION

Wound healing necessitates a well-orchestrated integration of the intricate biological and molecular events of re-epithelialization, dermal matrix regeneration and angiogenesis[24]. Cellular responses to inflammatory mediators, growth factors, cytokines, and mechanical forces must be appropriate and precise[6]. These imperative processes of wound healing are analogous to those guiding embryogenesis tissue, organ regeneration, and even neoplasia[25]. In cutaneous injuries, the foremost evolutionary force may have accomplished prompt repair with the least amount of energy. Consequently, such wounds heal with a scar but no regeneration[27]. In wounds with pre-existing pathophysiological abnormalities (chronic wounds, such as diabetic ulcers), evolutionary adaptations have probably not occurred and impaired healing is the result[28].

Cigarette smoke contributes to the pathogenesis of a variety of diseases. The sequences of disorders are exemplified by insufficient tissue repair, including pulmonary emphysema, peptic ulcer, and poor wound healing[29]. It is distinctly understood that mainstream and side stream cigarette smoke delay wound healing[36], but very little is acknowledged concerning the effects TPM on wound healing. The aim of this study was to appraise the application of TPM with a view of its examination in the context of wound closure, re-epithelialization, dermal matrix regeneration, and neovascularization in wound healing.

In the current investigation, the mice in the treatment groups had larger wound boundaries than those in the control group on different days post-wounding (Fig. 1). Continuous application of TPM at wound sites instigated progressive delay ($P<0.001$) in wound closure from day 6 to day 12 post-wounding (Fig. 6a), indicating that application of TPM meticulously engenders undesirable effects that might prolong the process of wound closure. These finding are compatible with our previous findings demonstrating delayed wound closure after exposure to mainstream smoke solution[31]. It was reported that delayed wound closure has also been observed in C57BL/6, BALB/c and Swiss mice exposed to cigarette smoke[30]. In this study, histological appraisal at wound sites also revealed delayed re-epithelialization in TPM-treated wounds (Fig. 2a). Re-epithelialization is accomplished by

keratinocyte proliferation and migration over an extracellular matrix[32]. It is alleged that cigarette smoke contains numerous toxic chemicals that hamper re-epithelialization[33].

Wound healing is a highly dynamic process and implicates complex communications between extracellular matrix molecules. Migrating epithelial cells intermingle with a provisional matrix of fibrin cross-linked to fibronectin and collagen. Especially, fibronectin emerges to endorse keratinocyte adhesion to direct these cells crossing the wound base[34]. The results of this, however, validate that these activities might be inhibited by toxins in TPM. Extending findings in a previous study demonstrated that smoke can inhibit lung fibroblast repair responses[35]. In this study, different preparations of TPM produced anomalous blueprints of the fibrillar material in wounds (Fig. 2b), which might be associated with the digression of interstitial collagens (types I and III) in the extracellular matrix. Our results are consistent with previous findings that explicate the identical destructive effect of cigarette smoke exposure on extra cellular matrix[36] and support that collagen metabolism is fundamental in controlling wound healing and either an excessive or deficient amount of collagen or inappropriate processing of collagen altars the normal wound healing process. Throughout the wound healing process, the extracellular matrix endures compositional transformation that is acknowledged to be imperative in influencing blood vessel development[37].

During wound healing, angiogenic capillary sprouts invade the fibrin/fibronectin-rich wound clot and within a few days assemble into a microvascular network throughout the granulation tissue. A dynamic communication transpires in endothelial cells, angiogenic cytokines, and extracellular matrix environment[38]. Thus restoring blood flow to the site of wounded tissue is a prerequisite for escalating a victorious repair response[39]. In this study, quantification of the Abbott curve revealed a dramatic decrease (P<0.001) in the surface height of blood vessels in TPM treated groups on day 6 post-wounding, while a significant increase (P<0.001) in the Abbott curve was observed in TPM-treated groups on days 10-12 post-wounding (Fig. 6b). A significant increase in the height of Abbott curve was documented by the end of experiment, which evidently demonstrated the commencement of angiogenesis at the subsequent phases of wound healing resulting in postponed wound healing. Angular distribution of blood vessels in different wounds after treatment with TPM was more rigorously retarded (P<0.001) from day 6 to day 8 post-wounding (Figs. 4a, b), suggesting that TPM negatively affects the growth and uniform distribution of blood vessels at wound sites. Consequently, it is recommended that depression of angiogenesis at wound sites is one of the pathologic mechanisms involving the delayed wound healing[40]. Indistinguishable events of primary depression and successive activation of 3D surface roughness in TPM treated wounds unveiled the delayed cellular activities at wound sites (Fig. 5), while utmost activities were observed at control wounds. Additionally, a very significant diminution (P<0.001) in Sci values for all TPM-treated groups (Fig. 6d) imitated a decrease in fluid retention at wound sites referring to retarded neovascularization. Thus, these parameters enlighten the discrepancy of 3D surface roughness between normal and TPM-treated wounds. Likewise, histological exploration also explicated identically delayed activation of neovascularization in TPM-treated wounds on day 12 post-wounding (Fig. 7), while quiescent blood vessels were observed in the control group. Our results are consistent with a preceding study by Ma *et al.* who explained the delayed ulcer healing due to retarded angiogenesis in rats exposed to cigarette smoke[41]. Tobacco smoke is a complex combination of over 4000 different chemical constituents in cigarette smoke that are toxic to endothelial cells (for example, cadmium and reactive oxygen species)[25], resulting in retarded angiogenesis.

In conclusion, various characteristic of wound healing have different sensitivities to TPM. Moreover, it is obligatory to let people know the detrimental effects of smoking on health, since they may cause delayed wound healing, leading to chronic wounds and oral ulcer.

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(*Received December* 12, 2008 *Accepted May* 20, 2009)