Sustained Wound Healing Activity of Curcumin Loaded Oleic Acid Based Polymeric Bandage in a Rat Model

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ABSTRACT: Wound healing is an intricate multistage process that includes inflammation, cell proliferation, matrix deposition and remodeling phases. It is often associated with oxidative stress and consequent prolonged inflammation, resulting in impaired wound healing. Curcumin has been reported to improve wound healing in different animal models. In order to increase the efficacy of curcumin in the healing arena a curcumin loaded oleic acid based polymeric (COP) bandage was formulated. The in vivo wound healing potency was compared with void bandage and control (cotton gauze treatment) in a rat model. Biochemical parameters and histological analysis revealed increased wound reduction and enhanced cell proliferation in COP bandage treated groups due to its efficient free radical scavenging properties. Comparative acceleration in wound healing was due to early implementation of fibroblasts and its differentiation (increased level of α-smooth muscle actin). Western blotting and semiquantitative PCR analysis clearly indicate that COP bandage can efficiently quench free radicals leading to reduced antioxidative enzyme activity. Further evidence at mRNA and protein level indicates that our system is potent enough to reduce the inflammatory response mediated by the NFκB pathway during wound healing. With this background, we anticipate that such a versatile approach may seed new arena for topical wound healing in the near future.

KEYWORDS: dermal wound healing, oleic acid, curcumin, antioxidant, anti-inflammatory, polymeric bandage

INTRODUCTION

Wound healing is a complex physiological response to injury. It is a very systemic biological, chemical and mechanical event, where the invaded pathogens are removed from the damaged wound site for complete or partial remodeling of injured tissue. In general, it proceeds in a very orderly and efficient manner characterized by three interrelated dynamic and overlapping phases, namely, inflammatory phase (consisting the establishment of homeostasis and inflammation); proliferative phase (consisting of granulation, contraction and epithelialization) and finally the remodeling phase. However, in severe pathological conditions this cascade of healing process lost and the wounds are locked in a state of chronic inflammation characterized by abundant neutrophil infiltration with the associated release of inflammatory mediators including reactive oxygen species (ROS), reactive nitrogen species (RNS) and their derivatives. These radicals result in oxidative stress leading to lipid peroxidation, DNA breakage and enzyme inactivation ultimately causing local and distant pathophysiological inflammatory effects. Mitigation of this deregulated chronic inflammation (the major cause of impaired wound healing) and finding a safe and efficacious anti-inflammatory agent is a frontier challenge in modern medicine. The role of oxidants in the pathogenesis of many inflammatory diseases suggests that antioxidant therapy could be an effective strategy for therapeutic approaches to such disorders. To this end, antioxidant activities of traditional medicine with free radical scavenging properties have been proven a new horizon for better healing treatment. In this regard, curcumin is one such drug which will certainly be beneficial against oxidative damage, due to its antioxidant properties, and be helpful to the better healing of the wound.

Curcumin (diferuloylmethane) is a naturally occurring phytochemical polyphenol derived from the rhizome of turmeric (Curcuma longa). Preclinical and clinical studies indicate that curcumin has potential therapeutic value against most chronic diseases because of its antioxidant, anti-inflammatory and anti-infective properties. Several in vitro and in vivo studies have demonstrated the effectiveness of curcumin in decreasing the release of inflammatory cytokines like interleukin-8 and tumor necrosis factor-α from monocytes and macrophages, and further it has been shown to inhibit enzymes associated with inflammation, such as cyclooxygenase and lipooxygenase. Besides its anti-inflammatory activity, it has the ability to scavenge free radicals, which is the major cause of inflammation during wound healing activity. Recently, a plethora of experimental data using animal models has established that treatment of curcumin may assist wound healing by increasing the formation of extracellular matrix proteins and granulation tissue and neovascularization. Studies to date have demonstrated that topical application of...
curcumin has more pronounced effects on wound healing compared to its oral administration owing to the greater accessibility of the drug at the wound site.\textsuperscript{11–14} Conversely, the intrinsic problem associated with parenteral delivery of curcumin is its extremely low water solubility and degradation in alkaline medium, which limits its bioavailability and clinical efficacy. In this view, several groups have developed new approaches like delivery with alginate chitosan sponge and poly(ε-caprolactone) nanofibers to achieve better topical administration of curcumin at the wound site.\textsuperscript{11,15} Much interest is currently focused on the development of a cell penetrating immune modulating delivery system to enhance the drug's bioavailability as well as to modulate inflammation at the wound site as it is the major problem associated with impaired wound healing.\textsuperscript{16} Recently, several groups have demonstrated that oleic acid (OA), an unsaturated fatty acid, may modulate the inflammation by downregulating COX2 expression and stimulating the production of cytokine-induced neutrophil chemoattractant in inflammation 2 alpha/beta.\textsuperscript{16,17} This immunomodulation response of OA (approved by the Food and Drug Administration for biomedical applications) at the wound site is anticipated to enhance faster wound reparative processes. Additionally, its viscous properties could provide a platform for sustained release of the drug by slow drug diffusion in solubilized form which can enhance the therapeutic efficacy. Furthermore, it has an enhanced cell penetrating and inflammatory modulation property.\textsuperscript{16,17} Consequently, OA was used in this study to achieve better administration and sustained delivery of curcumin at the wound site. We hypothesize that this delivery strategy, which combines better administration of curcumin with its immune modulating activity, may produce better wound healing.

In a quest for a fast and healthy wound healing process, we have prepared curcumin loaded OA based polymeric (COP) bandage (composed of chitosan and sodium alginate). We hypothesize that the sodium alginate and chitosan will cross-link to form a net-like structure which will allow the release of curcumin in a sustained manner for better therapeutic activity. Furthermore, the large surface area of the polymeric bandage may facilitate the interaction with the healing tissue, thereby serving as a platform for the sustained delivery of curcumin as well as improved wound healing by providing a substrate for proliferation of epidermal cells. Therefore, our further objective was to evaluate the therapeutic efficacy of COP bandage in dermal wound healing in a rat model as well as to study the key mechanism involved in promoting wound healing under an in vivo experimental setup.

\section*{Materials and Methods}

Materials. Curcumin used in the current study was taken from purified curcumin capsule, CUR-500, manufactured and purchased from UNICO Pharmaceuticals, Ludhiana, India. Each capsule contained the extract of \textit{Curcuma longa} containing curcumin (>95\%) and other curcinoids (~4\%) per 500 mg. Chitosan (degree of deacetylation = 85\%, average molecular weight 650 kDa) derived from crab shells, in the form of fibrillar flakes, sodium alginate (medium viscosity ≥ 2000 cP, molecular weight between 75 and 100 kDa) and oleic acid were obtained from Sigma-Aldrich, Germany. All other chemicals used were purchased from Sigma Aldrich (St. Louis, MO) without further purification.

Preparation of Curcumin Loaded and Void OA Based Polymeric Bandages. Briefly, alginate solution (0.5\% w/v) was prepared by dissolving sodium alginate powder (0.1 g) in 20 mL of deionized water at room temperature. Chitosan solution (0.5\% w/v) was prepared by dissolving chitosan powder (0.1 g) in 20 mL of deionized water containing 1\% acetic acid at room temperature. To prepare the COP bandage, 50 mg of curcumin was incorporated into a fluid phase of 1.75 mL of OA. The suspension of OA and curcumin was then emulsified with the above chitosan solution for 2 min with homogenization (Biospec Products Inc., Bartlesville, OK). The resultant solution was further subjected to homogenization (Biospec Products Inc., Bartlesville, OK) for 3 min with alginate solution. In this way, COP bandage solutions with different blend ratios of alginate:chitosan (1:1, 1:2 and 1:3) were prepared (keeping curcumin and OA content constant). The above suspensions were poured in a six well plate (Corning, NY, USA) and lyophilized for three days (~80 °C and <10 μm mercury pressure, LYPHLOCK, Labconco, Kansas City, MO) to obtain lyophilized COP bandage for further use. For the preparation of void OA based polymeric (VOP) bandage (without curcumin) a similar procedure was followed except the addition of curcumin into the OA solution during the preparation procedure.

Physicochemical Characterization of COP Bandages. Scanning Electron Microscope (SEM) Study. The surface morphologies of COP and VOP bandages were characterized by SEM (JEOL JSM7200A scanning electron microscope, MA) operating at an accelerating voltage of 10–30 kV. The polymeric bandages were sputtered with gold to make them conductive and placed on a copper stub prior to the acquisition of SEM images.

In Vitro Swelling Ability Study and Degradation Study. Different formulated VOP bandages of size 1 cm × 1 cm were used for in vitro swelling ability and degradation study. The above studies were done by following the protocol of Dai et al.\textsuperscript{15}

In Vitro Release Kinetics of Curcumin from Different Formulations of COP Bandage. Briefly, different formulations of COP bandage of size 5 cm × 5 cm containing ~50 mg of curcumin was suspended in 5 mL of PBS (0.01 M, pH 7.4) and kept in a shaker at 37 °C, rotating at 150 rpm in an orbit shaking incubator (Wadegati Lab equip, India). At predetermined time intervals, the samples were collected and replaced with the same volume of fresh PBS (0.01 M, pH 7.4). The collected samples were then subjected to centrifugation at 13,800 rpm, 4 °C for 10 min (SIGMA 3K30, Germany) to obtain the supernatant containing released curcumin. The released curcumin concentration was analyzed using reverse phase isocratic mode (RP-HPLC) system of Waters 600 (Waters Co., Milford, MA, USA) as described earlier.\textsuperscript{7}

In Vivo Wound Healing Test. For this experiment Sprague–Dawley male rats (160–180 g, 6 weeks) were used. All the in vivo experiments were carried out with the permission of the Institutional Animal Ethics Committee of Institute of Life Sciences, Bhubaneswar, India. In brief, the rats were divided into three groups, each group comprising 12 rats: group I, control; group II, VOP bandage (1:2) treated group; group III, COP bandage (1:2) treated group. The animals were anesthetized intramuscularly by ketamine (100 mg/kg) and xylazine (10 mg/kg). The dorsal hair of the rats was removed. A full-thickness wound of 1.5 × 1.5 cm\textsuperscript{2} was excised from the back of the rats. Each wound was covered with an equal size of COP bandage, or a VOP bandage, or cotton gauze as a control for comparison. Treated rats (n = 6) were observed and...
photographed on the 0th, 4th, 8th and 10th days using a digital camera (Sony, cyber-shot, DSC-H9). The area of the wound was calculated by measuring the length and breadth of the wound with digital slide calipers (Fisher Scientific). Percentage of wound reduction was calculated by observing the wound area at the present time.

In Vivo Detection of Apoptosis by Gel Electrophoresis and TUNEL Assay. To evaluate apoptotic and necrotic cell death at healing site, 4th and 10th day postoperative wounded tissues were collected and its 10% homogenate was prepared with lysis buffer (Promega, Madison, WI). After 5 min incubation at 37 °C, 10 μL of RNase (10 mg/mL) was added and incubated for 1 h, and proteinase K (final concentration 100 μg/mL) was added and incubated at 50 °C for 1 h. To the incubated solution an equal volume of phenol was added and centrifuged for 10 min at 4 °C. The aqueous phase of supernatant was taken and equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) was added. The DNA was then precipitated by addition of two volumes of absolute ethanol and 0.1 volume of sodium acetate (0.3 M) and kept at −20 °C for 24 h and centrifuged at 12000 rpm for 10 min at 4 °C. The collected precipitates were dissolved in 20 μL of T<sub>2</sub>El. The pattern of DNA fragmentation and smear was visualized by electrophoresis run at 80 V on a 1% (w/v) agarose gel. Gels were examined under an ultraviolet light source and photographed.

Figure 1. Physicochemical characterization of different formulations of COP bandages (1:1, 1:2 and 1:3) and void bandage (VOP) without curcumin (1:2). (a) Photograph of different formulations of COP and VOP bandages. (b) Photographs of respective bandage as observed in scanning electron micrograph. (c) In vitro water uptake ability of different formulations of VOP bandages with alginate to chitosan in different molar ratio, i.e., 1:1, 1:2 and 1:3. (d) In vitro degradation of different formulations of VOP bandages (alginate to chitosan molar ratio 1:1, 1:2 and 1:3) in PBS lysozyme solution. (e) The in vitro release kinetics of curcumin from COP bandages with alginate to chitosan 1:1, 1:2 and 1:3 molar ratio. All experiments were conducted three times.

Using the TUNEL (terminal deoxy nucleotidase transferase mediated dUTP nick end labeling) technique following the manufacturer’s instructions (TRAPeze XL, Millipore, India) and the protocol of Sidhu et al. Histology and Immunohistochemical Study of Granulation Tissue. Wounded tissues from different groups of rats were excised after the 4th and 10th days postwounding and fixed with paraformaldehyde (4% in PBS, 0.01 M, pH 7.4) for two days at 4 °C. Their cryosections were then taken and stained with hematoxylin and eosin (H&E) to assess the predominant stages of healing and Masson’s Trichrome stains to study the extent of collagen deposition in healed tissue during the course of wound healing. Similarly, immunohistochemical staining was performed using anti-alpha smooth muscle actin (α-SMA) antibody (AbCAM, Cambridge, MA, USA) with an aim to study the rate of wound contraction in different treated groups by an indirect avidin–biotin–immunoperoxidase technique (Vectastain ABC Elite, Vector Laboratories, Burlingame, CA), as specified by the manufacturer’s protocol.

Estimation of DNA, Protein, Aldehyde and Collagen. For the biochemical analysis of protein and DNA, 100 mg of wet granulation tissues (4th and 10th days postwounding) were taken and its 10% homogenate was prepared with lysis buffer. It was subsequently suspended in 5 mL of 5% trichloroacetic acid and kept at 90 °C in a water bath for 30 min. After cooling,
samples were centrifuged at 12000 rpm for 30 min in a water bath to extract the DNA and protein.\(^{18}\) The derived supernatants were used to estimate DNA by using the protocol of Burton et al.,\(^{19}\) and protein was quantified with the help of Bradford reagent (Sigma-Aldrich India). The aldehyde content was measured by a spectrophotometric method with N-methyl benzothiazolone hydrazone (MBTH).\(^{20,21}\) Further, the soluble fibrillar collagen of 4th and 10th day postwounded granulation tissue was estimated by Sircol collagen assay (Sircol Assay; Biocolor, U.K.) following the manufacturer’s instructions.

**Study of Lipid Peroxidation.** This assay was done to study the potency of COP bandage, in inhibiting cell membrane damage (due to oxidative stress) during the process of wound healing. In brief, wounded tissues of rat from different groups were collected after the 4th and 10th days postwounding and its 10% (w/v) homogenate was prepared in phosphate buffer (50 mM, pH 7.4) with a homogenizer. The crude homogenate was centrifuged at 1000 rpm for 10 min at 4 °C to pellet down nuclei and other cell debris. In the resultant supernatant, lipid peroxidation (LPx) was determined following the method of Ohkawa et al.\(^{22}\)

**Preparation of Tissue Homogenate and Western Blot Analysis.** The molecular mechanism of inhibition of inflammation at the wounded area and the mechanism involving the wound healing process following treatment with polymeric bandage was studied by Western blot analysis as per a previously standardized protocol using the following antibodies: anti-PI3K, pAKT, \(\alpha\) actin served as an internal control. The PCR products were subjected to electrophoresis on ethidium bromide stained 2% agarose gel.\(^{23}\) All samples were analyzed in triplicates.

**Statistical Analysis.** The data are presented as mean ± SEM. Statistical analysis was performed by using one-way ANOVA with the Tukey’s test applied post hoc for paired comparisons of means (SPSS 10, SPSS Inc., Chicago, IL, USA). Values of \(p < 0.05\) were indicative of significant differences and \(p < 0.005\) were indicative of a very significant difference.

### RESULTS

**Physicochemical Characterization of Polymeric Bandages.** Different formulations of COP bandages were successfully prepared by varying alginate to chitosan in different molar ratios (1:1, 1:2 and 1:3), and these were formed as a result of interaction between positively charged chitosan and negatively charged sodium alginate (Figure 1a). Cross section morphology was studied by SEM and appeared to be a porous and fibrillar structure (Figure 1b). Topology characterization revealed the polymeric bandages were soft, light and fibrous in textures with adequate flexibility, which will inevitably be required for in vivo applications.\(^4\) The water uptake study was conducted with PBS (0.01 M, pH 7.4) and degradation study was conducted in PBS (0.01 M, pH 7.4) lysozyme solution, demonstrating that all formulated bandages showed better water uptake as well as good degradation properties (Figure 1c,d). The therapeutic efficiency of drug loaded polymeric bandages primarily depends on its dose and release of the entrapped drug from its matrix at the wound site. In this view, while observing the in vitro release profile, we observed a sustained release of entrapped curcumin from different formulations, which could provide very accurate dosing, more availability of drug at the disease site, and enhancement of drug stability by protecting curcumin from hydrolytic degradation and biotransformation (Figure 1e).

**In Vivo Wound Healing Test.** COP and VOP polymeric bandage containing alginate to chitosan molar ratio 1:2 were used for wound healing test. At the initial point of observation (4th postoperative day) more healing of the wounds was observed in both COP and VOP polymeric bandage treated with the help of Sircol collagen assay (Sircol Assay; Biocolor, U.K.) following the manufacturer’s instructions.

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Figure 3. Detection of apoptosis in different treated wounded tissue (n = 3). (a) Square area is the representative location for collection of wounded tissue, which was excised from four sides of the wound for perusing various studies. (b) Agarose gel electrophoresis of genomic DNA from control (C), void (V) and COP bandage treated (T) wounded tissue at different postwounding period. (c) TUNEL assay for detection of apoptosis in wounded tissue after the 10th day postwounding. Brown color cells (arrow) are TUNEL positive, indicating apoptotic cells. COP bandage treated wounds were almost devoid of apoptotic cells, however, untreated and VOP bandage treated wounds showed a higher number of apoptotic cells. Bars length: 10 μm represents the magnification 10× (Leica QWin, Germany).

Figure 4. Histological study showing the effect of COP and VOP bandage on wound healing in rats (n = 3). (a) Histological findings of granulation tissue of control, VOP and COP bandage treated on the 4th and 10th days postwounding (hematoxylin−eosin (HE) stain). At day 4, in control, the infiltration of polymorphonuclear cells (PMN) was mild, the scab was thin, and the wound was hypocellular with lack of epidermis, whereas VOP and COP bandage treated groups showed enhanced infiltration of PMN cells. On the 10th day of wound healing, the control group showed a loose crust over the area of epithelial loss, and the VOP bandage treated wound demonstrated migration of the epithelium (E) over the dermis including granulation tissue formation but the dermis still under the process of remodeling, whereas the COP bandage treated wounds are covered completely with the well-formed and differentiated epithelium, and organization of the granulation tissue is more advanced with significant increased deposition of connective tissue between dermal epithelium (E) and muscular layer (M). Bar length: 20 μm represents the magnification 10× (Leica DM LS2, Germany). (b) Masson’s Trichrome staining for detection of collagen on control, VOP and COP bandage treated wounded tissue at the 4th and 10th days postwounding. Bar length: 25 μm represents the magnification 5X and 10X respectively. (c) Immunohistochemical staining of smooth muscle α actin to detect myofibroblasts in control, VOP and COP bandage treated wounds. Immunostaining was performed using anti smooth muscle α actin monoclonal antibody and avidin−biotin−immunoperoxidase on 4th day postwounded tissue. A greater intensity of staining was observed in the COP bandage treated wound compared to other groups. Bar length: 20 μm represents the magnification 5X (Leica QWin, Germany).
groups compared to control (Figure 2a). However, we have not noted any significant difference of wound closure in VOP and COP bandage treated groups on the same day of our observation, suggesting that irrespective of curcumin content our formulated bandage is a good absorbent, suitable substrate moreover to modulate inflammation (due to OA) for better wound healing. However, on the 8th day postwounding, it was observed that the COP bandage treated wounds showed more healing response compared to VOP bandage and cotton gauze treated wounds. While measuring the wound area, we found the wound area of the COP bandage is almost half and one-third of the void and cotton gauze treated wounds, respectively (Figure 2b). On the 10th postoperative day, we observed the control, VOP and COP bandage treated wounds contracted 70%, 80% and 94% respectively. Thus, it can be said that, although the formulated OA based bandage is a good substrate showing better healing, the antioxidant and anti-inflammatory properties of curcumin accelerate the healing ability more profoundly with time. To validate the role of COP bandage in the healing cascade, we have collected the healed tissue at different time intervals from different treated groups, Figure 3a, and conducted various experiments with a view to study the progress of wound healing as well as its associated mechanism.

In Vivo Detection of Apoptosis by Gel Electrophoresis and TUNEL Assay. In order to access apoptosis, necrosis and healthy cell population in different treated wounded tissue, agarose gel electrophoresis DNA fragmentation assay was conducted. As evident from Figure 3b, in the early postwounding period (4th day) the control tissue shows genomic DNA smear, indicating most of it was partially degraded (necrotic) and in the inflammatory phase. The appearance of DNA laddering in VOP and COP bandage treated tissue suggested that most of the tissues were in the apoptotic phase. However, this apoptosis is more prominent in the COP bandage treated case compared to VOP bandage treatment. Similarly, we have observed DNA fragmentation in control as well as void treated wound tissue on the 10th postoperative day, indicating that in both cases tissues are still present in the apoptotic stage. However, we did not observe signs of apoptosis in COP bandage treated wound at the 10th day as assessed by DNA smear and fragmentation study. To further supplement the above result, we have done the TUNEL assay to see the extent of apoptosis on the different treated groups on 10th day postwounding. This assay showed significant increased levels of apoptotic cells on controls and void treated group. Conversely, the COP bandage treated wounds were almost lacking in apoptotic cells, suggesting its increased healthy state compared to the control and void treated groups (Figure 3c).

Histology and Immunohistochemical Study of Granulation Tissue. In control specimens, the 4th day postwounded histology result showed that the infiltration of polymorphonuclear leukocytes (PMN) was mild, the scab was thin and the wound was hypocellular with lack of epidermis, whereas enhanced infiltration of PMN cells with moderate scab and lack of epidermis was clearly observed in the VOP and COP bandage treated groups. In all the cases the underlying dermis showed an ill-formed granulation tissue with sparse collagen (Figure 4a). However, on the 10th day of analysis the COP bandage treated group showed significant healing response compared to other groups (Figure 4a). The control group showed a loose crust over the area of epithelial loss with nonviable necrotic areas and inflammatory cells. Void groups demonstrated the migration of the epithelium (E) over the dermis, granulation tissue formation, and the dermis still under the process of remodeling, whereas COP bandage treated groups showed complete re-epithelialization of the wound with well-formed and differentiated epithelium, good organization of the granulation tissue and significant increased deposition of connective tissue between dermal epithelium and muscular layer (M) (Figure 4a). Further, we have studied the organization of tissues at the wounded area and the degree of collagen deposition as well as its alignment by staining the tissue section with Masson’s Trichrome stain. The result showed that COP bandage treatment resulted in greater collagen content in the wounds as compared with void and control wounds on the 4th as well as 10th postwounding days (Figure 4b). The control wounds had a loose reticular arrangement of collagen, whereas collagen was compact and well aligned in the COP bandage treated wounds. The 10th day postwounding tissue showed a loose reticular arrangement of collagen with large void space between the collagen fibers in the case of control and VOP bandage treated groups. However, collagen fibers were relatively compact, well aligned in a bundle shape in COP treated wound. The stained tissue section clearly pronounced the collagen content was greater in the COP treated group compared to the VOP bandage and control groups. Curcumin promoted wound healing activity has been well studied before. It is mostly due to the early influx of the resident dermal fibroblasts toward the wound site. This follows the expression of smooth muscle cell contractile proteins including α SMA, their modulation into myofibroblasts, and the reorganization of the extracellular matrix.13 This process eventually results in skin tissue regeneration and wound contraction.13,24 In this view, at an earlier period (4th day) of wound healing, tissues were collected from different groups and their sections were stained with anti α SMA for explaining the early infiltration of fibroblast and subsequent activation of α SMA during the healing cascade. It is apparent from Figure 4c that many of the infiltrating cells in the dermis were positive for α SMA staining, indicating that myofibroblasts were deposited early in the COP bandage treated group compared to the void and control groups.

Estimation of DNA, Protein, Aldehyde and Collagen. Wound healing is often regulated by ROS, and its high expression (by inflammatory cells) generates oxidative stress.6 This oxidative stress leads to damage in the cellular components, i.e., DNA, proteins and aldehyde in wounded tissue.25 The total DNA, protein and aldehyde content of wounded tissue in different treated groups are listed in Table 1. In all studies, we found COP bandage treatment increased the above parameter considerably compared to void treated and control groups, suggesting hyperplasia of the cell. Further, an increase in collagen content in COP bandage treated wound was confirmed by quantification of collagen by Sircol assay. The study demonstrated that COP bandage treated wound showed 3.1- and 2.1-fold more collagen on the 4th day and 1.7- and 1.2-fold more collagen on the 10th day compared to control and void treated tissue respectively (Figure 5a). This result is in accordance with the Masson’s Trichrome collagen staining studies, which also demonstrated an increased trend of collagen deposition in the COP bandage treated group compared to others (Figure 4b).

Study of Lipid Peroxidation. LPx was quantitated by monitoring the formation of TBARS in presence of 0.02% (w/v) butylated hydroxytoluene in the TBA reagents to suppress
peroxidation during the assay. Our studies on the LPx status reveal that the COP bandage showed a minimum of LPx level compared to the other groups, i.e., 0.18 and 0.11 nmol of TBARS formed/mg protein in 4th and 10th days postwounding (Figure 5b).

Study on Antioxidant Enzyme Protein and mRNA Expression. During the process of wound healing, the generated ROS play a central role for oxidative stress. However, their activity is highly regulated by antioxidative enzymes, i.e., SOD, CAT, GPx, and also by the action of various antioxidants. In the present study, we observed a substantial downregulation in the expression of antioxidative proteins and genes at translational as well as transcriptional level respectively following COP bandage treatment compared to the void and control at both 4th day as well as 10th day postwounding (Figure 6a,b).

Inhibition of PI3K/AKT/NFkB Signaling Pathway Following Treatment with COP Bandage. Most of the anti-inflammatory effects can be explained by the efficient inhibition of PI3K/AKT/NFkB signaling pathway. Our Western blot results as shown in Figure 6c demonstrated a decreased expression of PI3K and pAKT and increase in expression of IkBα in COP treated wound compared to control group (Figure 6c). The results demonstrated that COP bandage can more efficiently block the PI3K/AKT/NFkB signaling pathway in wounded area compared to control. Further, in order to substantiate the above finding, PCR amplification of NFkB was performed and the result demonstrated significant low NFkB expression at mRNA level in COP bandage treated wound with respect to control group (Figure 6d).

DISCUSSION

The rationale behind the use of OA in our COP bandage formulation is to deliver the drug topically in a sustainable way from its matrix structure at the wound site. Additionally it could modulate inflammation, the major problem associated with impaired wound healing. In this view, Pereira et al. demonstrated that topical treatment of OA showed an increase in vascular endothelial growth factor-alpha which promoted neovascularization and increased interleukin-1beta and CINC-2α/β, which facilitated the recruitment and accumulation of phagocytes in the inflamed tissue. In a similar approach Edwards et al. shown that topical application of OA in cotton wound dressing material significantly inhibited cathepsin G (a serine protease secreted by activated neutrophils that play a role in the inflammatory response), suggesting its usefulness in topical application for chronic inflammatory pathogenesis. To justify our formulated bandage’s persuasive healing efficacy, a comparative in vivo wound healing study was conducted. Our observation showed oleic acid containing VOP and COP bandage showed better wound healing compared to control. However, the added agent curcumin made COP bandage more potent compared to VOP owing to the anti-inflammatory activity of curcumin. It is anticipated that in our formulation oleic acid may afford sustained release of curcumin in solubilized form and the sustained availability of solubilized curcumin metabolites (sulfates and glucoronates) at the wound site may facilitate better healing activity. With a view to evaluate the progress of wound healing in different treated groups, we have studied the commencement of apoptosis, during the course of wound healing. It is well-known that apoptosis or programmed cell death plays a crucial role in the maturation of the wound. It is the universal pathway for the elimination of unneeded cells and inflammatory cells during the course of wound healing. In this regard, Brown et al. reported that during the course of wound healing the earlier evidence of apoptosis indicates a more rapid switch from the inflammatory phase to the proliferative phase of healing. In this view, our
DNA fragmentation assay showed that COP bandage treatment caused a greater amount of apoptosis (DNA ladder) in an earlier phase (4th day) of the wound healing compared to VOP bandage treatment, suggesting a faster progression from the inflammatory to the proliferative phase. Conversely, at the same time point low apoptosis with DNA smearing in the control case indicated a prolongation in the inflammatory phase of healing and delay in the progression to the next phase of healing. Sidhu et al. in their studies demonstrated that curcumin treatment promotes an increased level of apoptosis in earlier phases of wounds healing, with no trace of apoptosis in wounded tissue at the 11th day postwounding, whereas, in the control group, they found that the commencement of apoptosis was delayed and still observed up to the 11th day of their observation. Similar trends were also observed in our study where the 10th day apoptosis results (of both DNA fragmentation and TUNEL assay) demonstrated that COP bandage treatment showed the absence of apoptosis with well-organized granulation tissue (as observed by TUNEL assay), indicating that treated wounds have healthy texture. However, void and control wounds showed a higher number of apoptotic cells, suggesting that these tissues were still in the process of remodeling (Figure 3c).

We further studied the progress of wound healing by histological examination of H&E stained sections prepared from different treated groups. In all groups, we have observed moderate to high infiltration of PMN cells at the initial healing period (4th day postwounding). In this regard, Silva et al. and Mori et al. reported that wound exudates contained high quantity of complement proteins and interleukin-8 (produced by fibroblasts), which act as a chemoattractant for PMN cells and macrophage. However, we have observed that the extent of infiltration of PMN cells was high in both void and COP bandage treatment compared to control (Figure 4a). In this context, Uneo et al. reported that chitosan is a wound healing promoter and it accelerates the infiltration of PMN cells toward the healed site. In our study, we hypothesized that the polymeric bandage containing chitosan may efficiently activate the infiltration of the PMN cells into the wound area which formed thick fibrin compared to control (Figure 4a). This in vivo wound healing study clearly showed that the treatment of COP bandage accelerates the wound closure more efficiently compared to void and control groups. In view of the fact that, at an early wound healing period, enhanced myofibroblast differentiation can explain this acceleration of wound closure in COP bandage treatment, we stained 4th day wound tissues with an antibody against α SMA, a differentiation marker of smooth muscle cells. The results demonstrated enhanced myofibroblast differentiation (increased level of α SMA) occurred in the COP bandage treated wounds compared to VOP bandage and control groups. Furthermore, studies conducted by Sidhu et al. proved the point that native curcumin has the potential to facilitate early fibroblast accumulation in the wounded bed and subsequently produced abundant amounts of procollagen and various extracellular matrix molecules. In this regard, the extent of collagen deposition in the wounds was examined by Masson’s Trichrome staining (Figure 4b). The observed 4th and 10th day postwounding tissue treated with COP bandage showed more collagen deposition, suggesting an earlier organization of wound compared to other groups. This result is in accordance with biochemical quantification of total collagen derived from 4th and 10th day postwounding tissue treated with COP bandage. Further, the increased aldehyde content of collagen in COP bandage treated wound confirms that the collagen is highly cross-linked when compared with that of void and control. In the same context, George et al. demonstrated that newly formed collagen is always associated with an increase in aldehyde content. Besides the above reason, another cause like increased amount of DNA and proteins and inhibition of LPx also explained the advancement of healing in COP bandage.
Bandage treated wounds as compared with void treated and control wounds.

A series of recent studies have highlighted the important role of ROS in the wound healing process. On the one hand, they are required for efficient defense against invading pathogens. However, excessive production of ROS or impaired detoxification of these aggressive molecules causes oxidative stress, and this has been identified as an important feature in the pathogenesis of chronic, nonhealing wounds. Therefore, a balance regulation of ROS production and detoxification is crucial for the normal repair process. In fact, the use of antioxidants like curcumin has been a recent and effective strategy for therapeutic approaches to such disorders by inhibiting LPx. Our studies on the LPx status reveal that curcumin possesses significant antioxidant activity, which would help to prevent oxidative damage and promote the wound closure process. In fact, the use of antioxidants like curcumin has been a recent and an effective strategy for therapeutic approaches to such disorders by inhibiting LPx. Our studies on the LPx status reveal that curcumin possesses significant antioxidant activity, which would help to prevent oxidative damage and promote the wound closure process. The above finding was substantiated by work conducted by Phan et al., where curcumin acted as a powerful inhibitor against hydrogen peroxide damage in human keratinocytes and fibroblasts. Several groups have reported that curcumin exhibits its antioxidative function by activating the major antioxidative enzymes. In the present study we found that treatment with COP bandage results in a reduced expression (mRNA and protein level) of antioxidative enzymes (SOD, CAT and GPx) compared to void and control groups. This reduced expression of the above antioxidative enzyme in our study could be attributed to the ROS scavenging activity of curcumin. As curcumin has the ability to trap ROS, it quenched the major ROS and subsequently reduced the LPx during dermal wounding. This phenomenon subsequently leads to less activation of cellular antioxidative enzymes compared to void and control groups. Thus, in our study we anticipate that the implementation of the nonenzymatic antioxidative pathway by COP bandage to inactivate ROS may have resulted in reduced expression of antioxidative enzymes (Figure 6a,b).

The rationale behind the incorporation of curcumin in polymeric bandage is to target NFκB activation. NFκB, a transcription factor that regulates the expression of a number of genes involved in immune and inflammatory responses, has long been considered oxidant responsive. Generally, ROS phosphorylate PI3K/AKT and consequently activate NFκB by causing the release of the inhibitory subunit IκBα from the NFκB–IκBα complex. Several studies have documented that curcumin following the PI3K/AKT pathway inhibits the activation and translocation of NFκB into nucleus and subsequently downregulates transcription of NFκB regulated gene. A similar result is also observed in our study where a downregulation in expression of PI3K, pAKT proteins, following COP bandage treatment was observed compared to control group (Figure 6c). Further, COP bandage treated wound also exhibited a significant upregulation in expression of IκBα protein, which is a clear indication of inhibition of NFκB signaling pathway. Further, our result documents the inhibition of NFκB at the mRNA level in wounded tissue treated with COP bandage. This clearly indicates that COP bandage is more efficient to suppress the inflammation at wounded area caused by activated NFκB signaling pathway.

The proposed mechanism of PI3K/AKT/NFκB inhibition following the treatment of COP bandage in wounded tissue compared to control is depicted schematically in Scheme 1.

Scheme 1. Schematic Representation of Molecular Mechanism of Antiinflammation Induced by Curcumin Due to Downregulation of the PI3K/AKT/NFκB Pathway

"It is a well-known fact that the PI3K/AKT/NFκB pathway contributes to inflammation during wound healing. Activation of NFκB regulates inflammation and consequently delays the wound healing process. Wounds dressed with the COP bandage facilitate slow release of curcumin resulting in sustained blocking of the NFκB pathway."
Unlike control, in COP bandage treated wound, sustained release of curcumin from polymeric matrix with time exhibits successful quenching of ROS and enables reduction of the level of LPx (generated during oxidative stress) compared to void and control groups. The decreased ROS produced by curcumin polymeric bandage treatment activate NFkB pathway less efficiently compared to control.

Hence, the discussed results justified topical treatment with OA based polymeric dressing bandage loaded with curcumin for wound healing, at least in the rat model, by quenching the ROS generated through dermal wounding, as well as by inhibiting inflammation through downregulation of the NFkB pathway. However, a detailed investigation is warranted to elicit the molecular mechanism of curcumin in modulating the NFkB pathway.

**CONCLUSION**

In summary, the present study represents a complete investigation of curcumin loaded polymeric bandage and its therapeutic potential in dermal wound healing in a rat model. The observed comprehensible results demonstrated that dressings with curcumin-loaded polymeric bandage comparatively showed more healing response than control and void preparations. We hypothesize that this may be due to greater sustained intercellular curcumin retention and its subsequent enhanced anti-inflammatory effect by quenching ROS. Further studies are underway to determine the efficacy of our formulated curcumin loaded polymeric bandage in enhancing wound repair in diabetic impaired healing. We further envisioned that the curcumin loaded polymeric bandage may open new therapeutic application in clinic settings for impaired cutaneous wound healing.

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We would like to thank Dr. Sarita Jena, Scientist, and Mr. Sudhir, Institute of Life Sciences, for their generous help during wound healing animal experiments. The financial support from the core grant of Institute of Life sciences, Bhubaneswar, Odisha, India, and from TATA Innovative Fellowship from the Department of Biotechnology is gratefully acknowledged.

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