ORIGINAL ARTICLE

Wound healing activity of carbon monoxide liberated from CO-releasing molecule (CO-RM)

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Abstract Wound microenvironment presents widespread oxidant stress, inflammation, and onslaught of apoptosis. Carbon monoxide (CO) exerts pleiotropic cellular effects by modulating intracellular signaling pathways which translate into cellular protection against oxidative stress, inflammation, and apoptosis. CO-releasing molecules (CO-RMs) deliver CO in a controlled manner without altering carboxyhemoglobin levels. This study observed a potential therapeutic value of CO in the wound healing by using tricarbonyldichlororuthenium (II) dimer (CO-releasing molecule (CO-RM)-2), as one of the novel CO-releasing agent. The effect of CO-RM-2 treatment was studied on wound contraction, glucosamine, hydroxyproline levels, and mRNA of cytokines/adhesion molecule in rats using a full-thickness cutaneous wound model and angiogenesis in chick chorioallantoic membrane (CAM) model. CO-RM-2 treatment increased cellular proliferation and collagen synthesis as evidenced by the increase in wound contraction and hydroxyproline and glucosamine contents. The mRNA expression of cytokines endorsed fast healing, as was indicated by the inhibition of pro-inflammatory adhesion molecules such as ICAM-1 and cytokine TNF-α and upregulation of anti-inflammatory cytokine IL-10. An ELISA assay of IL-10 and TNF-α cytokines revealed pro-healing modulation in excision wound by CO-RM-2 treatment. CO-RM significantly promoted the angiogenesis as compared to the iCO-RM group in vitro in CAM model demonstrating pro-angiogenic effects of CO-RM-2 in wound healing process. These results indicate that CO-RM-2 may have a potential application in the management of recalcitrant/obstinate wounds wherein, active wound healing is desired. This study also opens up a new area of research for the synthesis of novel CO-releasing molecules to be used for such purposes.

Keywords Wound healing · CO-RM-2 · CO · Angiogenesis

Introduction

Wound healing is a natural restorative response to tissue injury and involves sequential influx of inflammatory cells, proliferation of stromal elements, ingrowth of blood vessels, and production of an extracellular matrix essential for efficient healing. Maximum tissue strength is achieved through the regulated remodeling and maturation of the extracellular matrix. Wound healing begins with a repair cascade, which culminates in the formation of new granulation tissue. Cutaneous wounds in addition to causing pain and discomfort and predisposing the patient to superficial and chronic infection, involve significant costs associated with the long-term treatment (Dal-Secco et al. 2003; Campos et al. 2008).

Carbon monoxide (CO), a low molecular weight gas and an infamous toxic air pollutant, is normally produced in the biological systems from degradation of heme catalyzed by heme oxygenase (HO-1 or HO-2) enzymes (Rodgers et al. 1994). HO-1 is a low molecular weight stress protein induced to invoke cellular protection in injury, inflammation, oxidative stress, etc. This protective response provided by HO-1 is ascribed to its end products namely, biliverdin and carbon monoxide (Stefan et al. 2007). The role of HO-1 in wound healing has also been shown previously (Wagener et al. 2003;

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Grochot-Przeczek et al. 2009; Ahanger et al. 2010). CO exerts pleiotropic cellular effects by modulating intracellular signaling pathways. CO elicits a cytoprotective, immunomodulator, and anti-inflammatory response by modulating cytokines and activating mitogen-activated protein kinases (Abraham and Kappas 2008). CO, when administered at 250 ppm, has shown inhibition of cytokine production, both in vivo and in vitro, in response to lipopolysaccharide insult (Otterbein et al. 2000; Moore et al. 2005). The administration of CO through inhalation tried during the systemic inflammatory response syndrome, demonstrated a protective effect but altered carboxyhemoglobin levels (Ott et al. 2005). Recently, transition metal carbonyls, CO-releasing molecules (CO-RMs), have been synthesized as novel group of substances with a unique ability to release CO in a controlled manner which is capable of modulating physiological functions, without disturbing the carboxyhemoglobin levels. These molecules have shown potential pharmacological use in conditions wherein controlled CO release is desired (Motterlini et al. 2003). In this perspective, CO delivered by tricarbonyldichlororuthenium (II) dimer (CO-RM-2) attenuated leukocytes infiltration in the kidney of burned mice (Sun et al. 2008). CO-RM-liberated CO has demonstrated inhibitory effects on myocardial ischemia–reperfusion damage (Clark et al. 2003; Guo et al. 2004); inflammatory response in LPS-stimulated macrophages (Sawle et al. 2005) and expression of CD11b in platelet-activating factor activated polymorphonuclear cells (Urquhart et al. 2007). In rat aortic and cardiac tissues, liberation of CO by CO-RM has shown vasorelaxant effects (Foresti et al. 2004; Motterlini et al. 2005).

The role of CO-RM-released CO in cutaneous excision wound healing has not been investigated so far. Therefore, in the present study, the use of CO-RM-2, one of the novel CO-releasing agents was proposed to assess the effects and potential mechanisms of CO in the modulation of wound healing in rats.

Methods

Animals

Healthy adult male Wistar rats (200–250 g) were procured from the Laboratory Animal Resource Section, Indian Veterinary Research Institute, Izatnagar (Uttar Pradesh), India. The animals were housed in polypropylene cages with free access to standard feed and water in divisional animal house for a week, as an acclimatization period. The experimental protocols involved in this study were approved by the Institutional Animal Ethics Committee and conforms to the guidelines for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication no. 85-23, revised 1996).

Drug and solutions

CO-RM-2, purchased from Sigma Chemicals, was dissolved in dimethyl sulfoxide (DMSO) and diluted with pre-autoclaved normal saline.

Wound model

The animals were anesthetized by an intraperitoneal (i.p.) injection of pentobarbitone sodium (50 mg/kg). A 2×2 cm^2 (400 mm^2) open excision-type wound was created to the depth of loose subcutaneous tissue. Animals, after recovery from anesthesia, were housed individually in properly disinfected cages and were divided in the following two groups:

Group I (Negative control): Inactive CO-RM-2 (iCO-RM-2) was prepared by dissolving CO-RM-2 in 0.75% v/v DMSO in normal saline. The solution was left for 18 h at 37°C in a 5% CO$_2$ humidified atmosphere to liberate CO. The iCO-RM-2 solution was finally bubbled with nitrogen to remove the residual CO present in the solution. iCO-RM-2 was administered i.p. at the rate of 5 mg/kg body weight to the rats once daily for 9 days.

Group II (Treatment): CO-RM-2 was prepared in 0.75% DMSO in normal saline and administered at a dose of 5 mg/kg i.p. once daily for 9 days. The dose selected for CO-RM-2 was decided on the basis of several reports citing a dose equivalent to 5 mg/kg body weight administered through i.p. route.

Wound contraction measurements

The surface area of the wound was measured by tracing its contour using a transparent paper on days 0, 2, 4, 7, and 10 post-wounding. The area (in square millimeters) within the boundaries of each tracing was determined planimetrically. The percent wound contraction was derived by using the mathematical expression as follows:

Percent wound contruction

$$= \frac{0 - \text{day wound area} - \text{unhealed wound area}}{0 - \text{day wound area}} \times 100$$

Biochemical parameters of wound healing

The rats were euthanized with an overdose of diethyl ether, and a granulation tissue from the healed wound, including 1–2 mm of normal skin surrounding it with full thickness,

was excised on day 10 after the creation of the wound. The granulation tissue was used to analyze biochemical parameters of healing, viz., hydroxyproline (Woessner 1961) and glucosamine (Rondle and Morgan 1955).

mRNA expression studies

The granulation tissue excised on day 10 after the creation of the wound was used to study the expression pattern of mRNA of various cytokines and adhesion molecule. The total RNA was extracted using the QIAGEN RNeasy Mini-extraction kit (QIAGEN Ltd., Valencia, CA, USA) as per the manufacturer's instructions. cDNA synthesis was carried out by using MMLV reverse transcriptase. The gene expression was determined using the real-time PCR technique. The published primer sequences specific for rat IL-10, TNF-α, intracellular cell adhesion molecule-1 (ICAM-1), and β-actin were employed (Table 1).

Real-time PCR reactions were conducted on a Stratagene Q-Cycler and analyzed using the Mx3000P software with SYBR Green as the reference dye. To ascertain the specificity of the amplified product, dissociation curves were generated at temperatures of 55 through 95°C. The results were expressed in terms of threshold cycle values (C_T). To study the relative change in gene expression, the $2^{-\Delta\Delta C_T}$ method was used (Livak and Schmitten 2001). This method enabled us to calculate the fold change in gene expression as "Fold change" $= 2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T = (C_T$ of target gene $- C_T$ of β-actin) treatment $- (C_T$ of target gene $- C_T$ of β-actin) control. β-Actin was used as a housekeeping gene required for the relative quantification of the mRNA of other genes.

ELISA assay for TNF-α and IL-10

The granulation tissue (50 mg) already stored at −80°C was pulverized in ice-cold lysis buffer containing 100 mM Tris–HCl, and 0.05 mM EDTA (500 μl of lysis buffer/50 mg tissue) with the help of chilled pestle and mortar and a pinch of glass wool. After the tissues were thoroughly pulverized into a homogenous mixture, they were transferred to 1.5-ml microcentrifuge tubes and centrifuged for about 10 min at 10,000 rpm, and a supernatant protein lysate was collected for the further protocols envisaged in the experiment. The enzyme-linked immunosorbent assay (ELISA) protocol was followed as per the manufacturer's instructions (eBioscience Inc. catalog nos. 88-7340-22-TNF-α and 88-7104-22-IL-10). The total protein content of the lysate was estimated as per the method of Lowry et al. (1951).

In vitro chick chorioallantoic membrane model to assess angiogenic activity

The chorioallantoic membrane (CAM) model was used to assess angiogenic activity of the treatments as described by Lobb et al. (1985). Nine-day-old embryonated chick eggs were screened by candling for the presence of live fully grown embryos. A small window of 10-mm^2 area was created in the shell, and the site was cleaned of shreds of shell membrane so that the chorioallantoic membrane is visible under the camera mounted on a microscope. A sterile methyl cellulose disk 10 mm in diameter loaded with CO-RM-2 and iCO-RM-2 (10–15 μg) was placed at the junction of large blood vessels. The window was sealed with a transparent tape, and the eggs were again incubated at 37°C in a well-humidified chamber for 72 h. Finally, the window was opened, and new blood vessel formation was observed and compared with the eggs containing inactive CO-RM impregnated disks. The blood vessel density was assessed by analyzing photographic images of CAM for morphometric analysis in terms of the number of red pixels per unit area of the photograph using the National Institute of Health Image J software (v1.38) and AngioQuant software (Niemisto et al. 2005).

Histological studies

Skin (granulation tissue) already preserved in 10% formalin was subjected to sectioning and 6-μm thickness sections were stained with hematoxylin and eosin. The stained slides

Table 1 Description of primers used

Sample no.	Gene	Primer sequences	Annealing temperature
1.	β-actin	F 5′-AGTGTGACGTTGACATCCGT-3′ R 5′-GACTCATCGTACTCCTGCTT-3′	52°C
2.	ICAM-1	F 5′-AGGTATCCATCCATCCCACA-3′ R 5′-GCCACAGTTCTCAAAGCACA-3′	50°C
3.	TNF-α	F 5′-ATGAGCACAGAAAGCATGATCC-3′ R 5′-GAAGATGATCTGAGTGTG-3′	50°C
4.	IL-10	F 5′-CCTGCTCTTACTGGCTGGAC-3′ R 5′-TGTCCAGCTGGTCCTTCTTT-3′	52°C

were visualized for histological changes under a light microscope.

Statistical analysis

Results are expressed as mean±SEM, with n equal to the number of replicates. The statistical significance between the experimental and control values was analyzed by applying an unpaired t test using the GraphPad Prism v4.03 software program (San Diego, CA, USA), and the differences between the experimental and control groups were considered statistically significant at $P<0.05$.

Results

CO-RM-2-enhanced wound contraction

The CO-RM-2-treated wounds contracted significantly faster than iCO-RM-treated group in this study. As is evident from Fig. 1, the mean percent wound contraction of the CO-RM-2-treated group (15.87±0.82, $n=6$) differed significantly from the iCO-RM group (5.45±1.21) on day 2, and this significant difference continued till the last observation (tenth day after wound formation), 85.73.40±1.49 (CO-RM-2) versus 75.73±1.88 (iCO-RM, $n=6$; $P<0.01$).

CO-RM-2 increased/upregulated pro-healing biochemical parameters

The CO-RM-2 treatment significantly increased the hydroxyproline content (15.80±0.34 mg/g tissue, $n=5$), as compared to the iCO-RM group (9.75±0.56 mg/g tissue, $n=5$) which too was significantly higher than the iCO-RM group (Fig. 2). The glucosamine content showed a similar trend as that of hydroxyproline, i.e., CO-RM-2 group revealed a significantly higher content of glucosamine (6.96±0.56 mg/g tissue, $n=5$) than the control group (4.78±0.45 mg/g tissue; Fig. 3).

Pro-healing modulation of CO-RM-2 on pro-inflammatory adhesion molecule (ICAM-1)

As shown in Fig. 4, the mRNA level of ICAM-1 in the CO-RM-2-treated group (0.390±0.007; $n=3$) was significantly lower than the iCO-RM group.

Pro-healing modulation of CO-RM-2 on pro-inflammatory TNF-α cytokine

The effect of CO-RM-2 on the mRNA expression of the pro-inflammatory cytokine TNF-α is presented in Fig. 5a. The CO-RM-2 treatment significantly reduced the expression of the mRNA level of TNF-α than the iCO-RM group. The ELISA assay revealed a significant reduction in the inflammatory cytokine TNF-α, in the granulation tissue of the CO-RM-treated group (54.65±4.79 pg/mg protein), as compared to the iCO-RM group (145.14±2.25 pg/mg of protein; Fig. 5b).

Pro-healing modulation of CO-RM-2 on anti-inflammatory IL-10 cytokine

The expression of mRNA of IL-10 in the CO-RM-2-treated group was significantly higher (6.48±0.024) than the iCO-RM group (Fig. 6a). The ELISA assay of IL-10, an anti-inflammatory cytokine, revealed significantly higher levels (1,599.3±95.41 pg/mg protein) in the CO-RM-treated group, as compared to the iCO-RM group (872.04± 35.86 pg/mg of protein; Fig. 6b).

Effect of CO-RM on the angiogenesis assayed by in vitro chick chorioallantoic membrane model

The pro-angiogenic effects of the treatments demonstrated by the CAM model are presented in Fig. 7a, b. The CO-RM significantly promoted the angiogenesis as compared to the
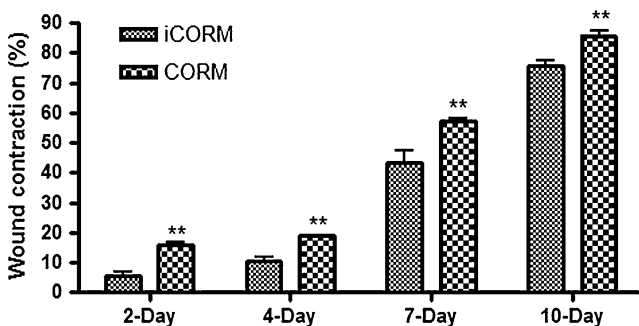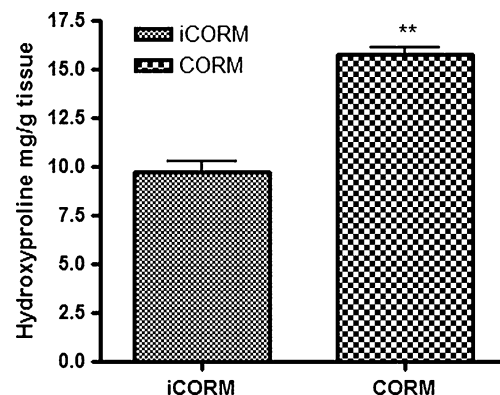
Fig. 1 Effect of CO-RM-2 (5 mg/kg, i.p. once daily for 9 days) on the percent wound contraction recorded on different days after wound creation. Values are depicted as mean±SEM, $n=6$. **$P<0.01$, as compared with control

Fig. 2 Hydroxyproline content (mg/g of tissue weight) of the granulation tissue excised on day 10 after the creation of wound. Values are depicted as mean±SEM, $n=5$. **$P<0.01$ as compared with control
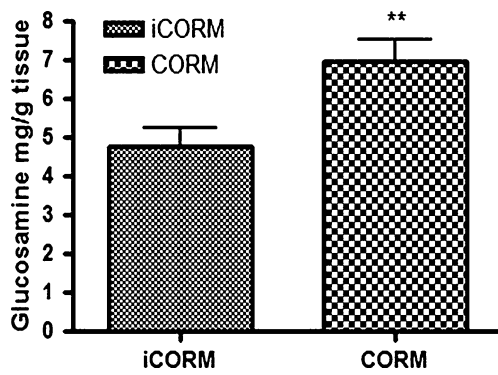
Fig. 3 Glucosamine content (mg/g of tissue weight) of the granulation tissue excised on day 10 after the creation of wound. Values are depicted as mean±SEM, $n=5$. **$P<0.01$, as compared with control

iCO-RM group. The maximum pro-angiogenic effects as demonstrated by the maximum number of red pixels ($3,431.6\pm165.2$) as against $1,563.5\pm76.7$ per unit area in the iCO-RM group are shown in Fig. 7. Figure 7b represents the photographs of chick chorioallantoic membrane taken pre- and post-drug impregnation.

Histological findings

The histological sections of the healed skin showed that tissue regeneration is much greater in the CO-RM-2-treated group. Evidently, the granulation tissue of the CO-RM-2 group (Fig. 8b) was compact and mature with less edema, inflammatory cell infiltration, and necrosis. The CO-RM-2 treatment also revealed enhanced epithelialization, fibroplasia, and neovascularization (Fig. 8b). Wound sections of the iCO-RM (Fig. 8a) group revealed loose granulation tissues with necrotic patches and sparse blood capillary formation.
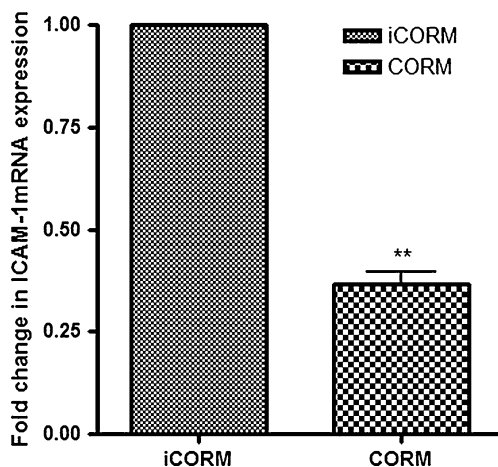

Fig. 4 Effect of CO-RM-2 (5 mg/kg, i.p. once daily for 9 days) on ICAM-1 mRNA expression in the granulation tissue excised on day 10 after the wound creation. Values are depicted as mean±SEM, $n=3$. **$P<0.01$, as compared with the control

Discussion

Wound healing involves a complex and well-orchestrated interaction of cells, extracellular matrix, and cytokines. The sensitive balance between the stimulating and inhibitory mediators of the wound repair process is crucial to achieve an early and fast healing following injury. The fibroblasts are responsible for the synthesis, deposition, and remodeling of the extracellular matrix. After migrating into the wounds, the fibroblasts initiate the synthesis of the extracellular matrix. Collagen is a major protein of the extracellular matrix and contributes to the wound strength (Singer and Clark 1999). The production of CO as a result of the increased activity of HO could be a novel defense mechanism of the cells beset with inflammation and other related stress factors present in the microenvironment of a wound, which may stimulate the process of wound healing (Abraham and Kappas 2008). The increased rate of wound contraction in the CO-RM-2-treated group might be attributed to the anti-apoptotic, anti-inflammatory, and cytoprotective action of CO which results in conditions conducive for proliferation, transformation of fibroblasts into myofibroblasts, and reepithelialization (Brouard et al. 2000; Zhang et al. 2003). CO potentially attenuates reactive oxygen species and reactive nitrogen species which are invariably present in the microenvironment of wounds; therefore, the presence of CO at such places may synergize the healing process (Cepinkas et al. 2008). The early reepithelialization and faster wound closure in the CO-RM-2-treated group might be ascribed to increased keratinocyte proliferation and their migration to the wound surface (Moulin et al. 2000). The observed enhanced levels of hydroxyproline, as an index of collagen deposition and glucosamine, a major building block of proteoglycans, in CO-RM-2-treated rats might be attributed to the protective and proliferative effects of CO on fibroblasts.

Various studies have demonstrated the role of exogenous CO in modulation of inflammation (Goebel et al. 2008). Recently, the transition metal carbonyl has been identified as a CO-RM with potential to facilitate the pharmaceutical use of CO by delivering it to the tissue and organs (Motterlini et al. 2003) without altering carboxyhemoglobin levels (Motterlini et al. 2003). The vasoactive, antihypertensive, cardioprotective, and anti-rejection effects of CO-RM have been attributed to CO released by CO-RM (Guo et al. 2004; Motterlini et al. 2005). CO-RM-released CO has been shown to produce anti-inflammatory effects both in vitro and in vivo, and anti-inflammatory action is beneficial for the resolution of acute inflammation (Otterbein et al. 2003). To determine the effect of CO-RM-2 on the inflammatory process, we analyzed the expression of mRNA of pro-inflammatory adhesion molecules, ICAM-1 and TNF-α cytokine and anti-inflammatory cytokine, IL-10.

Fig. 5 Effect of CO-RM-2 (5 mg/kg, i.p. once daily for 9 days) on TNF-α mRNA expression (a) and TNF-α cytokine (b) in the granulation tissue excised on day 10 after the wound creation. Values are depicted as mean±SEM, $n=3$. **$P<0.01$ as compared with the control
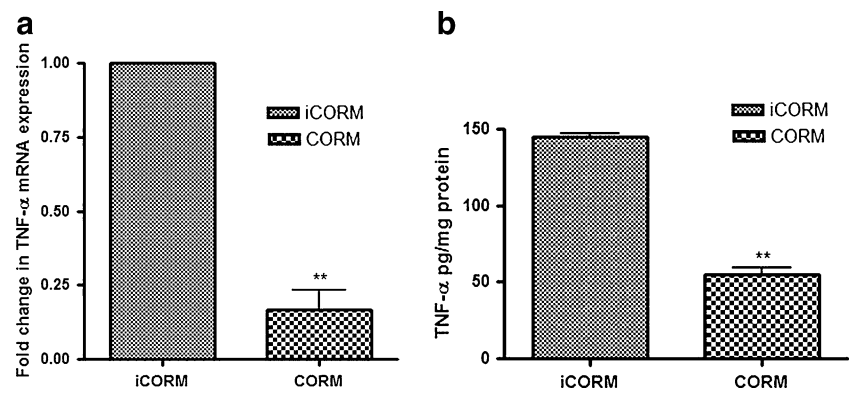
Fast and early healing process involves a balanced expression of pro-inflammatory and anti-inflammatory cytokines. In this study, the CO-RM-2-treated group showed markedly low levels of pro-inflammatory ICAM-1 adhesion molecule and TNF-α cytokine and high levels of anti-inflammatory IL-10 cytokine mRNA as compared to the iCO-RM group.

The adhesion molecule ICAM-1 mediates leukocyte adhesion and correlates with the infiltration of leukocytes into inflammatory lesions (Defazio et al. 2000; Rahman et al. 2002). ICAM-1 and other adhesion molecule expressions are the initial markers of inflammatory reactions and are involved in the acute inflammatory reactions following injury (Mileski et al. 2003). The present results showed that the expression levels of ICAM-1 in the granulation tissue of the iCO-RM group were higher and markedly inhibited by CO-RM treatment. Though we do not have data to support that CO-RM-2 inhibits the expression of ICAM-1 mRNA in excision wounds in rats, in related studies: (a) CO-RM-2 in thermally injured mice significantly reduced the expression of the ICAM-1 protein in renal tissues suggesting that the CO-RM-2 attenuates the leukocyte infiltration in skin wound (Sun et al. 2008) and (b) CO-RM-2 treatment prevented ICAM-1 expression in hepatic ischemia reperfusion injury in rats (Wei et al. 2010).

CO has been proved to be an important mediator that exerts a potent anti-inflammatory effect at low concentrations. In the murine model, CO preconditioning had

resulted in the reduced production of serum TNF-α, IL-1β, and IL-6 (Morse et al. 2003). Additionally, CO confers tissue protection by modulating intracellular signaling pathways to account for its anti-inflammatory, anti-apoptotic and antioxidant effects (Kim et al. 2006; Ryter et al. 2006). The entire quantum of cellular mRNA may not get translated into the target protein owing to the short half-life of the mRNA within the cell, particularly under the hostile conditions of a wound. To ascertain the actual quantity of the protein translated from the expressed mRNA, an ELISA assay was conducted for two important cytokines which regulate the level of inflammation within the cells. The anti-inflammatory cytokine IL-10 was markedly increased, and in contrast, pro-inflammatory cytokine TNF-α was significantly decreased suggesting a pro-healing trend in response to CO-RM-2. The loss of TNF-α in TNF-α null mice promotes granulation tissue formation and retards reepithelialization in healing of mouse cutaneous wound (Shinozaki et al. 2009). TNF-α regulates matrix metallopeptidase-2 (MMP-2) activation in human skin and inflammation and is found during matrix remodeling in many clinical situations including wound healing and cancer metastasis. High levels of TNF-α are found in chronic wounds and explains the destructive role of excessive inflammation in tissue. Since high levels of TNF-α are found in chronic wounds, TNF-α activation of pro-MMP suggests a mechanism for the destructive role of

Fig. 6 Effect of CO-RM-2 (5 mg/kg, i.p. once daily for 9 days) on IL-10 mRNA expression (a) and on IL-10 cytokine (b) in the granulation tissue excised on day 10 after the wound creation. Values are depicted as mean±SEM, $n=3$. **$P<0.01$ as compared with the control
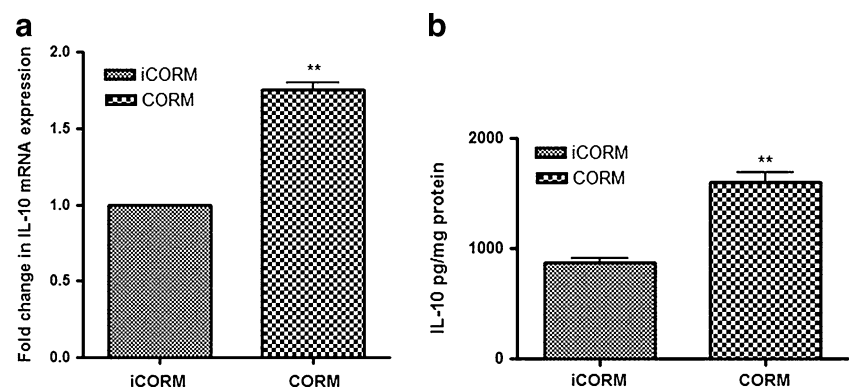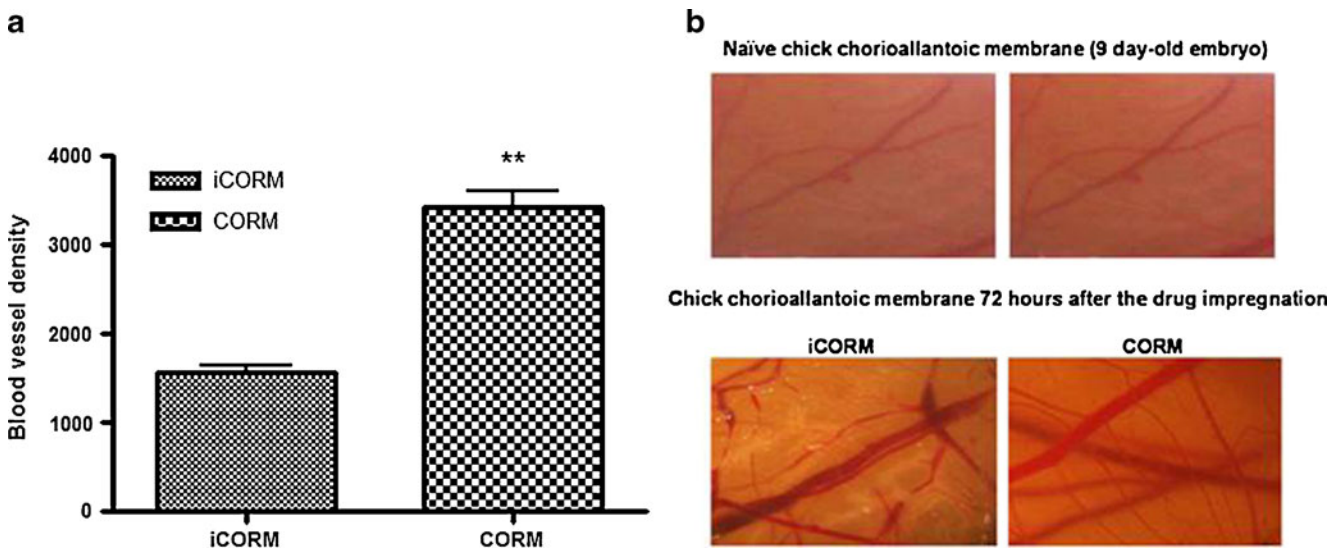
Fig. 7 Density of blood vessels per unit area assessed by in vitro chick CAM model (**a**) and photograph of chick chorioallantoic membrane before and after the impregnation with drugs (**b**). Values are depicted as mean±SEM, $n=6$. **$P<0.01$ as compared with control

excessive inflammation in tissues (Han et al. 2001). In this study, the CO-RM-2 treatment which caused a significant reduction of TNF-α mRNA expression may explain the reduced TNF-α protein expression favoring early wound closure and tissue regeneration. In addition to pro-inflammatory cytokines, anti-inflammatory cytokine IL-10 is thought to play a major role in the limitation and termination of inflammatory responses. In addition, it regulates growth and/or differentiation of various immune cells including keratinocytes and endothelial cells (Moore et al. 2001). IL-10 is an immunomodulatory cytokine with potent anti-inflammatory effects suppressing the most facets of innate and T cell-mediated inflammation. In a variety of cell types, including macrophages, neutrophils, etc., IL-10 blocks the pro-inflammatory functions and mediates downregulation of pro-inflammatory mediators (Moore et al. 2001). IL-10 decreases inflammatory response to injury and helps in the restoration of normal dermal

architecture (Peranteau et al. 2008). In the present study, CO-RM-2 treatment resulted in higher levels of IL-10 mRNA expression which may be associated with the reduced levels of pro-inflammatory cytokines and growth factor ICAM-1 favoring a shift to regenerative adult wound healing.

Neovascularization in the wounds is central to healing and involves the growth of new capillary blood vessels. This process is clinically manifested as "granulation tissue" (Rees et al. 1999; Li et al. 2001). Immediately following injury, angiogenesis is initiated by multiple molecular signals, including hemostatic factors, inflammation, cytokines, growth factors, and cell matrix interactions. Growth factors are critical mediators of wound neovascularization, expressed during healing in a temporal and orchestral manner. The growth factors refer to a broad family of proteins that promote cell proliferation and migration. At least 20 growth factors that stimulate angiogenesis have
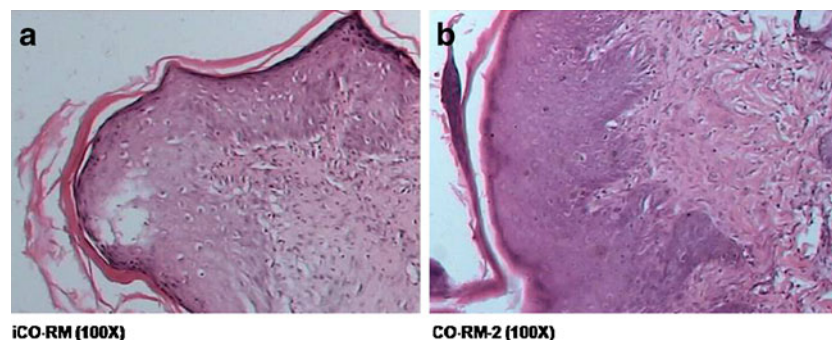

Fig. 8 Hematoxylin and eosin-stained tissues of iCO-RM-treated group (**a**) revealed loose granulation tissue with necrotic patches and sparse blood capillary formation and CO-RM-2-treated group under ×100 magnification (**b**) which was compact and mature with less edema, inflammatory cell infiltration, necrosis, and enhanced epithelialization, fibroplasia, and neovascularization, respectively

been identified and sequenced, and their genes have been cloned. Among these are platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), and transforming growth factors (TGFs; Li et al. 2003). One day after wounding, PDGF is detected on vascular endothelial cells of injured skin, where its presence is minimal in normal intact skin (Hanahan and Folkman 1996). CO decreases platelet-derived growth factor-stimulated vascular smooth muscle cell migration via the inhibition of Nox1 enzymatic activity, which reveals a novel mechanism by which CO may mediate its beneficial effects in arterial inflammation and injury (Rodriguez et al. 2010). Between 3 and 7 days after wounding, VEGF expression peaks coincide with the clinical appearance of granulation tissue (Asahara et al. 1999). At day 5, FGF is expressed at its peak level, which by day 7 returns to the baseline level (Carmeliet and Luttun 2001). CO treatment to myofibroblasts increased FGF15 expression which enhanced colonic epithelial cell restitution (Uchiyama et al. 2010). The most salient feature of CO-mediated cytoprotection is the suppression of inflammation and cell death. CO results in the regulated expression of TGF-β, a potent anti-inflammatory cytokine. CO-induced hypoxia-inducible factor 1α and TGF-β expression are necessary to prevent anoxia/reoxygenation-induced apoptosis in macrophage (Chin et al. 2007). Cross talk too takes place between various signal transduction pathways used by growth factors (Amano et al. 2002).

The CAM assay is the most widely used assay for screening angiogenesis in vivo. It allows for studying the mechanism of action of several angiogenic factors and inhibitors. It allows a quantitative analysis of new blood vessel formation, and it is amiable to both intravascular and topical administration. The valuable features of the CAM assay are the relative ease of carrying out the assay, the ready availability of the experimental materials which are inexpensive and suitable for large screening, and also the reliability of the procedure (Auerbach et al. 2003; Staton et al. 2004; Favia et al. 2008).

Angiogenesis during wound repair serves the dual function of providing the nutrients required by the healing tissue and contributes to the structural repair through the formation of granulation tissue (Martin et al. 2003). VEGF improves angiogenesis during wound healing by stimulating the migration of endothelial cells through the extracellular matrix (Ferrara 1999). The application of by-products of HO-1, i.e., CO and/or bilirubin, has also been demonstrated to upregulate VEGF in endothelial cells (Jozkowicz et al. 2003). In the present study, the CO-RM treatment group revealed significantly increased angiogenesis as is evident from the CAM model findings. Additionally, a histological evaluation of granulation tissue from the treated group of rats also indicated enhanced blood vessel

density as compared to the iCO-RM group of rats. Moreover, a pro-angiogenic effect of HO-1/CO has already been shown in different models of angiogenesis (Dulak et al. 2008; Loboda et al. 2008).

Conclusions

In conclusion, the treatment with CO-RM-2 showed a potent pro-healing activity which may be attributed to the cytoprotective, immunomodulatory, and anti-inflammatory effects of CO, invoked by modulating cytokines thereby, favoring an early and fast healing process.

This conclusion is based on several evidences. (a) CO enhances the wound contraction markedly better than the iCO-RM group in the excision wound, (b) CO augments the hydroxyproline and glucosamine contents of granulation tissue and (c) the mRNA expression of pro-inflammatory adhesion molecule (ICAM-1) and cytokine TNF-α and anti-inflammatory cytokine (IL-10) is modulated to favor early healing, (d) CO-RM-2 significantly promoted the angiogenesis as compared to the iCO-RM group in vitro chick CAM model demonstrated pro-angiogenic effects of CO-RM-2 in the wound healing process.

CO as a promoter of wound healing provides two advantages. Firstly, it offers the precise correlation between CO-RM-2 treatment and wound healing parameters. Secondly, the discovery of CO-RM-2 as promoter of wound healing permits the use of other CO-releasing molecules in the management of wound healing. This has obvious clinical applications in the management of immunocompromised/obstinate wounds.

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