

# Hyperbaric Oxygen Reduces Matrix Metalloproteinases in Ischemic Wounds through a Redox-Dependent Mechanism

Qixu Zhang<sup>1,2</sup> and Lisa J. Gould<sup>2,3</sup>

Little is known about the impact of hyperbaric oxygen treatment (HBOT) on matrix metalloproteinase (MMP) production in pre-existing high-oxidant wounds. This study aimed to investigate whether HBOT modulates reactive oxygen species (ROS) and MMP regulation in ischemic wound tissue. Using a validated ischemic wound model, Sprague–Dawley rats were divided into four groups for daily treatment: HBOT, *N*-acetylcysteine (NAC), HBO and NAC, and control (normoxia at sea level). High levels of inducible nitric oxide synthase (iNOS), gp91-phox, and 3-nitrotyrosine were detected in ischemic wounds, indicating high-oxidant stress. HBOT not only increased antioxidant enzyme expression, such as Cu/Zn-superoxide dismutase, catalase, and glutathione peroxidase, but also significantly decreased pro-oxidant enzyme levels, such as iNOS and gp91-phox, thereby decreasing net oxygen radical production by means of negative feedback. This effect was blocked by NAC treatment in ischemic wounds. HBO-treated ischemic wounds also manifested reduced phosphorylation of extracellular signal-regulated kinases 1/2, c-Jun N-terminal kinase, and c-Jun, indicating downregulation of mitogen-activated protein kinases (MAPKs). Furthermore, HBOT decreased the expression of several MMPs while simultaneously increasing tissue inhibitor of MMP (tissue inhibitor of metalloproteinase 2). These results indicate that HBOT acts via the ROS/MAPK/MMP signaling axis to reduce tissue degeneration and improve ischemic wound healing.

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## INTRODUCTION

Despite multiple inciting etiologies, the final common pathobiology of chronic wounds includes oxidative stress caused by high levels of reactive oxygen and nitrogen species (ROS/RNS; Abd-El-Aleem *et al.*, 2000; James *et al.*, 2003; Wlaschek and Scharffetter-Kochanek, 2005; Chen and Rogers, 2007) and excessive matrix degradation caused by upregulation of proteolytic enzymes, especially matrix metalloproteinases (MMPs; Wysocki *et al.*, 1993, Weckroth *et al.*, 1996; Mirastschijski *et al.*, 2002; Gill and Parks, 2008; Rayment *et al.*, 2008; Moor *et al.*, 2009). Although ROS have been

shown to be critical for MMP regulation in several cell lines (Eberhardt *et al.*, 2000; Chakraborti *et al.*, 2003; Turchi *et al.*, 2003; Nelson and Melendez, 2004; Woo *et al.*, 2004; Nelson *et al.*, 2006; Spallarossa, *et al.* 2006), the molecular link between ROS and MMP has not been investigated as a cause of delayed healing in ischemic cutaneous wounds.

Hyperbaric oxygen treatment (HBOT) is an effective adjunct for wound healing that has both systemic and local effects (Kessler *et al.*, 2003). At the tissue level, HBOT modulates cytokine release, accelerates microbial oxidative killing, reduces apoptosis, and modulates leukocyte activation and adhesion (Jon, 2000; Thackham *et al.*, 2007; Zhang *et al.*, 2008; Sen, 2009). Systemically, hyperbaric oxygen (HBO) increases bone marrow-derived endothelial progenitor cell mobilization. Although direct evidence of homing to the wound is lacking, there may be a correlation between increased circulating EPC and enhanced wound healing in diabetic patients (Goldstein *et al.*, 2006; Thom *et al.*, 2006; Gallagher *et al.*, 2007, Milovanova *et al.*, 2009). It is understood that ROS signaling is fundamental to HBOT (Thom, 2009) but the exact manner in which HBOT modulates ROS signaling pathways in the wound and protects ischemic tissue from oxidative stress remains unclear. Although upregulation of antioxidant enzyme activity by HBO preconditioning has an important role in the generation of tolerance against brain and heart ischemia-reperfusion injury (Kim *et al.*, 2001;

<sup>1</sup>Plastic Surgery Department, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA; <sup>2</sup>Plastic Surgery Division, The University of Texas Medical Branch, Galveston, Texas, USA and <sup>3</sup>Department of Molecular Pharmacology and Physiology, University of South Florida Morsani College of Medicine, Tampa, Florida, USA

Correspondence: Qixu Luke Zhang, Plastic Surgery Department, the University of Texas MD Anderson Cancer Center, 8010 El Rio Street, Unit 602, Houston, TX 77054, USA. E-mail: lukeqixu@yahoo.com

Abbreviations: ERK, extracellular signal-regulated kinase; GPx, glutathione peroxidase; HBO, hyperbaric oxygen; HBOT, hyperbaric oxygen treatment; H/N, HBO and NAC; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NAC, *N*-acetylcysteine; 3-NT, 3-nitrotyrosine; ROS, reactive oxygen species; RNS, reactive nitrogen species; SA, superoxide anion; TIMP, tissue inhibitor of metalloproteinase

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Zhang *et al.*, 2005; Li *et al.*, 2008), a clear understanding of HBOT's cellular and molecular mechanisms in cutaneous wound healing is lacking. HBOT-induced MMP9 is known to be an important mediator for endothelial progenitor cell migration at the systemic level (Liu and Velazquez, 2008). However, because high levels of proteolytic enzymes inhibit wound healing and many topical wound-healing modalities are designed to reduce MMPs, it will be beneficial to know how HBOT affects MMP expression at the tissue level.

In this study, we investigate the hypothesis that HBOT modulates the ROS/mitogen-activated protein kinase (MAPK)/MMP pathway to improve ischemic wound healing. Using our validated ischemic wound model, we compare the expression of antioxidant enzymes, MMPs, the MMP inhibitor TIMP2 (tissue inhibitor of metalloproteinase 2) and MAPKs in the wound bed of rats exposed to HBOT, *N*-acetylcysteine (NAC), or normoxia at sea level. Treatment with the potent ROS scavenger, NAC with and without HBOT was used to tease out the role of HBO-induced ROS.

**RESULTS**

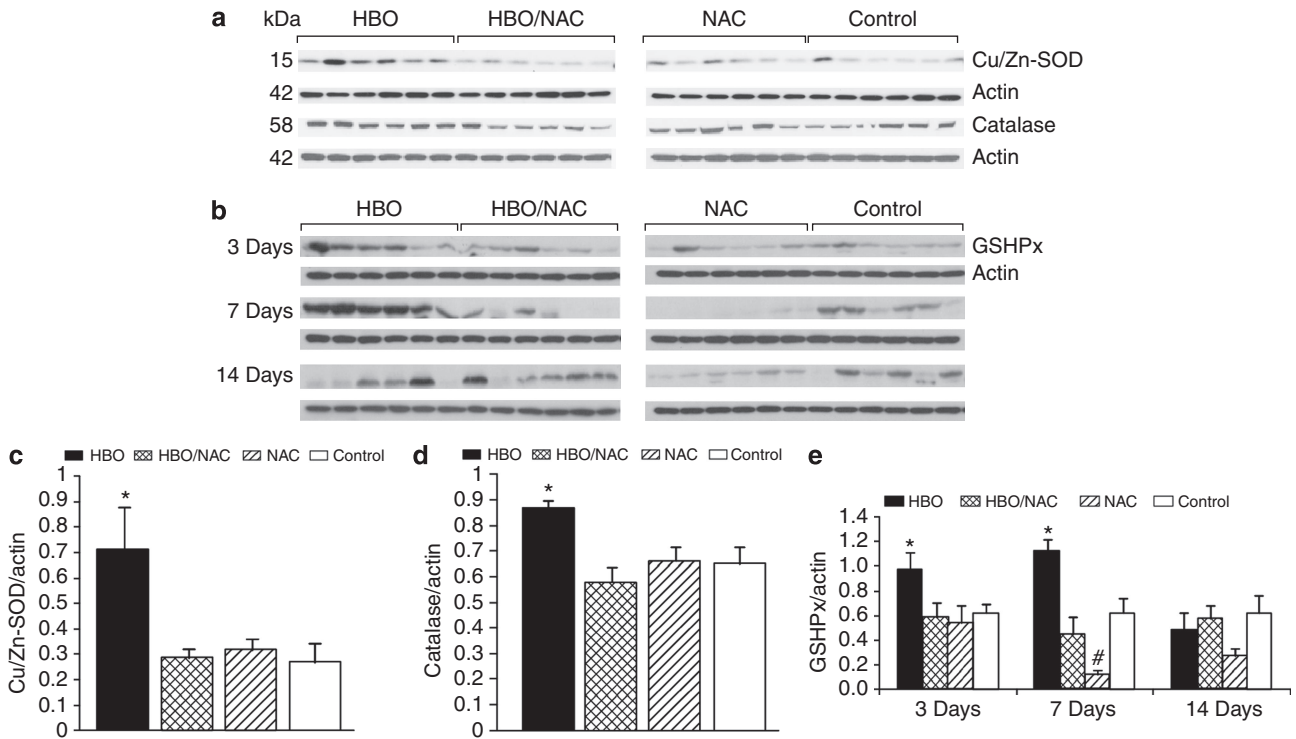
**HBOT-induced antioxidant enzyme expression in ischemic wound tissue**

Superoxide dismutase (SOD) eliminates cytotoxic ROS by catalyzing the dismutation of superoxide anion (SA) to oxygen and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). All three isoforms of SOD in

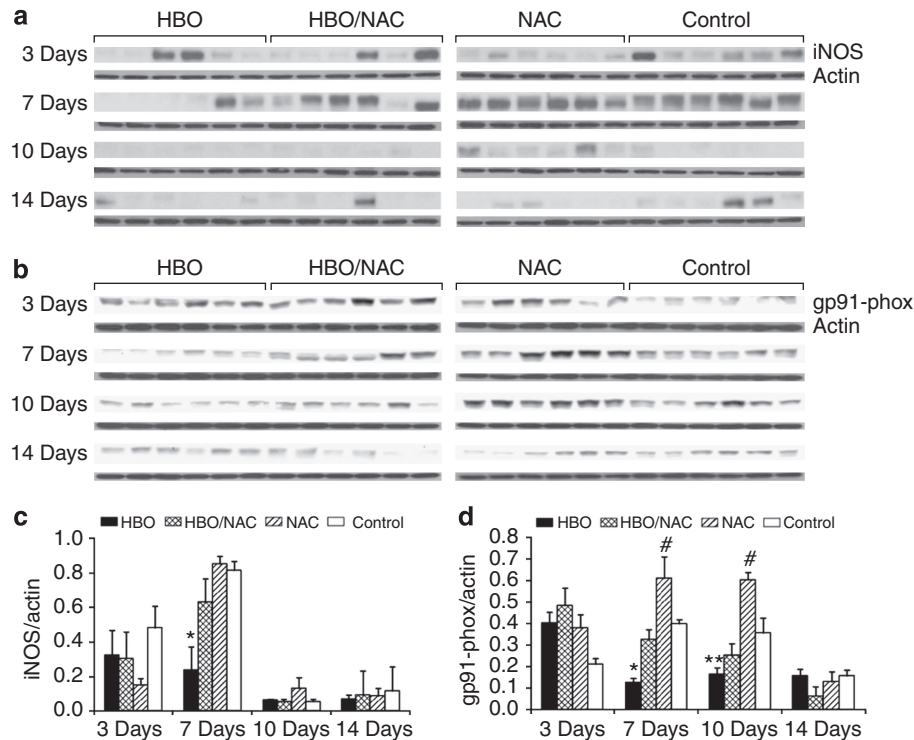
wound tissue were examined by western blot. HBOT significantly increased Cu/Zn-SOD at day 7, compared with the other three groups (Figure 1a and c). In contrast, manganese superoxide dismutase and extracellular superoxide dismutase expression was not significantly different in ischemic wounds (Supplementary Figure S1 online). In non-ischemic wounds, all three SOD isoenzymes were seen at high levels with no difference between groups from days 3 to 14 (Supplementary Figure S2 online). Catalase and glutathione peroxidase (GPx) catalyze the decomposition of H<sub>2</sub>O<sub>2</sub> to water and oxygen. Catalase and GPx levels were the same in all four non-ischemic wound groups at all time points (Supplementary Figure S2 online). In ischemic wound tissue, HBOT significantly increased catalase (day 7) and GPx (days 3 and 7) compared with the other three groups (Figure 1).

**HBOT reduced ROS and RNS synthase in ischemic wound tissue**

Expression of inducible nitric oxide synthase (iNOS) peaked at day 7 in the ischemic wound tissue of the HBO and NAC (H/N), NAC, and control groups (Figure 2a and c). iNOS expression in the wound bed was significantly decreased by HBOT. Conversely, endothelial NOS expression was at constitutive levels from days 7 to 14 in all four groups, with no significant difference between groups (Supplementary Figure S1 online). Thus, HBOT primarily decreased iNOS expression, and did not alter endothelial NOS function. Compared with



**Figure 1. Hyperbaric oxygen treatment (HBOT) induces antioxidant enzyme expression.** (a, b) Representative results of western blot analysis of Cu/Zn-superoxide dismutase (SOD) and catalase at day 7, GSHPx in ischemic wound tissue of all samples in each group from days 3 to 14. (c) Quantification with densitometry relative to actin shows that HBOT significantly increased Cu/Zn-SOD (\**P*<0.05 hyperbaric oxygen (HBO) vs. HBO and *N*-acetylcysteine (NAC) (H/N), NAC, and control) and (d) catalase (\**P*<0.05 HBO vs. H/N) at day 7, and (e) increased GSHPx at days 3 and 7 in ischemic wound tissue. \**P*<0.05 HBO vs. H/N, control, and NAC; #*P*<0.05 NAC vs. HBO and control.



**Figure 2. Hyperbaric oxygen treatment (HBOT) reduces reactive oxygen species (ROS) and reactive nitrogen species (RNS) synthase.** (a, b) Representative results of western blot analysis of inducible nitric oxide synthase (iNOS) and gp91-phox in ischemic wound tissue of all samples in each group from days 3 to 14. (c) Densitometry analysis shows hyperbaric oxygen (HBO) significantly decreased iNOS at day 7, and (d) gp91-phox at days 7 and 10, relative to the other treatments. \* $P < 0.05$  HBO vs. H/N, N-acetylcysteine (NAC), and control; \*\* $P < 0.05$  HBO vs. NAC and control; # $P < 0.05$  NAC vs. HBO and NAC (H/N), HBO, and control.

ischemic wounds, both iNOS and endothelial NOS levels were much lower in the non-ischemic wound tissue, with no difference between the four groups (Supplementary Figure S3 online). The protein gp91-phox (Nox2) is an important nicotinamide adenine dinucleotide phosphate oxidase latent in phagocytes that is activated during the respiratory burst. gp91-phox was detected in all ischemic wound tissue but was decreased markedly by HBOT and increased by NAC (Figure 2b and d). Like NOS, gp91-phox was not significantly different between the non-ischemic wound groups (Supplementary Figure S3 online).

#### HBOT decreased oxidative stress damage in ischemic wound tissue

Induction of iNOS results in high levels of NO that rapidly reacts with SA, leading to peroxynitrite formation and cell toxicity (Morris and Billiar, 1994; Horton, 2003). The compound 3-nitrotyrosine (3-NT) is a well-known marker of peroxynitrite damage (Viera *et al.*, 1999; Wilt *et al.*, 2000). High levels of 3-NT were detected in most cell types of the epidermis and dermis in ischemic wound tissue in the H/N, NAC, and control groups. HBOT significantly decreased 3-NT from days 3 to 14 whereas 3-NT was increased in NAC-treated wounds at days 7 and 10 (Figure 3). Compared with ischemic wounds, 3-NT was significantly less in non-ischemic wounds at all time points in all groups (Supplementary Figure S4 online).

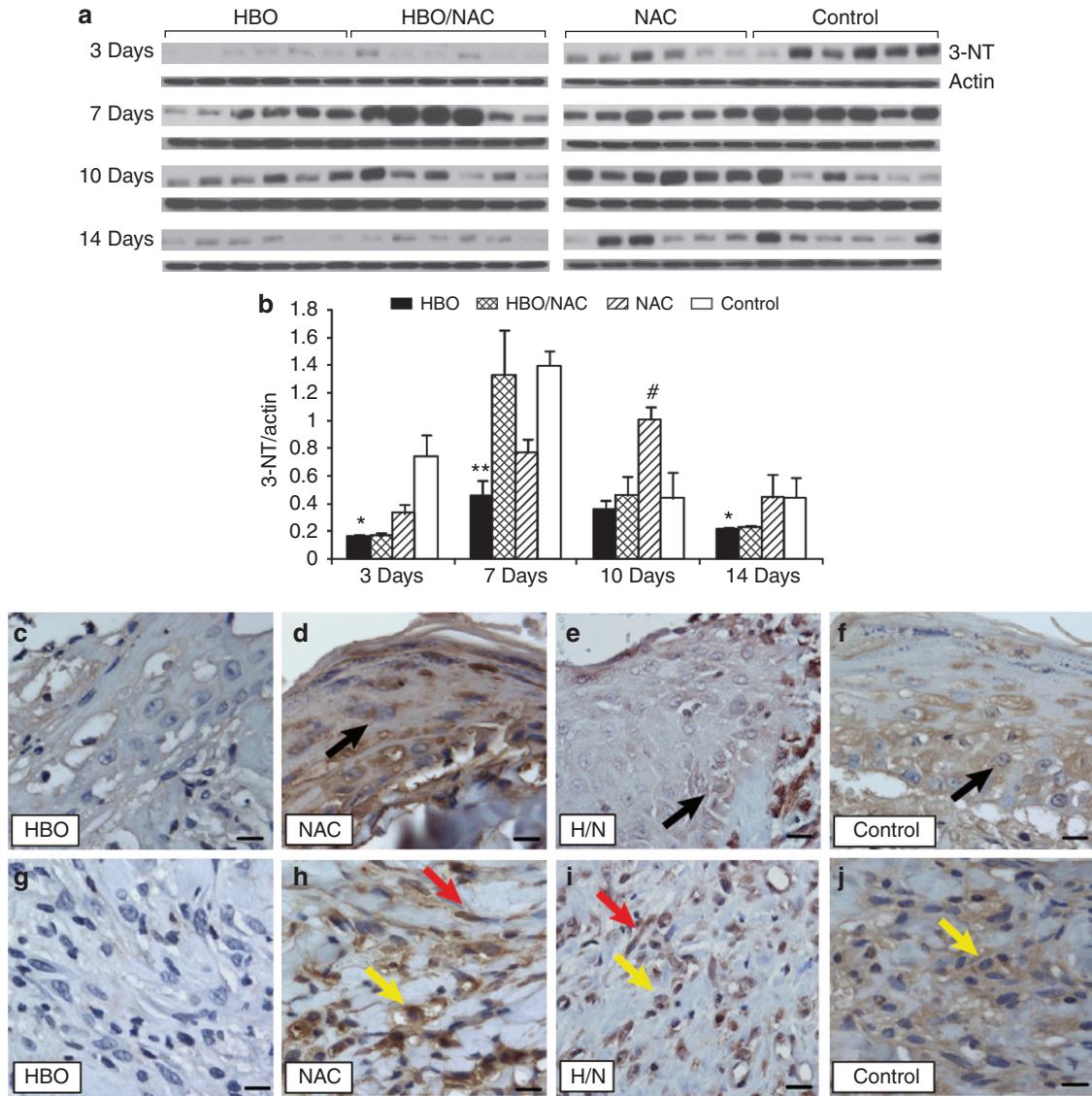
#### HBOT reduced MAPK activation in ischemic wound tissue

Oxidative stress results in tissue damage through induction of its downstream signals. Among these, MAPKs are unique to regulating MMP expression. Compared with HBOT, phosphorylation of c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinases 1/2 (ERK1/2) was significantly greater in ischemic wound tissue of H/N, NAC, and control groups from days 3 to 10 (Figure 4). It is particularly interesting that pJNK continues to increase through day 10 in the NAC-treated group. Epidermal keratinocytes, dermal fibroblasts, macrophages, and some endothelial cells in the three control groups exhibited strongly positive pJNK staining with localization to the cell nucleus. Phosphorylation of c-Jun was also markedly increased in the H/N, NAC, and control groups (Figure 5). Like pJNK, phosphorylation of c-Jun was predominantly expressed in keratinocytes (epidermis), fibroblasts, macrophages, and some endothelial cells (dermis) and was also localized to the cell nucleus, where it regulates transcription of its target genes (Figure 5c-j). Compared with ischemic wounds, expression of pERK, pJNK, and phosphorylation of c-Jun was low in all non-ischemic wounds (Supplementary Figure S4 online). These results indicate that HBOT significantly inhibited activation of MAPKs and the AP-1 family member c-Jun in ischemic wounds.

#### HBOT decreased MMP protein levels in ischemic wound tissue

The MMP-TIMP balance is critical for regulating cell-matrix composition. Many studies have documented the important





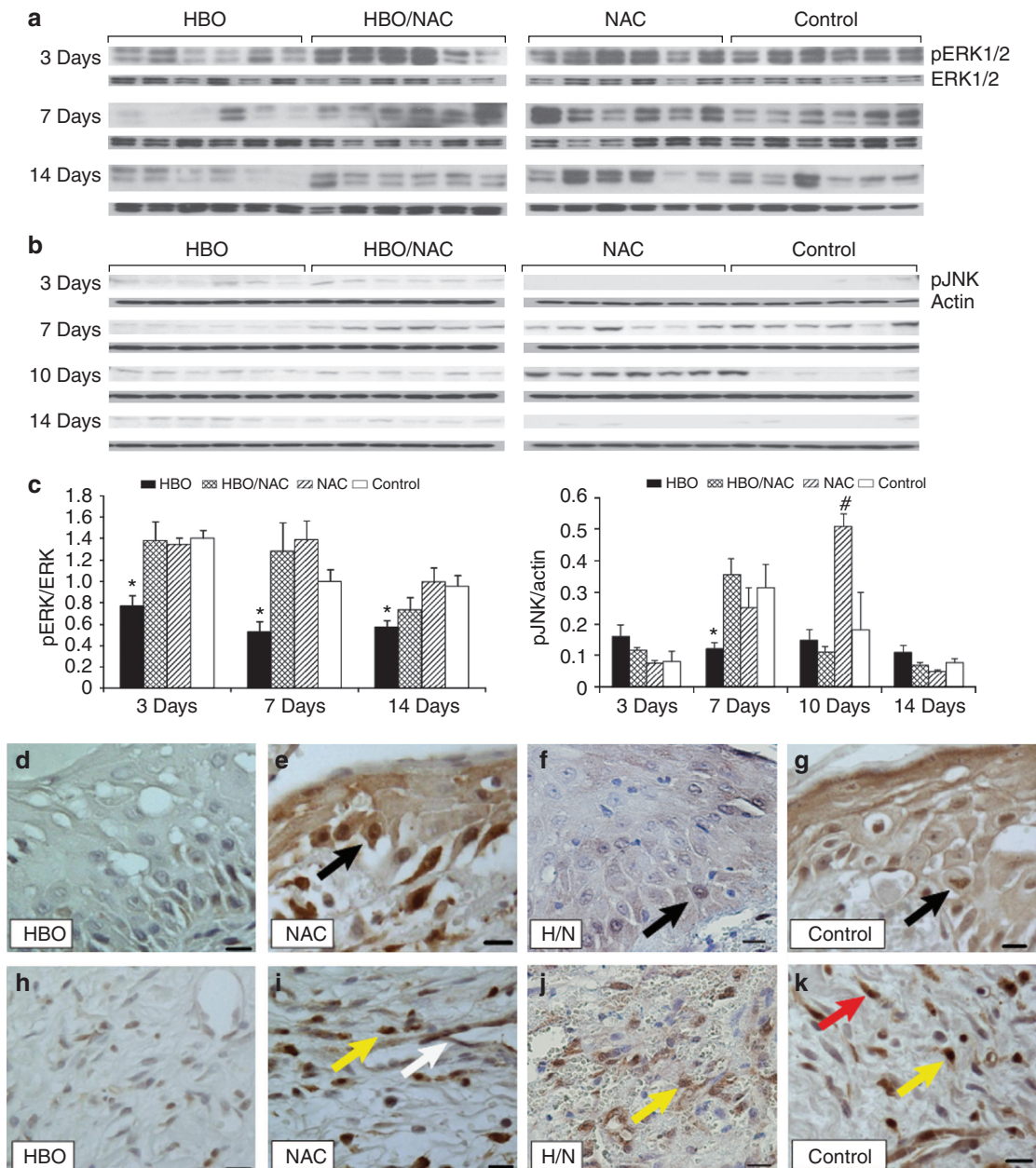
**Figure 3. Hyperbaric oxygen treatment (HBOT) decreases 3-nitrotyrosine (3-NT) production.** (a) Representative results of western blot analysis of 3-NT in ischemic wound tissue of all four treatment groups from days 3 to 14. (b) Densitometry analysis shows HBOT significantly decreased 3-NT in ischemic wound tissue at days 3 and 14 compared with the *N*-acetylcysteine (NAC) and control groups and at day 7 compared with the hyperbaric oxygen (HBO) and NAC (H/N), NAC, and control groups (\* $P < 0.05$  HBO vs. NAC and control; \*\* $P < 0.05$  HBO vs. H/N, NAC, and control; # $P < 0.05$  NAC vs. H/N, HBO, and control). (c–f) 3-NT immunostaining was seen in the epidermis and dermis (g–j) of ischemic wounds in the H/N, NAC, and control group. Positive 3-NT cell numbers were significantly decreased in the HBO group (black arrow: keratinocytes; red arrow: fibroblasts; yellow arrow: macrophages; bars = 20  $\mu$ m).

role of this balance in the pathophysiology of chronic wounds (Eming *et al.*, 2007; Toriseva and Kahari, 2009). We show that HBOT significantly decreased MMP1, MMP2, and MMP8 in ischemic wound tissue at day 7 (Figure 6). MMP9 gradually decreased from days 3 through 14 in HBOT ischemic wounds while the level of the MMP inhibitor TIMP2 was significantly increased. In contrast, MMP9 markedly increased in the other three groups between days 3 and 7, and remained elevated through day 10 in NAC-treated wounds. MMP9 was predominantly expressed in macrophages, but also in some fibroblasts in the granulation tissue of ischemic wounds (Figure 6d–g). In non-ischemic wounds, very low levels of

MMP9 protein were detected at days 3 and 7 in all four groups (Supplementary Figure S4 online). Consistent with reduced matrix degradation, HBOT also significantly increased collagen deposition and improved the ischemic wound closure rate (Supplementary Figure S5 online).

**NAC blocked the effects of HBOT on ischemic wound tissue**

The H/N and NAC treatment groups were included to confirm that HBOT is modulating lethal ROS production during ischemic wound healing. NAC acts primarily as a glutathione precursor. Although known to rapidly scavenge hydroxyl radicals and hypochlorous acid, NAC does not scavenge SA



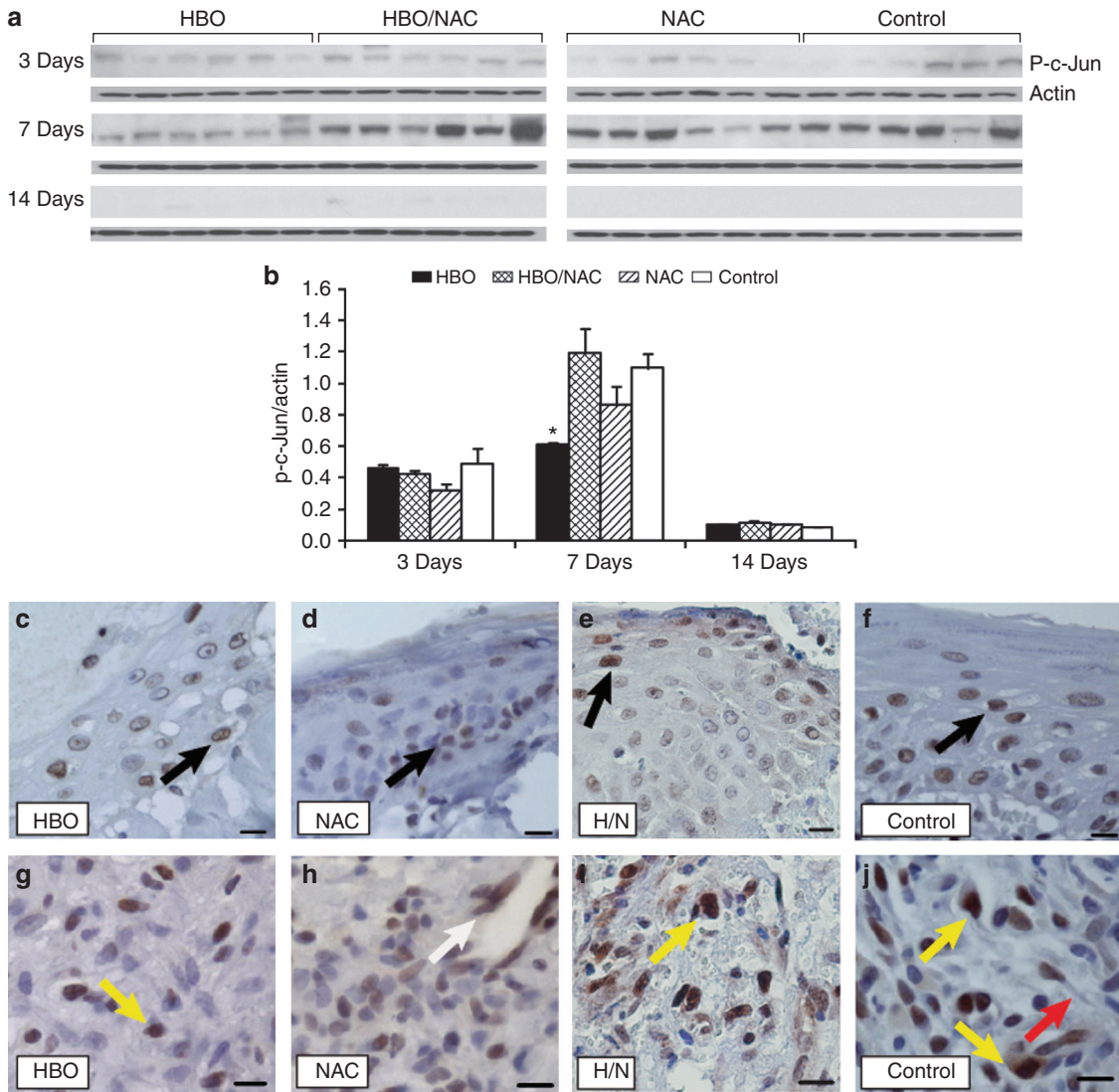
**Figure 4. Hyperbaric oxygen treatment (HBOT) reduces mitogen-activated protein kinase (MAPK) phosphorylation.** (a, b) Representative results of western blot analysis of extracellular signal-regulated kinase (ERK), pERK, pJNK in ischemic wound tissue of all groups from days 3 to 14. (c) Densitometry analysis shows hyperbaric oxygen (HBO) significantly decreased the pERK, pJNK in ischemic wound tissue compared with the HBO and NAC (H/N), NAC, and control groups (\* $P < 0.05$  HBO vs. H/N, NAC, and control; # $P < 0.05$  NAC vs. H/N, HBO, and control). (d–g) Immunohistochemistry (IHC) staining shows pJNK expression in the epidermis and dermis (h–k) of ischemic wounds. Abundant staining of pJNK was located in both the cytoplasm and the nucleus of H/N, NAC, and control groups. Positive cell numbers were significantly decreased in the HBO group (black arrow: keratinocytes; red arrow: fibroblasts; yellow arrow: macrophages; white arrow: endothelial cells; bars = 20  $\mu$ m).

(Aruoma *et al.*, 1989). In this study, NAC completely blocked the effects of HBOT on ROS/RNS regulation. NAC alone caused significant oxidative stress in ischemic wound tissue by decreasing GPx, while increasing gp91phox and iNOS (Figures 1–3). Consequently, pJNK and MMPs were also increased significantly over a prolonged period in ischemic wounds treated with NAC (Figures 4–6 and Supplementary Figure S6 online).

## DISCUSSION

HBOT is indicated for treating compromised flaps and grafts and to enhance healing in selected problem wounds, i.e., delayed effects of radiation and refractory diabetic wounds. Although it has been reported that HBOT has multiple effects on wound healing, the mechanism(s) by which HBOT improves healing remains unresolved. The rat ischemic wound model was designed to isolate the condition of tissue



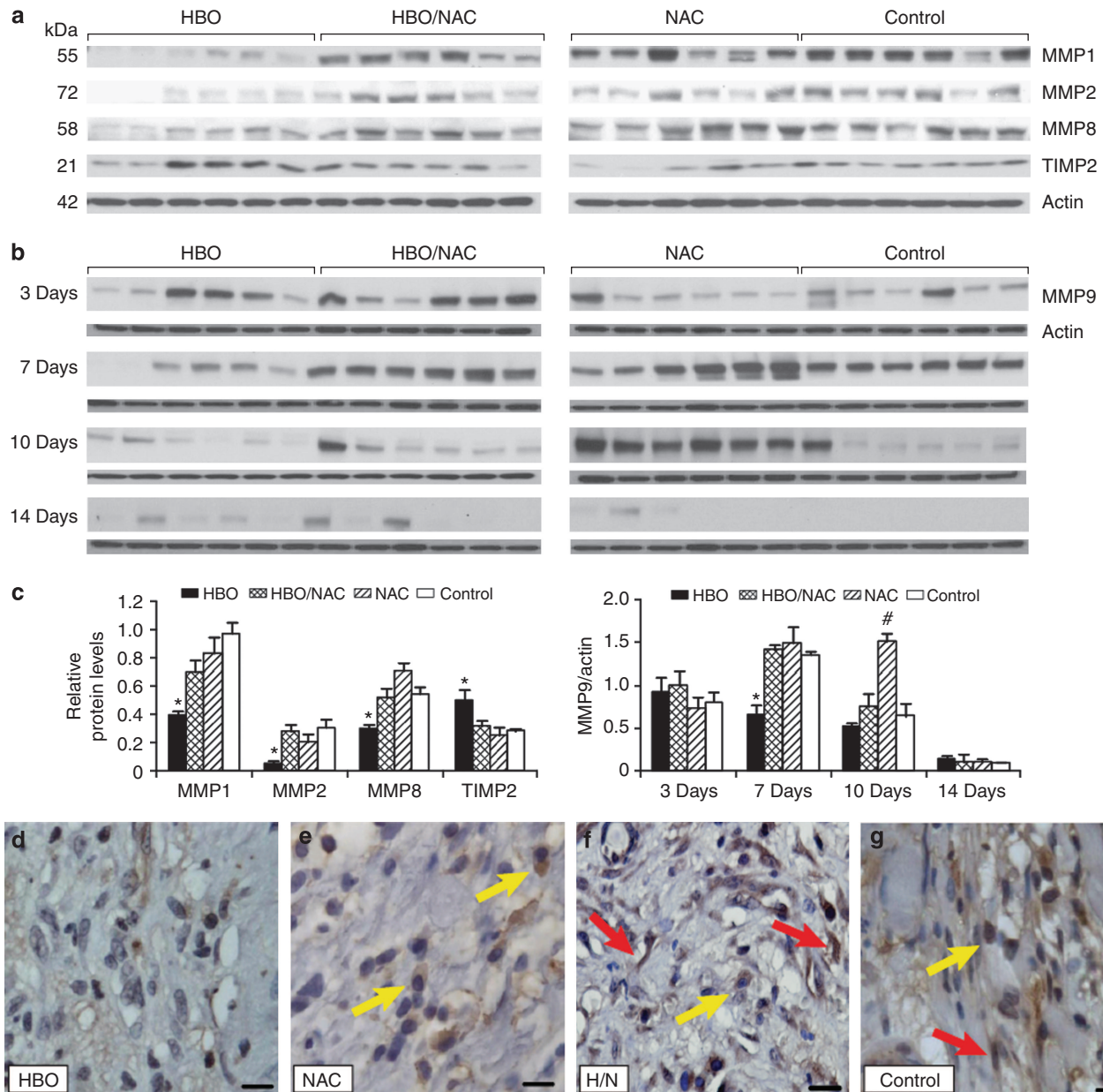


**Figure 5. Hyperbaric oxygen treatment (HBOT) reduces c-Jun phosphorylation.** (a) Representative results of western blot analysis of phosphorylation of c-Jun (p-c-Jun) in ischemic wound tissue of all groups from days 3 to 14. (b) Densitometry analysis shows hyperbaric oxygen (HBO) significantly decreased the p-c-Jun in ischemic wound tissue compared with the H/N, NAC, and control groups (\* $P < 0.05$  HBO vs. HBO and NAC (H/N), N-acetylcysteine (NAC), and control). (c–f) Immunohistochemistry (IHC) staining shows p-c-Jun expression in the epidermis and dermis (g–j) of ischemic wounds. Most of the p-c-Jun-positive staining was in the cell nucleus. Positive cell numbers were significantly decreased in the HBO group (black arrow: keratinocytes; red arrow: fibroblasts; yellow arrow: macrophages; white arrow: endothelial cells; bars = 20  $\mu$ m).

hypoxia from other comorbidities that contribute to chronic wounds. The ischemic wounds within the compromised flap have molecular parallels with human chronic wounds, including elevated proteases and increased inflammatory cytokines (Chen *et al.*, 1999). In this study we found that the oxidant-rich environment of the ischemic wound is closely linked to MAPK activation. Moreover, we found that HBOT reduces MMP overexpression in these highly oxidative wounds. Given the known regulation of MMP expression by MAPK, we propose that HBOT modulates MMP expression through the ROS/MAPK signaling pathway.

In ischemic wounds, neutrophils and macrophages are the major source of ROS/RNS, with gp91-phox (Nox 2) in phagocytes being the primary source of SA and iNOS producing

high levels of NO (Wlaschek and Scharffetter-Kochanek, 2005). Unless removed by SOD, SA rapidly combines with NO to form peroxynitrite, a powerful oxidant and inducer of cell death, lipid peroxidation, protein nitration, and oxidation. The high levels of 3-NT noted in the ischemic controls confirms this process and suggests that over-production of ROS/RNS in ischemic wounds overwhelms the antioxidant defenses resulting in oxidative stress (Figure 3). Compared with the control group, HBOT not only significantly decreased iNOS and gp91-phox but also upregulated three major antioxidant enzymes (Cu/Zn-SOD, catalase, and GPx) effectively reducing ROS/RNS production while enhancing ROS detoxification (Figures 1 and 2). The western blots demonstrate the inter-animal variability, particularly with respect to iNOS.



**Figure 6. Hyperbaric oxygen treatment (HBOT) decreases matrix metalloproteinase (MMP) protein.** (a) Representative results of western blot analysis of MMP1, MMP2, MMP8, and tissue inhibitor of metalloproteinase 2 (TIMP2) in ischemic wound tissue of all groups at day 7. (b) Representative results of western blot analysis of MMP9 in ischemic wound tissue of all groups from days 3 to 14. (c) Densitometry analysis shows hyperbaric oxygen (HBO) significantly decreased MMP1, MMP2, and MMP8 and significantly increased TIMP2 in ischemic wound tissue at day 7 compared with the HBO and NAC (H/N), N-acetylcysteine (NAC), and control groups (left panel). HBO significantly decreased MMP9 in ischemic wound tissue compared with the H/N, NAC, and control groups at day 7 and compared with the NAC group at day 10 (right panel). (d–g) Strongly positive MMP9 immunohistochemistry staining was seen in the dermis and granulation tissue of ischemic wounds in the H/N, NAC, and control groups. MMP9 was expressed predominantly in fibroblasts (red arrow) and macrophages (yellow arrow). MMP9-positive cell numbers were decreased in the HBO group. \* $P < 0.05$  HBO vs. H/N, NAC, and control; # $P < 0.01$  NAC vs. HBO, H/N, and control; bars = 20  $\mu\text{m}$ .

It appears that there are two non-responders to HBOT-induced suppression of iNOS. Clinically, approximately 25% of wounds treated with HBOT fail to respond, suggesting that further study of this phenomenon may be beneficial in identifying human non-responders (Löndahl *et al.*, 2010).

NAC was used to control for the effect of HBOT-induced ROS in ischemic wounds. Although NAC is known to increase

reduced glutathione, thereby restoring the total intracellular sulfhydryl pool, we found that NAC markedly increased gp91-phox expression (increasing ROS production) while simultaneously decreasing GPx. The combined effect of increased SA production, inability to scavenge SA, and lack of enzymatic reduction of  $\text{H}_2\text{O}_2$  despite adequate substrate seriously disrupts the balance of ROS, blocking cell signaling that

promotes wound repair (see Supplementary Figure S7 online). In contrast, based on our data showing increased SOD, GPx, and catalase, HBOT promotes resolution of oxidative stress and modulates the level of ROS/RNS in the wound bed. This is also how HBOT is thought to activate bone marrow-derived endothelial progenitor cell migration from the bone marrow (Thom, 2009) and how HBOT preconditioning protects against damage from oxidative stress in the brain and heart during ischemic-reperfusion injury (Kim *et al.*, 2001; Zhang *et al.*, 2005; Li *et al.*, 2008).

Our data suggest that negative feedback regulation could be an important mechanism to maintain the dynamic balance of ROS/RNS production and removal at the tissue level. We propose that HBO treatment of ischemic wounds transiently increases ROS levels, activating the negative feedback loop that downregulates the inducing enzymes (less production of ROS/RNS) and upregulates the antioxidant enzymes (increased removal of ROS) thereby limiting subsequent higher levels of ROS production. These results further imply that ROS/RNS-related enzymes, such as iNOS and nicotinamide adenine dinucleotide phosphate oxidase, rather than the ROS itself, could be important therapeutic targets for inhibiting oxidative stress.

Excess proteolytic activity leads to uncontrolled and off-target matrix destruction in chronic wounds. MMPs are expressed at low levels in normal skin but are activated in wound tissue. The transcription factor AP-1 has binding sites on a number of MMP promoters and is known to modulate MMP gene expression (Chakraborti *et al.*, 2003; Nelson and Melendez, 2004). The DNA binding and transactivation capacity of AP-1 is regulated by MAPK phosphorylation that is activated by a large variety of extracellular signals. A number of studies show that ROS are key regulators of MMP induction through AP-1 phosphorylation activated by ERK1/2 and JNK pathways (Brenneisen *et al.*, 2002; Turchi *et al.*, 2003; Woo *et al.*, 2004; Nelson *et al.*, 2006). In this study, MMP expression correlated well with the ROS production and MAPK activation in the ischemic wounds. Critical to understanding the difference between the HBOT and NAC effect is to recognize that Nox-derived H<sub>2</sub>O<sub>2</sub> regulates kinase-driven signaling (Gough and Cotter, 2011). H<sub>2</sub>O<sub>2</sub> exerts its effect through reversible oxidation of cysteine residues. Most cysteine residues are protonated at the low pH of the cytosol and therefore cannot react, however, the cysteine residues of protein tyrosine phosphatases are highly susceptible to oxidation, which inactivates the enzyme. Thus, when H<sub>2</sub>O<sub>2</sub> is increased, protein tyrosine phosphatase action is blocked allowing kinase activity to increase (ERK, AKT, and MAPK). The effects of H<sub>2</sub>O<sub>2</sub> depend on cell type, sub-cellular location, and concentration. High levels are known to cause catalase degradation with subsequent apoptosis and to increase MMP-9 through JNK signaling. Low levels activate signaling pathways to stimulate cell proliferation, differentiation, and migration. Low levels of H<sub>2</sub>O<sub>2</sub> also promote phosphorylation of catalase, which activates the enzyme and further reduces the H<sub>2</sub>O<sub>2</sub> concentration. Our data suggest that the molecular link between ROS, MAPKs, and MMPs at the tissue level revolves around the concentration of H<sub>2</sub>O<sub>2</sub> and

that a major effect of HBOT is to decrease the concentration of H<sub>2</sub>O<sub>2</sub> in the ischemic wound. NAC negates these effects (Supplementary Figure S7 and S8 online).

In addition to transcriptional regulation, MMP activity is inhibited by TIMPs (Chakraborti *et al.*, 2003). It has been reported that TIMPs are also spatially and temporally regulated by inflammatory cytokines, ROS, and antioxidants (Lu and Wahl, 2005; Byun *et al.*, 2006; Campo *et al.*, 2006). Imbalance between MMPs and TIMPs will lead to excessive proteolysis and impaired wound healing. In this study, HBOT significantly increased TIMP2, providing another level of MMP control. The details of how HBOT modulates other members of TIMP family (TIMP 1, 2, 3, and 4) remains to be investigated although we did not see significant differences in expression of TIMP 3 or 4 between groups (data not shown).

The signaling that occurs in response to wounding is dynamic and complex, orchestrating a series of events that must be resolved as the wound heals. As shown in Supplementary Figure S6 online, in the acute non-ischemic wounds, the pattern of MMP9, pJNK, gp91-phox, and iNOS protein levels was closely linked: they were all elevated at day 3 and gradually decreased from days 3 to 14 during the healing process. HBOT appears to 'jump start' healing even in the non-ischemic wounds (Supplementary Figure S5e and S5f online). Our data suggest that this might be related to the relative level of pJNK (lower in non-ischemic control and NAC groups), however, the effect is not sustained and does not alter the rate of closure. An alternative explanation is that this early effect is due to reduction of edema by HBOT. Although this was not measured, it is a known effect of HBOT and could account for the transient 'jump start' in these rapidly healing wounds. The most notable finding in comparing the pattern of protein expression in non-ischemic with ischemic wounds, is that MMP9, pJNK, gp91-phox, and iNOS increased between days 3 and 7 in the ischemic wounds. HBOT corrected the dysfunction of ROS-related signals in ischemic wounds, changing the molecular pattern to closely resemble the acute non-ischemic wounds.

#### **Limitations of the study**

We recognize that signaling occurs very early in wound healing and that measuring signaling molecules at day 3 and beyond may miss important events. The molecular markers discussed in this study were not elevated before day 3 in the ischemic wounds (unpublished observations). This is substantiated by the finding that these markers increase between days 3 and 7. The levels of ROS/RNS-related enzymes shown here only indirectly reflect ROS/RNS quantitation. Directly measuring ROS/RNS in the wound bed is difficult because of their very short half-life. To confirm the mechanism we propose here, we have begun to study MAPK and AP-1 silencing with small interfering RNA approaches in our laboratory. These techniques will be combined with real-time ROS quantitation *in vitro* along with increased focus on the very early-stage events in wound healing.

In conclusion, high oxidative stress exists in ischemic cutaneous wound tissue. Although the complex and often contradictory nature of ROS/RNS signaling is difficult to explain and



is highly dependent on local concentration and timing, we have demonstrated that HBOT provides a stimulus that promotes endogenous antioxidants to establish a therapeutic balance of oxidants and antioxidants, decreasing net oxygen radical production by means of negative feedback. The effect of HBOT on the ROS/MAPK/MMP axis alters the MMP/TIMP balance to increase extracellular matrix deposition. Our findings suggest that both ROS synthetic enzymes and MAPK pathways could be important therapeutic targets for chronic non-healing wounds. Further understanding of these pathways may provide insight into useful adjuncts to augment the efficacy of HBOT.

## MATERIALS AND METHODS

### Primary antibodies

Commercially available antibodies were purchased from the following vendors: anti-Cu/Zn-SOD, anti-manganese superoxide dismutase from Research Diagnostics (Flanders, NJ); anti-catalase, anti-GSHPx from Abcam (Cambridge, MA); anti-iNOS, anti-endothelial NOS, anti-gp91<sup>phox</sup> from BD Transduction Labs (Franklin Lakes, NJ); anti-EC-SOD, anti-3-NT from Upstate Cell Signaling Solutions (Lake Placid, NY); anti-ERK1/2, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-phospho-p38 MAPK (Thr180/Tyr182), anti-phospho-JNK (Thr183/Tyr185), anti-phospho-c-Jun (Ser73) from Cell Signaling Technology (Beverly, MA); anti-MMP1, anti-MMP2, anti-MMP8, anti-MMP9, anti-TIMP1, anti-TIMP2, anti-TIMP3, anti-TIMP4 from Chemicon International (Temecula, CA); anti- $\beta$ -actin from Sigma-Aldrich (St Louis, MO).

### Ischemic tissue animal model and HBOT

All procedures were approved by the institution's Animal Care Committee and abided by all requirements of the Animal Welfare Act. Ninety-six 8-week-old male Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA) weighing 250–300 g were used to create the ischemic wound model previously described (Zhang *et al.*, 2008). Daily treatments started on post-op day 1, including the day of wound procurement. The rats were placed into an HBO chamber (model number 800-45, Dixie Manufacturing, Baltimore, MD, maximum working pressure 45 psig) at 2.4 atmospheres absolute for 90 minutes. They did not require anesthesia and breathed spontaneously during HBOT.

### Experimental design

Ninety-six rats were randomly divided into four groups for daily treatment with HBO (90 minutes, 2.4 atm), NAC (150 mg kg<sup>-1</sup> injected intraperitoneally), H/N (both HBO and NAC), and control (normoxia at sea level). Control and HBO groups received an intraperitoneal saline injection with volume equivalent to the NAC group. On days 3, 7, 10, and 14, six rats from each group were anesthetized and the wounds were traced on clear plastic. Wound surface area was calculated using Sigma scan image software (Jandel Scientific, Cort Medera, CA). Non-ischemic and ischemic wounds were harvested, with one wound preserved in 10% buffered formalin for histology and another snap frozen in liquid nitrogen and stored at –80 °C for protein analysis.

### Western blot

Equal amounts of protein (40  $\mu$ g) from tissue homogenates were separated by 4–15% SDS-PAGE (Bio-Rad, Hercules, CA) and

transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked by 10% non-fat powdered milk in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20). Primary antibody incubation was in 5% non-fat milk in TBST overnight at 4 °C, followed by extensive washing with TBST, and secondary antibody incubation for 40 minutes at room temperature. Bands were visualized with enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). Relative quantities of protein were determined by densitometry (Kodak Digital Science 1D Analysis Software, Rochester, NY) and compared with  $\beta$ -actin expression.

### Histology and immunohistochemistry

Tissue was fixed with 10% formalin, embedded in paraffin, and sectioned into 5- $\mu$ m slices. Sections were deparaffinized, rehydrated, washed in distilled H<sub>2</sub>O, and placed in 95 °C antigen retrieval citrate buffer (Biogenex, Fremont, CA) for 10 minutes. Endogenous peroxidases were blocked with Peroxide Block (Innogenex, San Ramon, CA). Nonspecific binding was blocked with normal goat serum (Vector Laboratories, Burlingame, CA). Sections were incubated overnight with primary antibody at 4 °C, washed and incubated with biotinylated secondary antibody for 30 minutes, followed by streptavidin-horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories), diaminobenzidine (DAB kit, Vector Laboratories), and counterstained with hematoxylin. Slides were dehydrated, mounted, and viewed using Image-Pro Plus v.4.5 software (Media Cybernetics, Silver Spring, MD).

### Statistical analysis

To determine the sample size, we assumed that the effect size was 0.75. Six rats in each time point of each group could provide 91% power to detect a difference between treatment groups. Sample size justification was performed in nQuery Advisor 7.0 (Statistical Solutions, Cork, Ireland). Final data are presented as mean  $\pm$  SEM. One-way analysis of variance with *post hoc* comparisons using Tukey's honestly significant difference test was used for between-group comparisons (InStat v. 3.0, GraphPad Software, San Diego, CA). A *P*-value of <0.05 was considered significant.

### CONFLICT OF INTEREST

The authors state no conflict of interest.

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### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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