Beneficial effect of gaseous nitric oxide on the healing of skin wounds

Anatoly B. Shekhter a, Vladimir A. Serezhkov b, Tatiana G. Rudenko a, Alexander V. Pekshev c, Anatoly F. Vanin b,*

a Sechenov Medical Academy, Bolshaya Pirogovskaya Str. 2, bld. 4, Moscow 119992, Russian Federation
b Semenov Institute of Chemical Physics, Russian Academy of Sciences, Kosygin Str. 4, Moscow 119991, Russian Federation
c Bauman State Moscow Technical University, 2nd Baumanskaya Str. 5, 105005 Moscow, Russian Federation

Received 30 September 2004; revised 28 February 2005
Available online 25 April 2005

Abstract

Intermittent daily exposures (60 s) to NO-containing gas flow (NO dose of 500 ppm) generated by air-plasma unit “Plason” improves healing of skin wounds in rats. The gas flow treatment shortened the recovery time of both aseptic and purulent wounds (300 mm² area) by nearly a third. The treatment allows to achieve a marked improvement in the histological, histochemical, and electron-microscopic characteristics of the affected tissue. The mechanism of this phenomenon was studied by spin trapping method. The NO status of the wound tissue was investigated with EPR by following the formation of paramagnetic mononitrosyl complexes with iron-diethyldithiocarbamate, or with the heme groups in hemoglobin or myoglobin. For the first 5 min after a gas treatment with the exposure of 60 s, detectable NO levels in the affected tissue were slightly lowered with respect to untreated controls. At subsequent times, treated tissues showed the formation of large quantities of nitroso–iron complexes: At 30–40 min after gas exposure, their levels were nearly two orders of magnitude higher than soon after (15 s–5 min) the exposure. The data demonstrate that the accumulation of nitrosyl–iron complexes reflects a sharp rise in endogenous NO production inside the affected tissue. Paradoxically, the beneficial effect of gaseous NO treatment can be mediated by the formation of limited quantities of peroxynitrite due to the reaction between exogenous NO and superoxide anions generated in high amount in wound tissue. This peroxynitrite has a strong prooxidant effect and can activate various antioxidant systems which diminish the amount of superoxide anions in wound tissue. The reduced superoxide levels allow to increase the contents of endogenous NO in gas-treated tissues. Therefore, the beneficial action of the treatment is attributed to enhanced NO bioavailability.

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Keywords: Nitric oxide; Wound healing; Nitric oxide spin trapping

Wound healing processes are known to involve a sharp increase of nitric oxide generation in wound tissue. This increase is caused by the activation of constitutive isoforms of NO-synthase (eNOS and nNOS) and by a markedly enhanced synthesis of inducible NOS (iNOS) [1–5]. These observations suggest a possible therapeutic use of various NO donors for the acceleration of the wound healing. Previous studies [6–10] indicated that gaseous NO flow produced by air-plasma generator “Plason” acts beneficially on the wound healing. The mechanism of this effect remained unclear and will be the topic of this paper.

Our current findings suggest that the gaseous NO treatment changes the amount of NO in wound tissues and exerts its beneficial effect on wound healing indirectly by invoking a strong antioxidant response from the treated tissue.

The NO levels in the tissues of rats were studied using exogenous NO spin traps, Fe²⁺ complexes with
diethylthiocarbamate (DETC)\(^1\) or N-methyl-D-glucamine dithiocarbamate (MGD). NO binding with these traps results in the formation of paramagnetic mononitrosyl iron complexes, MNIC--DETC or MNIC--MGD, which are soluble in lipophilic or hydrophilic media, respectively. Due to its hydrophobic properties the MNIC--DETC complexes localize in cell membrane whereas MNIC--MGD is mainly found in blood and can be cleared from the body with urine [11–15]. Both complexes are paramagnetic and were quantified with electron paramagnetic resonance (EPR) at 77 K [11–15]. Alternatively, NO levels in the wound tissues were studied via the formation of well-known EPR detectable nitrosyl–heme–iron complexes in hemoglobin or myoglobin [16].

Materials and methods

Materials

Sodium diethylthiocarbamate (DETC) (Serva, USA), ferrous sulphate (Fluka, Switzerland), N\(^\ominus\)-nitro-L-arginine (NNLA), sodium ascorbate (both from Sigma, USA), and calyposal (Gedeon Richter, Hungary) were used. Sodium N-methyl-D-glucamine dithiocarbamate synthesized according to [17] was a kind gift of Dr. S. Kuprin (Karolinska Institute, Sweden).

The therapeutic NO-containing gas flow was generated by a "Plason" plasma generator (Fig. 1) designed at the Bauman Moscow State Technical University. It generates NO from the atmosphere by the plasmochemical reaction promoted by the high plasma temperature of an electric arc discharge:

\[
\text{N}_2 + \text{O}_2 \rightarrow 2 \text{NO}
\]

After leaving the plasma chamber, the NO-containing gas flow is cooled to 18–20 °C. The NO content of the gas flow can be controlled in the range between 0 and 2500 ppm. In this work, the NO levels of the gas flow were set to 800–1000 ppm. NO content in gas flow is measured with gas analyser Kane-May analyser (Model KM 9006 Quintox), production of Kane, Lim, UK. The dependence of NO content in gas flow from the distance from outlet of the manipulator is shown in Fig. 2. The similar content values are obtained when NO gas flow is directed towards the aqueous solution of Fe\(^{2+}\). MGD complex. That results in the formation of paramagnetic MNIC--MGD detected with EPR method (data not shown).

\[1\] Abbreviations used: EPR, electron paramagnetic resonance; DETC, diethylthiocarbamate; MGD, N-methyl-D-glucamine dithiocarbamate; MNIC--DETC(MGD), mononitrosyl iron complexes with DETC(MGD); NO, nitric oxide; NNLA, N\(^\ominus\)-nitro-L-arginine.

Fig. 1. Air-plasma unit "Plason."

![Graph showing the dependence of NO content in gas flow from the distance from outlet of the manipulator.](image)

Fig. 2. Dependence of NO content in gas flow from the distance from outlet of the manipulator.

Animals, surgical procedures, and wound care

One hundred and twenty white Wistar male rats weighting between 200 and 250 g were used in this study, carried out with the approval of the Moscow Medical Academy Ethics Committee. All surgical interventions were performed under calyposal anaesthesia (intraperitoneal injection, 80 mg per 1 kg of the body weight) in sterile conditions with surgical instruments sterilized with hot stream.

A 300-mm\(^2\) full-thickness flap of the skin was removed from the interscapular space of the rat's back. A Teflon (polytetrafluoroethylene) ring covered above by a fine perforated cellophane film was inserted intraoperationally into the edges of the wound to protect it from desiccating. This device was taken away from the wound on the fifth day after surgery.
diethylidithiocarbamate (DETC)\(^1\) or \(N\)-methyl-\(d\)-glucamine dithiocarbamate (MGD). NO binding with these traps results in the formation of paramagnetic mononitrosoyl iron complexes, MNIC–DETC or MNIC–MGD, which are soluble in lipophilic or hydrophilic media, respectively. Due to its hydrophobic properties the MNIC–DETC complexes localize in cell membrane whereas MNIC–MGD is mainly found in blood and can be cleared from the body with urine [11–15]. Both complexes are paramagnetic and were quantified with electron paramagnetic resonance (EPR) at 77 K [11–15]. Alternatively, NO levels in the wound tissues were studied via the formation of well-known EPR detectable nitrosyl–heme–iron complexes in hemoglobin or myoglobin [16].

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Fig. 1. Air-plasma unit “Plason.”

![Graph showing the content of NO vs distance from outlet](image)

Fig. 2. Dependence of NO content in gas flow from the distance from outlet of the manipulator.

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Further all animals were divided into four subgroups of 30 ones. Different interventions and wound care practices were employed in each group.

In the first and second subgroups the wounds were kept aseptic. In the other 60 animals (third and forth subgroups) wound were infected with 0.5 ml of 1-day culture of the Staphylococcus aureus (strain 25923, 1 billion microbial bodies per ml).

Group I (aseptic wounds, treated). The animals with aseptic wounds were exposed to NO-containing gas flow on the second, third, fourth, and sixth postoperative day. The duration of each treatment was 60 s.

Group II (control, aseptic wounds, non-treated). The animals with aseptic wounds were exposed to the warm air in the such terms and expositions.

Group III (infected wounds, treated). The animals with infected wounds were exposed to NO-containing gas flow under conditions identical to the first group.

Group IV (control, infected wounds, non-treated). The animals with infected wounds were exposed to the warm air under conditions identical to the second group.

Wound area measuring

The wound area was calculated every other day by transparent measuring lattice from the fifth day (after the Teflon ring removal) to the end of experiment. The index of acceleration of wound healing ($T_a$) was calculated by the formula:

$$T_a = (T_c - T_{exp}) * 100/T_c \%,$$

where $T_c$ is the time of complete wound healing in control conditions, and $T_{exp}$ is the time of wound healing in corresponding experimental group.

Morphological studies

The full-thickness wound tissue samples were obtained from the wounded area on the 4, 7, 10, 14, 21, and 28 day after surgery from three animals in each group. Before harvesting of the tissue samples these animals underwent the calypsol injection in toxic (lethal) dose. These tissue samples were fixed in ethyl spirit (70 °C), dehydrated in alcohols of increasing concentrations, cleared in toluene, and embedded in paraffin blocks. Slices 6 μm in thickness were prepared and stained with hematoxylin–eosin, picrofuchsin by Van-Gieson, toluidine blue, PAS-reaction, and pironin G by Brachet.

Electron microscopy

For routine electron microscopy study, samples were fixed in 2.5% glutaraldehyde and osmium tetroxide 1% solution, processed, and embedded in epon-araldite. Ultrathin sections were cut using a LKB Nova ultramicrotome (Sweden), stained with uranil acetate and lead citrate, and observed by a Phillips 301 electron microscope.

EPR assay

The experiments were performed on an additional group of 60 white Wistar male rats (the EPRA group). This EPRA group was used to investigate the mechanism for nitric oxide production in the skin tissue. The first 30 rats in this population were subjected to operative skin wounds but not treated by NO gas flow. On the fifth postoperative day, Fe$^{2+}$–citrate and DETC was injected into the granulation tissue formed on the bottom of the wound (10 rats) in 0.5 ml of physiological saline at the following doses: DETC, 500 mg/kg; FeSO$_4$, 40 mg/kg; and sodium citrate, 200 mg/kg. To determine the endogenous NO, granulation tissue was sampled 20 min after injection of NO trap and frozen in liquid nitrogen for subsequent EPR assay. The liver and blood samples were taken in all the animals for determination of NO also. Three animals were injected with the NOS inhibitor (NNLA) into the wound tissue in 0.4 ml of physiological solution at the dose of 200 mg/kg 30 min before injecting the NO trap. The other animals (10 rats) were injected with the Fe–citrate complex subcutaneously in the thigh and DETC intraperitoneally avoiding the wound (by conventional way [11,13]) in 0.5 ml of physiological saline at the following doses: DETC, 500 mg/kg; FeSO$_4$, 40 mg/kg; and sodium citrate, 200 mg/kg. Three wounded animals were injected with NNLA intraperitoneally in 0.4 ml of physiological saline at the dose of 200 mg/kg 30 min before addition of NO trap. In three control (non-wounded) animals, the NO content in the subcutaneous fat and muscular tissue, as well as in the liver and the blood, was determined after hypodermic injection of the NO trap. Three control rats (the EPRA-control group) were injected with the Fe–citrate complex subcutaneously in the thigh and DETC intraperitoneally at the doses mentioned above. The same protocol and similar molar dosages were used for NO trapping in the animals (four rats) with water soluble iron complexes with N-methyl-d-glucamine dithiocarbamate (MGD).

The EPR measurements were performed at 77 K using EPR spectrometer ESC-106 operating in X-range (Bruker, Germany). Recordings were made with a modulation amplitude of 0.5 mT, microwave power of 20 mW, and time constant 0.32 s. To estimate the concentration of MNIC–DETC detected in animal tissues by EPR method the solutions of MNIC–DETC complexes in dimethyl sulfoxide with known concentrations were used as a standard samples. The synthesis of the standard sample was performed as described in [13].
Results

The effect of gaseous NO flow on the aseptic wounds

The measuring of area of aseptic skin wounds in experimental group I showed that the reducing of wound area proceeds at a much faster rate than in the control group II (Fig. 3A). While the average time of complete wound healing in the control group was equal to 29.25 ± 1.6 days, such term of the experimental treated wounds decreased to 22.24 ± 1.02 days. So the wound healing under NO gas treatment accelerates to 7.5 days compared to that in control group, and $T_a$ is equal to 24.6%. On the fourth day after surgery, the histological, histochemical, and electron-microscopic studies of NO gas-treated animals showed a significant acceleration of the wound healing process compared to the control group. At this term it had passed from the inflammatory into the reparative phase. These changes were observed in the experimental groups apparently earlier than in the control groups. At the bottom of the wound in the experimental group was revealed a well-developed granulation tissue (but only its foci in the inflamed fatty tissue in the controls), a markedly pronounced fibroblast proliferation (many mitoses) and capillary growth, formation of collagen fibers, the accumulation of proteoglycans, and PAS-positive glycoproteins (Figs. 4A and B). The fibroblasts were distinguished by an increased RNA content in the cytoplasm and, in ultrastructural study by the developed granular endoplasmic reticulum (GER) and Golgi apparatus (GA) (Fig. 4C). The macrophagal reaction, including the phagocytic activity of macrophages, was more markedly pronounced in the experimental group. This was evidenced by an increased content of phagosomes and lysosomes in their cytoplasm (Fig. 4D). Microbial phagocytosis by macrophages and neutrophils was also increased.

There were great differences in the microcirculatory reaction. The vasodilatation of newly formed capillaries in the experimental group was revealed; however, such phenomena as endothelial destruction, erythrocytic sludge, aggregation of thrombocytes, microthrombosis, adhesion, and increased migration of neutrophils were significantly less notable than in the controls and subsequently disappeared.

At 7 days after surgery the granulation tissue in the experimental group wounds quickly matured and at 14 days it was underwent fibrotic and cicatricial transformation. The reduction of the capillaries and fibroblasts and the appearance of large areas of collagen fibrils with increasing of marginal epithelization and differentiation of the epithelium were observed (Fig. 4E). In the control group at this term was revealed the immature granulation tissue and expressed infiltration by the inflammation-associated cells (Fig. 4F).

At 21 day after surgery the epithelization of whole wound surface in the great majority of animals NO treated group was accomplished, and the remodelling of the scar tissue with the absence of inflammatory manifestation was started. At the same time in the control group the epithelization was not yet completed.

The effect of gaseous NO flow on the purulent wound

In the second series of experiments, in which the wounds were infected with a Staphylococcus aureus, the wound area in the experimental group reduced quicker than in the control (Fig. 3B). The average time of the complete healing of the wounds reduced to 9.3 days ($T_a = 31.6\%$) in comparison to that in the control group. In the control group IV wound healing lasts on the average $33.6 ± 1.8$ days, and in the experimental group III this term was equal to $24.3 ± 1.3$ days.

The morphological study in the control group III at 4–10 days after surgery revealed severe and prolonged signs of microcirculatory disorders, neutrophilic infiltration, oedema, vasculitis, the formation of primary and secondary necroses, microabscesses, expressed wound infection, suppression of phagocytosis of germs, and necrotic detritus as well as inhibition of the fibroblast proliferation, capillary growth, maturation of the granulation tissue, macrophagial activity, and epithelization (Fig. 5A).

In the experimental group IV all these signs were significantly less severe even at 4 days and quickly disappeared. The granulation tissue developed at 4 days. At the 10 days after surgery it was observed the maturated
Fig. 4. Healing of the aseptic wounds: (A) The mature granulation tissue on the fourth day after the exposition to NO-containing gas flow; fibroblast proliferation, increased number of the macrophages, growth of new capillaries, and vasodilatation. The decrease of inflammation. Staining with hematoxylin-and-eosin, 400x. (B) The slow growth of immature granulation tissue on the fourth day after exposition to warm air flow (control group): there are the fibrin and tissue detritus on the wound surface, inflammatory infiltration. Staining with hematoxylin-and-eosin, 200x. (C) The experimental group, fourth day after the exposition to NO-containing gas flow. An active mature fibroblast: the hyperplasia of the granular endoplasmic reticulum (GER) and Golgi apparatus (GA), 10,000x. (D) The experimental group, fourth day after the exposition to NO-containing gas flow. A macrophage in state of phagocytosis: numerous lysosomes, phagosomes, and vacuoles, 10,000x. (E) The experimental group, 14th day after the exposition to NO-containing gas flow. Fibroic transformation of the granulation tissue and wound epithelization. Staining with hematoxylin-and-eosin, 200x. (F) The control group, 14th day after the exposition to NO-containing gas flow. The immature granulation tissue and inflammation infiltration. Staining with hematoxylin-and-eosin, 400x.

granulation tissue (Fig. 5B), and at 14 days, in contrast to the control, its fibrosis and contraction with subsequent epithelization were occurred.

NO detection by EPR method

Experiments on animals of the EPRA group served to study the mechanism of formation of MNIC–DETC complexes in tissue. Previous work has shown the feasibility to use Fe–DETC or Fe–MGD complexes for detection of NO in tissues of living animals [11–15].

Representative EPR spectra in wound tissue from the EPRA group are shown in Fig. 6. Fe–citrate and DETC were injected directly into the wound tissue or administrated to the animals conventionally, i.e., in the thigh (iron) and intraperitoneally (DETC). Strong EPR signal
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Fig. 5. Healing of infected skin wounds: (A) The control group on the 10th day after exposition to warm air flow. The oedema, neutrophilic leukocyte infiltration, decreased number of macrophages, and low fibroblast proliferation. Staining with hematoxylin-and-eosin, 200x. (B) The experimental group, 10th day after the exposition to NO-containing gas flow. The mature granulation tissue with minimal inflammatory infiltration. Staining with hematoxylin-and-eosin, 200x.

Fig. 6. Representative EPR spectra from wound tissue samples. Fe-citrate and DETC were injected directly into the wound tissue (spectrum B) or administrated to the mouse subcutaneously in the thigh (Fe-citrate) and intraperitoneally (DETC) (spectrum A). The EPR signals from Cu\(^{2+}\)-DETC with the components a, b, c, and d and MNIC–DETC at \(g = 2.035\) composed spectrum B are presented separately in C and D, respectively. The EPR signal from heme–protein–nitrosyl complex with the component at \(g = 2.07\) and 1.98 is dominant in the spectrum A. Recordings were made at 77 K with microwave power of 20 mW and modulation amplitude of 0.5 mT. The amplification of spectrum A is four times more than that for spectra B–D.

with triplet hyperfine structure (HFS) at \(g = 2.035\) characteristic of MNIC–DETC complexes was observed in wound tissue in the first type of the experiments (Fig. 6B). Additively small EPR signal with quartet HFS (labelled a, b, c, and d) from Cu\(^{2+}\)-DETC complexes made contribution to the spectrum. Both signals are separately shown in Figs. 6D and C, respectively. The formation of MNIC–DETC and Cu\(^{2+}\)-DETC complexes in wound tissue was decreased when iron and DETC were injected into the body of wounded rats beyond the wound. The EPR signal from heme–protein–nitrosyl complex with the components at \(g = 2.07\) and 1.98 was dominant in the spectrum in the wound tissue recorded at higher amplification (Fig. 6A).

Fig. 7 demonstrates the influence of ascorbate or the NOS inhibitor NNLA on MNIC–DETC levels in wound tissue treated directly with Fe and DETC. In accordance with the intensity of the EPR signal of the complexes (Fig. 7A) the amount of MNIC–DETC

Fig. 7. EPR measurements of NO using Fe-DETC injected into the granulation tissue of the wound in all animals. (A) The sample of the granulation tissue frozen after isolation from the wound. (B) The sample (A) after thawing, further addition of ascorbate (10 mM) and freezing. (C) The samples of the granulation tissue from the wound of wounded animals injected with NNLA into the wound 30 min before addition of NO trap. (D) The sample (C) after thawing, further addition of ascorbate (10 mM) and freezing. (E) The sample of the liver frozen after isolation from the body. (F) The sample of the liver isolated from the rat injected with NNLA intraperitoneally 30 min before the addition of NO trap. Arrows a, b, c, and d show the positions of the components of hyperfine structure from Cu\(^{2+}\)-DETC EPR signal. Representative spectra are shown from 3 to 7 measurements. Recording conditions are shown in Fig. 6.
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complexes in wound tissues was equal to $1.3 \pm 0.3$ nmol/g wet tissue. Upon thawing and treatment with 10 mM ascorbate solution, the samples show a 3-fold increase in MNIC–DETC levels, corresponding to $4 \pm 1$ nmol of the MNIC–MGD per g wet tissue in the preparation (Fig. 7B). We attribute this increase to the reducing action of ascorbate on nitrite ions accumulated in the tissue. An alternative mechanism can operate here. It was earlier established that Fe$^{3+}$–DETC complexes injected into the animals and being oxidized to ferric state remain the capacity to trap NO molecules resulting in the formation of EPR silent, diamagnetic NO–Fe$^{3+}$–DETC complexes [18,19]. Ascorbate addition can reduce them to paramagnetic NO–Fe$^{3+}$–DETC form.

Application of NNLA to the wound 30 min before suppletion of Fe–citrate and DETC led to sharp attenuation of the MNIC–DETC level (Fig. 7C). In the presence of NNLA, the MNIC–DETC yields in tissue could not be enhanced by the addition of ascorbate (Fig. 7D). So the data demonstrate that NO molecules included into MNIC–DETC complexes were generated in wound tissue via enzymatic L-arginine-dependent pathway.

In EPRA wound tissue, MNIC–DETC complexes were also observable when Fe–citrate complexes and DETC were administered to the animals conventionally, i.e., subcutaneously in the thigh and intraperitoneally, respectively (Fig. 8A). However, the yield of MNIC–DETC complexes was 10-fold less than that when Fe–citrate and DETC were injected directly into the wound tissue (Fig. 7A). The addition of 20 mM ascorbate solution to the preparation did not significantly increase the yield of the MNIC–DETC complexes (data not shown).

Animals from the EPRA group also had detectable levels of MNIC–DETC complexes in liver tissue (Figs. 7E and 8E). As in wound tissue, the MNIC–DETC yield in liver was higher with injection of traps directly in to the wound than with conventional injection. The MNIC–DETC yields are $7 \pm 1$ nmol versus $5 \pm 1$ nmol/g wet liver tissue for direct and conventional injection, respectively (Figs. 7E and 8E). Preliminary administration of NNLA to the wound attenuated the MNIC–DETC yields by an order of magnitude (Fig. 7F). The MNIC–DETC amount in liver from control (non-wounded) animals is equal to $1.5 \pm 1.0$ nmol/g wet tissue for conventional injection of iron and DETC solutions (Fig. 8F). Much lower MNIC–DETC yields are found in kidney or heart tissues when NO traps were injected with conventional way (Figs. 8C and D). Here, yield from direct and conventional injection is not significantly different. Strong EPR signal at $g = 1.94$ characteristic of reduced iron sulphur proteins was also recorded in liver, heart, and kidney samples (Figs. 7E and F, and 8 C–F). The signal was much smaller in wound tissue preparations.

We now return to the effects of the NO gas treatment. The Fe–DETC complexes were added by direct wound injection 15 min prior to NO treatment. At various time points up to 60 min after gas treatment, tissue samples were extracted from the wound tissue and analysed with EPR. These samples revealed EPR signals from MNIC–DETC ($g = 2.035$) as well as paramagnetic NO–Hb or NO–Mb complexes ($g = 2.07$ and $1.98$) (Fig. 9A). The total amount of all the nitrosyl–iron complexes in wound tissue reached a maximum at 30–40 min after the treatment. This maximum was 10– to 100-fold higher than the amount at 15–300 s after treatment. (Unfortunately, the large scattering in the data was characteristic of the estimation for various animal groups.)

Interestingly, that for the first 5 min after a gas treatment of 60 s duration, detectable levels of nitrosyl–iron complexes in affected tissue are slightly lowered with respect to untreated controls. After passing through the maximum at 30–40 min after the treatment, the nitrosyl–iron content slowly decreased (data not shown). The addition of the NOS inhibitor LNNA to wound tissue 30 min prior gaseous NO treatment completely suppressed the effect of NO gas on the formation of nitrosyl–iron complexes (Fig. 9B). This observation demonstrates the endogenous origin of the NO in these nitrosyl–iron complexes.

The gas treatment even enhances MNIC–DETC accumulation in the liver and intestine when the gaseous NO flow is not directed to the wound, but towards intact skin above the animal abdomen for 30 s (Figs. 9C and E). Experiments on unwounded animals showed that these tissues had accumulated 5 nmol versus 1.5 nmol/g wet tis-

![Fig. 8. EPR measurements of NO using Fe–DETC injected into the body of wounded rats beyond the wound.](image-url)
Fig. 9. (A,B) Representative EPR spectra from wound tissue treated with gaseous NO 30 s without (A) or with NNLA (B). (C,D) Representative EPR spectra from liver (C), kidney (D) or intestine (E) when the gaseous NO flow is directed towards intact skin above the animal abdomen for 30 s. The spectra were recorded at the conditions shown in Fig. 6.

Discussion

Our EPR data demonstrate the formation of nitrosyl–iron complexes in wound tissue. Various potential sources of the NO ligand should be considered: (1) Endogenous from NOS enzymes. (2) Exogenous from the gas flow. (3) Released from an internal storage pool of nitrosyl–iron complexes or S-nitrosothiols. (4) Internally generated from nitrite. Recently it was reported that the formation of MNIC–MDG complexes is not conclusive proof of enzymatic synthesis of NO: in an alternative pathway, Fe$^{2+}$–MDG may react with nitrite to produce MNIC–MDG complexes [20]. Whether this mechanism be physiologically relevant or whether the analogous pathway exists at all for the DETC ligand is unknown at present. With the Fe$^{2+}$–DETC traps used in this work, we have not detected any contribution from this alternative pathway. In particular, inhibition of the NOS enzyme with LNNA completely reduced the level of nitrosyl–iron complexes to below the detection limit of the EPR spectrometer. It proves that the NOS enzyme is the dominant source of the NO ligand of these nitrosyl–iron complexes. Our data show that straightforward detection of this enzymatic NO may be achieved by injecting the Fe–DETC complexes directly into the wound tissue.

The liver tissue of wounded animals shows enhanced NO production. This seems to reflect the general inflammation response of the organism to the large wound injury of skin. The inflammation response increases the systemic cytokine levels, thereby activating the constitutive NOS isoforms and promoting iNOS synthesis in the liver [21,22]. It is noteworthy that this up-regulation of NO in wound tissue persists over a few days. A more detailed study of the kinetics of NO production in liver tissue is in progress now.

It was recently shown that NO molecules bind both with Fe$^{2+}$–DETC and Fe$^{3+}$–DETC complexes that result in the formation paramagnetic and diamagnetic MNIC–DETC, respectively [18,19]. In biological tissues we expect efficient oxidation of iron from Fe$^{2+}$ to Fe$^{3+}$ in the Fe–DETC complexes. Therefore, it is plausible to expect that trivalent Fe$^{3+}$–DETC complexes are the main traps for NO in animal tissues. The resulting mononitrosyl–Fe(II) complexes are diamagnetic and EPR silent. According to [19], the reduction into paramagnetic mononitrosyl–Fe(II) complexes may proceed through reductive nitrosation as shown in Scheme 1.

As it was shown in [19] that the rate of this process in organic media is much more efficient than that of the reactions of reductive nitrosation of hydrophilic complexes. We propose that the lipid compartment in animal tissues provides the proper hydrophobic environment to allow such rapid reductive nitrosylation from diamagnetic to paramagnetic form. It should be noted that alternative pathways for this transformation have been proposed in the literature [18]. The reaction is accompanied by the release of dithiocarbamate disulphide in accordance with Scheme 2.

Though certainly feasible in chemical assays, the relevance of this alternative pathway under conditions in living tissue is unknown. The second-order dependence on the trap concentration makes it improbable to us that the reaction rates of Scheme 2 will be high enough to explain the observed MNIC formation in the usual protocols with laboratory animals.

Returning to the physiology of the gas flow treatment of skin wounds, one of the main results of the present

\[
(\text{DETC})_2\text{Fe}^{2+}\text{NO} \rightleftharpoons (\text{DETC})_2\text{Fe}^{2+}\text{NO} + B \rightarrow (\text{DETC})_2\text{Fe}^{2+} + \text{B-NO}^\cdot
\]

where B- is a nitrosated agent.

Scheme 1.

\[
2\text{Fe}^{2+}(\text{dithiocarbamate}) + \text{NO} \rightarrow \text{Fe}^{2+}(\text{dithiocarbamate}) + 1/2 \text{dithiocarbamate disulfide}
\]

Scheme 2.
investigation is the sharp increase of MNIC-DETC and nitrosyl–heme–iron complexes in wound tissue as a response to successive short-lasting exposures to gaseous NO flow. We showed that endogenous NO is responsible for the formation of these complexes, and that this NO is synthesized by NOS enzymes. The EPR experiments gave direct proof that gaseous NO treatment increased the stationary level of endogenous NO molecules in the wound tissues. We propose that this beneficial effect be caused by mechanism involving peroxynitrite as an intermediate. Usually, peroxynitrite is considered a harmful substance and many harmful consequences of its formation have been well documented. But recent publications have reported that small doses of peroxynitrite may actually have protective action in cell cultures and tissues. These results were attributed to a mobilization of various antioxidant defences by the presence of peroxynitrite [23,24]. This mobilization could diminish the amount of superoxide anions in wound tissue, thereby protecting endogenous NO molecules against superoxide attack. It actually enhances the stationary NO level of the affected tissue. We have established beyond doubt that the beneficial effects of the gas treatment gaseous NO are accompanied by enhanced NO levels in the wound tissue. By implication, more endogenous NO molecules become available as signalling molecules to regulate the metabolic processes in wound tissue.

We feel that our results from the animal model have significance for the mechanism of using NO gas flows to treat skin wounds in humans as well. Up to now, the air-plasma unit “Plason” has been used to treat more than 10,000 patients with a wide variety of skin wounds and other disorders. The patient group included individuals with complicated, protracted postoperative wounds, purulent and gunshot wounds, burns, and trophic ulcers associated with venous insufficiency, atherosclerosis, diabetes mellitus, radiation therapy, bedsores, necroses of the skin grafts, osteomyelitis, etc. The gas treatment markedly accelerated the healing process throughout this group [6–10]. The microcirculation, roughly damaged in complicated wounds, was rapidly (after 2–6 sessions of NO-therapy) normalised as proved by Laser-Doppler Flowmetry and measurement of transcutaneous oxygen tension. The signs of tissue hypoxia were strongly reduced. The results of microbiological studies indicate that the infection in the tissue decreased quickly.

Biopsic samples were harvested from the patients and investigated with histochemical, cytological, and electron-microscopic studies [6,7,9,10]. The results showed notable decrease of inflammation and microbial infection, together with activation of macrophages and phagocytosis intensification. The treatment accelerated the normalisation of microcirculation, growth of new microvessels, and the clearing of necrotic detritus. We also observed proliferation of fibroblasts, growth and maturation of the granulation tissue, as well as normalization of tissue structure and rapid contraction and epithelization of the wounds. On a histological level, the effects of the gas flow treatment are surprisingly similar for the different wounds in different patients.

Experimental, theoretical, and clinical studies have shown that the new procedure of the NO-therapy can be used as an effective treatment of a wide variety of different wounds. Despite the methodical difficulties and gaps in our knowledge, we feel that the NO-therapy may give a valuable contribution to patient care in modern human medicine. Moreover, the present study has demonstrated that gaseous NO may penetrate the intact skin of our test animals. It proves the validity of the principle that external gaseous NO may have an effect on the internal organs of the animals.

Acknowledgments

The work was supported by Russian Foundation of Basic Researches (Grant: 02-04-48265) and Russian Foundation “Integration” (Grant No. 0580/2134).

References


