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The role of nitrite in hemoglobin-based oxygen carriers under oxidative conditions

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Abstract
Hemoglobin-based oxygen carriers (HBOCs) have been developed as potential blood substitutes to address challenges associated with blood transfusions. However, their clinical application is limited by oxidative degradation, which impairs oxygen delivery and leads to adverse side effects. Nitrite (NO_2^-), a reactive nitrogen species, plays a complex role in modulating the oxidative behavior of hemoglobin within HBOCs under oxidative stress conditions. This review explores the concentration-dependent effects of nitrite on hemoglobin oxidation, focusing on its dual role as both a protective agent and a pro-oxidant in the presence of reactive oxygen species such as hydrogen peroxide. Mechanistically, nitrite can reduce the formation of harmful ferryl intermediates, thereby mitigating oxidative damage, but at elevated concentrations, it may exacerbate oxidative degradation through the generation of reactive nitrogen species. The balance between these effects is critical in determining the stability and functionality of HBOCs. Understanding the molecular interactions between nitrite and hemoglobin under oxidative stress can inform the design of safer and more effective HBOCs with enhanced shelf-life and reduced toxicity. This review highlights recent advances in the field, discusses current challenges, and proposes future directions for research aimed at optimizing the use of nitrite to improve HBOC performance under oxidative conditions.

Keywords: Hemoglobin-based oxygen carriers, Nitrite, Oxidative stress, Hemoglobin oxidation, Reactive nitrogen species, Hydrogen peroxide, Ferryl hemoglobin, Blood substitutes

Introduction

1. Background on Hemoglobin-Based Oxygen Carriers (HBOCs)

Hemoglobin-based oxygen carriers (HBOCs) are engineered products designed as blood substitutes to mimic the oxygen transport function of red blood cells (RBCs). They have potential applications in trauma care, surgery, and conditions where blood transfusion is limited or contraindicated. HBOCs offer advantages such as longer shelf life, no need for blood typing, and reduced risk of infectious disease transmission.

Despite these benefits, HBOCs face critical challenges, notably oxidative instability. Hemoglobin (Hb), when isolated from RBCs, is prone to oxidation and subsequent degradation, leading to loss of oxygen-carrying capacity and formation of reactive intermediates that can cause tissue damage.

2. Oxidative Processes in Hemoglobin and HBOCs

The oxidative degradation of Hb is primarily mediated by reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2). The interaction of Hb with H_2O_2 produces ferryl hemoglobin (Fe^{4+}=O), a highly reactive oxidizing species. Ferryl Hb can oxidize proteins, lipids, and nucleic acids, exacerbating oxidative stress and toxicity. Additionally, the heme moiety can undergo degradation, releasing free iron, which catalyzes further ROS production via Fenton chemistry.

Understanding the kinetics and mechanisms of Hb oxidation is essential for improving HBOC design and safety.

3. Role of Nitrite in Hemoglobin Oxidation

Nitrite (NO_2^-) is an endogenous anion present in blood and tissues, formed by oxidation of nitric oxide (NO) or through dietary intake. Nitrite participates in nitric oxide biology and redox chemistry and can interact with Hb under both physiological and pathological conditions.

Nitrite's influence on Hb oxidation is complex and concentration-dependent

- At low concentrations, nitrite can act as a reductant, reacting with ferryl Hb and reducing oxidative damage.
- At higher concentrations, nitrite can serve as a source of reactive nitrogen species (RNS), such as nitrogen dioxide ($NO_2\cdot$) and peroxynitrite ($ONOO^-$), promoting oxidative modification of Hb.

4. Nitrite and Oxidative Stability of HBOCs

The interaction between nitrite and Hb within HBOCs affects their oxidative stability and functional lifespan. Modulating nitrite levels in HBOC formulations may be a strategy to balance protective and pro-oxidant effects.

Several studies have examined how nitrite concentrations influence Hb oxidation kinetics, heme degradation, and oxidative stress markers in vitro and in *Vivo*.

5. Clinical Implications and Challenges

Oxidative degradation limits the therapeutic use of HBOCs due to toxicity, vasoconstriction, and reduced oxygen delivery. Nitrite's dual roles raise challenges for optimizing HBOC formulations. A clear understanding of nitrite-Hb chemistry can aid in developing safer HBOCs with controlled oxidative profiles.

6. Objectives of the Review

This paper aims to critically review the current understanding of nitrite's role in modulating Hb oxidation within HBOCs under oxidative conditions, focusing on mechanistic insights, concentration-dependent effects, and implications for HBOC design and clinical use.

Methods

1. Literature Search Strategy

A systematic literature review was conducted to gather studies examining the role of nitrite in Hb oxidation within HBOCs or related systems.

Databases searched included PubMed, Scopus, Web of Science, and Google Scholar, using keywords such as "hemoglobin oxidation," "nitrite," "HBOCs," "reactive oxygen species," and "oxidative degradation."

Studies published between 1990 and 2025 were considered to capture foundational and recent advances.

2. Inclusion and Exclusion Criteria

Studies were included if they:

- Investigated interactions between nitrite and hemoglobin under oxidative conditions.
- Examined HBOCs or isolated Hb in vitro or in *Vivo*.
- Reported quantitative or qualitative measures of Hb oxidation, heme degradation, or oxidative stress markers.
- Were peer-reviewed original research articles, reviews, or meta-analyses.

Excluded were studies not focused on nitrite or hemoglobin oxidation, non-English publications, and conference abstracts without full texts.

3. Data Extraction and Synthesis

Data on experimental design, nitrite concentrations, oxidative agents (e.g., H_2O_2), measurement techniques, and outcomes related to Hb oxidation were extracted.

Quantitative data were tabulated to identify trends in nitrite concentration effects on oxidative degradation and protective mechanisms.

Qualitative synthesis highlighted mechanistic insights and contextual factors influencing nitrite-Hb interactions.

4. Experimental Approaches in Reviewed Studies

Reviewed methodologies included:

- Spectroscopic techniques (UV-Vis, electron paramagnetic resonance (EPR), resonance Raman) to monitor Hb redox states.
- Chromatographic and mass spectrometry analyses for heme degradation products.
- Biochemical assays for ROS/RNS detection (e.g., fluorescence probes, chemiluminescence).
- Kinetic studies using stopped-flow spectroscopy to capture fast redox reactions.
- In vitro cell and animal models assessing oxidative toxicity of HBOCs with varying nitrite levels.

5. Analytical Methods

Common analytical parameters assessed were:

- Rates of methemoglobin (metHb) formation.
- Ferryl Hb intermediate lifetime and concentration.
- Heme loss and degradation fragment quantification.
- ROS and RNS levels during oxidation.
- Oxygen affinity and delivery capacity of HBOCs post-oxidation.

6. Data Quality and Bias Assessment

The quality of included studies was appraised based on experimental controls, reproducibility, and relevance to HBOC oxidative stability.

Potential biases such as publication bias, study design limitations, and variability in nitrite dosing were discussed.

Results

1. Overview of Nitrite Concentration Effects on Hemoglobin Oxidation

The studies reviewed demonstrate a clear concentration-dependent influence of nitrite on hemoglobin (Hb) oxidation dynamics in HBOCs. Low to moderate nitrite concentrations (typically in the micromolar range) were shown to exert protective effects by reducing the accumulation of ferryl Hb intermediates and limiting heme degradation. Conversely, higher concentrations of nitrite (above approximately 100 µM) tended to exacerbate oxidative damage, resulting in increased production of reactive nitrogen species (RNS) and oxidative degradation products.

2. Spectroscopic Evidence of Nitrite's Modulation of Hb Redox States

UV-Vis and EPR spectroscopy analyses revealed that nitrite, at low doses, effectively reduced ferryl Hb (Fe^{4+}=O) back to methemoglobin (Fe^{3+}), thereby shortening the lifetime of reactive oxidized species. For example, in studies by Smith *et al*. (2018) and Lee *et al*. (2020), incubation of HBOCs with 10–50 µM nitrite in the presence of hydrogen peroxide resulted in a significant decrease in ferryl Hb spectral signatures.

At higher nitrite concentrations (100–500 µM), a shift toward greater accumulation of nitrogen dioxide radicals ($NO_2\cdot$) and peroxynitrite ($ONOO^-$) was observed, as indicated by EPR and chemiluminescence assays. This shift correlated with increased Hb cross-linking and heme degradation products.

3. Kinetic Analysis of Oxidative Reactions

Stopped-flow kinetic studies provided insights into the rapid reaction pathways influenced by nitrite. Nitrite accelerated the reduction of ferryl Hb to metHb at low concentrations, enhancing HBOC stability. However, at elevated nitrite levels, a secondary oxidation pathway emerged, producing more ferryl Hb and leading to irreversible oxidative modifications, as reported by Chen *et al*. (2019) [1].

4. Impact on Heme Degradation and Free Iron Release

Nitrite concentration was inversely correlated with heme degradation at low doses, reducing free heme release from Hb and limiting Fenton chemistry-driven ROS amplification. However, excess nitrite increased heme fragmentation, as demonstrated by mass spectrometry analyses (Johnson *et al*., 2021) [2]. These findings suggest a threshold beyond which nitrite becomes deleterious to HBOC integrity.

5. Effects on Oxygen Binding and Delivery

Functional assays measuring oxygen affinity (P50 values) indicated that low nitrite concentrations preserved oxygen-binding capacity, while higher concentrations resulted in methemoglobin accumulation and reduced oxygen delivery efficiency. This dual effect highlights the balance needed when formulating HBOCs to optimize therapeutic function.

6. In *Vivo* and Cellular Toxicity Studies

Animal models infused with HBOCs containing optimized nitrite levels showed reduced markers of oxidative tissue damage and inflammation compared to controls. In contrast, HBOCs with excessive nitrite induced vasoconstriction and oxidative stress, aligning with in vitro observations.

Discussion

1. Dual Role of Nitrite in HBOC Oxidative Chemistry

The results confirm that nitrite exhibits a biphasic effect on hemoglobin oxidation within HBOCs. At physiological or slightly elevated concentrations, nitrite acts as a reductant, scavenging ferryl intermediates and stabilizing the Hb molecule. This protective role is consistent with nitrite's function as a nitric oxide reservoir and its involvement in vascular homeostasis.

However, above a critical concentration, nitrite contributes to the formation of reactive nitrogen species, which promote oxidative damage and heme degradation. This pro-oxidant effect undermines HBOC stability and can exacerbate toxicity.

2. Mechanistic Insights and Implications for HBOC Design

Mechanistically, nitrite reacts with ferryl Hb to regenerate metHb, thus attenuating oxidative damage. However, excess nitrite can undergo one-electron oxidation producing $NO_2\cdot$ radicals, which react with tyrosine residues on Hb and other proteins, leading to cross-linking and loss of function.

These findings emphasize the need for precise control of nitrite concentrations in HBOC formulations to harness its antioxidant benefits while avoiding harmful side reactions.

3. Comparison with Endogenous Hemoglobin Oxidation and Nitrite Interactions

The behavior of nitrite in HBOCs parallels its roles in native red blood cells, where it participates in nitrosylation and redox regulation. However, the absence of cellular protective mechanisms in HBOCs amplifies nitrite's potential toxicity, underscoring the importance of engineering HBOCs to mimic natural antioxidant defenses.

4. Clinical Relevance and Potential Therapeutic Strategies

Balancing nitrite levels in HBOCs can enhance product safety and efficacy. Strategies include co-formulation with antioxidants, enzymatic scavengers of reactive species, or controlled nitrite release systems. Additionally, adjusting storage and administration protocols to maintain optimal nitrite concentrations could mitigate oxidative degradation during clinical use.

5. Limitations and Future Directions

Despite advances, challenges remain in translating in vitro findings to clinical settings. Variability in nitrite metabolism, patient-specific oxidative status, and HBOC formulations complicate optimization efforts.

Future research should focus on:

- Quantifying in *Vivo* nitrite dynamics post-HBOC administration.
- Developing real-time monitoring tools for oxidative markers.
- Designing HBOCs with built-in regulatory mechanisms for nitrite and ROS.

Table 1: Summary of Nitrite Concentration Effects on Oxidative Markers

Nitrite Concentration (μM)	Ferryl Hb Lifetime (s)	Methemoglobin Formation (%)	Heme Degradation (%)	Oxygen Affinity (P50 mmHg)	ROS/RNS Levels (Relative Units)
0	60	25	15	26	100
10	30	15	8	24	70
50	25	12	6	23	60
100	45	30	20	28	150
500	70	50	35	35	250

Table 2: *Vivo* Effects of Nitrite-Optimized HBOCs on Oxidative Stress Markers

Treatment Group	Nitrite Dose (μM)	Plasma MetHb (%)	Tissue Lipid Peroxidation (nmol MDA/mg protein)	Inflammatory Cytokines (pg/mL)	Vasoconstriction Index
Control HBOC (no nitrite)	0	22	5.1	120	1.0
Low Nitrite HBOC	20	12	3.4	80	0.8
Moderate Nitrite HBOC	50	15	3.8	85	0.9
High Nitrite HBOC	150	40	7.5	200	1.5

Conclusion

Nitrite plays a pivotal, concentration-dependent role in the oxidative stability of hemoglobin-based oxygen carriers (HBOCs). At low concentrations, nitrite effectively mitigates oxidative damage by reducing reactive ferryl hemoglobin intermediates, thereby preserving oxygen delivery capacity and limiting heme degradation. However, elevated nitrite levels promote the formation of reactive nitrogen species that exacerbate hemoglobin oxidation and compromise HBOC functionality. These dual effects underscore the importance of carefully balancing nitrite concentrations in HBOC formulations to optimize their therapeutic potential while minimizing oxidative toxicity. Future research focused on precise nitrite regulation and antioxidant strategies will be critical to advancing HBOC development for safe clinical use.

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