Meat and Muscle Biology™

Short Communication

Effects of Light and Oxygen Conditions on Nonenzymatic Metmyoglobin Reduction *In Vitro*

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Abstract: Previous research indicated that inherently present electron donors and carriers contribute to nonenzymatic metmyoglobin reduction. However, limited knowledge is currently available on how light and oxygen influence nonenzymatic metmyoglobin reduction. The objective of this study was to evaluate the effect of light and oxygen levels on nonenzymatic metmyoglobin reduction *in vitro*. Two experiments were conducted using equine metmyoglobin solution at pH 5.6 (represents typical postmortem muscle pH) with different electron donors and electron carriers in a 96-well plate. A gas blend of 80% oxygen and 20% carbon dioxide or a pure 99.9% nitrogen gas was bubbled in phosphate buffers and myoglobin solutions to create 2 oxygen levels (low oxygen [4%] and high oxygen [160%]). The desired dissolved oxygen content was measured using a NeoFox oxygen probe (atmospheric oxygen level was 20% oxygen). There were significant effects of electron donors, carriers, and light on nonenzymatic metmyoglobin reduction. Ascorbate and nicotinamide adenine dinucleotide reduced form (NADH) in the presence of methylene blue and ethylenediaminetetraacetic acid (EDTA) exhibited a greater nonenzymatic metmyoglobin reduction in light than dark. An increase in nonenzymatic metmyoglobin-reducing activity ($P < 0.0001$) was observed for the combination of NADH + methylene blue + EDTA in the high-oxygen condition in comparison with the low-oxygen levels. The results indicate that light and oxygen levels increase nonenzymatic metmyoglobin reduction. Characterizing the cofactors required for nonenzymatic metmyoglobin reduction and understanding the conditions conducive to reduction enhance knowledge related to meat color changes.

Key words: meat color, nonenzymatic metmyoglobin reduction, myoglobin, cofactors
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Introduction

Oxymyoglobin imparts consumer-preferred bright-cherry red meat color, but discoloration of meat negatively impacts purchasing decisions and meat wastage (Ramanathan et al., 2022). Among the proteins primarily responsible for meat color, myoglobin determines the appearance of meat. Myoglobin is a monomer that is composed of 1 globin and 1 heme (King et al., 2023). The heme ring contains a centrally located iron that can form 6 bonds. Iron within the heme ring forms coordination bond with four pyrole nitrogens, the fifth site binds with histidine, and the sixth site is available to bind with ligands. The iron can exist in ferrous (Fe^{2+}) or ferric (Fe^{3+}) valence states. Oxymyoglobin is formed when the iron is ferrous and the ligand is oxygen. However, oxidation of the ferrous form results in metmyoglobin and discoloration of meat. Metmyoglobin-reducing activity is an inherent process that extends the color stability of meat during storage (Bekhit and Faustman, 2005; Mancini and Hunt, 2005; Ramanathan and Mancini, 2018). Metmyoglobin-reducing activity occurs via enzymatic, nonenzymatic, and mitochondria-mediated

metmyoglobin-reducing pathways (Brown and Snyder, 1969; Arihara et al., 1995; Tang et al., 2005; Elroy et al., 2015; Denzer et al., 2020). Hence, characterizing the metmyoglobin-reducing pathways is important for elucidating meat discoloration.

Brown and Snyder (1969) demonstrated that in a nonenzymatic metmyoglobin-reducing system, electron donors such as nicotinamide adenine dinucleotide reduced form (NADH) donate an electron, and an artificial electron carrier such as methylene blue will transfer an electron to metmyoglobin (no enzymes are involved in electron transfer). An electron carrier shuttles electrons between compounds based on the reduction potential. Recently, Denzer et al. (2020) compared the efficacy of electron donors such as NADH and ascorbate and electron carriers such as methylene blue and cytochrome *c* on nonenzymatic metmyoglobin-reducing activity *in vitro*. Their results suggest that the type of electron donor-carrier combination can influence nonenzymatic metmyoglobin-reducing activity. In addition, nonenzymatic metmyoglobin can occur at meat pH and storage temperature. The mechanistic basis for nonenzymatic metmyoglobin-reducing activity noted the role of free radicals. Interestingly, commonly present meat-processing conditions such as light or oxygen can promote free radicals. However, limited knowledge is available on the effects that common meat storage conditions such as light and oxygen levels have on reduction. Therefore, the objective of this study was to evaluate the impact of light settings and oxygen conditions on nonenzymatic metmyoglobin-reducing activity *in vitro*.

Materials and Methods

Materials and chemicals

Equine skeletal myoglobin shares 88.2% homology with bovine myoglobin (www.expasy.org) and has been used in various studies to understand metmyoglobin-reducing properties *in vitro*. Potassium phosphate dibasic (K_2HPO_4), potassium phosphate monobasic (KH_2PO_4), cytochrome *c* from bovine heart, methylene blue (Met Blue), sodium L-ascorbate, β-nicotinamide adenine dinucleotide reduced (NADH) dipotassium salt, and ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate were used in this study and purchased from Sigma-Aldrich (St. Louis, MO). Each chemical was of reagent grade or greater purity.

Myoglobin preparation

Equine metmyoglobin was prepared by dissolving freeze-dried myoglobin powder in phosphate buffer at pH 5.6 (representing the average postmortem muscle pH). Myoglobin concentration was determined by measuring the absorbance at 525 nm (isosbestic point for all forms of myoglobin) and dividing by 7.6 (the molar absorption coefficient of myoglobin; Tang et al., 2005). The initial level of metmyoglobin was determined using wavelength maxima at 503, 557, and 582 nm (Tang et al., 2005).

Nonenzymatic metmyoglobin reduction

The assay mixture contained physiologically present and artificial electron donors and carriers and metmyoglobin (Table 1). Cofactors are those compounds that are necessary for the catalysis of a reaction but go unchanged themselves by a reaction (Karp, 2000). The methodologies described by Brown and Snyder (1969) and Denzer et al. (2020) were utilized to determine nonenzymatic metmyoglobin reduction *in vitro*. The treatments contained different combinations of ascorbate (2 mM for methylene blue and 5 mM for cytochrome *c*), NADH (0.71 mM), cytochrome *c* (0.094 mM), methylene blue (0.025 mM), EDTA (0.71 mM), and 0.078 mM equine metmyoglobin in phosphate buffer (50 mM) in a clear bottom 96-well plate. NADH (0.71 mM) and ascorbate (2 or 5 mM) were added last to each treatment mixture to initiate the reaction at room temperature (23°C to 25°C). Denzer et al. (2020) noted that electron donors such as NADH and ascorbate could not be added in the same concentration to achieve adequate metmyoglobin reduction. Methylene blue and cytochrome *c* were electron carriers, whereas EDTA was a metal chelator. The absorbance at 582 nm using the kinetic option was measured using a Molecular Devices SpectraMax M3 Multi-mode microplate reader (San Jose, CA). Absorbance at 582 nm represents the wavelength maxima of oxymyoglobin (Tang et al., 2005). The nonenzymatic metmyoglobin-reducing activity for all treatments was determined during the linear phase of the reaction.

Table 1. Substrate combinations used to measure nonenzymatic metmyoglobin reduction

	Substrate combinations
1	NADH + Met Blue[1] + EDTA
2	NADH + Cyt-*c*[2] + EDTA
3	Ascorbate + Met Blue[1] + EDTA
4	Ascorbate Cyt-*c*[2] + EDTA

[1]Met Blue = methylene blue;

[2]Cyt-*c* = cytochrome *c*;

Substrate combinations were added to equine MetMb (0.08 mM).

Nonenzymatic metmyoglobin reduction involves simultaneous interaction of electron donors and carriers. In a separate experiment, changes in electron carriers (methylene blue) were determined spectrophotometrically. The reaction mixture contained NADH (0.71 mM), methylene blue (0.025 mM), EDTA (0.71 mM), and 0.06 mM equine metmyoglobin in phosphate buffer (50 mM). NADH (0.71 mM) was added last to initiate the reaction. The absorbance at 660 nm using the kinetic option was measured using a spectrophotometer. At 660 nm, methylene blue accepts an electron and becomes colorless. The micromolar change was calculated by dividing the change in absorbance by the extinction coefficient at 660 nm (71,547 $M^{-1} \cdot cm^{-1}$), 2.303, and absorbance.

Evaluation of the effect of light on nonenzymatic metmyoglobin-reducing activity

Light and dark conditions were created by placing a 96-well plate in an enclosed box with the only allowable light being sourced from a light-emitting diode bulb (870 to 1,090 lux) with readings taken every 5 min for 25 min using the Molecular Devices SpectraMax M3 Multi-mode microplate reader set to 582 nm. At the end of each 5-min interval, the plate was quickly removed and placed in the microplate reader to read the absorbance and then replaced under direct light. To evaluate the impact of dark storage, the 96-well plate was kept continually inside a closed spectrophotometer to avoid light.

Evaluation of the effect of oxygen on nonenzymatic metmyoglobin-reducing activity

Metmyoglobin dissolved in phosphate buffer (50 mM) at pH 5.6 was flushed with the high-oxygen gas blend (80% oxygen and 20% carbon dioxide; Stillwater Steel and Welding Supply, Stillwater, OK) for 10 min to increase oxygen content. Following bubbling, the buffer was kept covered and sealed to ensure a minimal change in oxygen content. A separate metmyoglobin dissolved phosphate buffer at pH 5.6 was flushed with 99.9% nitrogen gas for 15 min to create a low-oxygen condition (final oxygen level after bubbling was 4%). The dissolved oxygen content in low- and high-oxygen buffers was quantified using a NeoFox probe-type oxygen sensor (Ocean Optics, Orlando, FL). The NeoFox oxygen probe noted the oxygen content in the atmosphere was 20%.

Statistical analysis

The overall experimental design was completely randomized, with experiments 1 (objective 1) and 2 (objective 2) being replicated a total of 3 times ($n = 3$) with 6 technical replicates/subsamples per treatment (3 replications × 10 treatments × 6 subsamples per replicate = 180 observations). Each individual well within the plate was analyzed as an experimental unit. For the first objective, the fixed effects include dark (control) and light (treatment) storage. The second objective fixed effects included low (control) and high (treatment) oxygen atmospheres. For both objectives evaluated, fixed effects tests were performed using the MIXED procedure of SAS (v. 9.4, SAS Institute, Cary, NC). Least-squares means for protected F-tests ($P < 0.05$) were separated by using the *pdiff* option.

Results

Effects of light on nonenzymatic metmyoglobin reduction

There was a significant cofactor × light effect on the metmyoglobin reduction (Figure 1). The treatment

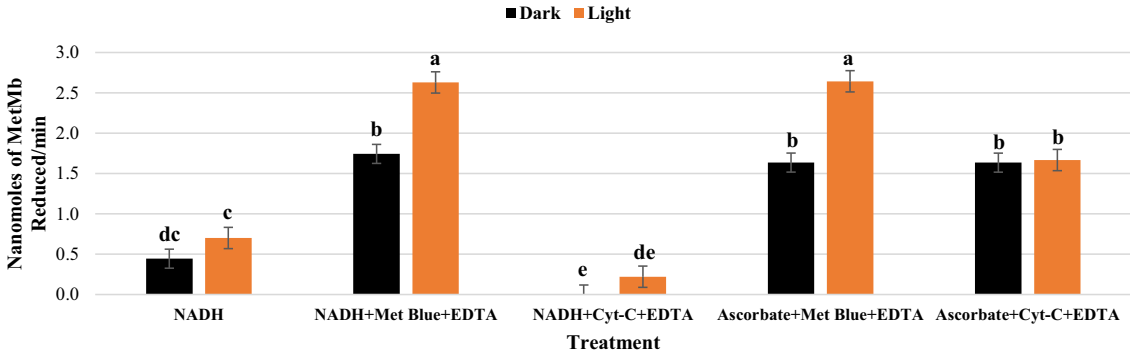

Figure 1. Least-squares means for each combination evaluating the effects of lighting conditions on nonenzymatic metmyoglobin reduction. Least-squares means with a different letter (a–e) are significantly different ($P < 0.05$). Standard error of mean = 0.13. Cyt-C= cytochrome *c*; Met Blue = methylene blue; MetMb = metmyoglobin. The main effects of light and dark were significant; dark = 1.1 and light = 1.6 nmol of metmyoglobin reduced per minute; $P < 0.05$, standard error = 0.13.

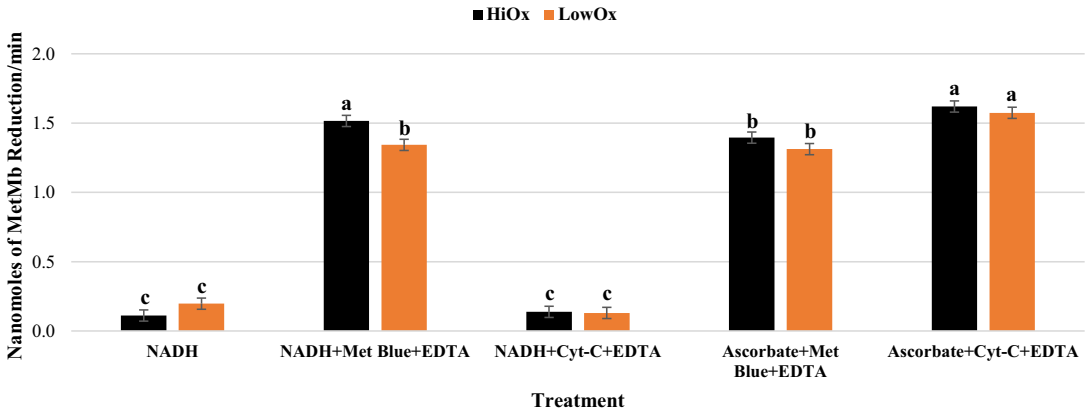

Figure 2. Least-squares means for each combination evaluating the effects of oxygen storage conditions on nonenzymatic metmyoglobin reduction. Least-squares means with a different letter (a–c) are significantly different ($P < 0.05$). Standard error of mean = 0.04. Cyt-C= cytochrome *c*; HiOx = high oxygen; LowOx = low oxygen; Met Blue = methylene blue; MetMb = metmyoglobin. The main effects of low and high-oxygen conditions were not significant for nonenzymatic metmyoglobin-reducing activity; low oxygen = 0.91 and high oxygen = 0.96 nmol of metmyoglobin reduced per minute; $P > 0.05$, standard error = 0.13.

containing NADH and metmyoglobin alone increased ($P < 0.0001$) metmyoglobin reduction in the presence of light more than dark. Metmyoglobin reduction in light for the combination of NADH and methylene blue was greater ($P < 0.0001$) than the same combination in dark conditions. Furthermore, in the presence of light, NADH + methylene blue + EDTA had an increase ($P < 0.0001$) in metmyoglobin reduction compared with dark. There was no significant difference observed in reduction when using methylene blue as an electron carrier coupled with either NADH or ascorbate as the electron donor in either light setting. The metmyoglobin reduction was limited in the presence of NADH + cytochrome *c* in both lighting conditions. Metmyoglobin reduction was significantly greater in the presence of cytochrome *c* and ascorbate compared with cytochrome *c* and NADH for light and dark conditions. In addition, metmyoglobin reduction increased ($P < 0.0001$) with light and the combination of ascorbate and methylene blue in comparison with dark conditions with ascorbate and methylene blue.

Effects of oxygen on nonenzymatic metmyoglobin reduction

The oxygen levels in reaction mixtures were 160% and 4% in high- and low-oxygen treatments, respectively. The percentage levels were relative to the amount of oxygen remaining in the solution following flushing (the atmospheric oxygen level was 20%). There was a significant cofactor by oxygen effect on the metmyoglobin reduction. An increase in nonenzymatic metmyoglobin-reducing activity ($P < 0.0001$) was observed for the combination of NADH + methylene blue + EDTA in a high-oxygen atmosphere in comparison with low oxygen. The metmyoglobin reduction was limited ($P > 0.05$) in the presence of NADH alone and within the combination of NADH + cytochrome *c* + EDTA for both oxygen atmospheres. Interestingly, when cytochrome *c* was used as an electron carrier, there was no ($P > 0.05$) oxygen level-specific effect.

Discussion

Metmyoglobin-reducing activity is an inherent biochemical process that occurs with the help of electron donors and carriers. Upon transfer of an electron from the donor, metmyoglobin with a ferric iron state reverts to a ferrous iron state after accepting an electron. However, nonenzymatic metmyoglobin reduction is a complex process that is more than a transfer of an electron to ferric heme. In the current study, light increased nonenzymatic metmyoglobin-reducing activity. Previous research noted that photocatalysis in the presence of flavin molecules contributes to nonenzymatic reduction (Brown and Snyder, 1969). Further light acted as a catalyst of reduction (Vernon, 1959), which could explain greater reduction in the present study. More specifically, we speculate that light projected onto myoglobin solution makes additional free radicals, creating more reduction opportunities.

Koizumi and Brown (1972) reported that metmyoglobin is nonenzymatically reduced in the presence of NADH under aerobic conditions. However, the addition of EDTA to NADH and metmyoglobin resulted in the greatest amount of reduction in aerobic conditions (Koizumi and Brown, 1972). These results align with the present study because the greatest differences were observed among the combination of myoglobin

with NADH + methylene blue + EDTA with the influence of the high-oxygen atmosphere rather than the low-oxygen condition. Furthermore, the combinations of ascorbate + methylene blue + EDTA and ascorbate + cytochrome *c* + EDTA resulted in numerically greater amounts of reduction under high-oxygen atmospheric conditions than low-oxygen conditions. Ascorbate is a free radical and oxygen scavenger commonly utilized in food products to inhibit or reverse oxidation (Rose and Bode, 1993). The nonenzymatic reduction involves the reciprocal involvement of electron donors and electron carriers. In the current research, methylene blue was used as an electron carrier, and its involvement in the reaction was confirmed with concurrent changes in the absorbance of methylene blue at 660 nm (0.4 micromole change per minute) during metmyoglobin reduction.

Both high-oxygen and light conditions favor free radical formation. Studies have shown that free radicals, such as superoxide ions, can donate an electron to ferric heme and form ferrous heme (Butler et al., 1982). A superoxide anion radical can transfer an electron to heme through amino acids close to the solvent-accessible heme edge (Butler et al., 1982). Nonenzymatic metmyoglobin is a reversible reaction and depends on cofactor concentration. Hence, reduced metmyoglobin might convert back to oxidized form, and limited research is available on the sustained reducing activity. Species specificity of myoglobin is another example of the effect of charge on nonenzymatic metmyoglobin reduction. Beef myoglobin has more nonenzymatic metmyoglobin reduction than porcine myoglobin (Elroy et al., 2015). More specifically, beef myoglobin has more histidine groups than porcine myoglobin; however, it is unknown whether histidine groups of beef myoglobin donate more electrons than histidine of porcine myoglobin (Nerimetla et al., 2017). Although several studies have demonstrated nonenzymatic metmyoglobin reduction *in vitro*, limited studies have shown its effectiveness *in situ*, which seems to be a future step to confirm the role of nonenzymatic metmyoglobin reduction in meat. Developing methodologies to quantify metmyoglobin reduction in meat will help to clarify the roles of enzymatic, mitochondria-mediated, and nonenzymatic pathways in meat color.

Conclusions

The current results suggest that light exposure and oxygen content increase nonenzymatic metmyoglobin reduction. However, the effects of light and oxygen levels depend on electron donors and carriers such as NADH and ascorbate. NADH, in combination with methylene blue and EDTA, increases nonenzymatic metmyoglobin reduction in the presence of light and oxygen compared with other electron donors and carriers. Therefore, optimizing the conditions for nonenzymatic metmyoglobin reduction has the potential to be utilized in ingredient-based or active packaging-based post-harvest processes to limit losses caused by meat discoloration.

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