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METMYOGLOBIN REDUCTION THROUGH LACTATE-NAD-LDH SYSTEM *IN VIVO* AND *IN VITRO*

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Introduction

Discoloration of meat surfaces due to brown metmyoglobin (MMb) formation significantly affects consumers' purchase decisions. Hood & Riordan (1973) reported that consumer discrimination against discolored meat was linearly correlated with increases in surface MMb. Renerre & Labas (1987) summarized that even at low levels of MMb, consumers begin to discriminate. Oxidized myoglobin can be converted to deoxymyoglobin (DMb) via metmyoglobin reducing activity (MRA). Then, it can be oxygenated back to oxymyoglobin (OMb). Ledward (1985) suggested that MRA is the most important intrinsic factor controlling the rate of metmyoglobin accumulation in beef. It is now well established that reduction of MMb occurs through both enzymatic and non-enzymatic reducing systems and that NADH is an ultimate reducing substrate for both pathways. However, where NADH comes from has not been established. Watts, Kendrick, Zipser, Hutchins, & Saleh (1966) hypothesized that since postrigor meat contains both lactate and lactate dehydrogenase (LDH), hydrogen may be transferred from lactate to nicotinamide adenine dinucleotide (NAD) by LDH. The reduction of NAD to NADH could be coupled with the reduction of MMb in the presence of intermediate electron carriers such as reductases, quinines, or methylene blue. Mancini, Kim, Hunt, & Lawrence (2004) further reported increased MRA and LDH activity of beef *longissimus* enhanced with 2% lactate. Therefore, we hypothesize that the lactate-NAD-LDH system is partially responsible for both (1) non-enzymatic and enzymatic MMb reduction of postmortem muscle and (2) the increased color life of lactate-enhanced beef. NADH may be replenished via the conversion of lactate to pyruvate by LDH, and the regeneration of NADH can be increased by the addition of substrates such as lactate and NAD.

Objectives

The objectives of this study were to: (1) examine the lactate-NAD-LDH system's ability to reduce MMb in a model system and (2) use an applied model to confirm the influence of lactate enhancement on intrinsic muscle biochemical traits related to color stability such as LDH enzyme activity, NADH contents, and metmyoglobin reducing activity (MRA) in enhanced strip loins.

Methodology

In two experiments, this study investigated the relationship between MMb reduction and the conversion of lactate and NAD to pyruvate with subsequent production of NADH via LDH.

Experiment 1: Lactate+NAD+LDH system in equine MMb model system

Assays of nonenzymatic MMb reduction were carried out at 22-23ºC in 10 mm path length cuvettes with 1.0 mL final reaction volume. The standard reaction mixtures at pH 8.0 contained: 0.3 mL of 0.5 mM equine MMb in 30 mM phosphate buffer pH 7.0, 0.1 mL of distilled water, 0.1 ml of 2.0 mM FMN, 0.1 mL of 6.5 mM NAD, 0.1 mL of 200 mM L-lactic acid with Tris 400 mM pH 8.0, 0.1 mL of 0.1 mM methylene blue, 0.1 mL of 50 mM citrate buffer, and 0.1 mL of LDH. The reaction was initiated by adding LDH to the mixture. Absorbance at 580 nm was recorded every 2 sec for 300 seconds. Nonenzymatic reducing activity was calculated as nanomole MMb reduced (equal to nanomole OMb formed) per min during the initial linear phase of the assay, using a difference in molar absorptive of 12000 l $mol^{-1}cm^{-1}$ at 580 nm (the wavelength at which the difference in absorption between MMb and OMb is maximal). Activity is expressed as the mean of triplicate samples. The effect of concentration of NADH, NAD, L-lactic acid and assay pH on the rate of MMb reduction was determined. The final pH of the assay was varied by altering the pH of the citrate and tris buffers. Oxalate or D-lactate (replaced L-lactate) was added to the mixture to investigate their inhibiting effects on LDH in the MMb reducing system.

Experiment 2: NAD+Lactate+LDH system in whole beef loin enhancement

Twelve USDA Select beef strip loins were divided into 4 equal-width sections. One of five treatments was assigned randomly to each loin section using an incomplete block design. Each loin section was enhanced 10% with aqueous solutions consisting of different combinations of lactate (1.5 or 2.5%), phosphate (0.3%), salt, and/or sodium acetate (0.1%). Steaks were packaged in 80% O_2 and 20% CO_2 and stored for 2 or 9 days and then displayed for 5 days at 1ºC. Visual and instrumental color were measured on d 2 and 9 to 14 and pH, metmyoglobin reducing activity (Hultquist, 1978), LDH activity (Vassault, 1983; Wahlefeld, 1983) in both directions (Lactate ↔ Pyruvate), and NADH (Klingenberg, 1974; McCormick & Lemuel, 1971) were measured on day 2, 9, and 14 (Fig. 1).

Results & Discussion

Experiment 1: Lactate+NAD+LDH system in equine MMb model system

All necessary constituents for generating NADH were mixed to test the reduction of horse MMb (Table 1).

Table 1. Nonenzymatic reduction of horse MMb with Lactate-LDH system in various mixtures[a] at 22ºC and pH 8.0

FMN	Methylene Blue	NAD[b]	L-lactic[c]	LDH	Oxalate[d]	D-lactic[c]	Activity (nmole/min)
+	+	+	+	+	-	-	0.69 ± 0.004
-	+	+	+	+	-	-	0.52 ± 0.012
+	-	+	+	+	-	-	0.17 ± 0.003
+	+	-	+	+	-	-	0.02 ± 0.004
+	+	+	-	+	-	-	0.03 ± 0.003
+	+	+	+	-	-	-	0.02 ± 0.000
+	+	+	+	+	+	-	0.40 ± 0.015
+	+	+	-	+	-	+	0.05 ± 0.002

[a]Substances present (+) or absent (-) in mixtures run in triplicate.
[b]4.5mM of NAD.
[c]200mM of L or D-lactic acid.
[d]200mM of oxalate.

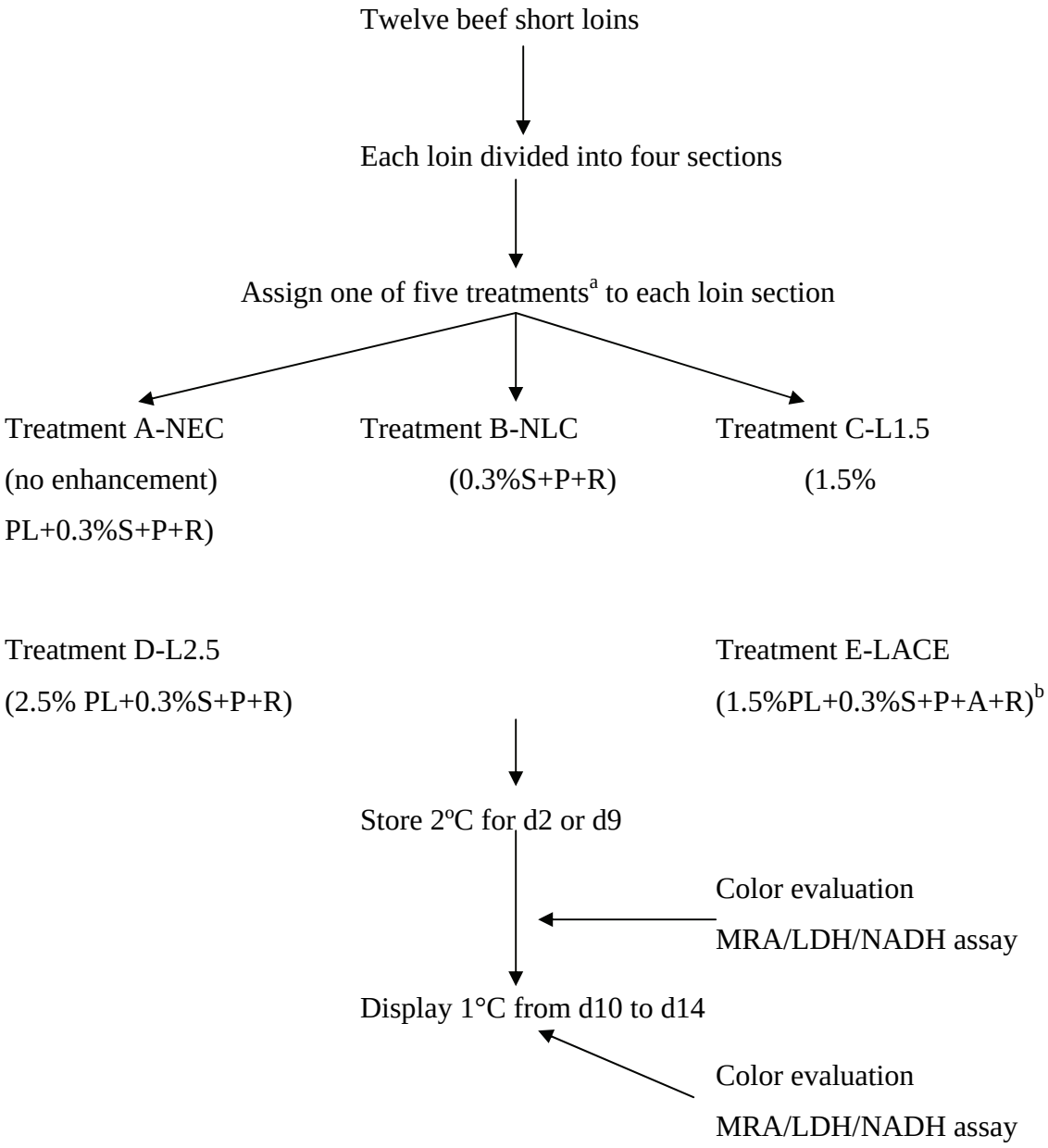

Figure 1. Flow diagram of experimental design.
[a]NEC: Non-enhanced control, NLC: No lactate control, L1.5: 1.5% lactate, L2.5: 2.5% lactate, LACE: lactate with acetate.
[b]S: Salt, P: Phosphate, PL: Potassium lactate, A: Acetate, R: Rosemary

The nonenzymatic reduction occurred effectively in the lactate-LDH system with NAD. Exclusion of any one necessary constituent (NAD, L-lactic acid, and LDH) reduced or eliminated reduction. Nonenzymatic reduction through the system increased to a level at 4.5 mM NAD in the reaction mixture (Fig. 2).

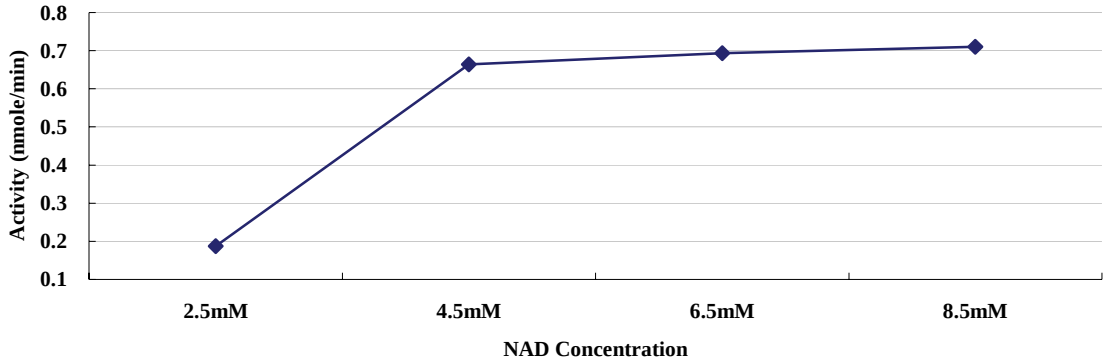

Figure 2. Effects of NAD concentration on horse MMb reduction at 22ºC and pH 8.0. All systems contained 200mM of L-lactic acid. Means are the average of 3 determinations. SEM=0.002 to 0.005.

Madhavi & Carpenter (1993) reported that NAD concentrations were directly related to meat color stability because NAD decreased rapidly in post-mortem muscle. They further reported lower NAD concentrations and less MRA in the *psoas major*, a less color stable muscle. Increasing the amount of L-lactic acid also resulted in greater MMb reduction (Fig. 3).

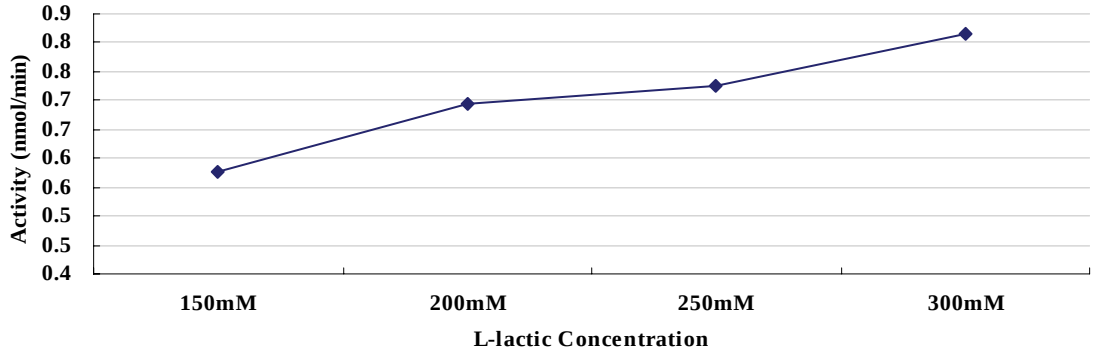

Figure 3. Effects of L-lactic acid concentration on horse MMb reduction at 22ºC and pH 8.0. All systems contained 6.5mM of NAD. Means are the average of 3 determinations. SEM=0.003 to 0.005.

Addition of oxalate, a known LDH inhibitor (Wahlefeld, 1983), to the reaction mixture tended to decrease reducing activity (Table 1). Replacing L-lactic acid with D-lactic acid in the assay mixtures reduced MMb reduction (Table 1), probably due to the selective interaction of LDH with L-lactic acid (D-lactic acid is not preferred for metabolism; Hall, 2000). As expected, the reduction reaction through lactate-NAD-LDH system was highly favorable in alkaline conditions (Fig. 4). The equilibrium of the LDH

reaction favors oxidation rather than reduction of NAD due to the acidic condition created by lactic acid (Vassault, 1983). However, although minimal, the nonenzymatic reduction through the lactate-NAD-LDH system still occurred at pH of 5.7, which is near the postmortem muscle pH.

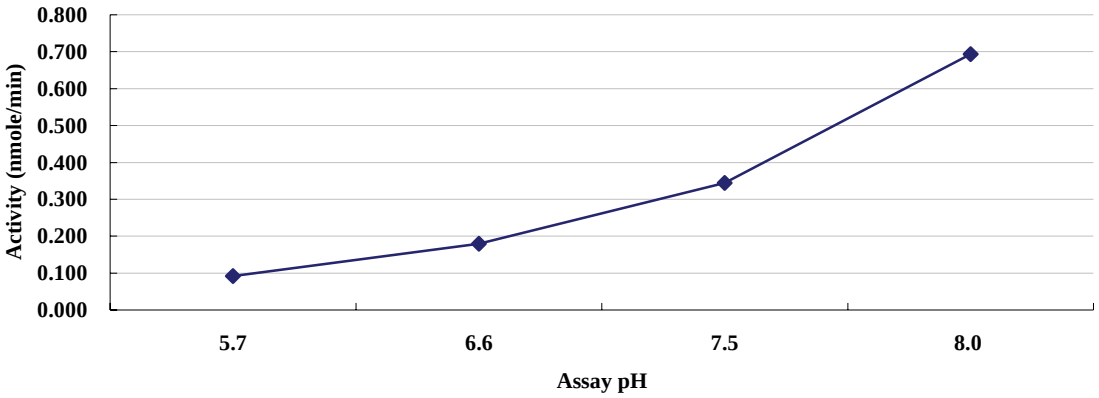

Figure 4. Effects of pH on horse MMb reduction at 22ºC.
All systems contained 200mM of L,-lactic acid and 6.5mM of NAD. Means the are average of 3 determinations. SEM=0.0001 to 0.0003.

Experiment 2: NAD+Lactate+LDH system in whole beef loin enhancement

Color, Color Stability and pH

Steaks enhanced with 2.5% lactate had the least visual discoloration, most color stability, and were most red (greatest a* values) at the end of display (Fig. 5). Non-enhanced controls and enhanced steaks without lactate were most discolored throughout display. Steaks treated with the combinations of lactate and acetate were less dark ($P < 0.05$) than steaks only treated with lactate. All enhanced meat samples had a slightly higher pH than non-enhanced controls at the end of display, likely due to the phosphate and/or lactate (Table 2).

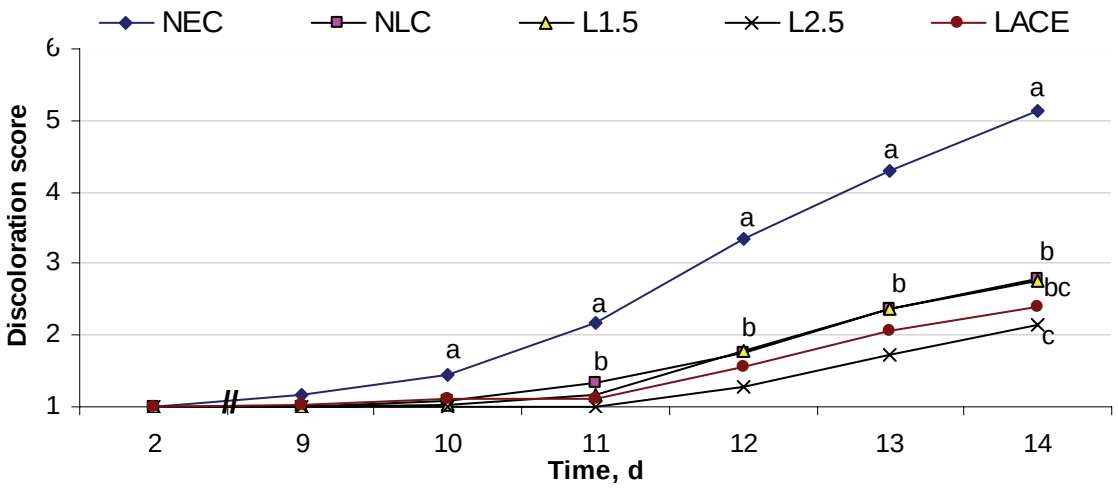

Figure 5. Effects of lactate enhancement on strip loin steak visual color. Discoloration evaluated as % MMb on the surface: 1 = no discoloration (0%), 2 = slight discoloration (1-19%), 3 = small discoloration (20-39%), 4 = modest discoloration (40-59%), 5 = modest discoloration (60-79%), 6 = extensive discoloration (80-99%), 7 = total discoloration (100%). [abc]Treatments within a display day with a similar letter do not differ (P > 0.05).

LDH Activity, NADH, and MRA

Enhancing steaks with 2.5% lactate significantly increased LDH activity in both directions (Lactate ↔ Pyruvate) compared to 1.5% lactate, no lactate controls, and non-enhanced controls on d 14 (Table 2). Consequently, increased LDH activity replenished more NADH, which was utilized for MMb reduction.

Table 2. Effects of lactate on pH, metmyoglobin reducing activity (MRA), lactate dehydrogenase (LDH 1 & 2) activity, and NADH content at d 14 of display

Treatment	pH	MRA[c]	LDH1[d]	LDH2[e]	NADH[f]
NEC (non-enhanced control)	5.83[a]	28.2[a]	145[a]	161[a]	1.0[a]
NLC (no lactate control)	5.94[b]	30.1[ab]	147[a]	162[a]	0.9[a]
L1.5 (lactate 1.5%)	5.94[b]	32.7[b]	151[a]	164[a]	1.1[ab]
L2.5 (lactate 2.5%)	5.93[b]	33.2[b]	172[b]	213[b]	1.4[b]
LACE (lactate + acetate)	5.90[b]	31.9[b]	161[ab]	182[a]	1.2[ab]

[ab]Least square means within a column with a similar letter do not differ (P > 0.05).
[c]Metmyoglobin: nmoles reduced/min/g of muscle.
[de]Lactate dehydrogenase activity: µmol/min/g sample.
[f]NADH: µg/ml.

NADH contents for steaks enhanced with 2.5% lactate increased during storage and display and were significantly greater than treatments without lactate on d14 compared to d2. Throughout storage and display, MRA decreased for all steaks. However, steaks

with 2.5% lactate enhancement retained more MRA compared with non-enhanced controls at the end of display. Increased MRA in 2.5% treated steaks was convincing evidence for increased NADH production via enhancement-stimulated LDH activity (Fig. 6).

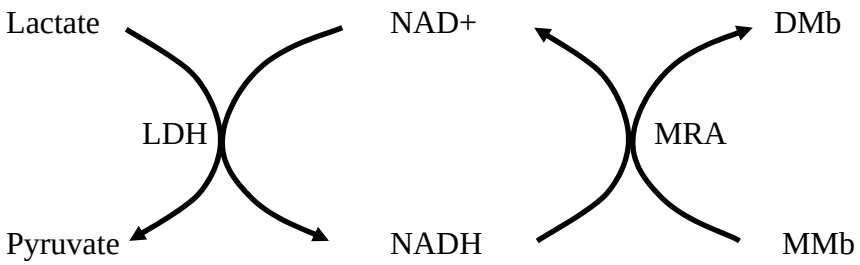

Figure 6. Proposed mechanism for lactate stabilization of meat color.

Conclusions

The results indicate that NADH can be regenerated through the lactate-NAD-LDH system *in vivo* and *in vitro.* Steaks enhanced with lactate had more color stability than steaks without lactate and were considerably more color stable than non-enhanced steaks. Lactate appears to promote color stability by the conversion of lactate to pyruvate via increased activity of lactate dehydrogenase and the concomitant regeneration of NADH. The NADH subsequently reduces metmyoglobin to either oxy- or deoxymyoglobin. The increased color stability due to lactate is accompanied by a darkening of muscle color. However, inclusion of acetate with lactate decreased muscle darkening and slightly improved color stability.

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