LACTATE DEHYDROGENASE REGULATION OF THE METMYOGLOBIN REDUCING SYSTEM TO IMPROVE COLOR STABILITY OF BOVINE MUSCLES THROUGH LACTATE ENHANCEMENT

A Dissertation

by

YUAN HWAN KIM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2008

Major Subject: Food Science and Technology

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Chair of Committee,	Jeffrey W. Savell
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ABSTRACT

Lactate Dehydrogenase Regulation of the Metmyoglobin Reducing System to Improve Color Stability of Bovine Muscles through Lactate Enhancement.

(August 2008)

Yuan Hwan Kim, B.S., Konkuk University;

M.S., Kansas State University

Chair of Advisory Committee: Dr. Jeffrey W. Savell

The primary objectives of this research were to characterize the involvement of lactate dehydrogenase (LDH) in color stability of physiologically different bovine muscles, and to investigate the influence of lactate enhancement on the myoglobin redox state of bovine muscles. In experiment 1, three different bovine muscles; *Longissimus lumborum* (LD), *Semimembranosus* (SM), *Psoas major* (PM) were (n=7 respectively) cut into steaks, and displayed for 7 days. Instrumental color, LDH-B, LDH isozyme expression, and NADH were measured. In experiment 2, strip steaks (n=8) were cut into half, and one side was injected with oxamate (LDH inhibitor), and the other was injected with water. Surface color, LDH, and NADH were measured after 10 days. In experiment 3, the three bovine muscles (n=10) were enhanced with solutions containing lactate and/or phosphate. Steaks were stored and displayed for 14 days. Instrumental color, LDH-B, total reducing activity (TRA), and NADH were measured. In experiment 4, fifteen beef strip loins were divided individually into four equal sections, and one of

six treatments containing phosphate and/or calcium lactate with or without irradiation (2.4 kGy) randomly assigned to each loin section (n=10). Steaks were packaged in high-oxygen modified atmosphere package, irradiated, stored in the dark at 1°C for 14 days. Instrumental color, TRA, lipid oxidation, and NADH were measured.

LD remained the most red, whereas PM was most discolored. LD had a significantly higher level of LDH-1 responsible for LDH-B activity as compared to SM and PM. Consequently, LD had a higher LDH-B, and more NADH ($p < 0.05$). Inclusion of oxamate inhibited LDH-B, decreased NADH, and consequently discolored more. Potassium lactate enhancement led to more NADH through elevated LDH flux and subsequently increased ($p < 0.05$) color stability of LD and PM throughout display. Loins with calcium lactate/phosphate maintained the most stable red color during display. Calcium lactate/phosphate in loins increased NADH concentration, TRA, and were the least oxidized over display. These results confirm the involvement of LDH in meat color stability through replenishment of NADH. Lactate enhancement promotes meat color stability by providing superior antioxidant capacity and increased reducing activity of myoglobin by elevating NADH concentration.

DEDICATION

This dissertation is dedicated to my wife, Jeongmin, who changed my life and made me want to be a better man.

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This might be the most exciting part throughout the entire chapters of my dissertation. I truly want to glorify God, my saver, my Lord, and my best friend, who has already made the perfect plan for my life, and who has given me the strength and wisdom to accomplish such a task as this. And I want to thank to my parents for their endless support and patience. Without your love and prayers, this could not have been done. Mom and Dad from Seoul and Daegu, you are truly co-authors of this dissertation!! I thank you so much.

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"He knows the way that I take; when he has tested me, I will come forth as gold. Job 23:10"

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CHAPTER I

INTRODUCTION

Maintaining bright red color during retail display time is vital in meat industry for maximizing attracting consumers' purchase decision, because the appearance of meat is the first factor that consumer determines meat freshness. It has been reported that shoppers' discrimination increased with increasing brownish-red color due to the formation of metmyoglobin ($MbFe^{3+}$) on the meat surface (*1*). Renerre and Labas (*2*) noted that at even low levels of metmyoglobin, consumers begin to discriminate. In addition, when 20% of the total myoglobin pigment is in the metmyoglobin form, the ratio of sales of discolored beef to bright red beef is about 1:2. The oxidized form of brown metmyoglobin can be converted to the purplish reduced deoxymyoglobin ($MbFe^{2+}$) through the metmyoglobin reducing system of muscle. Then, it is immediately oxygenated back to red oxymyoglobin ($MbFe^{2+}O_2$). It is now generally accepted that the metmyoglobin reduction in meat occurs primarily through enzymatic or non-enzymatic pathways with NADH as a coenzyme (*3*). NADH is the ultimate source of reducing equivalents, and increasing concentrations of NADH have been shown to result in more reducing activity (*2, 4-7*). Although the general mechanism of the metmyoglobin reduction system per se is well established, the origin of the pool of NADH has not been clearly established.

This dissertation follows the style of the *Journal of Agricultural and Food Chemistry*.

Watts et al. (*8*) originally proposed that lactate dehydrogenase (LDH) can catalyze the replenishment of NADH from lactate and NAD^+, and that the subsequent NADH could be utilized in the metmyoglobin reduction system. However, this hypothesis had not been tested until an *in vitro* study was performed by Kim et al. (*9*). They determined that nonenzymatic metmyoglobin reduction occurred in the lactate-LDH system with NAD^+, but that exclusion of NAD^+, L-lactic acid, or LDH eliminated the MMb reduction. They consequently proposed that the lactate-LDH system in post-mortem muscle can generate NADH by the reduction of NAD^+, and that a NADH-dependent reducing system, either enzymatic or non-enzymatic, can reduce metmyoglobin. It has been reported that different LDH activities depend on the dominant muscle fiber type in individual muscles, which implies different utilization rates of NADH (*10*). Therefore, it can be hypothesized that the variation in color stability of physiologically different muscles could be regulated by the different rates of replenishment of NADH via different LDH isozymes. Further, it also can be hypothesized that meat color stability could be enhanced through an external substrate inclusion, which might alter biochemical reaction of a specific substrate-enzyme coupling resulting in prolonging the reduced state of myoglobin.

The primary objectives of this research were to characterize the involvement of LDH isoenzymes in color stability of physiologically different bovine muscles, and to investigate influence of lactate enhancement on myoglobin redox state of bovine muscles packaged in a high-oxygen modified atmosphere packaging (MAP) system.

CHAPTER II

REVIEW OF LITERATURE

Myoglobin

Myoglobin is the primary intercellular protein responsible for meat color with minimal contribution from hemoglobin and cytochrome c. Most of the muscle hemoglobin is removed during exsanguination, but still constitutes between 6% and 16% of the total fresh meat pigments (*11, 12*). Myoglobin is a monomeric water soluble protein that has a high binding capacity to oxygen, and allows the exchange of O_2 and CO_2 within living muscle cell (hemoglobin has four subunits that carry O_2 and CO_2 in the blood system). Myoglobin consists of a single polypeptide protein or globin molecule, and the prosthetic heme group (**Figure 2.1**). The heme iron located in the center of the porphyrin ring can form six bonds. Four of these bonds are in the plane with pyrrole nitrogens, while the other two coordination sites connect perpendicular to this plane (*13*). The fifth coordinate is with the proximal histidine-93. The sixth position is referred to a free binding site opened for the formation of complexes with several compounds, such as oxygen, carbon monoxide, carbon dioxide, water, nitrous oxide or other ligands. Thus, various binding formations at the sixth position are responsible for differences in the color of meat. The globin portion of the molecules is a long protein containing a "pocket," which protects the heme iron group from oxidation (*14*). Therefore, denaturation of the globin moiety due to the high temperature and/or low pH can increase oxidation of myoglobin.

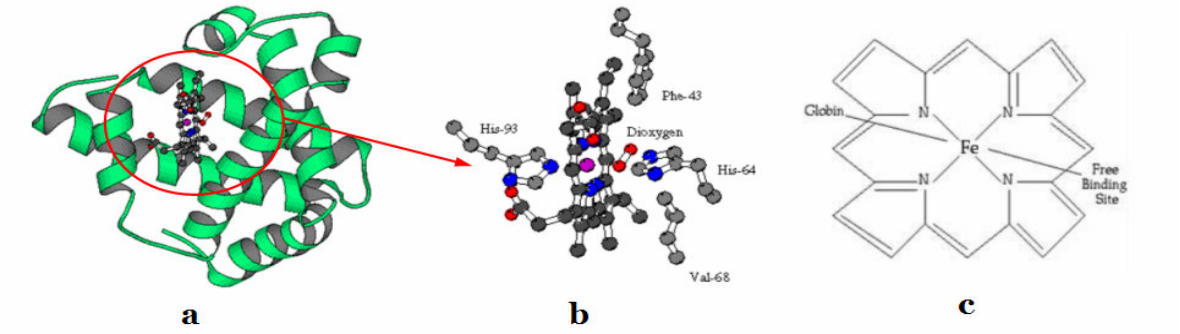

Figure 2.1. Myoglobin structure.
a. Ribbon representation of the myoglobin structure (http://metallo.scripps.edu/PROMISE/1MBO.html).
b. Heme-Oxygen plus proximal and distal residues.
c. Simplified structural representation of myoglobin.

The color of meat depends upon the nature of the ligands attached to the 6th binding site of the heme portion of myoglobin and the oxidation state of the iron molecule in the heme matrix (*15*). Freshly cut meat has native meat pigment form called deoxymyoglobin (DMb). Deoxymyoglobin (DMb), an oxygenated or reduced form of myoglobin, has a high speed ferrous (Fe^{++}) iron with no occupancy at the sixth ligand. DMb has a purplish-red color (usually seen in vacuum packaged meat) requiring very low oxygen pressure (less than 1.4 mm) for its presence (*16*). When DMb is oxygenated (oxygen binds to the sixth ligand), a bright-red color pigment designated as Oxymyoglobin (OMb) is formed. The heme iron state is still ferrous although the sixth coordination is now occupied by diatomic oxygen. Further, the distal histidine-64 interacts with bound oxygen resulting in the alteration of myoglobin structures and stability. Oxygenation, also called "bloom" in the meat industry, occurs within minutes

of exposure to air under partial pressure of oxygen greater than 25 mm Hg (*16*). The meat industry wants to hold this oxygenated state of myoglobin as long as possible to extend display time and keep meats attracting to consumers. After blooming, the outer layer of fresh cut muscle forms the bright red color of OMb, while the deepest layer extending to the center or to the bottom of the meat, retaining its purplish-red state of DMb due to the absence of oxygen or very low oxygen partial pressure. Depth of oxygen penetration and thickness of OMb layer depend upon extrinsic factors such as temperature, oxygen pressure, and storage condition, and intrinsic factors such as pH, and competition for oxygen by other respiratory processes (*15*).

Deoxygenation of the heme molecule resulting from the denaturation of the globin moiety of OMb will convert OMb to DMb, which then becomes oxidized to a brown metmyoglobin pigment form (*17*). The denaturation of the globin moiety due to extrinsic conditions, such as low pH, high temperature, ultra-violet light, and particularly low oxygen tension causes it to lose its biological function of protecting the heme from undesirable reactions (*18*). MMb is the major pigment found in discolored meat, which is an undesirable from the consumer's perception. Hood and Riordan (*1*) reported that consumers discriminate against discolored meat linearly with a corresponding increase in metmyoglobin formation. Renerre and Labas (*2*) noted that even at low levels of metmyoglobin, consumers begin to discriminate. MMb is normally formed at low oxygen partial pressure between 1.4 and 25 mm Hg (*16*). Therefore, when low oxygen tensions are present, a thin third layer of pigment, MMb, forms between the OMb and DMb layers (**Figure 2.2**). This intermediate myoglobin layer (MMb) becomes thicker

and moves toward surface with time, while the OMb layer becomes thinner and causes more surface discoloration. After the formation of MMb, further oxidative conditions in myoglobin induced by enzymes and bacteria cause meat spoilage and produce a series of brown, green, and faded appearing compounds.

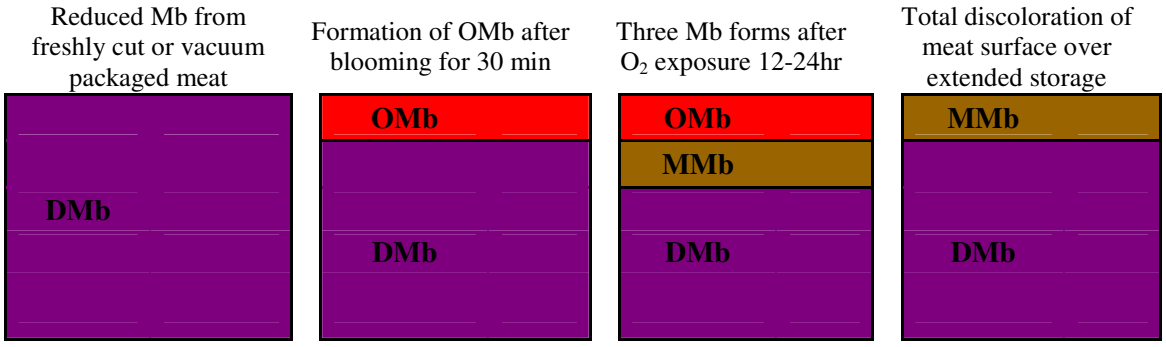

Figure 2.2. Illustration of DMb, OMb, and MMb formation in a perpendicular cut of meat depending upon the oxygen availability and display time.

Under normal display conditions, there is a mixture of OMb, DMb, or MMb. These three forms of myoglobin are interchangeable for a limited amount of time depending upon several intrinsic/extrinsic factors.

Intrinsic Factors Affecting Meat Color Stability

pH. The ultimate pH of muscle and the rate of pH decline postmortem have significant effects on meat color and discoloration. Muscle pigment forms are highly influenced by various muscle pH conditions. During the conversion of muscle to meat, the accumulation of lactic acid from anaerobic glycolysis lowers muscle pH from around 7 (living muscle) to 5.7 (ultimate pH). When the muscle pH drops far below the normal postmortem pH, it denatures the globin portion of the heme pocket and causes subsequent disassociation of oxygen from the heme as well as oxidation of the heme iron.

Hence, the incidence of heme iron oxidation is increased. The rate of autoxidation increases with continuous exposure to low pH (*16*). Shikama (*19*) reported that the half-life for OMb in buffer solutions to that of MMb at 35 °C is 3.3 days at pH 9.0, 11 h at pH 7.0 and less than 30 min at pH 5.0.

Further, the lower pH also causes protein denaturation, which reduces the water-binding capacity of muscle. Consequently, free-water molecules migrate to the meat surface, reflect light, and thus present much lighter color. It also has been reported that low pH reduces metmyoglobin reducing activity (MRA) of muscle (*20, 21*). On the other hand, high pH meat appears darker because it retains more water due to high water-binding capacity of the myofibrillar proteins and absorbs light resulting in a darker color. Further, oxygen-scavenging enzymes of muscle are more active and have longer survival at high pH. Thus, they compete against myoglobin for oxygen usage resulting in DMb formation rather than OMb (*22*). Consequently, oxygen penetration to muscle is weakened, and a darker color results.

Myoglobin concentration – age, species, sex, muscle type and location. Myoglobin concentration plays an important role in affecting the total meat pigments. Since myoglobin contains heme iron, it is rather susceptible to oxidation, and contributes to a darker color formation (*23*). As animals get older, the myoglobin content increases, but the affinity of oxygen for myoglobin decreases, and thus, they need to synthesize more myoglobin to store oxygen. Therefore, meat from older animals appears much darker in color due to the higher myoglobin concentration. Further, depending on muscle fiber type (slow twitch vs. fast twitch), myoglobin concentration is also different.

Red muscle (type I – slow twitch) has more oxidative metabolic activity, so it contains a higher myoglobin concentration as compared to white fibers (type II - fast twitch; glycolytic metabolism). Thus, red muscle is redder/darker in color (*24*). Following the same concept, a locomotive muscle appears redder/darker color than a supportive muscle. A locomotive muscle experiences more extensive muscle movements and requires more myoglobin concentration for oxidative metabolism (*25*). The myoglobin content also varies within different species. For example, a whale has a higher myoglobin concentration and is darker color followed by cattle, lamb, hogs, and poultry in decreasing order of darkness (*26*). Most sea animals including whales have relatively high myoglobin contents in their skeletal muscle tissue so as to store more oxygen during prolonged periods of diving. For a chicken, there is an apparent color difference between thigh (red muscle) and breast (white muscle) due to different myoglobin concentration.

Metmyoglobin reducing activity (MRA). Muscle has a limited ability to convert from the oxidized ferric state to the reduced ferrous state through its endogenous reducing activity called metmyoglobin reducing activity (MRA).

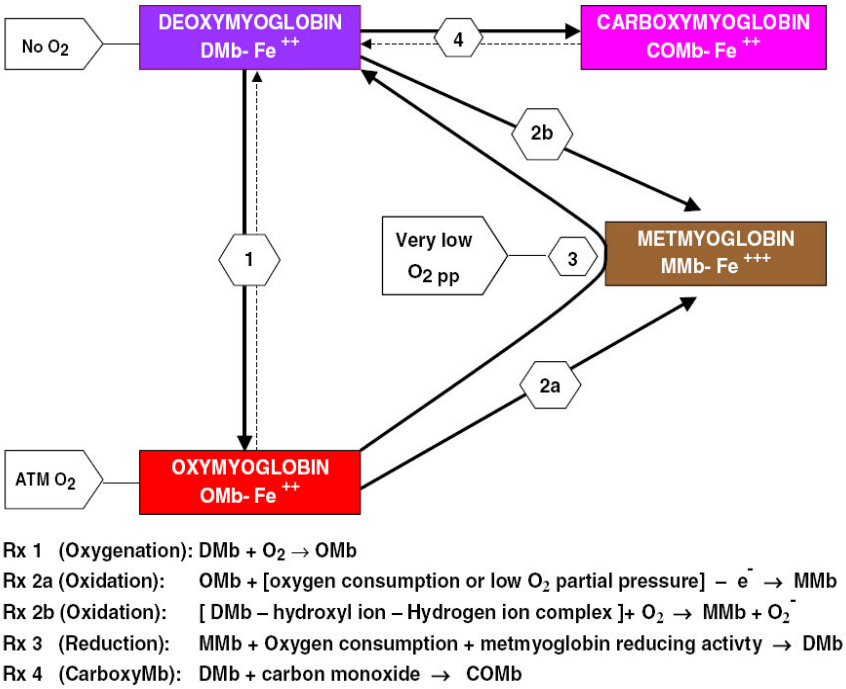

Figure 2.3. Visible myoglobin redox interconversions on the surface of meat (27).

With increased storage time, MRA gradually decreases and subsequently the color stability of muscle decreases. However, it is controversial among researchers whether more MRA in a muscle translates into greater color stability. Ledward (*28*) suggested that oxygen consumption rate (OCR) and MRA are the main factors affecting the rate of MMb formation at the surface of beef muscle, and MRA is the most important intrinsic factor controlling the rate of MMb accumulation in beef. It has also been found that different MRAs of muscles were highly related to differences in muscle color stability. Reddy and Carpenter (*29*) also supported the concept that the greater the MRA between muscles from the same animal the greater the color stability. In contrast, Renerre and Labas (*2*) and Echevarne et al. (*30*) claimed that MRA had no correlation to

color stability of various muscles when using muscle homogenates with methylene blue as electron-transfer mediator. They found that beef muscle of less color stability had highest OCR and the highest MRA. O'Keefe and Hood (*23*) concluded that OCR would be the most significant factor influencing color stability of muscles and MRA was of little consequence to meat color stability. The conflict between studies regarding MRA and meat color stability may be due to the lack of uniform of MRA measurement techniques.

Enzymatic reduction of metmyoglobin. It is now generally accepted that metmyoglobin reduction in meat is primarily through enzymatic pathways with NADH as a coenzyme (*3*). "Metmyoglobin reductase" often has been used for indicating all metmyoglobin reducing enzymes or the enzymes related to the metmyoglobin reducing system (*31-33*). The wide use of their terminology (MRA) appears to have contributed to the confusion of MMb reduction in many experiments. Without demonstrating or targeting a specific reductase, many researchers just adopted general MRA techniques, which might not be appropriate for measuring the enzymatic MMb reducing system.

To date, the major enzymes known to be related to the muscle pigment reduction are metmyoglobin reductase (muscle NADH-cytochrome b_5 reductase), methemoglobin reductase (erythrocyte NADH-cytochrome b_5 reductase), diaphorase, and cytochrome *c* reductase. The system of methemoglobin (MHb) reduction *in vivo* is well known. Under physiological conditions, less than 0.5% of Hb is in the Met-form in human erythrocytes (*34*). In addition, the accumulation of MHb was found in MHb reductase-deficient erythrocytes. Several MHb reducing enzymes have been described and thus

prevent MHb accumulation to any appreciable content. MHb reductase is variously described as NAD(P)H dehydrogenase or diaphorase utilizing reduced pyridine nucleotide as an immediate source of reducing equivalents. In model systems, there has generally been a requirement for a nonphysiological intermediate, such as methylene blue or 2,6-dichlorophenolindophenol, between the NAD(P)H-linked reductase preparations and ferrihemoprotein. Hegesh and Avron (*35*) reported that the most active system for reducing MHb directly required ferrocyanide activation.

Passion et al. (*36*) reported a MHb reducing system in human erythrocytes to consist of a soluble cytochrome b_5 hemoprotein and a soluble NADH-linked cytochrome b_5 reductase flavoprotein. Cytochrome b_5 is the physiological intermediate which directly reduces ferrihemoglobin. The cytochrome b_5 system appears to be rather ubiquitous among mammalian tissues (*6*). In liver cells, it is bound within the membranes of the endoplasmic reticulum and of the mitochondria, and it is present in solubilized form in erythrocytes. Kuma et al. (*37*) found that an antibody aginst NADH-cytochrome b_5 reductase from rat liver microsomes will interact with the MHb reductase from human erythrocytes by inhibiting the rate of erythrocyte cytochrome b_5 reduction by NADH. They also indicated that the soluble MHb reductase and cytochrome b_5 of human erythrocytes are immunochemically similar to NADH- cytochrome b_5 reductase and cytochrome b_5 of liver microsomes, respectively, except that no hydrophobic tail is present. Thus, MHb reductase is often referred as "erythrocyte NADH-cytochrome b_5 reductase."

Hemoglobin and myoglobin have a number of similar properties, including the capacity of reversible oxygenation to form OHb or OMb, or irreversible oxidation to MHb or MMb, respectively (*38*). Mb has greater susceptibility to oxidation than Hb, but it is generally accepted that MMb does not accumulate in healthy living muscle. However, it can be assumed that Mb in living tissue is continually being oxidized and reduced similar to Hb. In contrast to the Hb, the reduction system of MMb *in vivo* has not been fully understood. Because Hb still constitutes 10 to 30% of the total heme pigment in muscle (*12*), the MHb reductase may play a certain role in the reducing system of MMb in muscle.

The natural MMb reducing system in fresh meat was firstly recognized by observing that the loss of red color of fresh meat under vacuum packaging was restored subsequently during aerobic storage (*39*). Stewart et al. (*40*) determined enzymatic reduction of MMb by oxidizing ground beef with ferricyanide, followed by measurement of MMb reduction at 7 °C by the meat for 1 hour. They used the term "metmyoglobin reducing activity" to represent the reduction, because more than one enzyme appeared to be involved in the reducing system. They found that MRA increased with pH from 5.1 to 7.1 and with temperature from 3 to 35 °C. They also reported a significant relationship between total pigment concentration and tissue reducing activity. O'Keeffe and Hood (*23*) reported low myoglobin content in *M. psoas major* compared to *M. longissimus dorsi*, which has high myoglobin content, and concluded that more highly pigmented muscles have higher MRA. Although Stewart et

al. (*40*) described a method for estimating enzymatic reducing activity of MMb, they did not investigate the enzymatic reducing pathways or purify a MMb reductase.

Shimizu and Matsuura (*33*) purified "MMb reductase enzymes" from blue-white dolphin muscle, which was found to be similar in several respects to their "MHb reductase" isolated from dolphin erythrocytes. There were no significant differences in the absorption curve and enzyme properties between the muscle and erythrocyte enzymes. They both strongly reduce oxidized pigments such as metmyoglobin, ferric cytochrome c and methemoglobin in the presence of NADH and methylene blue. However, they also found several distinguishable points between muscle enzyme and erythrocytes for sedimentation and diffusion coefficients, pH-mobility curve, electrophoretic mobility, and the Michaelis constant (*Km*). Hence, they concluded that the muscle enzyme from blue-white dolphin was not the MHb reductase.

MMb reductase also was isolated from blue-fin tuna (*31*) and yellow-fin tuna (*41*). Enzyme activity was greatest at pH 6.5 and at 33 to 35 °C, and was highly NADH dependent. Hagler et al. (*38*) purified and characterized the first bovine MMb reductase from heart muscle. The enzyme was NADH-dependent and required the ferrocyanide ion for *in vitro* reduction of MMb. They also found that reduction rates of enzymes were far greater than any previously reported for nonspecific and nonenzymatic systems. Demonstration of enzymatic activity was dependent on a suitable myoglobin substrate, NADH, and ferrocyanide. Artificial electron carriers such as DCPIP and methylene blue were not required. They determined that an equimolar amount of cytochrome b_5 was more effective than ferrocyanide in the enzymatic reduction of MMb. They also

reported that the characteristics of the enzymatic activity and the assay system were similar to erythrocyte MHb reductase described by Hegesh et al. (*35*).

Livingston et al. (*42*) further classified a MMb reductase from bovine heart muscle as NADH-cytochrome b_5 reductase from the kinetic mechanism of the enzymic reduction. They found that MMb reduction by MMb reductase requires cytochrome b_5 as electron-transfer mediator, and the reduction rate is proportional to the cytochrome b_5 concentration. Unlike other enzymes or enzyme systems, NADH-cytochrome b_5 reductase can reduce MMb without artificial mediator or activator, such as methylene blue or ferrocyanide. Cytochrome b_5 reductase is a flavoprotein that reduces cytochrome b_5 by transferring electrons to it from NADH and subsequently, the reduced cytochrome b_5 then reduces ferric myoglobin to ferrous myoglobin.

Faustman et al. (*43*) also partially purified a MMb reductase from bovine liver and cardiac muscle. They reported that greater substrate reduction occurred at pH 6.3 versus 7.0 or 7.3 and at 37.5 °C compared to 22 °C using either partially purified cytochrome b_5 or potassium ferrocyanide as reaction mediator (**Table 2.1**). NADH was the ultimate source of reducing equivalents for all assay conditions. In the absence of MMb reductase preparation, the cytochrome b_5 preparation was as effective in reducing MMb at 22 °C and more effective at 37.5 °C. On the other hand, potassium ferrocyanide with NADH did not show reducing activity in the absence of the reductase preparation. It is possible that potassium ferrocyanide facilitates MMb reduction in a manner similar to its role in MHb reduction (*35, 38*). The ferrocyanide activates the reduction by

binding to the heme group protein in such a way as to make the heme group accessible to the MMb reductase (*43*).

Table 2.1. Initial velocities of MMb reduction at various assay condition

Assay Condition				Initial Velocity (nmol/min)	
MMb reductase	Cyt b5	$K_4Fe(CN)_6$	NADH	22℃	37.5℃
+	+	-	+	9.9[a]	17.4[a]
+	-	+	+	8.6[a]	14.7[b]
-	+	-	+	10.2[a]	20.2[c]
-	-	+	+	0.0[b]	0.0[d]
-	-	-	+	0.6[b]	0.3[d]

[a-d]Mean values in the same column with different superscripts differ significantly ($P<0.05$)

MMb reductase enzymes have been purified from bovine skeletal muscle to homogeneity and characterized (*29, 32*). The enzyme had a pH optimum of 6.5, isoelectric pH between 5.6-5.8, and molecular weight of 33,000 daltons. The enzyme was NADH-dependent and reduced MMb in the presence of either ferrocyanide or cytochrome b_5. Arihara et al. (*32*) found that the enzyme were flavoproteins and was immunologically indistinguishable from the NADH-cytochrome b_5 reductase of bovine erythrocytes (**Table 2.2**). Therefore, they regarded bovine skeletal muscle MMb reductase as "muscle NADH-cytochrome b_5 reductase."

Table 2.2. General properties of NADH-cytochrome b_5 reductase from bovine erythrocytes and metmyoglobin reductase from bovine muscles (32)

Source	Molecular weight	pI	pH optimum	Km for cytochrome b_5 (M)
Erythrocytes	33,000	5.7-5.9	6.5	4.5×10^{-8}
Skeletal muscle	33,000	5.7-5.9	6.5	3.2×10^{-8}
Heart muscle	32,000	5.6-5.9	6.5	

Further, the localization of the MMb reducing enzyme system components such as NADH-cytochrome b_5 reductase, cytochrome b_5, and outer mitochondrial membrane cytochrome *b* in bovine skeletal muscle was determined by Arihara et al. (*44*). NADH-cytochrome b_5 reductase was found predominantly in the mitochondrial fraction, but also was detected at low levels in the microsomal fraction. Outer mitochondrial (OM) cytochrome *b* was found mostly in the mitochondria, whereas cytochrome b_5, was detected in the microsomal fraction, which were principally the sarcoplasmic reticulum. Bekhit et al. (*5*) found NADH-dependent MMb reducing activity in the particulate fraction of meat following sedimentation of a meat homogenate at 35,000 x*g*. They also reported that there was 5.8 times more MMb reducing activity in the particulate fraction compared with that of the sarcoplasmic (supernatant) fraction. Therefore, NADH-cytochrome b_5 reductase mainly reduces MMb by using outer mitochondrial cytochrome b_5 and partially by using cytochrome b_5 at the sarcoplasmic reticulum . Levy et al. (*41*) suggested that mitochondria are the most likely site for MMb reduction because of its unique conditions. First, oxygen tension is lowest near the mitochondria, which favors myoglobin oxidation. Second, mitochondria are a ready source of energy for MMb reduction in muscle. The MMb reducing enzyme system involves steps at least:

enzymatic reduction of cytochrome b_5 through NADH-cytochrome b_5 reductase followed by nonenzymatic reduction of MMb.

Non-enzymatic reduction of metmyoglobin. The nonenzymatic reduction of MMb and MHb by NADH and NADPH in the presence of ethylenediaminetetraacetate (EDTA) was reported by Brown and Snyder (*45*). They determined that NADH alone can reduce MMb if EDTA is present and that the reduction can be increased considerably by the mediation of flavins or methylene blue. Flavins, particularly in the presence of EDTA, can catalyze the reduction of MMb. EDTA may act as a reductant, especially in systems containing flavins (*45*). The nonenzymatic reduction of MMb in porcine tissue also was reported (*46*). The rate of nonenzymatic reduction of porcine muscle was similar to that of enzymatic reduction. NADH was mandatory for both reducing systems in pork muscle and other animal species. The presence of an electron transfer mediator was less critical with bovine MMb reductase. However, other researchers have failed to detect nonenzymatic reduction of MMb in bovine and ovine muscle (*2, 29, 38, 43, 47*). The reduction with addition of NADH, methylene blue and MMb without muscle homogenates was either minimal to none. Equine MMb also was not reduced in the absence of MMb reducing enzyme (*4, 46*).

Oxygen consumption rate. The oxygen consumption rate (OCR) significantly affects meat color and color stability. Increasing the depth of oxygen penetration into the muscle prolongs the formation of OMb layer in muscle, which consequently retains the layer of MMb further below the surface. Depth of oxygen penetration into meat depends mainly upon oxygen partial pressure at the surface, oxygen diffusion, and

oxygen consumption rate (*48*). The respiration of tissue is primarily responsible for OCR in postmortem muscle. Mitochondria in postmortem muscle continue to metabolize oxygen and thus compete with myoglobin's ability to oxygenate. Renerre and Labas (*2*) stated that muscles with an elevated mitochondrial content will be highly oxidative and thus have lower color stability. Mitochondrial activity is highly favored by high storage temperatures and pH values (*3, 28, 49-52*). At higher temperature and pH, the respiratory enzymes utilize more oxygen, and thus limit oxygen penetration and diffusion into the muscle. Consequently, dark-colored muscle, which has only a very thin layer of OMb on the surface, will appear darker due to the subsurface DMb (*53*).

O'Keefe and Hood (*48*) reported that muscle with rapid discoloration displayed higher rates of oxygen consumption, which resulted in a greater proportion of DMb. The reduced myoglobin is less stable than OMb and has a great disposition toward pigment oxidation. They found that *psoas major* (less color stable) muscle had higher OCR rate associated with thinner depth of penetration of oxygen, whereas *longissimus dorsi* (more color stable) muscle had lower OCR with consequently greater oxygen penetration. Renerre (*3*) supported the relationship between OCR and color stability by reporting that muscle with highest color stability showed lowest oxygen consumption and mitochondrial content. McKenna et al. (*54*) determined that even though OCR was the same among various muscles, the ability to reduce MMb to DMb was quite different. Bendall and Taylor (*50*) found that mitochondrial respiration as the primal factor affecting post-rigor OCR. They also reported that OCR decreased with storage time and its rate was most rapid from 2 to 6d postmortem. Atkinson and Follett (*55*) observed

that beef with the lowest oxygen uptake had the best color stability, whereas lamb with the highest oxygen uptake had the worst color stability. They also found that oxygen uptake, metmyoglobin reducing activity (MRA), and nicotinamide adenine dinucleotide (NAD) content diminished with increasing time of storage. Madhavi and Carpenter (*56*) found lower NAD concentration in the the *psoas major* (poor color stability) than *longissimus dorsi* (good color stablity) and thus suggested that NAD concentrations were directly associated with color stability in regards its relation to MMb reductase activity. Cheah and Ledward (*57*) applied pressures of 80-100 MPa for 20 min to fresh beef muscles (2 days post-slaughter) prior to display and measured MMb formation rate. They found the significant inhibition of OCR by the pressure treatment reduced MMb formation of beef LD.

Extrinsic Factors Affecting Meat Color Stability

Temperature and display condition. Muscle pigment can be affected by different temperature conditions. High temperatures disrupt the globin structure resulting in the loss of the globin moiety's function of protecting the heme. Additionally, high temperatures elevate oxygen consumption rate by enzymes within the tissue (*49, 50*). All of these contribute to lower oxygen tension at the meat surface, and consequently, oxygen solubility in the meat is lower with increased temperature. This results in dissociation of oxygen from oxymyoglobin, and the formation of DMb, which has a great disposition toward pigment oxidation (*6, 18, 52*). Additionally, high temperature decreases oxygen penetration into the meat, and thus will move the intermediate MMb layer closer to the surface of meat (*16*). Furthermore, increased storage temperature

mainly due to temperature abuse results in enhanced microbial growth forming spoilage-type green pigment (sulfmyoglobin) and also accelerates lipid oxidation, elevating meat discoloration. Intense display lights or too close distance between light and the meat can cause photo-oxidation of meat discoloring meat surface during display time.

Animal handling, feeds, and stress. The beef industry widely adopts vitamin-E supplementation into the diets of feedlot cattle for the final 100 to 125 d prior to slaughter to enhance beef color stability (*43*). Due to vitamin E's anti-oxidant property, carcasses from cattle treated with vitamin-E supplement have a better color stability by reducing oxidation incidence (*43, 58*). Rough animal handling can cause bruises on carcasses, and can result in increased animal stress, which negatively affects meat quality. Ante-mortem stress related defects such as DFD (Dark, Firm, and Dry - long term stress) and PSE (Pale, Soft, and Exudative – short term excitement) can result in abnormal ultimate muscle pH (high pH or low pH), and thus affects muscle color. Further, different MMb reductase activity among fresh normal, PSE, and DFD pork was reported by Zhu and Brewer (*21*). DFD pork had higher MMb reductase activity and OCR than normal or PSE pork. The pH of DFD (6.16) was close to the optimal reduction rate (pH 6.0) reported by Mikkelsen et al. (*46*) compared to PSE (5.44) and normal (5.52) pork samples. The enzyme activity of PSE muscle was lower than that of normal muscle until the fifth day of storage after which there was no difference in enzyme activity between the two samples. MMb accumulation in samples occurred in the order of PSE > normal > DFD. The rapid accumulation of MMb on the PSE pork surface resulted in more rapid loss of pink color than that of normal and DFD samples.

Added ingredients. Addition of non-meat ingredients to fresh meat or processed meat affects color stability and causes alterations in pigment formation. It has been reported that the addition of phosphate and/or lactate improves color by increasing meat pH or other mechanisms (*59-65*). Tan and Shelef (*65*) reported that color stability and red color (a* value) were enhanced by 2% sodium lactate (SL) with sodium chloride in refrigerated and frozen fresh ground pork. Addition of SL reduced surface discoloration in fresh pork sausage during retail display (*62*). Eckert et al. (*60*) noted that SL enhanced ground beef patties and enhanced the development of a much darker red color that remained stable during display. Papadopoulous et al. (*64*) determined that an increased SL level decreased L* and b* values and increased a* values during display. Maca et al. (*63*) also reported that addition of SL improved the lean color of cooked, vacuum-packaged roast beef and the storage of up to 84 days at 4℃ did not change the visual appearance. They concluded that SL had a protective and color stabilizing effect because roasts with SL were darker and redder than controls and storage time and temperature did not affect color. This stabilization of meat color may be due to a pH effect on the myoglobin conversion to MMb in the cooked product. Raw muscle with higher pH has darker in color (*66*). In addition, high pH (>6.0) stabilizes myoglobin during heating and after cooking a red color remains (*67*). However, it has been questioned that there may be some mechanism other than pH, because less correlation between pH and the color stabilizing effects of SL has been reported (*64*). Therefore, the exact mechanism by which SL affects color is still unknown.

Kim et al. (*9*) proposed that increased color stability of lactate injected steaks might be due to the increased MRA through increased LDH flux and its concomitant regeneration of NADH, which is a reducing equivalent. L-lactic acid concentration increased with increasing levels of SL up to 3% and decreased during storage (*68*). The decrease in lactic acid content during storage possibly was due to loss of unbound lactate resulting from increased purge during storage. DePablo et al. (*69*) reported that L-lactic acid content remained constant during storage of vacuum packaged raw pork, with values ranging from 500 to 700 mg/100g meat. However, they further determined that D-lactic acid content increased during storage, which is mainly due to the numbers of lactic acid bacteria. Increased L-lactate through the addition of SL may be further metabolized by LDH, and consequently generates more NADH, which is used for reducing MMb.

Nitrite is commonly used in cured meat products. When nitric oxide binds to the sixth ligand of myoglobin, the unstable bright red color of nitrosylmyoglobin is formed. However, since it is unstable, the myoglobin is easily oxidized to nitrosylmetmyoglobin, forming brownish-red color. When heat is applied to the meat during thermal processing, it turns to nitrosylhemochromogen, a stable pinkish cured meat color. Some cross-contamination of nitrite or nitric oxide from cured meat to fresh meat especially pork during meat processing also can cause a heat-stable pink color development upon further cooking (*70*).

Packaging type and meat process. Different packaging conditions (available gaseous condition) also affect meat color stability. A low-oxygen condition like a fresh,

vacuum packaged cut of meat, or low-oxygen Modified Atmosphere Packaged (MAP) meat induces the formation of DMb, a purplish-red color, on the surface. When the meat is transferred to highly oxygenated environment (bloom, traditional PVC packaging, or High-oxygen MAP package), oxygen binds to the sixth ligand of heme ring forming OMb, a bright-cherryish red color. Especially, when muscle stores in high oxygen condition (80% oxygen and 20% carbon dioxide), oxygen penetrates deeper, and thus presents much thicker layer of OMb and consequently the red color is stabilized (*22*). Carbon monoxide has a strong affinity to heme iron forming carboxymyoglobin (COMb), a stable cherry red color. Currently, CO-MAP packaging mostly used in ultra-low oxygen systems includes gas mixtures of 0.4% CO (GRAS), 50-60% CO_2 (antimicrobial effect), 40-50% NO_2 (as a filler). Due to its high binding capacity to the heme iron, meat stored in a CO-MAP system retained a relatively redder appearance after cooking called "persistent pinking" (*22*). Persistent pinking occurs when thermal cooking process does not effectively denature the protein (*70*). Therefore, it may require applying a higher temperature for a longer time during cooking to have a well-done appearance, which consequently results greater moisture loss and a reduction in tenderness. Persistent pinking of cooked meat also can be developed under low-oxygen packing condition like low oxygen-MAP packaging or vacuum packaged meat. Low oxygen partial pressure results in forming DMb state, which is the most heat stable form among other Mb states especially at higher pH (*71*). On the other hand, meats with higher concentrations of OMb or MMb will develop brown color quicker than those with higher proportion of DMb resulting in more prevalence of "premature browning" (PMB)

in cooked meat (*71, 72*). PMB can be defined as developing a well-done appearance when cooked to unsafe-temperatures to destroy pathogenic bacteria (*73*). Seyfert et al. (*74*) found the PMB defects observed in steaks packaged in Hi-Ox MAP when cooked to a medium degree of doneness. However, steaks in Lo-Ox MAP appeared predictable color, and a slightly pink interior. Therefore, PMB defects would be a significant food safety issues if consumers rely on internal meat color for cooked meat (especially ground meat) as a degree of doneness. Repeated freezing-thawing induces "tired meat," which has lower MRA and can also cause a premature browning defect (*75*). Irradiation can cause color change in raw beef, and it is more evident when the beef is irradiated in the presence of oxygen. However, ionizing radiation under low oxygen condition such as vacuum packaged or in CO-MAP causes minimal color deterioration (*76*). Irradiation under aerobic conditions promotes lipid oxidation and forms free radicals such as hydroxyl (OH), which stimulates myoglobin oxidation (*76*) consequently forming MMb.

Grinding of meat can cause the increasing incidence of meat oxidation resulting in shortening of meat color life. Furthermore, different cutting methods (i.e., bandsaw vs. knife cut) can have a different rate of surface discoloration of steaks (*56*).

Lactate Dehydrogenase

Lactate dehydrogenase (LDH) has been purified and crystallized from yeast, bacteria, and various animal and plant tissues (*77, 78*). Kidney has the highest LDH activity followed by heart and skeletal muscle in human tissue extracts. Skeletal muscle LDH is a tetramic enzyme that exists in five isoforms due to the existence of two

subunits of either M (muscle) or H (heart) form, or also called either A or B form (*79*), both of which favor the production of lactate (*80*). As the names indicate, the M subunit (or A subunit) is produced most likely in tissues capable of anaerobic metabolism, such as glycolytic white skeletal muscle, while the H subunit (or B subunit) is mainly found in tissues with aerobic metabolism, such as oxidative cardiac muscle (*81*).

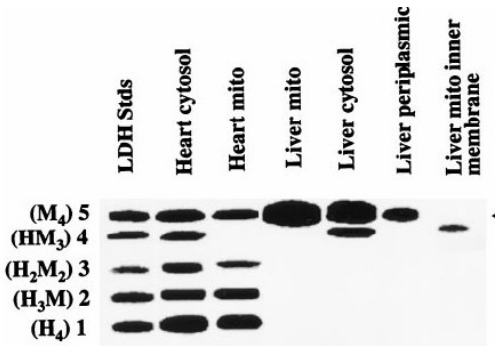

Figure 2.4. Agarose gel electrophoresis of LDH in mitochondria from rat liver and heart. LDH isoenzyme patterns differ between cytosol and mitochondria in both tissues (82).

Total LDH activity is regulated by specific subunit combinations of the LDH-A or LDH–B gene products. LDH-A_4 (or LDH5) favors the complete and rapid conversion of pyruvate to lactate, whereas LDH-B_4 (or LDH1) favors the complete and rapid conversion of lactate to pyruvate (*79*). LDH-A_3B (or LDH4) and LDH-A_2B_2 (or LDH3) are intermediate isoforms that have lower substrate affinity compared to LDH-A_4 and LDH-B_4 enzyme isoforms. Therefore, the combination of these different isoforms partially determines the total LDH activity in various tissue types (**Figure 2.4**). Isoenzyme LDH_5 is the predominant enzyme accounting for over 87% of the total LDH activity (*10*).

The enzyme is located in both cytosol and mitochondria of skeletal muscle cells (*78, 83, 84*). Lactate produced in the cytosol by glycolysis can be taken up directly into mitochondria, oxidized to pyruvate through LDH, and then utilized by the TCA cycle under aerobic exercise conditions. Through this intracellular lactate shuttle, the reducing equivalents (NADH) are produced and transported to the electron transport chain (ETC) (**Figure 2.5**).

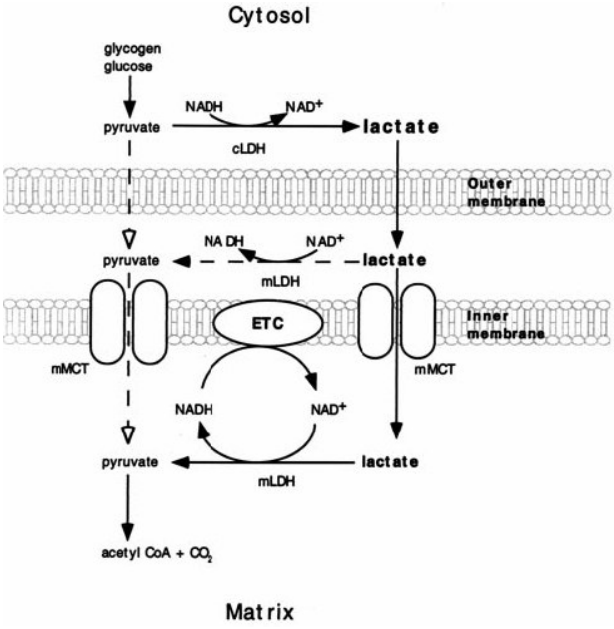

Figure 2.5. Illustration of the functional relationship between mitochondrial LDH and mMCT in operation of the intracellular lactate shuttle under aerobic exercise conditions (82). Entry of lactate and pyruvate into the mitochondrial matrix is facilitated by mMCT. Thus, lactate enters mitochondria; lactate is oxidized to pyruvate via mitochondrial LDH when mitochondrial Redox decreases, and pyruvate is oxidized via the TCA and ETC.

LDH rapidly binds NAD before binding lactate, which induces movement within LDH, trapping both substrate and coenzyme in the active site (*85*). Two major interactions are involved in the association between lactate and LDH (*85*). First, lactate forms a salt bridge with an arginine located on LDH. Second, oxygen binds to the center

carbon of lactate coordinated with the imadazole ring of a histidine located on LDH. These enzyme-substrate interactions are responsible for distinguishing between D-lactate, which is less likely to be metabolized by LDH, and L-lactate, the preferred substrate. The hydrogen on the center carbon of L-lactate is positioned close to nicotinamide, which allows easy transfer of hydrogen to NAD. In contrast, D-lactate orients with its methyl group toward NAD, therefore hydrogen points away from NAD.

The LDH isozymes differ depending on muscle types. White muscles contain primarily the M_4 isozyme of LDH, while the H_4 isozyme predominates in red muscles (*86*). Different LDH activity, which implies a different utilization rate of NADH, depends on which muscle fiber type is dominant within a muscle (*10*). *Semimembranosus* muscle (1476 U) has been shown to have a high level of LDH activity, the *longissimus* (1439 U) which is intermediate, and the *psoas major* has the lowest level of LDH activity (644 U) of the three muscles. Postmortem aging increased LDH activity (**Table 2.3**), while freezing and freezing-thawing markedly decreases in activity (*86*). During postmortem aging of muscles, enzymatic degradation of the muscle structure occurs, and thus soluble proteins including LDH may be released from sarcoplasmic protein. High temperature (> 63℃) and low pH (4.8) conditions significantly deactivate LDH mainly due to the denaturation of muscle proteins (*10*).

Table 2.3. Average LDH activity[a] in fresh, aged, frozen and thawed ham muscles (*86*).

Muscle	Fresh	Aged	Frozen	Frozen and thawed
Biceps femoris	460.67	1187.12	120.55	64.76
Rectus fermoris	757.53	949.86	337.79	101.53
Semimembranosus	743.95	802.52	501.38	191.72
Semitendinosus	653.27	493.91	113.41	20.82

[a]μmol/min/g sample

Lactate-LDH System

NADH is an essential cofactor in either enzymatic or nonenzymatic MMb reduction. Speculations exist about the pool where the NADH involved in the metmyoglobin reduction comes from. Watts et al. (*8*) hypothesized that since postrigor meat is well supplied with both lactate and lactate dehydrogenase (LDH), hydrogen may be transferred from lactate to nicotinamide adenine dinucleotide (NAD^+) by LDH. Then, the reduction of NAD^+ to NADH could lead to the reduction of MMb in the presence of intermediate electron carriers such as other enzymes, quinines, or methylene blue. In addition, many enzymes involved with glycolysis, the Krebs cycle, and the electron transport system remain potentially active in meat even after extended refrigerator storage (*87*). Therefore, the limiting factor in post-mortem muscle metabolism is likely the lack of adequate substrates rather than a loss of enzymes.

Saleh and Watts (*88*) tested several glycolytic and Krebs cycle intermediates for their ability to increase MMb reduction in ground beef. The addition of intermediates including glyceraldehyde-3-phosphate, fructose-1,6-diphosphate, α-glycerophosphate, malate, and glutamate increased MMb reduction. Unfortunately, they did not test for the

addition of lactate, because it was believed that the equilibrium of the lactate dehydrogenase reaction favors the oxidation rather than the reduction of NAD^+. They concluded that the reducing activity of most samples of meat can be increased by addition of appropriate substrates. NAD is too expensive to be considered as an additive to meat, but monosodium glutamate may be a cost-efficient addition. Although they suggested the hypothetical scheme for the role of substrates and intermediates in MMb reduction, the actual endogenous substrates responsible for reducing activity in meat were not described.

Arihara et al. (*89*) further proposed that the glycolytic pathway may have a significant role in the enzymatic reduction of MMb by supplying cytoplasmic NADH for the NADH-cytochrome b_5 reductase system in muscle. Nitrite ($NaNO_2$) was used to oxidize the intracellular myoglobin from rat hearts. The addition of 2-deoxy-D-glucose, an inhibitor of the glycolytic pathway, to the cell suspension completely inhibited the reduction of MMb. Conversely, an inhibitor of the citric acid cycle (malonic acid) did not inhibit MMb reaction. Therefore, they concluded that active glycolytic flux may be necessary for MMb reduction system in muscle cells.

Cheah (*90*) determined that the lactate-LDH system was the most likely system responsible for MMb reduction prior to nitrosylmyoglobin (NOMb) formation in cured bacon. The conversion of the induced MMb to NOMb in cured bacon could only occur after MMb reduction, indicating that a reducing system was still active in the bacon. They reported that bacon contained LDH capable of reducing exogenous NAD^+ to NADH (about 260 nmol NADH per gram of bacon), which in turn could be completely

re-oxidized stoichiometrically by exogenous MMb. In addition to LDH, bacon also contained NAD^+ (34 to 364 nmol per gram bacon) and lactate (69 to 99 μmol per gram bacon). Therefore, bacon contained all the necessary constituents for the generation of NADH for MMb reduction.

MRA in muscle is stable in the temperature range of 10° to 37.5 °C (*30*), but it negatively affected by temperatures above 40 °C. However, Bekhit et al. (*4*) reported that heating the ovine MMb preparations at 50 °C for 10 min did not decrease the reducing activity. In addition, reducing activity was not significantly affected by electrical stimulation or after 6 weeks of vacuum storage. Moreover, in aqueous muscle extracts there is a reducing system present which can, even at 60°C, convert MMb to OMb (*15*). Osborn et al. (*7*) reported that addition of NADH markedly improved the reducing system that operated at high temperatures of up to 70°C. Clearly, LDH activity is stable up to muscle endpoint temperatures of 63 °C. Collins et al. (*86*) reported that LDH in the *semimembranosus* steaks remained active up to 1 month postmortem. Therefore, Kim et al. (*9*) hypothesized that the lactate-LDH system in postmortem muscle can generate NADH by the reduction of NAD+, and then NADH dependent MMb reducing system utilizes supplied NADH to reduce MMb (**Figure 2.6**).

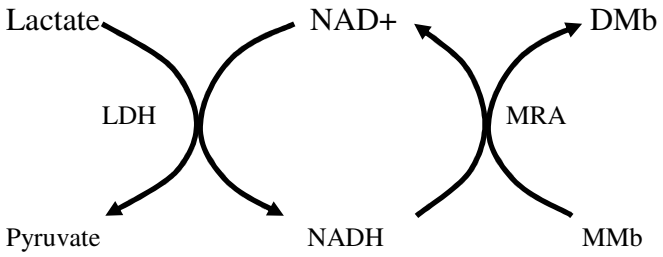

Figure 2.6. Proposed mechanism for lactate stabilization of meat color by Kim et al. (9).

They determined (**Table 2.4**) that nonenzymatic metmyoglobin reduction occurred in the lactate-LDH system with NAD^+, but that exclusion of NAD^+, L-lactic acid, or LDH eliminated the MMb reduction. They, consequently, concluded that the lactate-LDH system in post-mortem muscle can generate NADH by the reduction of NAD^+, and that a NADH-dependent reducing system, either enzymatic or non-enzymatic, can reduce metmyoglobin. Further, they determined that the variation in color stability of physiologically different muscles could be regulated by the different rates of replenishment of NADH via different LDH isozymes (*91*).

Table 2.4. Nonenzymatic reduction of horse MMb with Lactate-LDH system in various mixtures[a] at 22 °C and pH 8.0 (*9*).

FMN	Methylene Blue	NAD[b]	L-lactic[c]	LDH	Oxalate[d]	D-lactic[c]	Activity (nmole/min)
+	+	+	+	+	-	-	0.7 ± 0.004
-	+	+	+	+	-	-	0.5 ± 0.012
+	-	+	+	+	-	-	0.2 ± 0.003
+	+	-	+	+	-	-	0.0 ± 0.004
+	+	+	-	+	-	-	0.0 ± 0.003
+	+	+	+	-	-	-	0.0 ± 0.000
+	+	+	+	+	+	-	0.4 ± 0.015
+	+	+	-	+	-	+	0.1 ± 0.002

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

CHAPTER III

INVOLVEMENT OF LACTATE DEHYDROGENASE IN METMYOGLOBIN REDUCTION AND COLOR STABILITY AND WATER-HOLDING CAPACITY OF DIFFERENT BOVINE MUSCLES

Overview

The role of lactate dehydrogenase (LDH) in metmyoglobin (MMb) reduction and color stability of different bovine muscles was studied in two consecutive experiments. In experiment 1, three different bovine muscles – *Longissimus lumborum* (LD), *Semimembranosus* (SM), *Psoas major* (PM) – were fabricated (n=7 respectively), cut into steaks, PVC packaged, and then displayed for 7 days at 1 ºC. Instrumental color, surface MMb, water-holding capacity (WHC), metmyoglobin reducing activity (MRA), LDH-B activity (reaction toward NADH production), LDH isoform expression, and NADH were measured. LD showed the least MMb accumulation, and was most red over display time followed by SM. LD had more LDH-B activity and LDH1 isoform expression, thus producing more NADH, and had greater MRA. Although PM had higher pH and WHC, it had the least color stability and lowest MRA possibly due to lower LDH-B activity and LDH1 isoform, and subsequently lowered NADH regeneration. In experiment 2, beef strip steaks (n=8) were cut in half, one side syringe-injected with oxamate (LDH inhibitor), and the other injected with distilled water (control). Surface color, LDH, and NADH were measured after 10 days display at 1 ºC. Inclusion of oxamate inhibited LDH-B activity, decreased NADH, and discolored surfaces more ($p < 0.05$) than the control. These results suggest that variation in color

stability of physiologically different muscles is regulated by different replenishment rates of NADH via different LDH isozymes. LDH influences the metmyoglobin reduction system by replenishing NADH.

Introduction

Discoloration of raw meat cuts due to the formation of brown metmyoglobin (MMb) on the meat surface significantly affects consumers' purchasing decisions. Hood and Riordan (*92*) reported that consumers discriminate against discolored meat linearly with a corresponding increase in metmyoglobin formation. Renerre and Labas (*2*) noted that even at low levels of metmyoglobin, consumers begin to discriminate. In addition, when 20% of the total myoglobin pigment is in the metmyoglobin form, the ratio of sales of discolored beef to bright red beef is about 1:2. The oxidized form of brown metmyoglobin can be converted to the purplish reduced deoxymyoglobin (DMb) through the metmyoglobin reducing system of muscle; then, can be immediately oxygenated to red oxymyoglobin (OMb). This process is often referred to as metmyoglobin reducing activity (MRA). Ledward (*28*) suggested MRA to be the most important intrinsic factor controlling the rate of metmyoglobin formation in beef muscles. Metmyoglobin can be converted to deoxy-/oxy-myoglobin through the MRA of muscle. Although the general mechanism of the metmyoglobin reduction system, per se, is well established, the origin of the pool of nicotinamide adenine dinucleuotide (NADH), an ultimate reducing substrate for the MRA, has not been clearly established. Kim et al. (*9*) determined that nonenzymatic metmyoglobin reduction occurred in the lactate-LDH system with NAD^+, but that exclusion of NAD^+, L-lactic acid, or LDH eliminated the

MMb reduction. Consequently, they proposed that the lactate-LDH system in post-mortem muscle can generate NADH by the reduction of NAD^+, and that a NADH-dependent reducing system, either enzymatic or non-enzymatic, can reduce metmyoglobin. Therefore, we hypothesize that the variation in color stability of different muscles can be regulated by different rates of replenishment of NADH via different LDH isozymes. The objectives of this study were to characterize the involvement of LDH isoenzymes in MMb reduction, to determine color stability, NADH concentration, and MRA of different bovine muscles, and to investigate the relationship of water-holding capacity (WHC) and pH to color stability of physiologically different muscles.

Materials and Methods

Raw materials and processing. In experiment 1, three different bovine muscles – *Longissimus lumborum* (LD), *Semimembranosus* (SM), *Psoas major* (PM) – were obtained (n=7 for each muscle) from a local meat purveyor, and were transferred to the Rosenthal Meat Science and Technology Center at Texas A&M University. At 5d postmortem, all muscles were trimmed free of subcutaneous and seam fat and any visible connective tissue, and were portioned into 2.54 cm thick steaks by cutting perpendicular to the muscle fiber orientation. Steaks from each muscle were placed on styrofoam trays and over-wrapped with polyvinylchloride film and then displayed for 7 days at 1 °C under 2150 lux of fluorescent light. Instrumental color, pH, WHC, MRA, NADH concentration, LDH activity, and LDH isoform expression were evaluated through the display period.

In experiment 2, strip steaks (n=8) were cut into half, and one side was injected with a solution containing oxamate (180 mM at 10% injection rate, pH = 6.5), an LDH inhibitor and the other was injected with a distilled water. Surface color, LDH, and NADH were analyzed initially and after 10 days of display at 1 °C under 2150 lux of fluorescent light.

Instrumental color. Instrumental color (CIE L*a*b* for Illuminant A) was evaluated during display using a HunterLab MiniScan^{TM}XE Spectrophotometer (Model 45/0 LAV, Illuminant A, 3.18 cm diameter aperture, 10º standard observer; Hunter Associates Laboratory, Inc.; Reston, VA). Reflectance from 400 to 700 nm with 10 nm increment readings and CIE L* a* b* values (Illuminant A) were measured and used to calculate saturation index $[(a^{*2} + b^{*2})^{1/2}]$, hue angle $[(b^*/a^*)^{\tan-1}]$, and percentages of oxymyoglobin (OMb), deoxymyoglobin (DMb) and metmyoglobin (MMb) (*93*). Three different locations per steak were scanned and averaged for statistical analyses.

pH. On d 1, 4, and 7, a sample from each muscle was frozen in liquid nitrogen and pulverized in a Waring® table-top blender (Dynamics Corp. of America, New Hartford, CT). About 5 g of muscle tissue was mixed with 20 ml of distilled water for 20 sec using a homogenizer (Polytron Model PT 10/35, Kinematica, Luzernerstrasses, Switzerland), and pH values were measured with a standardized combination pH electrode attached to a pH meter (Accumet 50; Fischer Scientific, Fair Lawn, NJ).

Water-holding capacity. Water-holding capacity (WHC) was measured by using the Honikel gravimetric bag method (*94*) as described by Bertram et al. (*95*). For the Honikel bag method, a cube of meat weighing approximately 100 g was trimmed of

any visible fat and connective tissue and weighed. A metal clip was attached to the sample and the sample hung in an inflated plastic bag for 48 h at 4 °C, after which it was weighed again to determine moisture loss. Drip loss (%) was calculated using the formula:

Drip loss (%) = [(initial weight- weight after 48 h)/initial weight] x 100.

MRA-NO_2 method. The nitric oxide method of Watts et al. (*8*) was used to determine MRA on d 1, 4, and 7. A 3 × 2 × 1.27 cm^3 sample of muscle tissue with no visible fat or connective tissue was excised from a steak. Sample cubes were oxidized to form nitric oxide metmyoglobin in 50 ml of 0.3% sodium nitrite at room temperature (18-32 ºC) for 20 min with occasional stirring. Then, the samples were blotted to remove excess solution, vacuum packaged, and percent reflectance from 400-700 nm immediately taken using a Hunter LabScan 2000 (1.27 cm diameter aperture, Hunter Associates Laboratory, Inc., Reston, VA). Samples then were placed in an incubator at 30ºC for 2 hr and the amount of metmyoglobin remaining was re-measured. The MRA was calculated as: (observed decrease in nitric oxide MMb concentration ÷ initial nitric oxide MMb concentration) x 100.

NADH concentration. NADH concentration was measured by using a NADH quantification kit (Biovision, #K337) for the samples from d 1, 4, and 7. In brief, 20 mg of muscle tissue was homogenized with 400 µl of NADH extraction buffer, centrifuged at 14,000 rpm for 5 min, and then heated (200 µl aliquot) to 60 ºC for 30 min in a water bath to decompose NAD from extraction. Subsequently, 50 µl of heated aliquot was transferred into labeled 96-well plate in duplicate with a NADH developer. The plate

was read at OD 450 nm. The NADH concentration (ng/mg) was calculated using the equation obtained from the standard curve.

LDH-B activity. LDH-B activity was measured following the UV-method of Wahlefeld (*96*), which monitors the reduction of NAD^+ in the following reaction:

$$\text{L-Lactate} + NAD^+ \xrightarrow{\text{LDH}} \text{Pyruvate} + NADH + H^+$$

In brief, powdered frozen muscle tissue (2.0 g) was homogenized in 8 ml of a 0.01M sodium phosphate buffer (pH 7.5) for 30 s and held on ice. The homogenate was centrifuged at 13,823 x g for 30 min at 4ºC, and then filtered through Whatman No. 42 filter paper (Whatman Inc., Clifton, NJ). The filtered supernatant (0.1 ml) was added to a glass cuvette with 0.1 ml of NAD and 2.4 ml of Tris/L-lactate (pH 9.3). Activity of LDH was measured in duplicate by the continuous increase in absorbance at 339 nm for 2 min. Increased absorbance (increased NADH) between 30 and 120 s was used for calculation for LDH activity. Units of LDH activity were expressed as µmol/min/g sample.

LDH isoform expression. The cellular composition of LDH isoform was evaluated by using the Titan Gel LD Isoenzyme procedure (Helena lab. #3043). The filtered supernatant from a previous extraction step also was assayed for LDH isoform expression. Total protein concentration was determined in the supernatant by the Bradford protein assay (Bio-Rad). LDH isoforms were fractionated by running 3 µl of total soluble protein (5 µg) on an agarose gel. The protein was separated for 60 min at 100V using a LD Isoenzyme control (Helena lab. #5134) as an internal positive control. Bands that represented the five isoforms were revealed colorimetrically, and the gels

were fixed with 10% acetic acid. The LDH isoform bands were scanned and quantified with an imaging densitometer. LDH isoforms were expressed as a relative ratio compared to the control.

Data analysis. For instrumental color, myoglobin contents, MRA, WHC, NADH contents, and LDH activity, type-3 tests of fixed effects for muscle difference (LD, SM, and PM), display time, and their interaction were analyzed using the Mixed procedure of SAS (*97*) for ANOVA. LSMeans were separated (F test, $p < 0.05$) using least significant differences generated by the PDIFF option.

Results and Discussion

Experiment 1: Comparison of three physiologically diverse bovine muscles of color stability and biochemical properties.

Instrumental color and color stability. Lightness values (L*) of three different muscles were only affected ($p < 0.05$) by display time. The L* values for LD and PM decreased ($p < 0.05$) during display **(Table 3.1)**, but SM maintained stable lightness values for 7 d. PM had a lighter surface color than LD and SM on the initial display day; however, there were no significant differences in lightness values among different muscles at the end of display. There was a significant interaction between muscle and time ($p < 0.05$) for a* values (redness) and chroma values (**Table 3.1**). LD and SM had similar redness values, but PM was lower in a* value on 1 d. As display time progressed, a* values for all muscles decreased ($p < 0.05$), but LD maintained the reddest (highest a* value) surface color followed by SM, and PM had the lowest in redness on 7 d. Chroma

values of each respective bovine muscle followed the same trend as a* values indicating that LD had the most stable redness and color intensity.

LD had the least amount (%) of MMb accumulation on the surface **(Figure 3.1)** compared to SM and PM throughout the 7 d display period. Hue values (an indication of discoloration) of LD were not changed ($p < 0.05$) over the display period (**Table 3.1**), but PM hue values increased continuously followed by SM at the end of display time. Thus, of the three muscles, LD had the most color stability. Similar discoloration rates and patterns among different muscle types have been reported by several researchers (*2, 23, 30, 50, 54, 98*). McKenna et al. (*54*) classified LD as a "high" color stability muscle, SM as "intermediate", and PM as a "very low" color stability muscle, which corresponds to our findings.

pH and WHC. Muscle pH of PM and LD was significantly higher than that of SM throughout the display time (**Table 3.2**). Following the same trend of muscle pH, PM and LD had significantly higher WHC than SM confirming that higher pH meat retains more water molecules during storage (**Table 3.2**).

However, regardless of high pH and WHC, PM had the lowest color stability and most rapid discoloration, which indicates that pH does not solely dictate muscle pigment change under display conditions.

Metmyoglobin-reducing activity. An interaction between muscle and day of display occurred ($p < 0.05$) for MRA (**Table 3.3**). LD and SM had higher ($p < 0.05$) MRA compared to PM, which had almost no reducing activity throughout the display period. All muscles showed a decrease in MRA with increasing retail display time, with the exception of PM. The nitric oxide method used for measuring MRA of muscles is not an absolute indication of natural MRA, per se. The method could form surface MMb with the addition of an oxidizing agent, which might not exactly correspond to the mechanism of the natural formation of MMb. However, it does provide a relative measure of the MRA of muscles with high correlation to meat color stability as well as

Table 3.1. LSMeans for instrumental color scores of bovine muscles - *Longissimus lumborum, Semimembranosus,* and *Psoas major* steaks for displayed 7 days at 1 ºC

	Instrumental Color Scores							
	d of display							
	1	2	3	4	5	6	7	SE[a]
Longissimus lumborum								
L*	45 dxy	43.1 dex	43.3 dex	42.6 dex	42.6 dex	43.1 dex	42.4 ex	0.87
a*	32.1 dx	32.7 dx	32.1 dx	32.7 dx	31 dx	28.9 ex	29.4 ex	0.72
b*	24.3 dx	25.3 dx	24.7 dx	25.4 dx	23.7 dx	21.7 ex	22.6 dex	0.59
Hue[b]	37 dx	37.7 dx	37.6 dx	37.9 dx	37.3 dx	36.9 dx	37.4 dx	0.74
Chroma[c]	40.4 dx	41.6 dx	40.7 dx	41.3 dx	39.3 dfx	36.1 egx	37 fgx	0.86
Semimembranosus								
L*	44 dx	43.4 dx	42.6 dx	42.7 dx	42.3 dx	42.7 dx	42.7 dx	0.87
a*	31.7 dx	30.4 dy	29.6 ey	29.3 ey	27.1 fy	22.6 gy	22.4 dy	0.72
b*	24.3 dxy	23.7 dey	23.6 dexy	23.3 dey	22.1 efy	18.7 fgy	19 dy	0.59
Hue[b]	37.4 dx	37.7 dex	38.6 defxy	38.6 defxy	39.1 defy	39.6 efy	40.6 fy	0.74
Chroma[c]	40 dxy	38.6 dey	37.9 dey	37.4 ey	35 fy	29.4 gy	29.4 gy	0.86
Psoas major								
L*	47.4 dy	45 dex	44.1 ex	43.3 bx	43 ex	43.3 ex	41.3 ex	0.87
a*	29 dy	26 dz	22.6 fz	22.1 fz	19.1 gz	16 hz	16.3 hz	0.72
b*	21.7 dy	20.1 dez	18.7 efy	18.6 efz	17.3 fgz	15.7 gz	16.4 gz	0.59
Hue[b]	36.4 dx	37.7 dex	39.7 efy	40.3 fgx	41.9 gz	44.9 hiz	45.3 iz	0.74
Chroma[c]	36.1 dy	32.9 ez	29.6 fz	28.7 fz	25.9 dz	22.7 hz	23.3 hz	0.86

[a] Standard errors. [b] Hue angle$(b^*/a^*)^{tan-1}$ and [c]saturation index $(a^{*2}+b^{*2})^{1/2}$ were calculated. [defghi] Means in a row within day having different letters are different ($p < 0.05$). [xyz] Means in a column of muscle types with different letters are different ($p < 0.05$).

Table 3.2. LSMeans for pH and water-holding capacity of steaks from bovine *Longissimus lumborum* (LD), *Semimembranosus* (SM), and *Psoas major* (PM) at 1, 4, 7 d of display at 1 ºC

Trait	Day	Muscle			
		LD	SM	PM	SE[c]
pH	1	5.73ax	5.56bx	5.77ax	0.02
	4	5.71ax	5.54bx	5.74ax	0.02
	7	5.70ax	5.51bx	5.69ay	0.02
WHC[d]		0.93a	1.81b	0.83a	0.22

[ab] Means within a row with different letters are different ($p < 0.05$). [xy] Means within a column with different letters are different ($p < 0.05$). [c] Standard errors. [d] Water-holding capacity (WHC) of three different muscles was measured at d 1.

Table 3.3. LSMeans for LDH-B activity, NADH concentration, metmyoglobin-reducing activity (MRA) of steaks from bovine *Longissimus lumborum* (LD), *Semimembranosus* (SM), and *Psoas major* (PM) at 1, 4, and 7 d of display 1 ºC

Muscle	Day	Trait		
		LDH-B (U)[d]	NADH (ng/mg)	MRA (%)
LD	1	9.66ax	16.81ax	15ax
	4	8.94ax	15.60ax	13ax
	7	8.66bx	14.74bx	5bx
SM	1	9.49ax	17.55ax	14ax
	4	8.66bx	12.37by	12ax
	7	8.66bx	13.71bx	2bxy
PM	1	6.31ay	11.89ay	1ay
	4	6.23ay	10.59aby	0ay
	7	6.63ay	9.46by	0ay
SE[c]		0.22	0.78	1.17

[ab] Means within a muscle at different display days with different letters are different ($p < 0.05$). [xy] Means between muscles on the same day with different letters are different ($p < 0.05$). [c] Standard errors. [d] µmol/min/g.

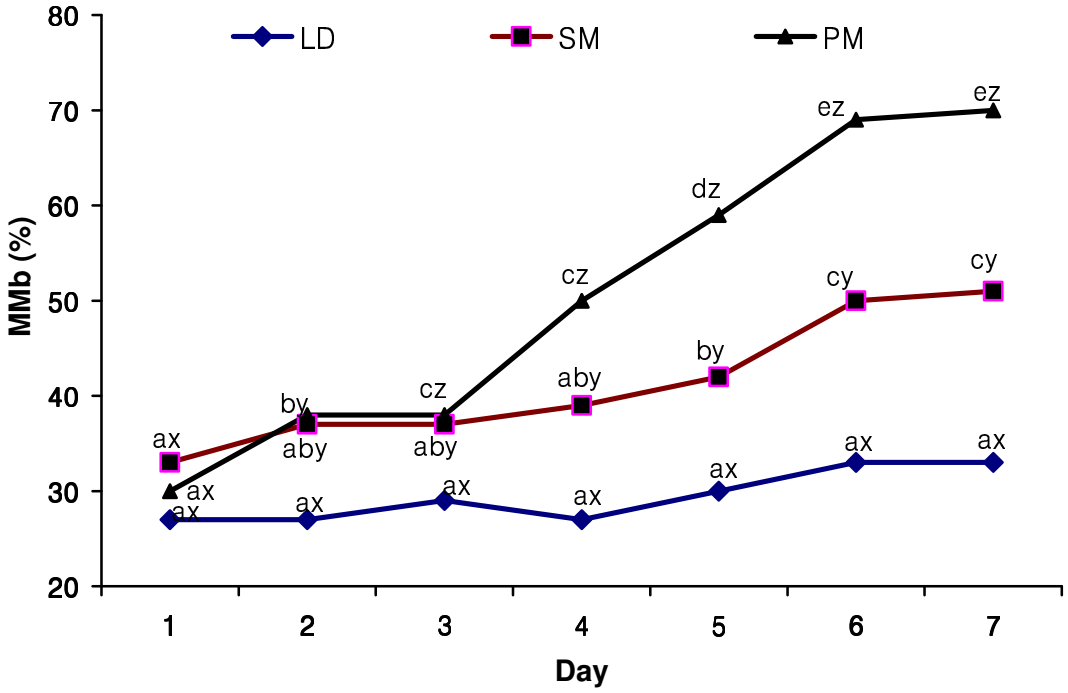

Figure 3.1. LSMeans for surface metmyoglobin accumulation (%) in bovine *Longissimus lumborum* (LD), *Semimembranosus* (SM), and *Psoas major* (PM) steaks displayed 7 days at 1 ºC. ^{abcde}Means within the same muscle across display days with different letters are different ($p < 0.05$). ^{xyz}Means between muscles within the same display day with different letters are different ($p < 0.05$).

being fast and non-destructive way (*8, 54, 99*). Therefore, although PM showed almost no reducing activity initially to the end of display, it does not necessarily suggest that PM had virtually no reducing power to convert MMb to DMb. Madhavi and Carpenter (*56*) also found that PM had greater MMb accumulation and lower MMb reductase activity, whereas LD had the most stable red color and higher MRA. McKenna et al. (*54*) observed an identical trend, decreasing nitric oxide-MRA (NORA) of all muscles (19 bovine muscles) with increasing days of retail display. They determined that LD had the highest NORA percentages throughout display time, but PM showed a rapid decrease (62.1%) and had the lowest MRA at the end of retail display.

NADH concentration. LD had a significantly higher NADH concentration (**Table 3.3**) than PM throughout the display period. SM was not significantly different from LD in NADH, but on 4 d, SM had lower NADH than LD. NADH concentration of all muscles decreased ($p < 0.05$) with increasing days of retail display. Sammel et al. (*20*) also found reduced NADH concentrations in SM muscle during vacuum storage at 1°C. For the reduction of MMb in muscle, the presence of NADH has been shown to be essential for both enzymatic and non-enzymatic reduction pathways. NADH-cytochrome b_5 reductase, suggested as the main reducing enzyme (*4, 32*), is absolutely specific for NADH. In the MMb reducing system, NADH acts as a coenzyme and electron carrier in the conversion of ferric myoglobin to its ferrous form (*3*). Mikkelsen et al. (*46*) found no MMb reduction without NADH in porcine LD, whereas they found increased the rate of MMb reduction by increasing the amount of NADH in an *in vitro* study. Kim et al. (*9*) also reported increased MMb reduction of equine MMb with

incremental concentrations of NADH. Further, they observed nonenzymatic MMb reduction when they replaced NADH with lactate, NAD, and LDH in their *in vitro* system concluding that the lactate-LDH system in postmortem muscle can regenerate NADH by the reduction of NAD, and that a NADH-dependent reducing system can reduce MMb **(Figure 3.2)**.

Lactate dehydrogenase activity-B. There was a significant muscle by day interaction for LDH-B activity (reaction toward NADH production). LDH-B activity of all muscles followed the same trend as the generation of NADH. LD had a significantly higher LDH-B activity (**Table 3.3**) than PM throughout the display period, while SM was not significantly different from LD in LDH-B activity. As storage progressed over 7 d, LDH-B activity of LD and SM decreased ($p < 0.05$), but PM did not change over display time and had lower LDH-B activity. Due to the equilibrium constant of the LDH reaction ($K = 2.7 \times 10^{11}$ mol/l), LDH activity strongly favors to the formation of lactate and NAD^+ instead of the oxidation of lactate by NAD^+ (*96*). However, although minimal, muscles still have LDH-B activity, and we found LD had higher LDH-B activity than PM. Thus, it is reasonable to assume that the higher concentration of NADH from LD may be due to the higher LDH-B activity, thus favoring the reaction to replenish NADH, which was used for donating an electron for MRA. Consequently, LD maintained higher color stability by lowering MMb accumulation on the surface throughout display time. In other words, LDH activity of individual muscles may be directly related to the color stability of those muscles postmortem.

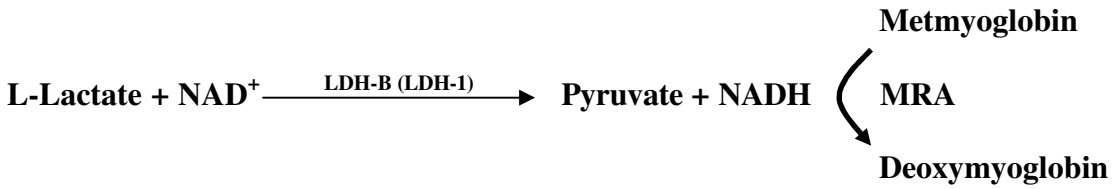

Figure 3.2. Depiction of the functional role of lactate dehydrogenase (LDH) in metmyoglobin-reducing activity (MRA). LDH1 isoform regulates LDH-B activity, which catalyzes oxidation of lactate to pyruvate generating NADH. Then, a NADH-dependent metmyoglobin reducing system utilizes the generated NADH to reduce metmyoglobin to deoxymyoglobin, consequently maintaining color stability of muscles.

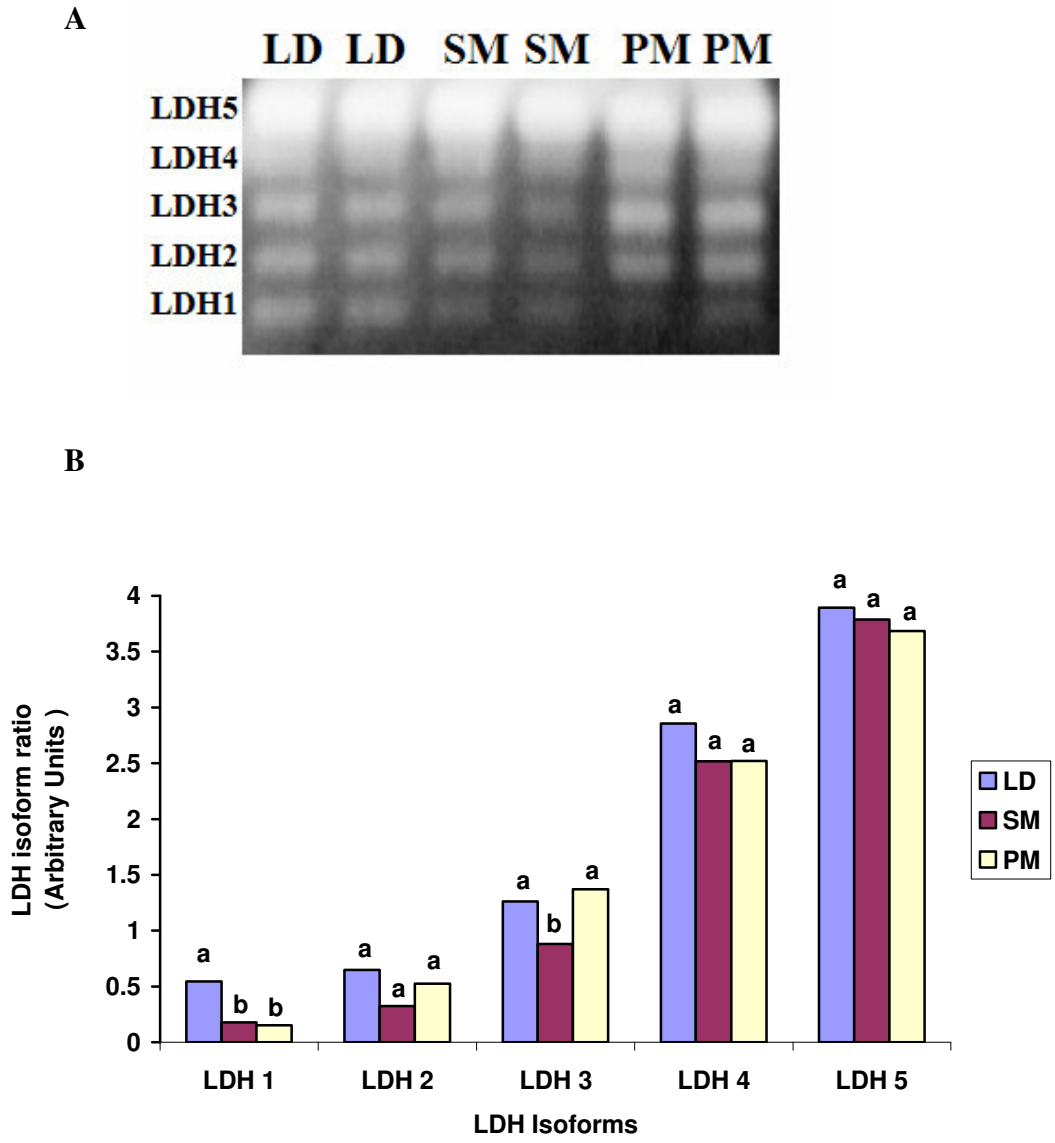

Figure 3.3. A: Agarose gel electrophoresis zymogram of LDH isoform expression from three bovine muscles (LD, SM, and PM) at d 7 (end of display). B: LDH isoform of three different bovine muscles. LDH isoforms were evaluated by densitometer color reading and were expressed as a relative unit over the control. [ab]Means with different letters are different ($p < 0.05$).

Lactate dehydrogenase isoform expression. Total LDH activity is regulated by specific subunit combinations of the LDH-A or LDH–B gene products (*79*). The two polypeptides can be arranged into five possible LDH tetramers such as LDH-A_4 (or LDH5), LDH-A_3B (or LDH4), LDH-A_2B_2 (or LDH3), LDH-AB_3 (or LDH2), and LDH-B_4 (or LDH1). LDH-A_4 (or LDH5) favors the complete and rapid conversion of pyruvate to lactate, whereas LDH-B_4 (or LDH1) favors the complete and rapid conversion of lactate to pyruvate, producing NADH (*79*). LDH-A_3B (or LDH4) and LDH-A_2B_2 (or LDH3) are intermediate isoforms that have lower substrate affinity compared to LDH-A_4 and LDH-B_4 enzyme isoforms (*79*). Therefore, the combination of these different isoforms partially determines the total LDH activity in various tissue types. All muscles had significantly higher LDH5 compared to LDH1 isoform expression (**Figure 3.3**). Stalder et al. (*10*) also reported that LDH5 isoenzyme was the predominant enzyme accounting for over 87% of the total LDH activity. There was no difference ($p < 0.05$) in amount of LDH5 isoform among muscles. However, LD had a significantly higher LDH1 isoform expression than SM and PM, confirming that LD had a higher LDH-B activity due to the higher LDH1 protein expression.

Experiment 2: Effect of LDH inhibition on color stability of bovine muscle.

Instrumental color and color stability. An LDH inhibitor, oxamate, was injected with a syringe into bovine LD steaks to determine the involvement of LDH in color stability of muscle. There was no treatment difference ($p > 0.05$) in L* values (**Table 3.4**) between steaks injected with oxamate (OM steak) and distilled water (DW steak).

Table 3.4. LSMeans for instrumental color scores of *Longissimus lumborum* steaks injected with distilled water and oxamate and displayed 1 and 10 d at 1 ºC

	Control[c]		Oxamate[d]		
	d1	d10	d1	d10	SE[e]
L*	46.3ax	42.7bx	44.7ax	43.2ax	0.91
a*	33.2ax	26.2bx	33.7ax	19.8by	1.22
b*	24.1ax	18bx	24.7ax	17.6bx	0.72
Hue[f]	36.3ax	35.1ax	36.3ax	41.6by	1.6
Chroma[g]	40.66ax	31.3bx	41.9ax	26.3by	1.4

[ab]Means within a treatment at different display day with different letters are different ($p < 0.05$). [xy]Means between a treatment at same display day with different letters are different ($p < 0.05$). [c]Syringe-injected (10% rate) with distilled water. [d] Syringe-injected (10% rate) with oxamate solution (180 mM). [e]Standard errors. [f]Hue angle$(b^*/a^*)^{tan-1}$ and [g]saturation index $(a^{*2}+b^{*2})^{1/2}$ were calculated.

However, OM steak had significantly lower a* values compared to DW steak on 10 d. Further, with increasing display time, hue values for OM steaks increased ($p < 0.05$), whereas DW remained the same indicating that steaks with oxamate addition caused more MMb accumulation (**Table 3.4**). Data for chroma values of OM steaks also confirmed that oxamate addition to the beef steaks decreased ($p < 0.05$) color intensity over retail display time compared to steaks with distilled water (**Table 3.4**).

NADH and LDH-B activity. OM steaks had significantly lower NADH concentration and LDH-B activity compared to DW steaks (**Figure 3.4** and **3.5**). Thus, it can be logical to conclude that oxamate addition to beef LD steaks inhibited LDH-B activity resulting in lower NADH production, and consequently decreasing color stability.

The results of two consecutive research experiments in this study suggest that the variation in color stability of physiologically different muscles could be regulated by different rates of replenishment of NADH via different LDH isozymes, namely LDH1. LDH1 influences the metmyoglobin reduction system by replenishing NADH. LD maintained the most stable red color, and had the highest MRA, NADH, and LDH-B activities. Although, PM had a higher pH and WHC, it showed the least color stability and lowest MRA possibly due to lower LDH-B activity (lower LDH1 isoform expression) and subsequently lower NADH regeneration. Oxamate addition to beef steaks inhibited LDH-B activity, decreased NADH concentration, and as a consequence it resulted in more discoloration (**Figure 3.6**). Thus, it can be concluded that LDH plays a specific role in metmyoglobin reduction and color stabilization of bovine muscles.

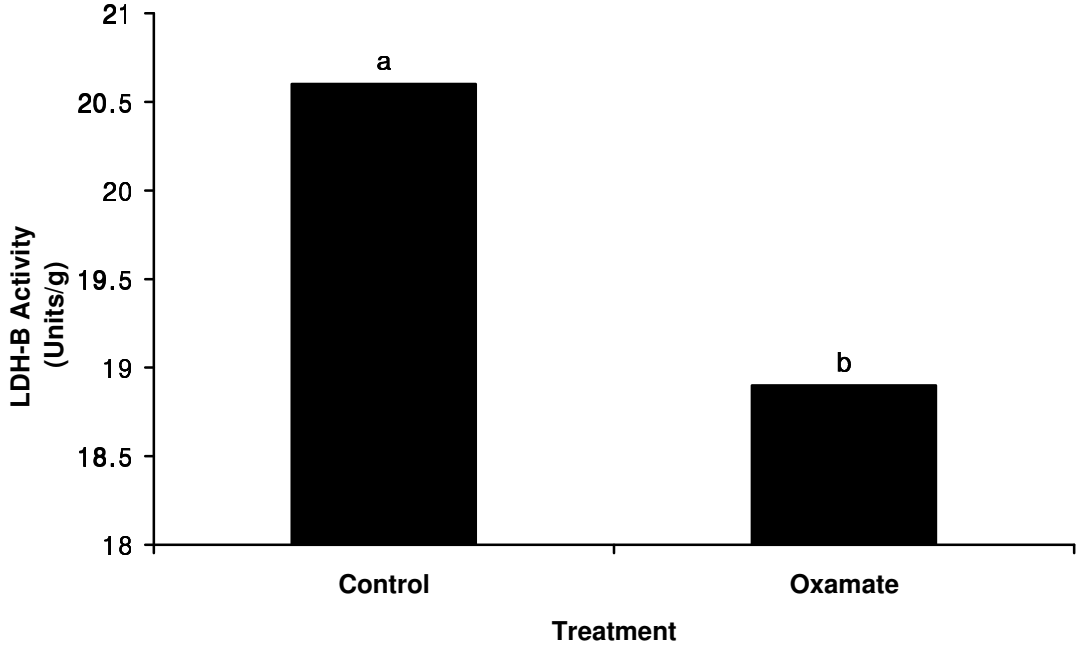

Figure 3.4. LSMeans for LDH-B activity of *Longissimus lumborum* steaks injected with distilled water (control) and oxamate at d 10 (end of display). [ab]Means with different letters are different ($p < 0.05$).

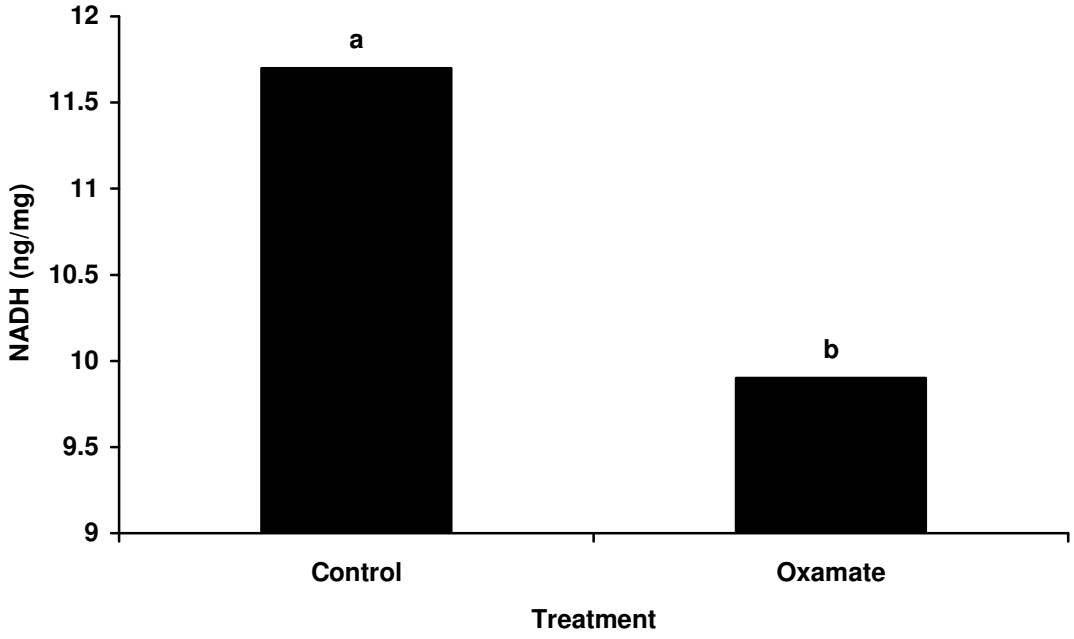

Figure 3.5. LSMeans for NADH concentration of *Longissimus lumborum* steaks injected with distilled water (control) and oxamate at d 10 (end of display). [ab]Means with different letters are different ($p < 0.05$).

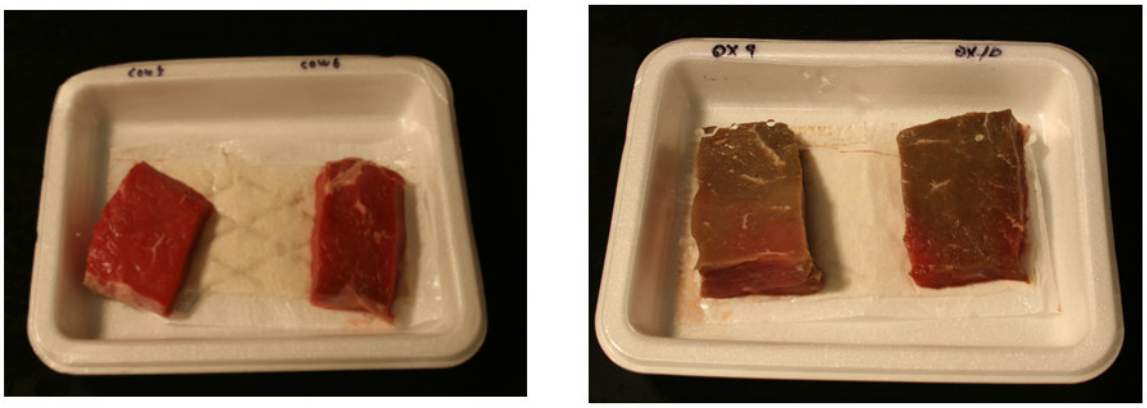

Figure 3.6. Pictures for *Longissimus lumborum* steaks injected with distilled water (control; left) and oxamate (right) at d 10 (end of display).

CHAPTER IV

COLOR STABILITY AND BIOCHEMICAL CHARACTERISTICS OF DIFFERENT BOVINE MUSCLES WHEN ENHANCED WITH LACTATE

Overview

Three different bovine muscles – *Longsissimus lumborum* (LD), *Semimembranosus* (SM), and *Psoas major* (PM) – were injection enhanced (n = 10 repectively) with solutions containing phosphate and/or potassium L- or D-lactate, cut into steaks, packaged with a high-oxygen (80% O_2) modified atmosphere packaging system, stored 9 days and then displayed for 5 d at 1ºC. Instrumental color, total reducing activity (TRA), lactate dehydrogenase – B (LDH-B) activity (reaction toward NADH production), and NADH were measured. Enhancement with potassium L-lactate resulted in less color deterioration, and higher a* and chroma values ($p < 0.05$) than non-enhanced control of the three bovine muscles. L-lactate/phosphate enhancement significantly increased NADH concentration and TRA of LD and PM than the non-enhanced control at the end of storage and display. Although LDH-B activity of the lactate enhanced steaks was not significantly different from that of the non-enhanced control ($p = 0.08$), PM steaks injected with the L-lactate and phosphate had a higher ($p < 0.05$) LDH-B flux compared to the control steaks. Enhancement with lactate/phosphate solution did not affect ($p > 0.05$) NADH, TRA, and LDH-B activity of SM steaks at the end of display.

Introduction

Overall appearance of retail meat cuts is the primary factor consumers consider when determining meat freshness and making purchasing decisions. It has been reported that the U.S. beef industry could prevent $520 million in lost revenue annually from retail sales by retarding surface discoloration of meats during retail display (National Cattlemen's Association, 1993 as cited by Wheeler et al. (*100*)). Hence, prolonging the natural bright-red color that consumers consider desirable in fresh red meat has been a major goal of the meat industry. Meat discoloration due to formation of metmyoglobin ($MbFe^{3+}$) can be retarded by reducing equivalents, such as NADH through NADH-dependent reducing systems, maintaining a reduced form of myoglobin ($MbFe^{2+}$) such as deoxymyoglobin or oxymyoglobin. Kim et al. (*91*) reported different color stability, metmyoglobin reducing activity (MRA), and NADH concentration of different bovine muscles. They concluded that the variation in color stability of physiologically different muscles could be regulated by different rates of replenishment of NADH via different lactate dehydrogenase (LDH) activities. LDH influences the metmyoglobin reduction system by replenishing NADH.

Improved color stability of both raw and cooked processed meat by lactate enhancement has been reported by many researchers (*9*), (*63, 101-103*). However, the exact mechanism by which lactate enhancement maintains the reduced form of myoglobin longer period of retail display time has not been clearly established. Kim et al. (*9*) previously found improved color stability and elevated MRA in beef *longissimus* muscles injected with 2.5% potassium lactate (10% pump) over that of non-injected

muscles. They determined that lactate enhancement promoted color stability by the conversion of lactate to pyruvate via increased flux through LDH and the concomitant regeneration of NADH. However, they investigated the effect of lactate enhancement on color stability only for *Longissimus* muscle, which is generally considered as a "high" color stability muscle (*2, 23, 30, 50, 54, 98*). Further, because Kim et al. (*9*) proposed the coupling mechanism of L-lactate and LDH for replenishing NADH, incorporating the D form of lactate (D-lactate) to beef muscles will allow an effective comparison to determine a proposed mechanism because LDH only metabolizes L-lactate. Therefore, our objectives were to evaluate influence of lactate enhancement on color stability of metabolically different bovine muscles and to determine the effects of lactate inclusion on LDH enzyme activity, NADH content, and reducing capacity in enhanced muscles packaged in a high-oxygen modified atmosphere packaging (MAP) system.

Material and Methods

Raw materials and processing. Three different USDA Select beef muscles (n=10 each of *Longissimus lumborum (LD), Semimembranosus (SM),* and *Psoas major* (PM)) at 3 day of postmortem were obtained form a commercial meat facility, and were transferred to the Rosenthal Meat Science and Technology Center at Texas A&M University. At 4 day post-mortem, each muscle was divided into four equal-length sections (40 total sections), and one of four enhancement solutions (**Table 4.1**) containing distilled water, potassium L-lactate (PURASAL HiPure P, 60% potassium L-lactate/40% water; PURAC America, Inc., Lincolnshire, IL), potassium D-lactate (SKr-2007-125, 60% potassium D-lactate/40% water; PURAC America, Inc., Lincolnshire,

IL), and/or sodium tripolyphosphate (Brifisol® 512; BK Giulini Corp; Simi Valley, CA) was assigned randomly to each of the muscle sections resulting in 10 treatment replications. The muscle sections were weighed individually before and after injection with a multineedle injector (model N30, Wolftec, Inc., Werther, Germany) to calculate actual injection levels (12%). Each enhanced product was sliced (2.54 cm thick steaks) into steaks for packaging.

Packaging. Each steak was placed in preformed trays (polypropylene/ethylene vinyl alcohol, 22.5 cm x 17.3 cm x 4.1 cm; Rock-Tenn.; Franklin Park, IL). Trays were flushed with a high-oxygen atmosphere (80% O_2/20% CO_2, Certified Standard, Airgas Specialty Gases; Austin, TX) and sealed by a ROSS INPACK JR (Model S3180; Ross Industries, Inc.; Midland, VA) and a shrinkable barrier sealing film (MAP-Shield AF; 1.5 mil high-barrier nylon/ethylene vinyl alcohol/methallocenece polyethylene with an oxygen-transmission rate of 0.02 cc/645.16 cm^2/24 h at 10ºC and 80% relative humidity and a water vapor transmission rate of 0.92 g/645.16 cm^2/24 h at 37.8ºC and 100% relative humidity; Honeywell, Morristown, NJ) was used for MAP. Packages were stored in the dark at 2ºC for 10 d before display for 5 d at 1°C under 2150 lux of fluorescent light.

pH. On d 14, a sample from each muscle was frozen in liquid nitrogen and pulverized in a Waring® table-top blender (Dynamics Corp. of America, New Hartford, CT). About 5 g of muscle tissue was mixed with 20 ml of distilled water for 20 s, and pH values were measured with a calibrated (pH 4.0 to 7.0) combination pH electrode attached to a pH meter (Accumet 50; Fischer Scientific, Fair Lawn, NJ).

Table 4.1. Composition of experimental injection-enhancement solutions for beef muscles

	Treatment[a]			
	CON	PHS	LAC	DLAC
Sodium tripoly phosphate	0	0.3	0.3	0.3
Potassium L-lactate	0	0	2.5	0
Potassium D-lactate	0	0	0	2.5

[a] Non-enhanced control (CON); no lactate control (PHS); 2.5% potassium L-lactate (LAC); 2.5% potassium D-lactate (DLAC).

Instrumental color. Instrumental color (CIE L*a*b* for Illuminant A) was evaluated during storage and display periods using a HunterLab MiniScan^{TM}XE Spectrophotometer (Model 45/0 LAV, Illuminant A, 3.18 cm diameter aperture, 10º standard observer; Hunter Associates Laboratory, Inc.; Reston, VA). Reflectance from 400 to 700 nm with increment readings at 10 nm and CIE L* a* b* values (Illuminant A) were determined and used to calculate saturation index $[(a^{*2} + b^{*2})^{1/2}]$ and $(K/S)_{572}/(K/S)_{525}$ values (*93*). Three different locations per steak for d 3, 9, and 14 were scanned and averaged for statistical analyses.

LDH-B activity. LDH-B activity was measured following the UV-method of Wahlefeld (*96*), which monitors the reduction of NAD^+ in the following reaction:

$$\text{L-Lactate} + NAD^+ \xrightarrow{\text{LDH}} \text{Pyruvate} + NADH + H^+$$

In brief, powdered frozen muscle tissue (2.0 g) from d 14 steaks (LD, SM, and PM) was homogenized in 8 ml of a 0.01 M sodium phosphate buffer (pH 7.5) for 30 s on ice. The homogenate was centrifuged at 13,823 x g for 30 min at 4ºC, and then was filtered through Whatman # 42 filter paper (Whatman Inc., Clifton, NJ). The filtered supernatant (0.1 ml) was added to the glass cuvette with 0.1 ml of NAD and 2.4 ml of Tris/L-lactate (pH 9.3). Activity of LDH was measured in duplicate by the continuous increase in absorbance at 339 nm for 2 min. Increased absorbance (increased NADH) between 30 and 120 s was used for calculating LDH activity. Units of LDH activity (U) were expressed as µmol/min/g sample.

NADH concentration. Alkaline extraction of NADH as described by Klingenberg (*104*) was used to measure NADH concentration of muscle tissue from d 14

steaks (LD, SM, and PM). In brief, 1 g of frozen pulverized muscle tissue in 8 ml of 0.5 M alcoholic KOH solution was vortexed 30 s, placed in agitating water bath for 5 min at 90°C, and rapidly cooled to 0°C in a -80 °C ultra freezer. The muscle mixture was neutralized (pH 7.8) by adding 6 ml of a triethanolamine-HCl-phosphate mixture. After holding at room temperature for 10 min to flocculate the denatured protein, the mixture was centrifuged at 25,000 g for 10 min at 4°C, and filtered the supernatant through Whatman # 42 filter paper (Whatman Inc., Clifton, NJ). Then, a modified assay of McCormick and Lemuel (*105*) was used to determine NADH by measuring the reduction of 2, 6-dichlorophenolindophenol (DCIP) by muscle extracts using absorbance at 600 nm (UV-2010; Hitachi Instruments, Inc., San Jose, CA). The NADH concentration (nmol/g) was calculated using the equation obtained from the standard curve using known NADH concentration.

Total reducing activity. The method of Lee et al. (*106*) was used to determine total reducing activity (TRA) of muscle tissue from d 14 steaks (LD, SM, and PM). In brief, powdered frozen muscle tissue (2.0 g) was homogenized in 10 ml of 25 mM PIPES (piperazine-n,n-bis-2-ethane-sulfonic acid) buffer. Five ml of homogenate were mixed with 2 ml of 5 mM potassium ferricyanide, chilled at 2 °C for 1 h with occasional stirring, and 0.1 ml of 0.5% ammonium sulfamate and 0.2 ml of 0.5 M lead acetate were added. After holding at room temperature for 5 min, 2.5 ml of 20% trichloroacetic acid was added to the mixture and the solution was filled up to volume (10 ml) with distilled water. After 5 min, the solution was filtered through Whatman # 42 filter paper (Whatman Inc., Clifton, NJ). A solution of 1 mM potassium ferricyanide was used as a

standard. The absorbance of the filtrate was read at 420 nm using a Hitachi spectrophotometer. TRA, an arbitrary value, was expressed as absorbance of 1 mM potassium ferricyanide minus absorbance of sample filtrate.

Data analysis. The experimental design was arranged as a split-plot. In the whole-plot portion, 10 muscles were served as blocks to which 1 of 4 treatments are assigned to sections within a muscle [n=10; (10 muscle x 4 sections per muscle) /4 treatments]. In the subplot, each steak from the muscle section was considered an experimental unit. Type-3 tests of fixed effects for injection treatment, muscle difference (LD, SM, and PM), display time, and their interaction (3-way) were analyzed by using the Mixed procedure of SAS for ANOVA. LSMeans was separated (F test, $p < 0.05$) by using least significant differences generated by the PDIFF option.

Results and Discussion

pH. The pH of the enhancement solutions ranged from 8.0 to 8.2 **(Table 4.2)**. All enhanced meat samples had a greater ($p < 0.05$) pH than that of the non-enhanced control (CON) at the end of storage and display. There were no differences ($p > 0.05$) in pH among the enhanced LD steaks, but SM and PM had higher pH ($p < 0.05$) for the potassium L-lactate (LAC) treatment compared to the CON and phosphate enhanced (PHS) steaks. PM had a greater pH compared to LD and SM for the both non-enhanced and enhanced treatments, which might be due to the difference in muscle fiber types between PM (oxidative) and LD (glycolytic). The higher pH of the enhanced meat samples was mainly due to the inclusion of phosphate in the enhancement solutions (*9, 102*).

Table 4.2. LSMeans for pH of treatment solution and steaks from bovine *M. - Longissimus lumborum* (LD), *Semimembranosus* (SM), and *Psoas major* (PM) at d 14 (end of display) at 1 ºC

	Treatment[e]				
	CON	PHS	LAC	DLAC	SE[d]
Injection-Solution		8.2	8.0	8.0	
LD	5.47ax	5.7bx	5.78bx	5.76bx	0.03
SM	5.39ax	5.59by	5.72cx	5.65bcy	0.03
PM	5.58ay	5.84bz	5.94cy	5.89bcz	0.03

[abc] Means within a row with different letters are different ($p < 0.05$). [xyz] Means within a column with different letters are different ($p < 0.05$). [d] Standard errors. [e] Non-enhanced control (CON); no lactate control (PHS); 2.5% potassium L-lactate (LAC); 2.5% potassium D-lactate (DLAC).

Instrumental darkening. CIE L* (lightness) values were affected ($p < 0.05$) by muscle, treatments, and day. The potassium L-lactate (LAC) enhancement darkened (lower L* values, $p < 0.05$) the meat surface of LD and SM throughout storage and display (**Table 4.3**). The potassium D-lactate (DLAC) enhancement also darkened LD at d 3, but there was no significant difference in L* value compared to the control (CON) and phosphate injected (PHS) LD at d 14. The LAC and DLAC treated PM steaks were not different in lightness as compared to the CON, but were darker ($p < 0.05$) than the PHS treated steaks at d 14. The darkening effect of lactate enhancement on meat color has been reported by several researchers (*9, 107-109*). However, other than the increased pH effect by the enhancement solutions, there were no clear explanations for the muscle darkening. Because the lactate enhanced steaks were darker ($p < 0.05$) than the phosphate treated steaks of all muscles despite of no differences in pH for the both treatments, there must be other physiochemical reactions associated with the darkening effect of lactate enhancement. The surface darkening of meat with lactate salts might be due to the increased binding reaction of myoglobin and myofibrillar protein, which is induced by elevated ionic strength from lactate salts. Chaijan et al. (*110*) found a decreased whiteness value for fish myoglobin as the bound formation of myoglobin-natural actomyosin (NAM) increased when ionic strength was increased to a concentration of 0.6 M KCl. They suggested that myoglobin could bind with myofibrillar proteins, which were released from the myofibrils as salt concentration increased, dispersed in the solution as monomers, and partially unfolded (*111, 112*), thus

leading to the formation of a larger molecular weight aggregate. They found the decreased whiteness was consistent with greater aggregation (*110*).

Instrumental color and color stability. There were muscle x treatment and muscle x day interactions ($p < 0.001$) for a* values (indication of redness). Initial redness values (d 3) for the LAC treated LD steaks were lower ($p < 0.05$) than other treatments (**Table 4.3**), but LAC maintained higher redness ($p < 0.05$) compared to the CON and DLAC at the end of storage and display (d 14). The LAC enhanced steaks from SM and PM also had a similar effect in redness as LD. There were no significant differences in a* values between lactate treatment and control at 3 d; however, as storage time and display period progressed, the redness values of the CON steaks decreased ($p < 0.05$) sharply, and were significantly lower than the LAC enhanced steaks. The DLAC treated steaks from LD were not different ($p > 0.05$) from the CON steaks in redness at 14 d, whereas the DLAC steaks from SM and PM were redder than the CON steaks most likely due to the increased muscle pH by phosphate inclusion in the DLAC solution. Chroma (color intensity) values and $(K/S)_{572}/(K/S)_{525}$ values (indication of metmyoglobin (MMb) accumulation) also followed similar trends as a* values for all muscles in each respective treatment. The enhanced steaks had higher ($p < 0.05$) chroma and $(K/S)_{572}/(K/S)_{525}$ values over the non-enhanced CON steaks at d 14. The PM steaks enhanced with the LAC had the least MMb accumulation on the surface of meats among treatments at the end of storage and display period. In general, PM is considered as "very low" color stability muscle (*54*).

Table 4.3. LSMeans for instrumental color scores of Bovine *M - Longissimus lumborum, Semimembranosus,* and *Psoas major* steaks for stored 9 d and displayed 14 d at 1 ºC

			Treatment[f]				
Muscle	Trait	Day	CON	PHS	LAC	DLAC	SE[d]
LD							
	L*	3	43.2ax	43.8ax	39.5bx	41bx	0.97
		9	43.7ax	43.8ax	39.7bx	40.2bx	0.97
		14	42.4ax	44.1ax	37.8bx	41.6ax	0.97
	a*	3	32.2ax	33.6ax	29.7bx	32.1ax	0.86
		9	27.8ay	30.3by	27.2axy	29.1aby	0.86
		14	23.5az	25.9bz	26.9by	25.4az	0.86
	Chroma[e]	3	38.9ax	41.2ax	35.4bx	38.8ax	0.97
		9	33.3ay	37.3by	32.3ay	35by	0.97
		14	28.7az	32.1bz	32.4by	31bz	0.97
	$(K/S)_{572}/(K/S)_{525}$	3	1.42ax	1.46ax	1.4ax	1.46ax	0.03
		9	1.33ay	1.4ax	1.34axy	1.37axy	0.03
		14	1.23az	1.3by	1.32by	1.31by	0.03
SM							
	L*	3	44.9ax	45.9ax	43.5ax	43.8ax	0.97
		9	44.3abx	46.3bx	43.5ax	43ax	0.97
		14	42.9ax	44.6ax	39.6by	42.5ax	0.97
	a*	3	31.9ax	34.5bx	31.5ax	32.2ax	0.86
		9	26.2ay	29.4by	26.5ay	27.5aby	0.86
		14	20.5az	24.8bz	24bz	23.5bz	0.86
	Chroma[e]	3	39.2ax	43.5bx	39.3ax	40ax	0.97
		9	32.5ay	37.3by	33.3ay	34.2ay	0.97
		14	26.8az	32.3bz	30.5bz	29.9bz	0.97
	$(K/S)_{572}/(K/S)_{525}$	3	1.36abx	1.43bx	1.36abx	1.34ax	0.03
		9	1.24ay	1.3ay	1.23ay	1.25ay	0.03
		14	1.01az	1.21bz	1.17by	1.2by	0.03
PM							
	L*	3	43.9abx	45.5bx	43.5abx	42.2ax	0.97
		9	43ax	44.5axy	41.9axy	41.9ax	0.97
		14	41.2abx	43.1ay	39.9bx	40.4bx	0.97
	a*	3	28.6ax	30.1ax	30.4ax	31.9ax	0.86
		9	20.7ay	24.7by	25.5by	26.2by	0.86
		14	12.4az	20.1bcz	21.7bz	18.3cz	0.86
	Chroma[e]	3	34.3abx	36.8abx	36.9ax	34.2bx	0.97
		9	25.3ay	30.1by	29.5by	28.3by	0.97
		14	18.8ay	26bz	26.7bz	23.2cz	0.97
	$(K/S)_{572}/(K/S)_{525}$	3	1.31ax	1.35ax	1.35ax	1.32ax	0.03
		9	1.12ay	1.22by	1.23by	1.2by	0.03
		14	0.8az	1.05bz	1.13cz	1.04bz	0.03

Table 4.3. Continued.

[abc] Means in a row within day having different letters are different ($p < 0.05$). [xyz] Means in a column of muscle types with different letters are different ($p < 0.05$). [d] Standard errors. [e] saturation index $(a^{*2}+b^{*2})^{1/2}$ were calculated. [f] Non-enhanced control (CON); no lactate control (PHS); 2.5% potassium L-lactate (LAC); 2.5% potassium D-lactate (DLAC).

A greater MMb accumulation, lower MMb reductase activity (*56*), and lower nitric oxide-MRA (*54*) of PM have been reported. However, based on our findings, the injection enhancement improved color stability of PM steaks, and the potassium L-lactate/phosphate combination was more effective at retarding discoloration of PM over the phosphate treatment alone.

NADH concentration. There were no significant interactions for NADH concentration, but there were main effects ($p < 0.001$) differences for muscle and treatment (**Figure 4.1**). The LD steaks had significantly higher NADH concentration than the SM and PM steaks over the all treatments. The LAC enhanced steaks of LD had the highest NADH concentration ($p < 0.05$) among the other treatments. The LAC treatment increased NADH concentration ($p < 0.05$) of the PM steaks compared to the CON and PHS enhanced steaks. These findings are in agreement with the previous report of Kim et al. (*9*) who found a higher NADH concentration in beef *longissimus lumborum* enhanced with 2.5% potassium L-lactate solutions. They concluded that L-lactate reacted with LDH increasing the LDH flux to regenerate NADH. We also found that the DLAC steaks of LD and PM had a higher ($p < 0.05$) NADH concentration than the CON, but the concentration was not different with the PHS. This might be due to the increased pH effect by combining the DLAC solution with a phosphate. LDH-B activity is more active under alkaline conditions (*96*), and thus the PHS and DLAC/PHS treatments might create a more favorable condition for LDH to produce NADH. The antioxidant property of phosphate (*113*) and lactate (*114, 115*) might protect oxidation of NADH during storage and display period.

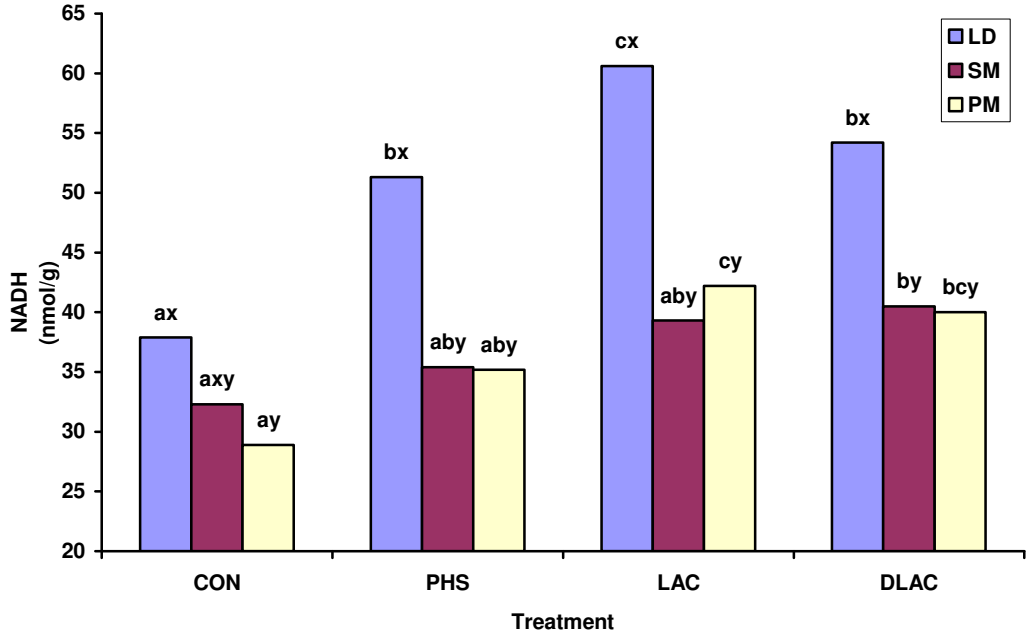

Figure 4.1. LSMeans for NADH concentration of bovine *M.- Longissimus lumborum* (LD), *Semimembranosus* (SM), and *Psoas major* (PM) steaks at 14 d (end of storage and display) at 1 ºC. ^{abc}Means within the same muscle across the treatments with different letters are different ($p < 0.05$). ^{xy}Means within the same treatment with different letters are different ($p < 0.05$). Non-enhanced control (CON); no lactate control (PHS); 2.5% potassium L-lactate (LAC); 2.5% potassium D-lactate (DLAC).

Mancini et al. (*116*) observed a suppression of MMb formation of equine oxymyoglobin (OMb) incubated with sodium lactate, and concluded that lactate itself may increase color stability through a non-enzymatic interaction with myoglobin.

LDH-B activity. There were significant main effects of muscle ($p < 0.0001$) and treatment ($p = 0.0009$) for LDH-B activity (**Figure 4.2**). The LD steaks had significantly higher LDH-B activity than the SM and PM among the CON steaks. Although LDH-B activity of the LAC enhanced steaks was not significantly different from that of the CON, there was a trend ($p = 0.08$) toward increasing LDH flux for the LAC enhancement. PM steaks enhanced with the PHS and LAC had a higher LDH-B flux compared to the CON steaks. The increased LDH-B flux for the PHS treatment might be due to the increased pH effect, which resulted in a more favorable reaction condition for the enzyme (*117*). The DLAC enhancement to PM did not significantly affect LDH-B flux, but there was a tendency to increase like the PHS treatment possibly due to the pH effect (**Table 4.2**). The enzyme-substrate reactions that occurred selectively distinguished between D-lactate, which can not be metabolized by LDH, and L-lactate, the selected substrate (*85*). Due to the conformational difference of L-lactate from D-lactate, the hydrogen on the center carbon of L-lactate is located near nicotinamide, which allows easy transfer of hydrogen to NAD. On the other hand, D-lactate is positioned with its methyl group toward NAD, and thus the hydrogen points away from NAD. Decreased L-lactate and increased D-Lactate concentrations of meat during storage have been reported (*69, 118*). The decrease of L-lactate during storage is due to its utilization by the microorganisms as a carbon source when glucose is no longer

available or limited (*119, 120*). In contrast, the increased production of D-lactate is the result of metabolic activity of microbial growth (*118*). Therefore, the structural differences of potassium L- and D- lactate allowed for different metabolic reaction rates with LDH-B in the lactate enhanced muscles, and consequently resulted in differences in meat color stability.

Previously, Kim et al. (*9*) determined improved color stability of LD steaks enhanced with 2.5% potassium lactate in combination with phosphate solutions by increasing NADH for MRA via LDH. Furthermore, Kim et al. (*91*) concluded that although PM had a higher pH, it had the least color stability and lowest MRA possibly due to lower LDH-B activity and subsequently lower NADH regeneration. However, based on our findings, lactate enhancement can improve color stability of PM muscles via increased NADH concentration through the LDH-B reaction.

Total reducing activity. Total reducing activity (TRA) of LD and PM also was increased by the LAC enhancement (**Figure 4.3**). There were significant main effects of muscles and treatments for TRA. The LD steaks for all treatments had higher ($p < 0.05$) TRA than the PM steaks. TRA of the LAC enhanced steaks for LD was significantly higher than the CON and PHS treated steaks. The enhancement did not affect ($p > 0.05$) TRA of SM steaks. The PHS and LAC enhancement of PM increased ($p < 0.05$) TRA.

The results of this experiment confirmed that potassium L-lactate enhancement improved color stability of physiologically different bovine muscles. Lactate dehydrogenase seems to distinguish between the two isomers of the enhanced lactate (L-

and D-lactate), increase its flux (LDH-B) to generate NADH, and consequently improve the reducing activity. This study suggests that PM, a very low color stable muscle, also can reduce its color deterioration through lactate enhancement by minimizing surface metmyoglobin oxidation (**Figure 4.4**).

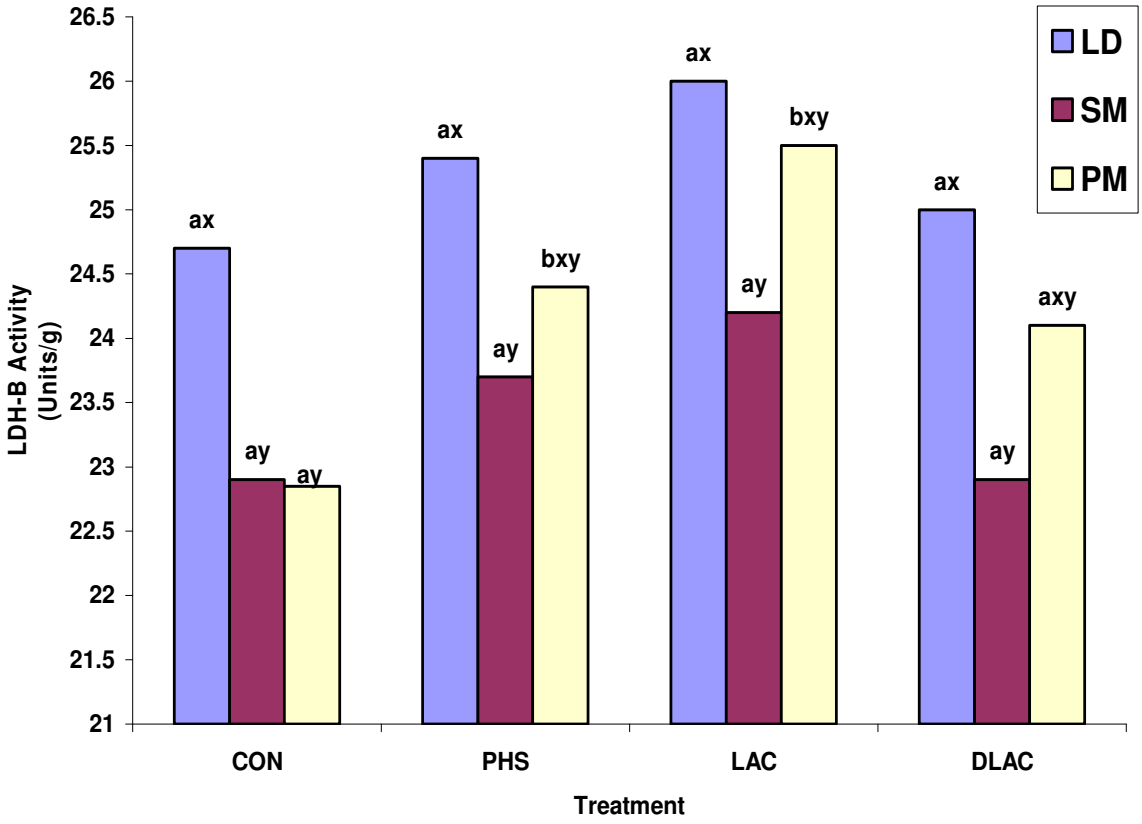

Figure 4.2. LSMeans for LDH-B activity of bovine *M.- Longissimus lumborum* (LD), *Semimembranosus* (SM), and *Psoas major* (PM) steaks at 14 d (end of storage and display) at 1 ºC. ^{ab}Means within the same muscle across the treatments with different letters are different ($p < 0.05$). ^{xy}Means within the same treatment with different letters are different ($p < 0.05$). Non-enhanced control (CON); no lactate control (PHS); 2.5% potassium L-lactate (LAC); 2.5% potassium D-lactate (DLAC).

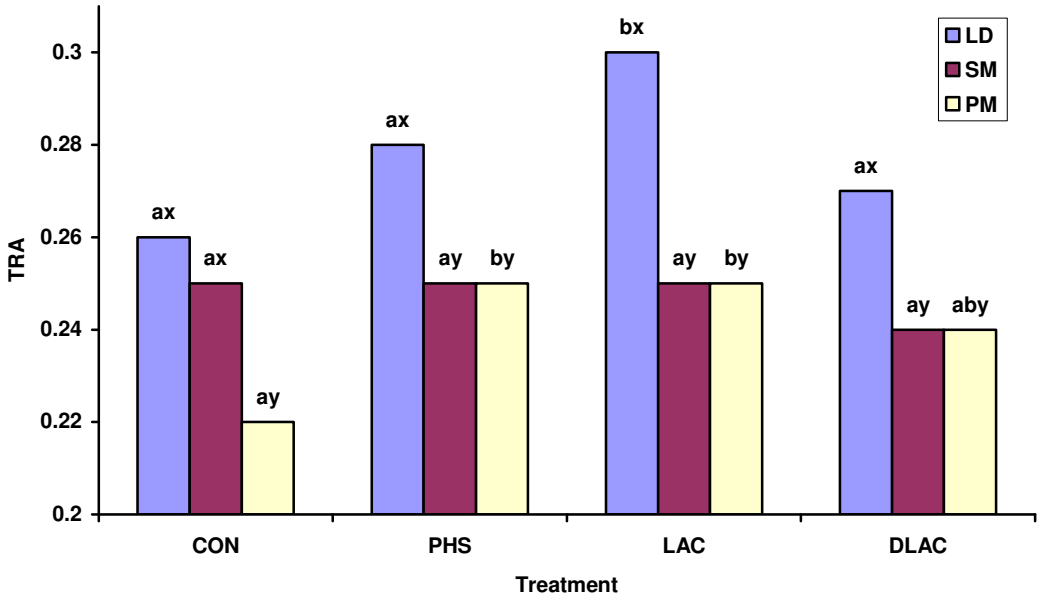

Figure 4.3. LSMeans for TRA of bovine *M.- Longissimus lumborum* (LD), *Semimembranosus* (SM), and *Psoas major* (PM) steaks at 14 d (end of storage and display) at 1 ºC. ^{ab}Means within the same muscle across the treatments with different letters are different ($p < 0.05$). ^{xy}Means within the same treatment with different letters are different ($p < 0.05$). Non-enhanced control (CON); no lactate control (PHS); 2.5% potassium L-lactate (LAC); 2.5% potassium D-lactate (DLAC).

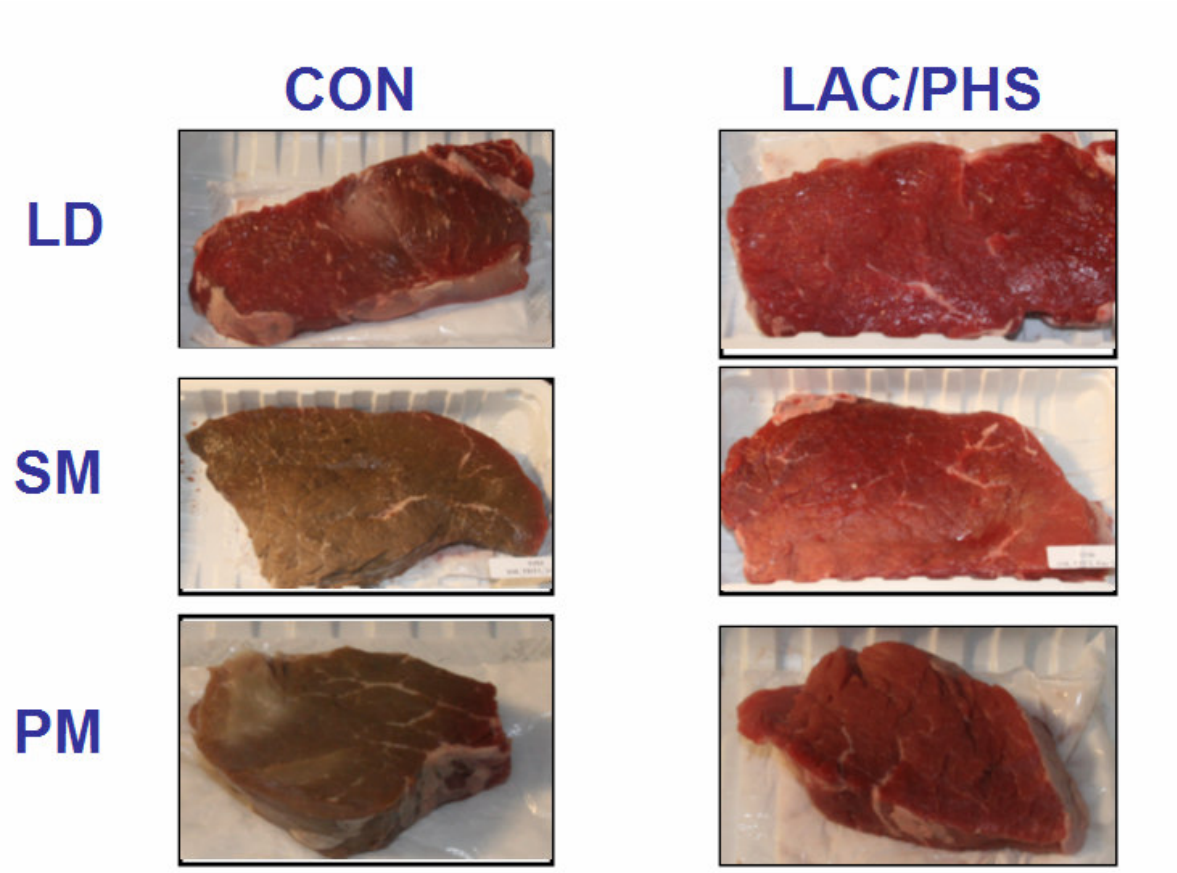

Figure 4.4. Pictures for bovine *M.- Longissimus lumborum* (LD), *Semimembranosus* (SM), and *Psoas major* (PM) steaks at 14 d (end of storage and display) at 1 ºC. Non-enhanced control (CON); 2.5% potassium L-lactate (LAC/PHS).

CHAPTER V

EVALUATION OF ANTIOXIDANT CAPACITY AND COLOR STABILITY OF CALCIUM LACTATE ENHANCEMENT ON FRESH BEEF UNDER HIGHLY OXIDIZING CONDITIONS

Overview

The objectives of this study were to determine the influence of calcium lactate enhancement on color stability and lipid oxidation of bovine muscles under highly oxidizing conditions. Fifteen USDA Select beef strip loins were divided individually into four equal width sections, and one of six treatments (control; 0.3% phosphate; 0.2% calcium lactate with 0.3% phosphate; irradiated (2.4kGy) non-enhanced control; irradiated with 0.2% calcium lactate; and irradiated with 0.2% calcium lactate plus 0.3% phosphate) randomly assigned to each loin section (n=10). Steaks from each loin section were packaged in high-oxygen MAP (80% oxygen), irradiated at 2.4 kGy, stored in the dark at 1°C for 10 d, and then displayed for 5 d at 1 °C under 2150 lux of fluorescent light. Instrumental surface color, total reducing activity (TRA), 2-thiobarbituric acid value (TBARS), and NADH concentration were measured. Loins with calcium lactate and phosphate maintained the most stable red color (highest a* values) during storage and display time compared to other treatments. Further, calcium lactate with phosphate in loins increased NADH concentration ($p < 0.05$), and were the least oxidized (lowest TBARS). Even under highly oxidizing conditions (hi-ox MAP packaging with irradiation), calcium lactate with phosphate treatment minimized lipid oxidation, increased NADH and TRA, and consequently had a higher a* value compared to other

irradiated treatments. These results suggest that lactate inclusion improves color stability of fresh beef by providing superior antioxidant capacity and increased reducing activity of myoglobin by elevating NADH concentration.

Introduction

Injection enhancement of beef and pork muscles with brine solutions or marinades has been widely practiced in the value-added meat industry. The calcium salts of lactic acid are commonly incorporated into both raw and cooked meat products as microbial inhibitors, as well as to minimize off-odors and flavors, increase water-holding capacity and cook yield, and improve juiciness and tenderness (*102, 121-123*). Infusion of lactate by injection enhancement also has been recognized to stabilize the color of both raw and cooked meat products (*9*), (*63, 101-103*). However, the exact mechanism by which lactate improves meat color stability has not been fully explained.

Meat color is the one of the most important factors affecting consumer purchasing decisions (*98*). Meat discoloration due to formation of metmyoglobin (MMb), the oxidized form of myoglobin, is considered to indicate an inferior or old product. Hood and Riordan (*1*) found a 50% reduction in retail sales of beef when MMb formation reached 20% of the total pigment present. The formation of MMb resulting from the oxidation of ferrous heme proteins is positively associated with lipid oxidation (*124*). It has been suggested that free radicals produced by lipid oxidation can initiate the reaction of oxymyoglobin (OMb) to MMb (*125*). Furthermore, 4-Hydroxynonenal (4-HNE), a secondary aldehyde product from lipid oxidation, accelerated MMb formation through the covalent modification of histidine residues of myoglobin (*126*).

The oxidized form of myoglobin can be converted to the reduced state through a nicotinamide adenine dinucleotide (NADH)-dependent muscle reducing system (*127*). Ledward (*28*) suggested that metmyoglobin reducing activity (MRA) is the most important intrinsic factor controlling the rate of MMb accumulation in beef. Reddy and Carpenter (*29*) reported that the higher the MRA of muscles had the greater the color stabilities. Because the reducing system is NADH-dependent, the depletion of NADH during storage and/or display periods results in decreasing MRA, and increased surface discoloration caused by MMb accumulation (*91*). However, it also has been reported that MRA can be increased with NADH regeneration by several dehydrogenases in the cytoplasm with appropriate substrates (*8, 44, 128, 129*). Kim et al. (*9*) determined increased color stability of beef longissimus steaks injection enhanced with 2.5% potassium lactate solution. They concluded that lactate enhancement improved color stability by replenishing NADH through the increased LDH flux (reaction toward to pyruvate), and consequently increasing MRA.

Thus, we hypothesize that calcium lactate improves color stability of fresh beef by hindering oxidation of myoglobin via increased reducing capacity and enhancing antioxidant properties. Thus, the objectives of this study in two experiments were: (1) to evaluate MMb reduction through the generated NADH via the reaction between calcium lactate and muscle LDH *in vitro*, and (2) to determine the influence of calcium lactate enhancement on color stability and lipid oxidation of bovine muscles under highly oxidizing conditions *in situ*.

Material and Methods

Experiment 1: Equine MMb reduction by calcium lactate-NAD-LDH System

Reagents. Horse heart metmyoglobin, NAD, calcium L-lactate, 2, 6-dichlorophenolindophenol (DCIP), and phenazine methosulphate (PMS) were obtained from Sigma (St Louis, MO, USA).

Reduction of equine MMb. The reduction of equine MMb through calcium lactate (CaLac) with muscle extract was tested by following the modification method of Kim et al. (*9*). Reactants in various combinations were added to 10 mm path length polystyrene cuvettes with 1.0 mL final reaction volume. The standard reaction mixtures at pH 5.7 contained one or more of the following (**Table 5.1**): 0.3 mL of 0.5 mM equine MMb in 30 mM phosphate buffer pH 7.0, 0.1 mL of citrate buffer (50 mM), 0.175 mL of distilled water, 0.025 mL of 30 mM PMS, 0.1 ml of bovine longissimus muscle extract, 0.1 mL of 6.5 mM NAD, 0.1 mL of 200 mM calcium lactate, and 0.1 mL of 0.6 mM DCIP.

The effects of different CaLac concentrations (50, 100, 150, 200 mM) and different assay pH on the rate of MMb reduction were determined. The final assay pH (4.9, 5.4, 5.7, and 6.7) was varied by altering the concentration of the citrate (100, 80, or 50 mM respectively) or by adding sodium phosphate buffer (pH 8.3, 30 mM). The bovine muscle extract was prepared previously; in brief, powdered frozen muscle tissue (2.0g) was homogenized (Polytron Model PT 10/35, Kinematica, Luzernerstrasses, Switzerland) in 8 ml of a 0.01M sodium phosphate buffer (pH 7.5) for 30s on ice.

Table 5.1. Reduction of horse MMb through calcium lactate+NAD+muscle extract system at 22 ºC and pH 5.7 (n = 3)

Solution components[a]							
MMb (0.5 mM)	DCPIP (0.6 mM)	Citrate (50 mM)	NAD (6.5 mM)	CaLac (200 mM)	Extract	PMS (30 mM)	Activity (nmole/min)
+	**+**	**+**	**+**	**+**	**+**	**+**	**0.091±0.006**
+	-	+	+	+	+	+	0.082±0.003
+	+	+	**-**	**+**	**+**	+	0
+	+	+	**+**	**-**	**+**	+	0
+	+	+	**+**	**+**	**-**	+	0
+	+	+	+	+	+	-	0
-	+	+	+	+	+	+	0

[a] Substances present (+) or absent (-) in mixtures. MMb, equine metmyoglobin; DCIP, 2, 6-dichlorophenolindophenol; NAD, nicotinamide dinucleuotide; CaLac, calcium L-lactate; PMS, phenazine methosulphate. Assays were conducted with 0.5 mM equine MMb in 30 mM phosphate buffer pH 7.0.

The homogenate was centrifuged at 13,823 x g for 30 min at 4 ºC, and then was filtered through Whatman No. 42 filter paper (Whatman Inc., Clifton, NJ). The reaction was initiated by adding 0.1 mL of the filtered supernatant. Absorbance at 580 nm was recorded every 2 sec for 120 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 absorption 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 was expressed as the mean of triplicate samples.

Experiment 2: Effects of calcium lactate enhancement on color stability, reducing activity, and anti-oxidant capacity of injection-enhanced beef under highly oxidized conditions.

Raw materials and processing. Fifteen USDA selected beef strip loins were obtained form a commercial meat facility, and were transferred to the Rosenthal Meat Science and Technology Center at Texas A&M University. On 4 day post-mortem, each muscle was divided into four equal-length sections (60 total sections), and one of six treatment combinations (control, CON; 0.3% phosphate, PHS; 0.2% calcium lactate with 0.3% phosphate, CLPS; irradiated (2.4kGy) non-enhanced control, I-CON; irradiated with 0.2% calcium lactate, I-CAL; and irradiated with 0.2% calcium lactate plus 0.3% phosphate, I-CLPS) randomly assigned to each loin section resulting in 10 replications per treatment. Enhancement solutions contained one or more of the following (**Table 5.2**): distilled water, calcium L-lactate (PURACAL; PURAC America, Inc., Lincolnshire, IL), and/or sodium tripolyphosphate (Brifisol® 512; BK Giulini Corp;

Simi Valley, CA). Sequential injections of calcium lactate followed by phosphate were applied at 12% of raw weight since phosphates chelate calcium in solution if mixed together. Loin sections were weighed individually before and after injection with a multineedle injector (model N30, Wolftec, Inc., Werther, Germany) to calculate actual injection levels (12%). Each enhanced product was sliced (2.54 cm thick steaks) into steaks for packaging.

Packaging. Each steak was placed in preformed trays (polypropylene/ethylene vinyl alcohol, 22.5 cm x 17.3 cm x 4.1 cm; Rock-Tenn.; Franklin Park, IL). Trays were flushed with a high-oxygen atmosphere (80% O_2/20% CO_2, Certified Standard, Airgas Specialty Gases; Austin, TX) and sealed by a ROSS INPACK JR (Model S3180; Ross Industries, Inc.; Midland, VA) and a shrinkable barrier sealing film (MAP-Shield AF; 1.5 mil high-barrier nylon/ethylene vinyl alcohol/methallocenece polyethylene with an oxygen-transmission rate of 0.02 cc/645.16 cm2/24 h at 10 ºC and 80% relative humidity and a water vapor transmission rate of 0.92 g/645.16 cm^2/24 h at 37.8 ºC and 100% relative humidity; Honeywell, Morristown, NJ) was used for MAP.

Irradiation. After packaging, samples assigned for the irradiation treatment (I-CON, I-CAL, and I-CLPS) were transported to the National Center for Electron Beam Food Research irradiation facility located at Texas A&M University's Research Park. The Hi-oxygen MAP packaged samples were irradiated to doses of 2.5 kGy using a 10 MeV, 15 KW electron beam linear accelerator (L-3 Communications, Pulse Sciences, San Leandro, Calf., U.S.A.). After irradiation, the samples were placed in cardboard boxes and were transferred immediately to the Rosenthal Meat Science and Technology

Table 5.2. Composition of experimental injection-enhancement solutions for beef muscles

	Treatment[a]					
	Non-irradiated			Irradiated		
	CON	PHS	CLPS	I-CON	I-CAL	I-CLPS
Sodium tripoly phosphate	0	0.3	0.3	0	0	0.3
Calcium lactate	0	0	0.2	0	0.2	0.2

[a] Non-enhanced control (CON); no lactate control (PHS); calcium L-lactate/phosphate (CLPS); non-enhanced irradiated control (I-CON); calcium lactate only w/ irradiation (I-CAL); calcium L-lactate/phosphate w/ irradiation (I-CLPS).

Center at Texas A&M University. Packages were stored in the dark at 2 ºC (35ºF) for 10 d before display for 5 days at 1 °C under 2150 lux of fluorescent light.

pH. A sample from each muscle on d 14 was frozen in liquid nitrogen and pulverized in a Waring® table-top blender (Dynamics Corp. of America, New Hartford, CT). About 5 g of muscle tissue was mixed with 20 ml of distilled water for 20 s, and pH values were measured with a standardized combination pH electrode attached to a pH meter (Accumet 50; Fischer Scientific, Fair Lawn, NJ).

Instrumental color. Instrumental color (CIE L*a*b* for Illuminant A) was evaluated during storage and display periods using a HunterLab MiniScan™XE Spectrophotometer (Model 45/0 LAV, Illuminant A, 3.18 cm diameter aperture, 10º standard observer; Hunter Associates Laboratory, Inc., Reston, VA). Reflectance from 400 to 700 nm with 10 nm increment readings and CIE L* a* b* values (Illuminant A) were measured and used to calculate saturation index $[(a^{*2} + b^{*2})^{1/2}]$ and $(K/S)_{572}/(K/S)_{525}$ values (*93*). Three different locations per steak for d 3, 9, and 14 were scanned and averaged for statistical analyses.

NADH concentration. Alkaline extraction of NADH as described by Klingengerg (*104*) was used to measure NADH concentration of muscle tissue from d 14 steaks. In brief, 1 g of frozen pulverized muscle tissue in 8 mL of 0.5 M alcoholic potassium hydroxide solution was vortexed 30 sec, placed in agitating water bath for 5 min at 90 °C, and rapidly cooled down to 0 °C in -80 °C ultra freezer. The muscle mixture was neutralized (pH 7.8) by adding 6 mL of triethanolamine-HCl-phosphate mixture. After holding at room temperature for 10 min to flocculate the denatured

protein, the mixture was centrifuged at 25,000 g for 10 min at 4 °C, and the supernatant filtered through Whatman # 42 filter paper (Whatman Inc., Clifton, NJ). Then, a modified assay of McCormick and Lemuel (*105*) was used to determine NADH by measuring the reduction of DCPIP by muscle extracts using absorbance at 600 nm (UV-2010; Hitachi Instruments, Inc., San Jose, CA). The NADH concentration (nmol/g) was calculated using the equation obtained from the standard curve using known NADH concentration.

Total reducing activity. The method of Lee et al. (*106*) was used to determine total reducing activity (TRA) of muscle tissue from d 14 steaks. In brief, powdered frozen muscle tissue (2.0g) was homogenized in 10 ml of 25 mM PIPES (piperazine-n,n-bis-2-ethane-sulfonic acid) buffer. Five 5 mL of homogenate was mixed with 2 mL of 5 mM potassium ferricyanide, chilled at 2 °C for 1 hour with occasional stirring, and 0.1 mL of 0.5% ammonium sulfamate and 0.2 mL of 0.5 M lead acetate were added. After holding at room temperature for 5 min, 2.5 mL of 20% trichloroacetic acid was added to the mixture and filled up the solution to volume (10 mL) with distilled water. After 5 min, the solution was filtered through Whatman # 42 filter paper (Whatman Inc., Clifton, NJ). A solution of 1 mM potassium ferricyanide was used as a standard. The absorbance of filtrate was read at 420 nm using a Hitachi. TRA, an arbitrary value, was expressed as absorbance of 1mM potassium ferricyanide minus absorbance of sample filtrate.

Lipid oxidation. Lipid oxidation of steaks from d 14 were measured using the 2-thiobarbituric acid method described by Yin et al. (*130*). In brief, powdered frozen

muscle tissues (5.0 g) were mixed with trichloroacetic acid solution (11%), homogenized for 30 s, and filtered through Whatman # 42 filter paper (Whatman Inc., Clifton, NJ). The filtrate (1 mL) was mixed with 1 mL of thiobarbituric acid (20 mM) and incubated at 25 °C for 20 h. The absorbance of samples was measured spectrophotometrically at 520 nm and reported as thiobarbituric acid-reactive substances (**TBARS**).

Data analysis. For experiment 1, tests were conducted in triplicate under a completely randomized design. Least square means and standard errors were used to determine the effects of lactate and lactic dehydrogenase on *in vitro* metmyoglobin reduction. The design for experiment 2 was a split plot. For the whole plot portion, 15 muscles was served as blocks to which 1 of 6 treatments were assigned to sections within a muscle as incomplete randomized block design [n=10; (15 muscle x 4 sections per muscle) /6 treatments]. For the subplot, each of the steaks from a loin section was considered an experimental unit. Type-3 tests of fixed effects for injection treatment, display time, and their interaction (2-way) were analyzed by using the Mixed procedure of SAS for ANOVA. LSMeans was separated (F test, $p < 0.05$) by using least significant differences generated by the PDIFF option.

Results and Discussion

Experiment 1: Equine MMb reduction by calcium lactate-NAD-LDH System.

The equine MMb was effectively reduced by the calcium lactate-NAD-muscle extract system. Exclusion of any substrate (NAD, calcium lactate, and muscle extract) for NADH generation resulted in no MMb reduction activity (**Table 5.1**). Omission of

PMS, an electron transfer catalyst, caused no MMb reduction through the system. PMS is often used to mediate electron transfer between NADH and the oxidizing cofactor (*131*). Our data suggest that the substrate mixture of calcium lactate and NAD with muscle extract containing LDH can generate NADH resulting in concomitant reduction of MMb to OMb through its electron donation. Increasing the concentration of calcium lactate resulted in increasing the equine MMb reduction (**Figure 5.1**). Kim et al. (*9*) found identical results of the non-enzymatic MMb reduction which increased with greater amounts of either NAD or lactate. The reduction of equine MMb was positively affected by pH condition (**Figure 5.2**). At pH of 4.9, there was no reduction through the lactate-NAD-LDH system, but at a normal pH range of postmortem muscle (5.3 to 5.7), the MMb reduction increased as well. The higher the pH, the more MMb was reduced by NADH generation via the lactate-LDH system. This is mostly due to the higher LDH-B activity (reaction toward pyruvate + NADH) at alkaline condition (*117*).

Experiment 2: Effects of calcium lactate enhancement on color stability, reducing activity, and anti-oxidant capacity of injection-enhanced beef under highly oxidized conditions.

pH. The pH of the enhancement solutions ranged from 6.5 to 8.2 **(Table 5.3)**. All enhanced steaks containing phosphate had higher pH values compared to the non-enhanced control (CON). Irradiated steaks with only calcium lactate enhancement (I-CAL) had lower pH compared to the steaks enhanced with a combination of calcium lactate and phosphate.

Table 5.3. LSMeans for pH of treatment solution and Longissimus lumborum (LD) steaks at d 14 (end of display) at 1 ºC

	Treatment[f]						
	Non-irradiated			Irradiated			
	CON	PHS	CLPS	I-CON	I-CAL	I-CLPS	SE[e]
Solution		8.2	8.0		6.5	8.0	
LD	5.48a	5.69c	5.73cd	5.42b	5.39b	5.74d	0.03

[abcd] Means within a row with different letters are different ($p < 0.05$). [e] Standard errors. [f] Non-enhanced control (CON); no lactate control (PHS); calcium L-lactate/phosphate (CLPS); non-enhanced irradiated control (I-CON); calcium lactate only w/ irradiation (I-CAL); calcium L-lactate/phosphate w/ irradiation (I-CLPS).

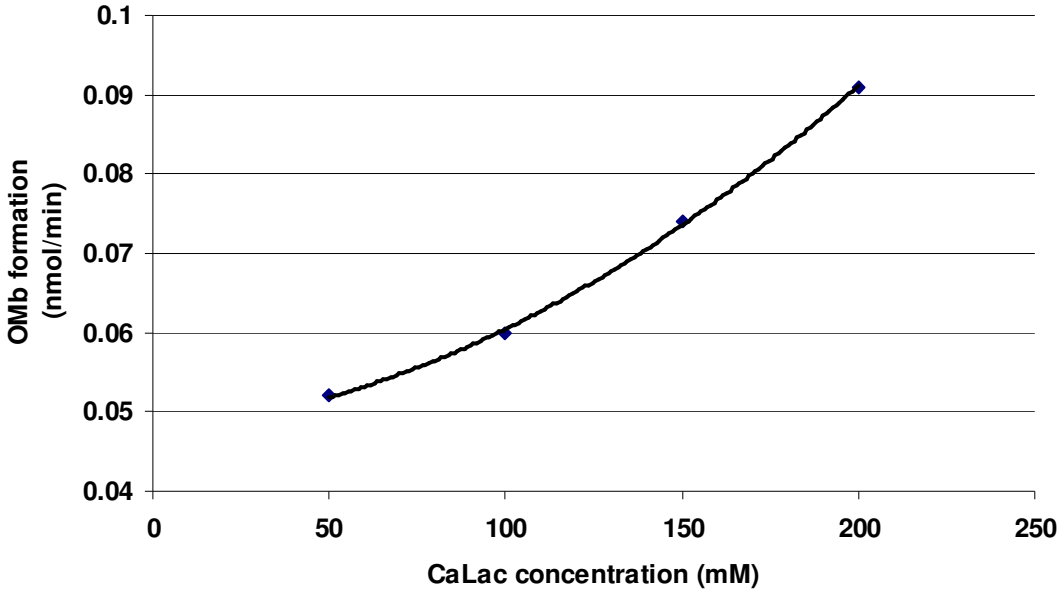

Figure 5.1. LSMeans for equine MMb reduction (equal to OMb formation, nmol/min) through lactate-NAD-muscle extract system. Assays were conducted with 0.5 mM equine MMb in 30 mM phosphate buffer pH 7.0 giving final pH 5.7 for a test cuvette .

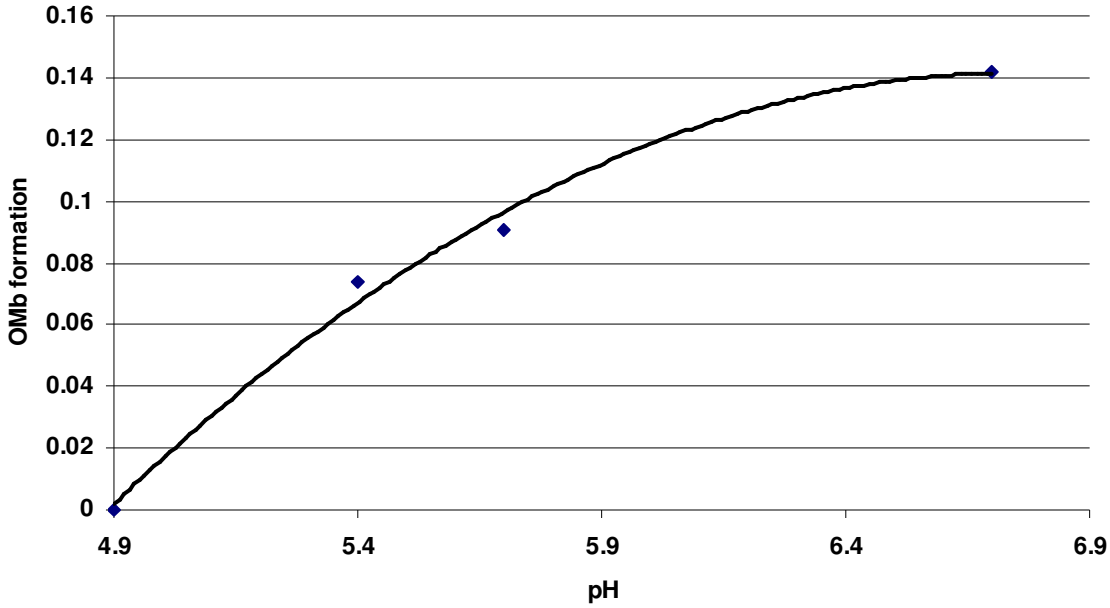

Figure 5.2. LSMeans for equine MMb reduction (equal to OMb formation, nmol/min) through lactate-NAD-muscle extract system within different assay pH by altering the concentration of the citrate (100, 80, or 50 mM respectively) or adding sodium phosphate buffer (pH 8.3, 30 mM). Assays were conducted with 0.5 mM equine MMb in 30 mM phosphate buffer pH 7.0, NAD (6.5 mM), and calcium lactate (200 mM) mixture in test cuvettes.

Instrumental color and color stability. CIE L* (lightness) values for the non-irradiated treatments (**Table 5.4**) were not different ($p > 0.05$). Calcium lactate enhancement did not cause any surface darkening, which was reported in other lactate enhancement studies (*9, 107-109*). Irradiated steaks (I-CON, I-CLPS) had lower L* values ($p < 0.05$) compared to non-irradiated steaks at the initial storage day. However, as storage and display time increased, lightness values for all irradiated steaks increased ($p < 0.05$). Lightness values for non-irradiated steaks did not significantly change during storage and display periods.

There were significant day x treatment interactions in redness values (a*). Initial redness values for non-irradiated steaks were not different ($p > 0.05$). However, during display periods, a* values for all steaks decreased significantly except the calcium/phosphate (CLPS) enhanced steaks. The CLPS treated steaks maintained higher redness values ($p < 0.05$) among all other irradiated-/non-irradiated steaks at the end of the storage and display period (d 14). Irradiation significantly decreased redness values for all enhanced- and non-enhanced steaks compared to the non-irradiated steaks throughout time. There were no differences ($p > 0.05$) in a* values among different treatments within the irradiated steaks at d 3. However, at the end of storage and display periods, the calcium/phosphate enhanced steaks (I-CLPS) had the highest redness value among other irradiated steaks.

Chroma values (indication of color intensity) for the treatments presented similar trends as a* values. The CLPS enhanced steaks maintained the higher color intensity ($p < 0.05$) compared to the CON and PHS treated steaks at d 14.

Table 5.4. LSMeans for instrumental color scores of *Longissimus lumborum* steaks for stored 9 days and displayed 14 days at 1 ºC

		Treatment[e]						
		Non-irradiated			Irradiated			
Trait	Day	CON	PHS	CLPS	I-CON	I-CAL	I-CLPS	SE[f]
L*	3	43.1ax	44.1ax	43.9ax	39.8bx	41.4abx	39.9bx	0.82
	9	43.8ax	43.8ax	44.8ax	43.1ay	44.9ay	42.5ay	0.82
	14	42.8bcx	44.13abx	42.9bcx	41.7cz	45.6ay	41.4by	0.82
a*	3	32.1ax	33.7ax	33.2ax	15.8bxy	16.4bx	17.6bx	0.77
	9	27.9ay	30.4by	30.3by	16.6cy	15.4cx	18.9dx	0.77
	14	23.1az	26.1bz	29.3cy	14.9dx	12.5ey	18.6fx	0.77
b*	3	21.8ax	24.1bx	23.6bx	12.1cx	12.7cx	12.7cx	0.46
	9	18.6ay	21.7by	21.4by	13.1cxy	13.4cxy	13.5cx	0.46
	14	16.4az	19.0bz	20.8by	13.4cy	13.9cy	13.7cx	0.46
Hue[g]	3	34.1ax	35.6ax	35.4ax	37.5ax	37.6ax	35.8ax	1.69
	9	34.1ax	35.6ax	35.2ax	39.1bxy	41.7by	35.6ax	1.69
	14	40.7acy	36.0bx	35.2bx	42.9cy	48.9dz	36.5abx	1.69
Chroma[h]	3	38.8ax	41.4ax	40.7ax	20.0bx	20.8bx	21.7bx	1.01
	9	33.5ay	37.3ay	37.1by	21.3cx	20.5cx	23.2cx	1.01
	14	25.4az	32.3bz	35.9by	20.2cx	18.8cx	23.1dx	1.01

[abcd] Means in a row within day having different letters are different ($p < 0.05$). [xyz] Means in a column of treatment with different letters are different ($p < 0.05$). [e] Non-enhanced control (CON); no lactate control (PHS); calcium L-lactate/phosphate (CLPS); non-enhanced irradiated control (I-CON); calcium lactate only w/ irradiation (I-CAL); calcium L-lactate/phosphate w/ irradiation (I-CLPS). [f] Standard errors. [g] Hue angle$(b^*/a^*)^{tan-1}$ and [h]saturation index $(a^{*2}+b^{*2})^{1/2}$ were calculated.

Although irradiation significantly decreased color intensity for steaks for all treatments, the I-CLPS steaks had ($p < 0.05$) higher chroma values among the other irradiated steaks at d 14. The hue angle values (indication of surface discoloration) for both the calcium lactate/phosphate enhanced steaks (CLPS, I-CLPS) were significantly lower than that of both non-irradiated-/irradiated controls (CON, I-CON). The CLPS and I-CLPS were not significantly different in hue angle throughout storage and display periods. The I-CAL steaks had the highest hue angle value among all other treatments.

Decreases in redness and color intensity of muscles due to irradiation were reported previously (*132, 133*). Free radicals generated by ionizing irradiation can oxidize myoglobin directly or they can oxidize lipids producing lipid radicals which subsequently oxidize myoglobin resulting in changing from a bright cherry-red to a dull red color (*132, 134*). Irradiation of oxygen permeable packaged beef decreases a* values and increases visual and brown colors inducing a metmyoglobin-like pigment (*132*). Although the steaks were exposed to a severe oxidizing conditions by applying irradiation to high-oxygen (80%) MAP, the calcium lactate/phosphate enhanced steaks maintained stable red color and resulted in less surface discoloration during display periods. Enhancement of lactate to fresh beef improves color stability during retail display (*9, 121, 135*). Our data suggest that lactate enhancement protects the meat pigment from myoglobin oxidation under strongly oxidizing conditions.

NADH concentration. The calcium lactate/phosphate enhancement increased ($p < 0.05$) NADH concentration (**Figure 5.3**) for both non-irradiated-/irradiated steaks compared to the controls (CON, I-CON, respectively). The phosphate enhancement

(PHS) and calcium-only injected steaks (I-CAL) did not affect NADH concentration ($p > 0.05$). Irradiation decreased NADH concentration ($p < 0.05$) of beef steaks probably due to oxidation. Kim et al. (*9*) reported increased NADH concentration of beef strip steaks injection enhanced with 2.5% potassium lactate/phosphate solution. They determined that endogenous NADH can be regenerated by exogenous substrate (lactate) addition via increasing LDH-B flux (reaction toward pyruvate and NADH). The replenished NADH was utilized for transferring electrons to the ferric state of heme maintaining the reduced state of myoglobin (*9, 91*).

Total reducing activity. Total reducing activity (TRA) of non-irradiated steaks was not significantly different (**Figure 5.4**). In a previous study (chapter IV), we found a higher TRA value for potassium lactate enhanced beef muscles. Irradiation significantly decreased TRA for the control (I-CON) and calcium-only injected (I-CAL) steaks. However, the I-CLPS enhanced steaks maintained higher TRA ($p < 0.05$) than other irradiated steaks (I-CON, I-CAL), and similar to other non-irradiated steaks ($p > 0.05$) suggesting that increasing muscle pH by adding a combination of phosphate and lactate provided higher reducing capacity of myoglobin under severe oxidizing conditions. Nam et al. (*136*) found that irradiation of high pH (> 6.2) pork meat caused less color change and lower lipid oxidation than low pH (< 5.4) pork. Sammel et al. (*99*) reported that TRA was correlated to NADH and visual color of beef *semimembranosus* muscle. Sepe et al. (*137*) determined that the addition of reducing agents (sodium erythorbate, sodium ascorbate, ascorbic acid, and ascorbyl palmitate) increased surface a* values, TRA, and decreased lipid oxidation of ground beef. Nam et al. (*133*) found

that incorporation of ascorbic acid (0.1%) to ground beef prior to irradiation prevented color changes in irradiated beef. They concluded that the addition of ascorbic acid accelerated MMb reduction by donating electrons to the ferric state of heme and subsequently resulted in the conversion of ferri-myoglobin to ferrous-myoglobin. It is not clear why the CLPS enhanced steaks were not higher than other non-irradiated steaks in TRA. However, under severe oxidizing conditions, the CLPS enhanced steaks maintained higher TRA, which was mostly due to the increased NADH concentration through calcium lactate enhancement with a higher pH.

TBARS value. The CLPS enhanced steaks had a significantly lower lipid oxidation (**Figure 5.5**) than non-irradiated control (CON). Irradiation significantly increased the TBARS values of the I-CON and I-CAL steaks, whereas the TBARS values of the I-CLPS enhanced steaks were not influenced by the irradiation treatment. The difference in TBARS values for the I-CAL (pH = 5.39) and I-CLPS (pH = 5.74) enhanced steaks suggests that the lower pH meat is more susceptible to lipid oxidation.

The antioxidant property of lactate has been reported in several studies (*114, 115*). Mancini et al. (*116*) found a less MMb formation of equine oxymyoglobin (OMb) incubated with sodium lactate, and concluded that lactate itself may increase color stability through a non-enzymatic interaction with myoglobin. However, our data indicated that calcium lactate-only enhanced steaks (I-CAL) did not protect myoglobin oxidation under severe oxidizing condition. Seyfert et al. (*74*) also reported adverse effects of using calcium lactate only on color stability of ground beef. They did not incorporate lactate with phosphate for their ground beef, and consequently it did not increase pH (5.4-5.5). In study, the PHS only enhanced steaks were not significantly different from the CON in TBARS values. However, when the combination of phosphate and calcium lactate was applied to steaks (CLPS), it decreased lipid oxidation whether irradiated or non-irradiated, suggesting that the combination of phosphate and lactate has a synergistic effect on reducing oxidation (**Figure 5.6**).

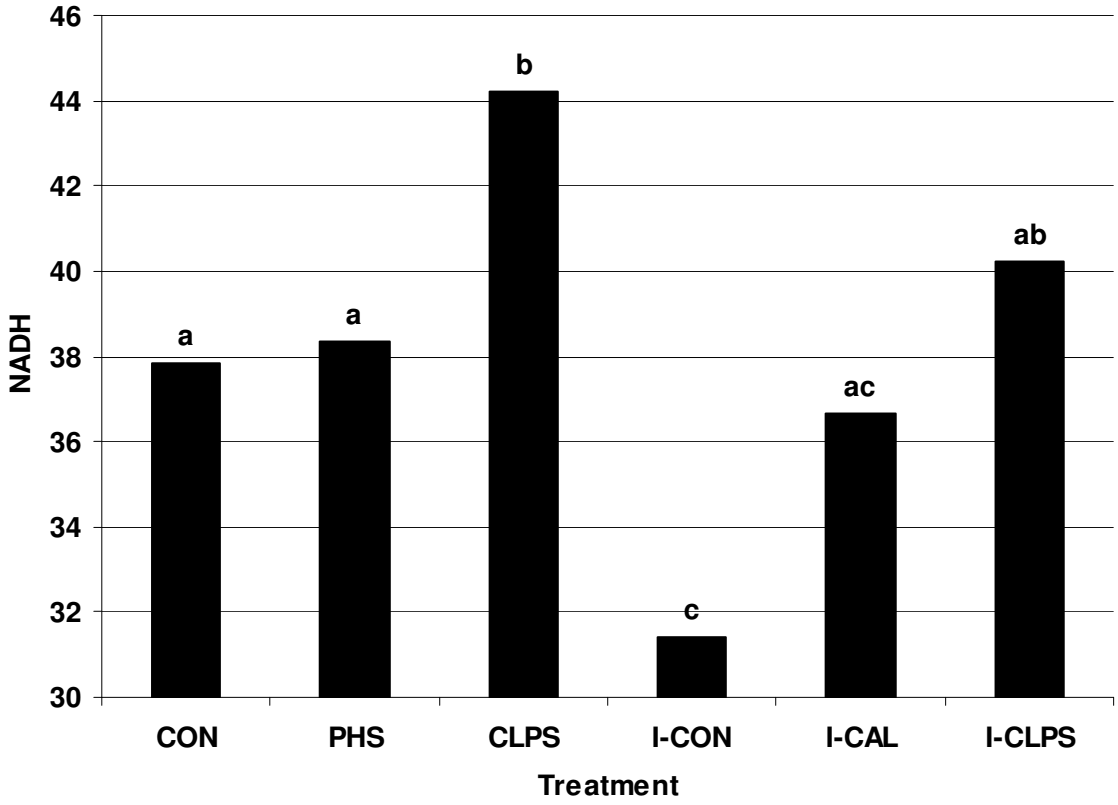

Figure 5.3. LSMeans for NADH concentration of bovine *M.- Longissimus lumborum* (LD) steaks for each treatment at 14 d (end of storage and display) at 1 ºC. ^{abc}Means with different letters are different ($p < 0.05$). Non-enhanced control (CON); no lactate control (PHS); calcium L-lactate/phosphate (CLPS); non-enhanced irradiated control (I-CON); calcium lactate only w/ irradiation (I-CAL); calcium L-lactate/phosphate w/ irradiation (I-CLPS).

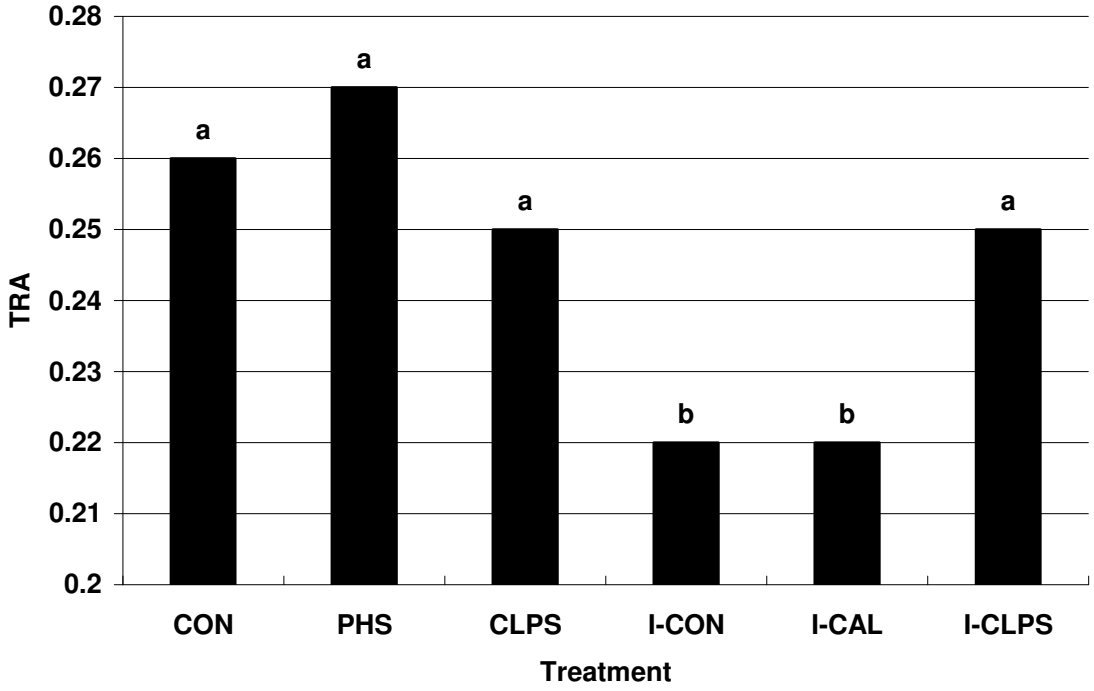

Figure 5.4. LSMeans for TRA values of bovine *M.- Longissimus lumborum* (LD) steaks for each treatment at 14 d (end of storage and display) at 1 ºC. ^{abc}Means with different letters are different ($p < 0.05$). Non-enhanced control (CON); no lactate control (PHS); calcium L-lactate/phosphate (CLPS); non-enhanced irradiated control (I-CON); calcium lactate only w/ irradiation (I-CAL); calcium L-lactate/phosphate w/ irradiation (I-CLPS).

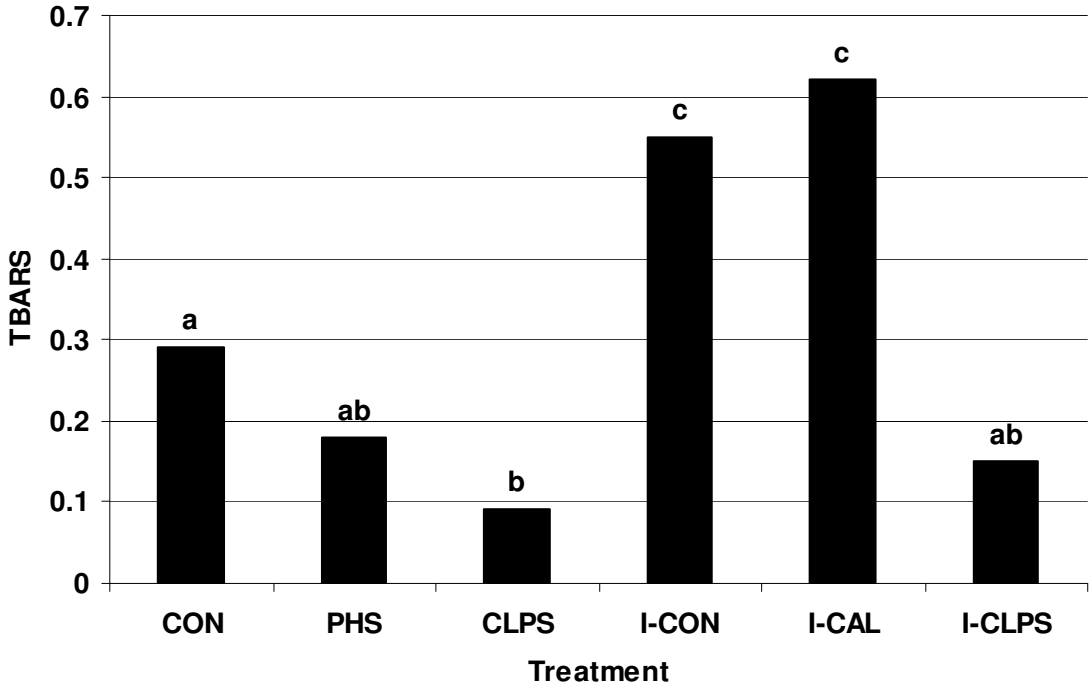

Figure 5.5. LSMeans for TBARS values of bovine *M.- Longissimus lumborum* (LD) steaks for each treatment at 14 d (end of storage and display) at 1 ºC. ^{abc}Means with different letters are different ($p < 0.05$). Non-enhanced control (CON); no lactate control (PHS); calcium L-lactate/phosphate (CLPS); non-enhanced irradiated control (I-CON); calcium lactate only w/ irradiation (I-CAL); calcium L-lactate/phosphate w/ irradiation (I-CLPS).

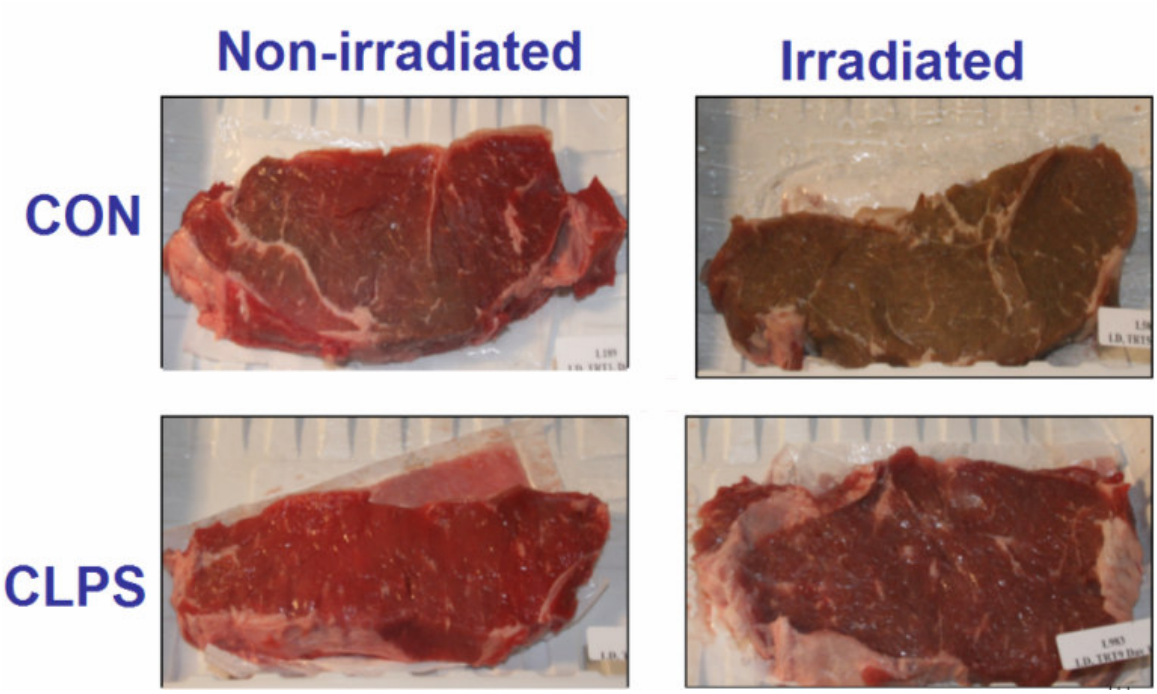

Figure 5.6. Pictures for bovine *M.- Longissimus lumborum* (LD) steaks for each treatment at 14 d (end of storage and display) at 1 ºC. Non-enhanced control (CON); calcium L-lactate/phosphate (CLPS).

These data suggest that calcium lactate/phosphate enhancement actively minimizes lipid oxidation, and subsequently protects myoglobin oxidation from either direct oxidation from hydroxyl radicals or indirect oxidation thorough lipid radicals (*124, 126, 134*). Further, the calcium lactate/phosphate combination induces an increase in NADH concentration, and concomitant increase in reducing capacity of muscle tissue resulting in maintenance of the ferrous state of heme for a longer period of time. This novel research determined improved color stability of fresh beef through lactate/phosphate inclusion by providing superior antioxidant capacity and increased reducing activity of myoglobin by elevating NADH concentration. Future research should focus on the addition of lipid oxidation catalase into the oxymyoglobin-lactate-NAD-LDH system, and combination of other reducing agents on myoglobin oxidation.

CHAPTER VI

CONCLUSION

These results demonstrated that variation in color stability of physiologically different muscles is regulated by different replenishment rates of NADH via different LDH isozymes, namely LDH1. LDH1 influences the metmyoglobin reduction system by affecting the rate of NADH supply. LD maintained the most stable red color, and had the highest MRA, NADH, and LDH-B activities, whereas although PM had a higher pH and WHC, it showed the least color stability and lowest MRA possibly due to lower LDH-B activity (lower LDH1 isoform expression) and subsequently lower NADH regeneration. Enhancement with potassium L-lactate/phosphate resulted in less color deterioration, and improvement of red color intensity of the three bovine muscles (LD, SM, and PM). L-lactate/phosphate enhancement significantly increased NADH concentration via the specific substrate-enzyme reaction of the lactate/LDH-B of LD and PM, and consequently increased the total reducing activity of myoglobin. Injection enhancement of loins with calcium lactate/phosphate maintained the most stable red color (highest a* values) during storage and display periods. Even under highly oxidizing conditions (high-oxygen MAP packaging with irradiation), calcium lactate with phosphate treatment minimized lipid oxidation, increased NADH and TRA, and consequently had a higher a* value compared to other irradiated treatments. These results suggest that lactate inclusion improves color stability of fresh beef by prolonging the reduced state of myoglobin via providing superior antioxidant capacity and increased reducing activity of myoglobin by elevating NADH concentration.

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