THE NITRIC OXIDE SYNTHASE SYSTEM PRODUCING NITRIC OXIDE IN PRE-RIGOR *SEMIMEMBRANOSUS* PORK MUSCLES

A Thesis

by

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ABSTRACT

Curing meat products requires sodium nitrite to generate residual nitrite for preservation and development of a pink cured meat color. This study investigated the use of the essential amino acid L-arginine to activate the Nitric Oxide Synthase (NOS) in pre-rigor porcine muscle to evaluate the efficacy of this system in generating nitric oxide (NO) and residual nitrite (NO_2). Pre-rigor porcine *Semimembranosus* muscles were collected from four separate carcasses at six different harvest intervals (N=24). Varying concentrations of L-arginine solutions were applied to the pre-rigor muscle samples in tubes containing 0.9% NaCl and 576 ppm sodium erythorbate in deionized water, immersed for two hours, and transferred into separate tubes for stabilization. Cooked samples were cooked to 62°C over one hour in a water bath. After stabilization the samples were homogenized, centrifuged, and frozen at -30°C for three weeks and then analyzed for residual nitrite in raw, cooked, and cooked pellet samples, and curing efficiency in cooked samples. Raw L-arginine treated samples overall had higher ($P<0.05$) levels of residual nitrite at 32 mM (14.34 ppm) compared to the control (0 mM; 0.08 ppm). The cooked samples had less residual nitrite compared to the raw samples, suggesting that the NOS system converted the nitrite to NO to form the cured meat pigment nitrosylhemochromagen. The highest concentration of residual nitrite in the cooked pellets muscle samples occurred at 32 mM (15.69 ppm) L-arginine concentration. For curing efficiency and NO-hemochrome values no differences were found with respect to L-arginine concentration in the cooked supernatant samples. Differences ($P<0.05$) in total heme pigmentation were observed for 2 mM (24.22 ppm),

4 mM (34.17 ppm), 8 mM (70.38 ppm), 16 mM (20.14 ppm), and 32 mM (31.67 ppm) L-arginine concentrations compared to the control (0 ppm) but no significant differences between L-arginine concentrations were detected. The pellets of cooked samples analyzed for curing efficiency were highest at 32 mM (156.72%) and were significantly different ($P<0.05$) compared to the control (0.00%) and to other concentrations. This data suggests that the NOS system can generate NO and residual nitrite with the addition of L-arginine in pre-rigor porcine *Semimembranosus* muscle.

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Contributors

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NOMENCLATURE

Acetyl-CoA	Acetyl coenzyme A
ASS	Argininosuccinate synthase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CFR	Code of Federal Regulations
CP	Carbamoyl Phosphate
Dmb	Deoxymyoglobin
eNOS	Endothelial NOS
ETC	Electron Transport Chain
GSNO	S-nitrosoglutathione
HNO_2	Nitrous acid
$H_2N_2O_2$	Hyponitrous acid
HNO_3	Nitric acid
iNOS	Inducible NOS
I/R	Ischemia/reperfusion
Mb	Myoglobin
mM	Millimolar
Mmb	Metmyoglobin
mtNOS	Mitochondrial NOS

N_2	Dinitrogen gas
Na^+	Sodium
NaCl	Sodium chloride
NaE	Sodium erythorbate
Ngb	Neuroglobin
NH_2OH	Hydroxylamine
NH_3	Ammonia
NH_4NO_2	Ammonium nitrite
nNOS	Isoform neuronal NOS
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NO_2	Nitrite
N_2O	Nitrous Oxide
NO(II)Mb	Nitric oxide myoglobin
OAA	Oxaloacetate
Omb	Oxymyoglobin
ppm	Parts per million
ROS	Reactive oxygen species
RSNO	S-nitrosothiol
USDA	United States Department of Agriculture

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1. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Sodium nitrate and nitrite are defined as a crystalline salt used as an oxidizing agent and meat curing agent that easily dissolves in water (1). Nitrite is a highly reactive compound that can function as an oxidizing, reducing or a nitrosylating agent, and can be converted to a variety of related compounds in meat including nitrous acid, nitric oxide and nitrate.[1] The ability of nitrite to act as a curing agent depends on its distribution and amount within a product, which increases product shelf life and stability. Current curing methods with sodium nitrite are efficient and safe, but recent concerns regarding the potential carcinogenic compounds (i.e., nitrosamines)[2, 3] that can be formed from cured meat products has increased consumer concern regarding the safety of consuming cured products. This concern has resulted in the development of alternative curing methods where nitrite is indirectly added to meat via a "high nitrite source" (i.e., vegetable/celery powder). However, these alternative curing methods are not as efficient as sodium nitrite and can develop less than desirable organoleptic properties (vegetable taste or aroma) and less intense cured meat color.[4]

The Nitric Oxide Synthase (NOS) system is vital for muscle function due to its ability to use L-arginine to convert nitrite to nitric oxide, which has been proven to improve vasodilation and muscle metabolism.[5-7] Little to no research has been conducted to evaluate the NOS system's ability to generate NO and nitrite post-harvest

(1) This thesis follows the style of the *Journal of Agricultural and Food Chemistry*

muscle/meat, thereby presenting an opportunity to investigate the feasibility of using the NOS system as an alternative curing method.

Arginine is a major component of activating and stabilizing the NOS system, suggesting the same mechanics in muscle may still be active in pre-rigor meat. The residual nitrite and curing efficiency were used in this study to establish a baseline of any curing ability capable by arginine. This study investigates the efficacy of an essential amino acid, arginine, as a component of an alternative curing system for pork muscle and evaluates the cure reaction efficiency of the amino acid as an alternative curing system.

1.2. Brief History of Sodium Nitrate and Nitrite

The use of sodium nitrates and nitrites is widely utilized in many products, with strict regulations on concentrations and balance with salt and cure accelerants depending on the type of product being produced for consumption. The history of nitrite starts as early 1200 BC, through its use as a meat preservative through use of saltpeter and establishment of using saltpeter for stable red color by the Romans in the 10th century.[8] By the end of the 19th and beginning of the 20th century, experiments for regulation and the use for concentrated nitrite instead of nitrate to cure meat products. Haldane [9] reported the formation of nitrosohemoglobin by adding nitrite to hemoglobin and its breakdown into nitrosohemochromagen as being responsible for the cured red/pink color of cooked meat. The reduction of nitrate to nitrite then to nitrous acid and finally nitric oxide was first studied by Haldane[8] and showed that nitrite is the primary compound for curing meat and enhancing its antimicrobial properties.[8, 9]

The levels of nitrite allowable for use in the manufacture of cured meat products were determined by research starting in 1925 by the USDA Bureau of Animal Industry. It was established that no more than 200 ppm (mg/kg) of nitrate, nitrite, or any combination based on meat weight could be used to cure meat to regulate the amount and form of ingoing nitrite to produce a more consistent product across manufacturers and ensure safe consumption levels of cured meats. These regulations were then clarified in 1970 by the USDA to include cure accelerators and separation of nitrites and nitrites usage based on the curing method and product being made.[10] This was amended in 1978 for bacon manufacturing, which stated that nitrate could not be used only nitrite for better control of nitrite concentrations.[11] Table 1 represents the amounts allowed for cured meat and poultry products in the United States.

Table 1-1. Curing Agent Regulations

Maximum allowed added levels of curing ingredients in meat and poultry in the United States.[a]

Curing agent	Curing method			
	Immersion cured (ppm)	Massaged or pumped (ppm)	Comminuted (ppm)	Dry cured (ppm)
Sodium nitrite	200	200	156	625
Potassium nitrite	200	200	156	625
Sodium nitrate	700	700	1718	2187
Potassium nitrate	700	700	1718	2187

[a]Limits are calculated by total formulation and brine weight for immersion cured, massaged, or pumped, and raw meat (green) weight for comminuted or dry cured products.

Adapted from USDA FSIS Directive 7620.3[12]

1.3. Meat Curing Mechanism

The conversion of nitrate to nitrite to nitric oxide is the fundamental process that allows for meat to be cured. Since nitrite is the active component of a cure, which usually contains salt, sugar, and nitrate and/or nitrite, the method of introducing the nitrite into the meat determines the concentration of nitrite needed for efficient and rapid distribution.[13] Dry curing is direct contact of the cure on the meat surface. This method is less efficient and requires a longer time to become active. Using a pickle by either immersion of product in the pickle or direct injection via needles allows for quicker absorption and distribution throughout the meat. Based on the uniformity of brine distribution, the curing process should produce a characteristic pink, heat stable color throughout the product, a typical cured meat flavor through the direct or indirect retardation of oxidative rancidity, and a texture different from fresh meat.[13] This unique flavor profile varies with type of meat used (e.g. pork, beef, poultry) and amount of sodium nitrite added, and can be described in cured meat lexicons based on the product type and administration of nitrite.[14] In addition to the specialized texture and color of cured meat products, nitrite provides some preservation against spoilage organisms resulting in a long product shelf life. Under aerobic conditions, NO bound to hemoglobin will convert to methemoglobin, which will lead to the conversion of metmyoglobin (Mmb) if not controlled.[15] Nitric oxide reacts very quickly with oxymyoglobin (Omb) to form metmyoglobin and nitrate, while nitric oxide myoglobin (NO(II)Mb) reacts very slowly with oxygen to form metmyoglobin and nitrate. This autooxidation of NO(II)Mb

is the result of slow dissociation, O_2 binding, and subsequent dioxygenation of the released NO, producing a low-spin ferric Mb dihistidyl hemichrome.[16]

1.3.1. Cured Meat Color Formation

Some meat color changes are undesirable, such as in fresh meat with undenatured myoglobin and oxymyoglobin, or reduced globin hemochromes of well-cooked meats that can result in a red or pink color when cooked.[17] In cured meats, the concentration, stability, or discoloration of the cured meat pigments or any cured color discoloration by nitroso-myoglobin and nitrosohemochromagen can be measured to determine the rate of fading of cooked meat pigments if no influence from oxidative changes take place.[18] Extraction of cured meat color pigments by acetone can assess the conversion to cured meat pigment and the efficiency of this conversion. While the extraction process is efficient, there is concern that these pigments can be negatively impacted when exposed to light, resulting in fading or color changes. The exposure of cured meat to light and oxygen can be abated by handling and storage of samples and conducting testing in diminished light conditions. Initial formation of cured meat color starts with the oxidation of deoxymyoglobin to metmyoglobin, where nitrite is then reduced to nitric oxide to react with metmyoglobin.[19] From here, formation of nitrosylmetmyoglobin and rapid autoreduction to nitrosylmyoglobin or simultaneous NO coordination through autoreduction forms nitrosylmyoglobin shows a conversion by the presence of iron (II) nitrosylmyoglobin radical cation. This reaction, when thermally processed, then produces the stable denatured hemochrome, nitrosylmyochrome, and nitric oxide myoglobin to produce the nitrosylhemochromagen characteristic “cured pink” color.

Nitrosylmyoglobin when heated denatures the protein for detaching from the heme, where a second mole of nitrite is bound to the denatured protein necessary to form nitrosylhemochromagen.[19]

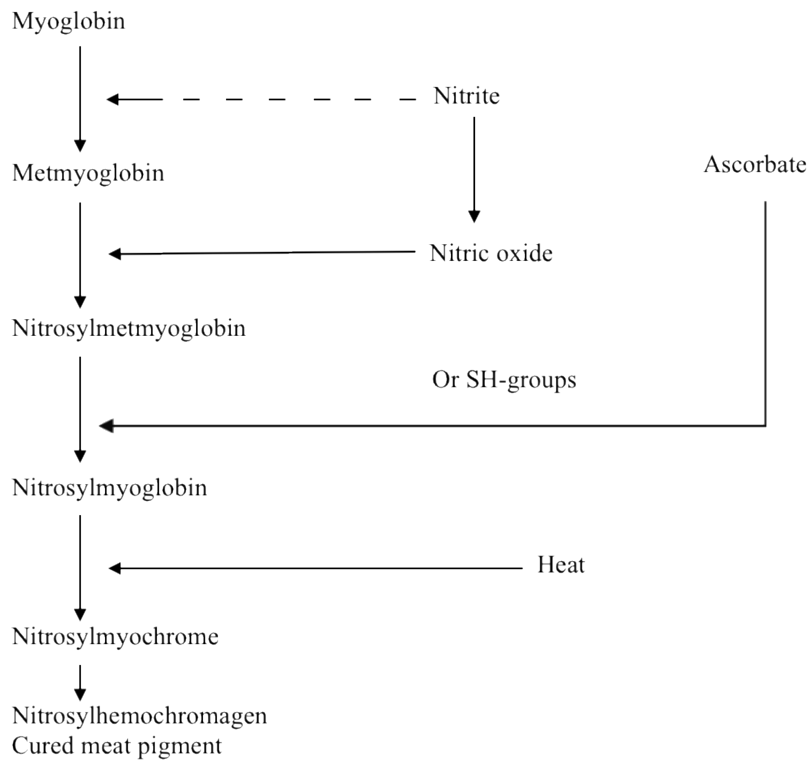

Figure 1.1 Cured Meat Color Formation (adapted)
Steps to form nitrosylhemochromagen (cured meat pigment) from myoglobin (fresh meat pigment). Ascorbate and SH-groups are included in this figure to indicate influence of color change reaction being accelerated or inhibited. The initial form of nitrite as nitrite or nitric oxide also influences the rate of color change reaction. Reprinted and adapted with permission from *Small-Scale Sausage Production* by IV Savic, 1985, Food and Agriculture Organization of the United Nations, Copyright 1985 by FAO.[20]

1.3.2. Curing Accelerators

Compound requirements for a nitrite accelerant includes its ability to speed up the cure reaction to provide antimicrobial and antioxidant stability for increased cured meat product shelf life. Sodium ascorbate and sodium erythorbate can reduce the effects of dissociation and reduce metmyoglobin to allow for reduction to deoxymyoglobin.[20] Phenolic compounds, organic acids, and flavonoids are three major compounds that can be added as natural antimicrobial and antioxidants via cranberry and tomato extracts. These organic acids and phenolic compounds naturally reduce product pH as well, creating acidic conditions that favor the conversion of nitrite to NO, resulting in an efficient curing process and reducing the amount of residual nitrite found in cooked products.[21] However, these natural accelerants may produce off flavors and colors at higher concentrations. Spice extracts from rosemary, thyme, sage, and garlic can play a dual role as an antibotulinal and carcinogen reducing agent when thermally processed. Reduction of carcinogenic compounds occurs through the reduction of heterocyclic aromatic amines in cured cook meat.

1.3.3. Residual Nitrite

Residual nitrite is defined as nitrite left in a product (roughly 10-20% of the originally added nitrite) after the curing process has been completed. It slowly recedes during the shelf life of the product until it is negligible or undetectable.[22] Residual nitrite is necessary to ensure cured meat color stability is maintained through regeneration of cured meat pigment lost due to oxidation and light induced iron-nitric oxide dissociation.[23] Residual nitrite from cured meat products has decreased considerably in

the past 20 years from 52.5 ppm in the 1970s to an average of 10 ppm in 1996 when tested from retail products that were not in the last week of their shelf life “sell by” date due to more efficient curing methods and control of curing reactions by regulated nitrite instead of nitrate.[24, 25]

Nitrite depletion occurs during the storage of cured meat products at a rate dependent on production formulation and pH, as well as the length of time and product temperature during processing and storage. The sulfhydryl-disulfide content in the meat plays an important role in the redox reactions when sulfhydryl groups are blocked by metallic ions and nitrite loss decreases.[26, 27] A correlation exists between low pH (results formation of S-nitroso cysteine) and nitroso-thiol content to maintain nitrite stability while decreasing total nitrogen content. When cured meat is subjected to thermal processing meat pH should be maintained from 5.5-6.0 using a stabilizer such as sodium erythorbate. This minimizes nitrite loss breakdown into intermediate nitroso-thiols, minimizing the formation of disulfides that can further increase the amount of lost nitrite.[27]

1.3.4. Shelf Life Stability

Antimicrobial safety is an integral part of nitrite curing methods by either inhibiting or controlling growth of food spoilage and pathogenic bacteria. Improved product stability against *Staphylococcus aureus* and *Clostridium botulinum* have been shown in cured meat products with the minimum ingoing nitrite levels at 50-60 ppm in conjunction with pH, salt concentration, reductants, and iron content.[28] The higher level of nitrite in most products is not for color development but for control of outgrowth of *C.*

botulinum spores and any toxin production, with levels higher than 70 ppm able to sustain a longer shelf life against pathogen spore germination and outgrowth.[29]

1.4. Health Concerns of Sodium Nitrite

The industry in the 1970s was able to eliminate most carcinogen concerns in cured meat consumption by eliminating the use of nitrate, reducing the levels of nitrite, and controlling manufacturing processes to better monitor any ingoing nitrite.[2] However, this does not negate the fact that most nitrate is ingested from the intake of food, especially vegetables, and ingestion of nitrite is mainly from nitrite by conversion of nitrate to nitrite from bacteria [2]. The most critical step in mammals for vasodilation is the synthesis of nitric oxide by nitric oxide synthase which catalyzes the oxidation of L-arginine to nitric oxide and L-citrulline [30]. However, since nitrate can convert to ammonia through reduction and oxidation processes, as well as reverse and/or form hydroxylamine and nitric oxide [29], the reduction and oxidation rates must be controlled with another substrate such as tetrahydrobiopterin or sodium erythorbate to be stable in a biological system or be used for an alternative curing system.

1.4.1. Nitrosamines and Carcinogenic Properties

Meat products cured with nitrite possess a unique flavor profile and are less susceptible to the creation of off flavors due to lipid oxidation. The established red-pink color of cure meat from nitrite addition adds a threshold for consumer acceptance and desirability. Nitrite in connection with secondary amines were the focus for elimination in order to control nitrosamine formation, causing awareness of nitrite and secondary amines in cured meats caused initial public health hazard concerns by studies conducted

in the early 1970s.[2] The most concern for nitrosamine formation comes with frying bacon since there are secondary amines present, nitrite is available for reaction, a near neutral pH, and a product temperature reaching above 130°C. This led to the regulation in 1978 reducing added nitrite in bacon from 200 to 125 ppm, the addition of sodium erythorbate or ascorbate at 550 ppm, and banned all nitrate addition during bacon processing. This regulation has led to present day levels of nitrosamine to be virtually eliminated in meat and poultry products.[2] Public controversy after four separate studies in 1979, 1980, 1981, and 1982[31-33] showing that nitrite usage in regulated settings had no effect on increasing carcinogenic tumors were quelled, and further studies in the 1990s showed that any epidemiological studies associating risk of childhood leukemia[34-36] were weak and unwarranted with consumption of nitrite.[37, 38] There is also the argument that swallowing any saliva in combination with any food could be a result of potential formation of nitrosated compounds since most ingested nitrite is formed in saliva.[2]

Nitrosamine formation in cured meats is a cause for concern, formed by the reaction of secondary or tertiary amines with a nitrosation agent such as nitrous anhydride from nitrite in an acidic aqueous solution.[39] Nitrosamines can cause DNA damage by the generation of nitric oxide which is an active nitrosating agent that can react with secondary amines in meat to form carcinogenic nitrosamines.[24] Drying processes and the application of heat used in the manufacturing of many cured meat products can aid in the formation of nitrosamines. However, nitrosamine formation can be inhibited by the addition of ascorbic acid, sulfur dioxide, and sodium erythorbate as part of the curing process to regulate the conversion of nitrite to nitric oxide. Bacon is

the only cured meat that consistently contains some type of nitrosamine after cooking.[13] This is due to the presence of secondary amines, readily available nitrite, a near neutral pH found in most bacon, and the high cooking temperatures used to cook bacon, such as frying at a temperature of or exceeding 130°C.

N-nitrosamines are possibly formed during production and storage of cured meat products in volatile and non-volatile compounds. The volatile nitrosamine compounds are mostly carcinogenic and nonvolatile nitrosamines can be toxic or carcinogenic if decarboxylated into their carcinogenic counterparts.[40] With ingoing nitrite levels, meat quality, fat content, processing, maturation, and handling with storage, the levels of nitrosamine formation are hard to predict.[40] Increasing nitrite does increase most nitrosamine formation linearly but using erythorbic acid or ascorbyl palmitate can control most formations along with antioxidants and polyphosphates.[41]

1.4.2. Replacing Nitrite

Direct replacement of nitrate and nitrite is the complete removal of nitrate and nitrite from a curing system.[42] Organic acid salts such a propionate, citrate, acetate, lactate, and pyruvate can inhibit *C. botulinum* growth but only as a secondary barrier and needs additional additives to enhance the safety of the product.[43] Sorbic acid and alkaline salts can control spoilage and inhibit *C. botulinum* spore outgrowth but can affect flavor.[44] Cured color development, spoilage due to lipid oxidation, pH, and residual nitrates and nitrites are all affected as well.

Indirect replacement of nitrate and nitrite is the process of removing some or all nitrate and nitrite from the curing system and replacing it with another source [45]. Starter

cultures such as lactic acid bacteria can be used to produce colors similar to cured meat color thresholds. Naturally occurring nitrate is found in cabbage, lettuce, celery, spinach, beets, and radishes; these are used to create juice or powdered concentrates to supply a source of nitrate/nitrite for curing in a non-traditional or alternative fashion.[45]

A challenge has been to identify an ingredient that provides the same functional benefits of nitrite without compromising food safety. Sorbic acid[44], short-chain alkynoic and organic acids [43], and cooked cured meat pigment[46] has been investigated but none were as effective as nitrite. Single ingredient alternatives may be able to replace a single functional attribute of nitrite. However, numerous scientific studies have not found a full replacement for nitrite that stabilizes cured meat color, produces desired cured meat flavor, prevents lipid oxidation, changes texture, and acts as a preservative in the same manner as nitrite.[45]

1.4.3. Alternative Curing Methods

Vegetable juice powder (VJP) with starter cultures containing *Staphylococcus carnosus* has been shown to be an effective replacement for nitrite.[47] However, no matter the replacement, there is still a need for a cure accelerant. Cherry and lemon powder can be utilized for cure accelerant and color stabilizers, but also can carry off flavors in higher concentrations. As previously stated, these alternative ingredients do not possess all the functional attributes of nitrite. Often a higher concentration of these ingredients is necessary to attain desirable curing reactions, resulting in flavors or bitterness. Additionally, since starter cultures are required to convert nitrate to nitrite, there is no accurate way to ascertain the exact amount of nitrite generated in the product.[48] Residual

nitrite levels can be determined but at lower levels than sodium nitrite-cured products, resulting in less available residual nitrite to generate nitric oxide to maintain antimicrobial properties throughout the product's shelf life.[47]

Bacteriocins are antimicrobial proteins or peptides produced by bacteria that can inhibit other bacteria [49]. These are considered safe and natural food preservatives, with nisin specifically used in heat processed food that increases sensitivity of spores to heat. As nisin offers the closest replacement efficiency to nitrite, it is considered to be a possible acceptable alternative to nitrite or at least an acceptable way to reduce nitrite levels without compromising on food safety.[49] Nisins in particular have been shown to reduce the number of *Listeria innocua* and *Listeria monocytogenes* during storage.[49]

New technology has resulted in the development of pre-converted celery juice powder. The celery juice powder has undergone conversion of nitrate to nitrite and the nitrite concentrations have been standardized to a greater degree. Pre-converted celery juice powder removes the fermentation/conversion step for meat processors and is readily incorporated into formulas already established for conventional curing.[4, 50] Often the amount of ingoing nitrite (amount of pre-converted celery juice powder) is added at lower concentrations, which may result in lower curing efficiency and shelf life stability as shown in emulsified cooked sausages studied by Sindelar.[47] There has been no great variation between cured meat and total meat pigment between celery juice powder and sodium nitrite, or lower concentrations of 50 to 100 ppm when used in manufacturing turkey processed meat logs when tested by Redfield.[4] However, source and concentration did affect cured meat color perception, acceptability, and flavor when

celery juice powder was used. Results of this study indicated that off flavors can be diminished, and cure color is optimal when celery juice powder is added at lower concentration levels, making the alternative curing process not as effective as curing with sodium nitrite.

1.4.4. Regulations of Alternatively Cured Products

The USDA does permit the manufacturing of uncured versions of typical cured meats according to 9 CFR 319.2[51], provided that the product is similar in size, flavor, consistency, and general appearance and labeled properly to display this process. There is also allowance for another category in natural, which is defined by 21 CFR 101.22[52] to not contain any artificial flavor or flavoring, coloring ingredient, or chemical preservative, or any other artificial or synthetic ingredient, and the product and its ingredients are not more than minimally processed. By definition, technically natural and organic products are uncured, but with indirect addition of nitrate or nitrite that are allowed by the industry the products are still considered cured [48].

1.4.5. Sources and Health Benefits of Sodium Nitrite

Nitrite plays an important role in human health by synthesizing nitric oxide via the nitric oxide synthase (NOS) system to control blood pressure, blood flow in cardiac muscles, immune response, wound repair, and neurological functions [53]. Exogenous sources for nitrate are primarily from consumption plants and water, while endogenous nitrite consumption is mostly from the ingestion of saliva that naturally produces nitrite.[54]

1.5. Nitric Oxide (NO) and the Nitric Oxide Synthase (NOS) System

1.5.1. IV.I Nitric Oxide Mechanisms

The basic conversion of nitrate to nitrite to nitric oxide is more commonly applied in the live system for use in vasodilation. Nitrate/nitrite is absorbed into the bloodstream and diverted to the lungs to be filtered to either form or decompose to nitric oxide. Nitric oxide (NO) is the product of the chemically equivalent guanidino nitrogens of L-arginine by the enzyme nitric oxide synthase (NOS).[55] The major breakdown product of NO in aqueous solutions is nitrite. It is sparingly soluble in water with a half-life in aqueous solution at 3.8 to 6.2 seconds.[56] Nitric oxide is unstable and can easily convert to nitrogen and oxygen gas when in concentrated states. In aqueous solution the nitric oxide can revert to nitrite to stabilize, with a longer half-life as nitric oxide becomes more dilute.[56] The metabolic pathways of NO and N-oxides have a rapid loss of antioxidants in human plasma, specifically in protein S-thiols. This causes the formation of S-nitrosothiol (RSNO), which does not allow NO to react directly with thiols. However, the nitrosonium side of NO that a possible storage area for NO can allow for direct reaction to thiols to bind. Cysteine by direct use is the lone thiol source in proteins that forms S-nitrosoprotein derivatives that have endothelium-derived relaxing properties and allows the plasma protein to become a reservoir for nitric oxide.[30] This cysteine source can overcome RSNO inhibition and allow for access to nitric oxide stores within the plasma protein.

1.5.2. NO Regulators and Inhibitors

S-nitrosothiols S-nitrosohemoglobin and S-nitrosoglutathione are NO products that regulate protein expression and function as well as act as sources for NO.[7] The creation can be produced by NOS and accept NO groups by nitrosylation in proteins, mainly storing in the mitochondria. This NO release from the S-nitrosothiols can act as a powerful vasodilator and survive with metal ion chelators and blood-like conditions for temperature and pH.

Deoxymyoglobin (Dmb) is a nitrite reductase that generates NO and is regulated by pH.[57] This helps with the regulation of mitochondrial function and reactive oxygen species production even at low oxygen concentration. Also, Dmb reduces nitrite to NO at a rate 36 times faster than deoxyhemoglobin in vitro.[57] As oxygen sensors hemoglobin and myoglobin shift from being NO scavengers to NO producers in hypoxia, this transition triggers a release of NO from the mitochondria. Deoxymyoglobin dependent nitrite reduction is able to produce NO, but with the use of metmyoglobin does not reduce nitrite.[57] As myoglobin deoxygenates, it is able to reduce nitrite to bioactive NO before mitochondria become oxygen limited. Thus, the NO formed from nitrite reduction can inhibit respiring mitochondria to conserve tissue oxygen.[57]

1.5.3. Nitric Oxide Synthase (NOS) Isoforms

There are three different main NOS isoforms: Inducible (iNOS), endothelial (eNOS), and neuronal (nNOS). Inducible NOS (iNOS) is not found in cells unless induced by cytokines, microbes, or microbial products but nearly every tissue in the

body can express this enzyme when stimulated. iNOS expression results in a sustained production of NO, which exerts both cytostatic/cytotoxic and cytoprotective actions in mammalian tissues and antimicrobial activity toward certain pathogens.[55] The iNOS isoenzyme overall is not regulated by Ca^{2+} or calmodulin once it is expressed, meaning arginine has more chance of transport and reaction.[6] If it is possible to keep this system intact after rigor, the enzymatic production of nitric oxide can be stabilized. The inducing of iNOS expression like in macrophages has been shown to increase L-arginine transport,[58] but when in vivo must compete with the same transport system used by L-lysine and L-orthinine that can alter cellular NO synthesis rates.[58, 59]

The blood vessel wall NO is mainly produced from L-arginine by endothelial NOS (eNOS).[7] eNOS is specifically located in the endothelial cells within the vascular system. Various cofactors such as tetrahydrobiopterin (BH_4), flavin adenine dinucleotide and flavin mononucleotide, calmodulin, and iron haeme help the monomer isoforms bind and catalyze NO production through generation of O_2^-.[60, 61] Agonists such as acetylcholine, bradykinin, and histamine increase the concentration of calcium that binds to calmodulin and bypasses the inhibitor site Thr495 to generate NO.[7]

Interestingly, it has been shown that purified constitutive, endothelial NO-synthase (ecNOS) forms simultaneously NO and O_2^- , the ratio of both depending on the concentration of the ecNOS substrate L-arginine and the redox conditions of the cofactors.[62] ecNOS and mitochondria are co-located suggesting that there is a mitochondrial NOS (mtNOS) that converts L-arginine to L-citrulline conversion within the mitochondria and is prevalent in skeletal muscle.[63] NO can interact with other heme-

containing proteins like cytochrome c oxidase or iron-sulfur clusters related to the respiratory chain and promotes enzyme inhibition.[64] NO competes with cytochrome c oxidase for binding at the O_2 site within the cell that decreases O_2 consumption and ATP formation, allowing for continual function at low O_2 concentrations with little energy.[65] The isoform neuronal NOS (nNOS) is the most prevalent form in the skeletal muscle due to its contact with the central and peripheral nervous systems.[66] nNOS is expressed in the surface membranes that surrounds the sarcoplasmic reticulum and increases in expression when induced by chronic hypoxia.[67]

1.5.4. NOS in Different Cells

Nitric oxide synthesis by eNOS and iNOS plays a crucial role in macrophage antimicrobial activity. The production of reactive nitrogen intermediates through macrophages has even been shown to inhibit the replication of tumor cells while slowing the nitrosamine metabolism in carcinogenesis.[68] This can only be done by the conversion of L-arginine to nitrite and L-citrulline without the loss of the guanidino carbon.[69] There is evidence that macrophages can generate broadly cytotoxic molecules from the guanidine molecule of L-arginine and form nitrite and nitrate.[68] However, there are no specific microbial targets that can be inhibited by macrophages, suggesting that although they have nitrite capabilities, there must be no interference in order for L-arginine to be used in a macrophage biosynthetic pathway.[69] Also, there is not significant evidence that reactive nitrogen intermediates (RNIs) are found within human mononuclear phagocytes that have antimicrobial properties.[70]

Mitochondria of cardiomyocytes are the primary focus of diffusion and NO binds to myoglobin for breakdown by reactive oxygen species for the function of vasodilation.[71] The different kinetics of the reaction of NO with the oxygen-derived radicals superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO^-) has been shown that all three compounds are produced by cells from mammalian species, especially from the endothelial cells and macrophages, both of which also capable of synthesizing NO.[56] The accumulation of NO from typical nitrite concentrations found in biological tissues rises about 100-fold when the pH falls from 7.4 to 5.5.[72] Enzyme independent NO (without any isoform of NOS) occurs through conversion of nitrate to nitrite in the mouth by anaerobic bacteria, in the very acidic conditions in the lumen of the stomach, and in biological tissues undergoing intracellular acidosis.[72]

1.5.5. Nitrite in Metabolism and NOS Activity

Concentrations of nitrite are found stored in micromolar levels in vascular and muscle tissues (1-20 micromolar) and mostly derived from the oxidation of NO Synthase (NOS)-generated NO.[73] In vascular and muscle tissues, the monomeric hemoglobins, myoglobin (Mb) and neuroglobin (Ngb), catalyze NO_2^- reduction by the same reaction as hemoglobin through hypoxic vasodilation in allosteric structural transition of the protein from relaxed to tense state.[74] However, the muscle tissues react at lower oxygen tensions (p50 Mb1⁄42.4 mmHg; p50 Ngb1⁄42.2 mmHg).[73] Mb-dependent NO_2^- reduction has been implicated in the protective effects of NO_2^- after ischemia/reperfusion (I/R) in the heart as well as in vasodilation.[73, 75, 76] Nitrite reduction, specifically controlled by the

mitochondrial electron transport chain (ETC), has been shown to occur in near anoxic conditions.[77] The best conditions for reaction occur at pH less than 7 and with relatively high (millimolar) concentrations of nitrite. Nitrite regulates mitochondrial function by modifying specific proteins in the mitochondria and inhibits reactive oxygen species (ROS) generation that creates nitrite dependent S-nitrosation.[78] These paths create a NO-dependent inhibition of mitochondrial oxygen consumption, even when oxygen tension is decreased and can stimulate hypoxic mitochondrial biogenesis.[79] Myoglobin can be used as a functional nitrite reductase that regulates the cellular response to hypoxia.[80] It is similar to the resting state of hemoglobin but reduces nitrite almost 60 times faster than T-state hemoglobin due to the low heme redox potential found in myoglobin that does not have an allosteric state.[73]

Nitrite is more inhibitory under acidic conditions as part of the biochemical oxidation-reduction chain that allows for nitrite to be reversible and have multiple intermediate compounds as demonstrated in Figure 1.2. Hydroxylamine and nitric oxide do possess anti-microbial properties, but not as high or in varying conditions as nitrite.[81]

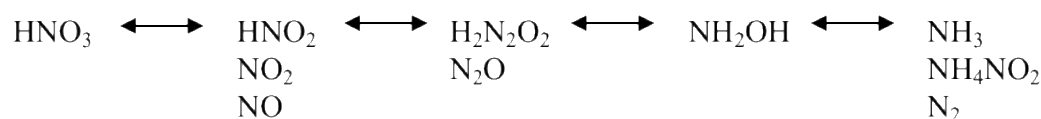

Figure 1.2 Variable conditions of nitrite with intermediate compounds depending on stability and state (adapted).

Nitric acid (HNO_3) can convert to nitrous acid (HNO_2) , nitrite (NO_2), or nitric oxide (NO) and back to nitric acid. These forms can convert to hyponitrous acid ($H_2N_2O_2$) or nitrous oxide (N_2O) and back. These states can convert to hydroxylamine (NH_2OH) and back, then to ammonia (NH_3), ammonium nitrite (NH_4NO_2) , or dinitrogen gas (N_2). Adapted from and reprinted with permission from "Proceedings of the International Symposium on Nitrite in Meat Products" by B. Krol and B. Tinbergen, 1974. *Proceedings of the International Symposium on Nitrite in Meat Products*, page 65, Copyright 1974 by Wageningen.[81]

Nitrite is the primary oxidative product of NO derived from the conversion of L-arginine to NO by the purified enzyme ecNOS.[82] N-labeled L-arginine has demonstrated that almost all circulating nitrite is mainly derived from the L-arginine-NO pathway and in smaller proportions from the NO-related adducts peroxynitrite or RSNO. Since L-arginine is the only physiological nitrogen donor for the NOS-catalyzed reaction [7, 55], the need for increased uptake and synthesis of L-arginine is imperative to generate higher NO concentrations.

1.6. L-Arginine in NO Generation and Metabolism

1.6.1. L-Arginine and the Nitric Oxide Synthase (NOS) System

L-Arginine is a conditionally essential amino acid in adult humans and other animals and in the synthesis of creatine, the precursor for mammalian nitrite/nitrate synthesis, and the generation of nitric oxide through the nitric oxide synthase (NOS) system.[6] Many cells utilize nitric oxide for vasodilation, immune responses, neurotransmission, and adhesion of platelets and leucocytes.[6] Arginine has multiple pathways for synthesis, such as L-citrulline cyclic regeneration by glutamine/glutamate, and the cell signaling molecules of nitric oxide, glutamate, and agmatine use the regulate key cellular processes (Figure 1.3).

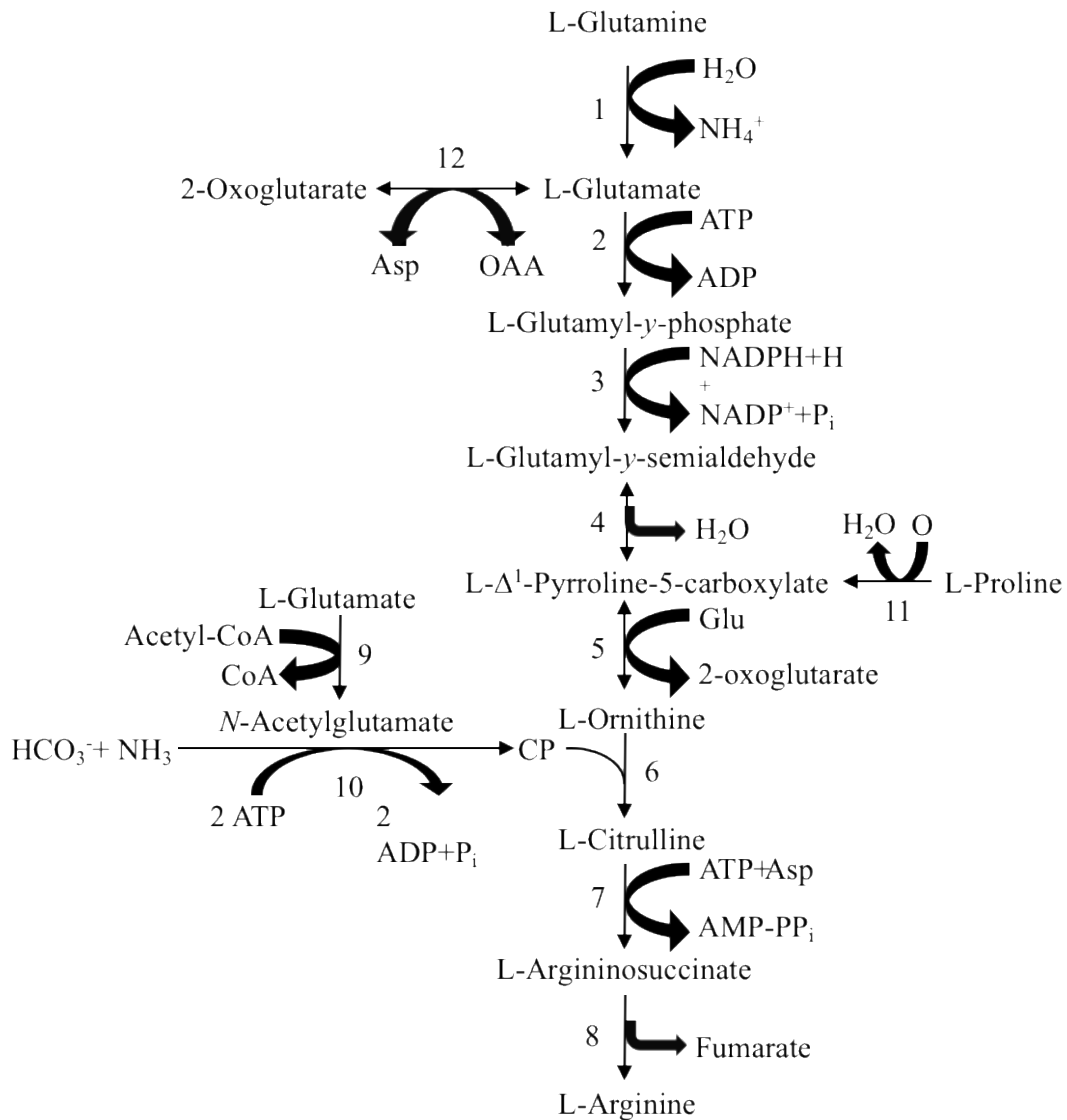

Figure 1.3. Arginine Synthesis Pathways (adapted)
The main metabolic pathway for arginine synthesis in mammals is done through P5C synthetase and proline oxidase. Reactions occur by a chemical equilibrium favoring P5C formation that is a bifunctional polypeptide. Reactions 1-6 and 9-11 take place in mitochondria, reactions 7 and 8 in the cytosol, and reaction 12 can occur in both. Abbreviations: OAA, oxaloacetate; CP, carbamoyl phosphate. Reprinted and adapted with permission from "Arginine metabolism: nitric oxide and beyond" by Guoyao Wu and Sidney M. Morris, 1998. *Biochemical Journal*, 336, 1-17, Copyright 1998 by Biochemical Society and Portland Press.[6]

1.6.2. Components of Arginine Synthesis

L-citrulline is responsible for most of the endogenous synthesis of arginine in adult humans by generation from glutamine or glutamate. The L-citrulline aids in circulating arginine to other proteins, assisting in storage in other systems beyond the small intestine, liver, and kidneys.[6] The highest rates of arginine synthesis occur within the hepatic urea cycle and directly correlate to NO synthesis.[6]

Argininosuccinate synthase (ASS) acts as a rate-controlling enzyme in L-arginine biosynthesis when iNOS is initiated, which regulates the L-arginine recycling pathway which in turn regulates iNOS synthesis.[6] L-glutamine and hypoxia inhibit L-arginine synthesis in NO producing cells, but can be countered if ASS activity is not hindered.[83] The most important mechanism for arginine uptake in most cell types is system y+, which is a high-affinity, Na+-independent transporter of arginine, lysine and ornithine.[6] Regulation of system y^+ presents a problem in the modulation of cellular L-arginine metabolism represented by the competing L-lysine, L-ornithine, canavanine, and NOS inhibitors N^G-monomethyl-L-arginine and N^G-iminoethyl-L-ornithine as shown in studies by Schmidt and Bogle.[84-87] However, system y^+ has been shown to co-induce with iNOS in most cell types allowing for L-arginine to flow in transport to promote the influx of NO synthesis to the induced cell.[84, 88, 89] Arginase can also be used as a regulatory enzyme for arginine availability, competing with iNOS for arginine and can

only be inhibited by N^G-hydroxyarginine if it is produced from iNOS and not oxidized to citrulline and NO.[6, 90]

1.6.3. L-arginine and L-citrulline

With the supplementation of L-citrulline in combination with L-arginine, there is a way to promote the L-citrulline-to-L-arginine recycling pathway to sustain localized L-arginine availability for eNOS-catalyzed NO production.[91] L-citrulline used as a precursor for L-arginine is an effective supplement to inhibit arginase and increase L-arginine plasma levels while simultaneously increasing plasma NO_x and cGMP. NO synthesis occurs in mostly all cell types through the arginine biosynthetic pathway and recycles citrulline to arginine by the arginine/citrulline cycle that can be looped in with the citric acid cycle as shown in Figure 1.4.[6, 92]

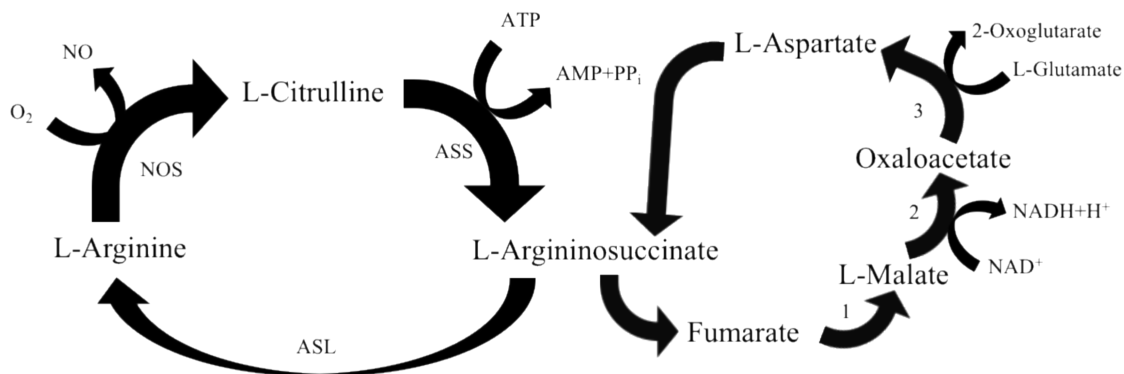

Figure 1.4 Citrulline/NO Cycle (adapted)
This particular cycle can be couple to the citric acid cycle (right). Fumarate produced in the cytosol then enters the citric acid cycle in the mitochondrion to convert into oxaloacetate. Enzymes to catalyze 1-3 reactions occur with fumarase, malate dehydrogenase and aspartate aminotransferase. The reactions are reversible, but the diagram only shows the unidirectional flow in NO-producing cells. Adapted from Nussler et al[92] and Wu and Morris[6] with permission.

1.7. I.VII NO in Muscle to Meat Conversion

There is little or no medical research investigating the NOS systems and the effect of NO in muscles after death to determine if the NOS system is still functional during the conversion of muscle to meat, or its viability in post-rigor muscle. Hypoxic/ischemic conditions that trigger NOS and NO production in the mammalian cell also occur in postmortem muscles. Modulation of NO level in pre-slaughter and post-slaughter muscle cells using NO donors and NOS inhibitors are reported to affect the meat quality attributes of tenderness, water holding capacity and color in a variety of animal species including beef, lamb, pork and chicken.[93] The combination of NO and protein S-nitrosylation is thought to be regulatory in postmortem aging and the development of meat quality, particularly affecting the myofibrillar proteins. [94]

Myofibrillar proteins have been shown to be endogenously S-nitrosylated in skeletal muscle with a high reactivity to S-nitrosoglutathione (GSNO).[95, 96]

1.7.1. Effects of NO on Meat Quality

Degradation of myofibrillar proteins during aging affects overall meat tenderness and is mainly caused by protease calpain-l. Since NOS can inhibit calpain-1 from proteolysis by modification and protein S-nitrosylation affects proteolysis by calpain-1 in myofibrillar proteins, there is a concern on the potential impact on meat quality. Increasing the levels of S-nitrosoglutathione (GSNO) modifies myofibrillar proteins by decreasing their thiol content while accumulating S-nitrosylated protein [97]. There is some evidence that the accumulation of S-nitrosylated protein by NO results in the unfolding of the tertiary structure of myofibrillar proteins allowing more degradation by calpain-1 to occur. In a study by Cook et al.[93] in bull *longissimus lumborum* muscles, there was an effect on tenderness early on, tenderizing meat faster in the NO enhanced samples than the NO inhibited samples and slowly decreasing by the end of aging at Day 8 after being measured at Day 1, 3, 6, and 8. Free radical activity is much higher immediately after slaughter, showing higher NO concentrations and effects calcium levels as well to activate proteolytic enzymes.[93]

1.7.2. Measuring NO and NOS Levels

Measuring NO is difficult due to its short half-life, but can be monitored when bonded to metal chelates measured by electron paramagnetic resonance (EPR) or the binding to intrinsic metallo-heme centers within the tissue such as myoglobin contained in muscle tissue.[72] Measuring nitrosyl-heme formation by spectrophotometry will

indicate if NO formation has occurred and its concentration within muscle samples. Concentrations of nitrite measured can indicate nitrite-mediated NO formation formed from ischemia from vascular occlusion.[98] Measurement by this residual nitrite is dependent on pH directly correlating with increased reduction caused by acidosis.

1.7.3. NOS System Potential in Pre- and Post-rigor Meat

NOS is mainly concentrated in fast twitch fibers, making it readily available for access in post rigor relaxation of muscle and more prone to stress effects pre-slaughter.[99] Low concentrations of NO when exposed to superoxides can inactivate NO in uncontracted skeletal muscles, while higher concentrations of both superoxides and NO can form peroxynitrite that decomposes to extremely reactive free radicals with fiber breakdown capability.[93]

Most importantly, ‘‘stress’’ physiology of any animal perimortem is important in determining quality through effects on ultimate pH, muscle glycogen and the dark-cutting condition, but also through independent mechanisms.[95] Not only does NO affect calcium uptake, but also its release from the sarcoplasmic reticulum, which affects meat tenderness.[100] Figure 1.5 demonstrates the positive and negative effects on protein degradation by calcium fluctuation.

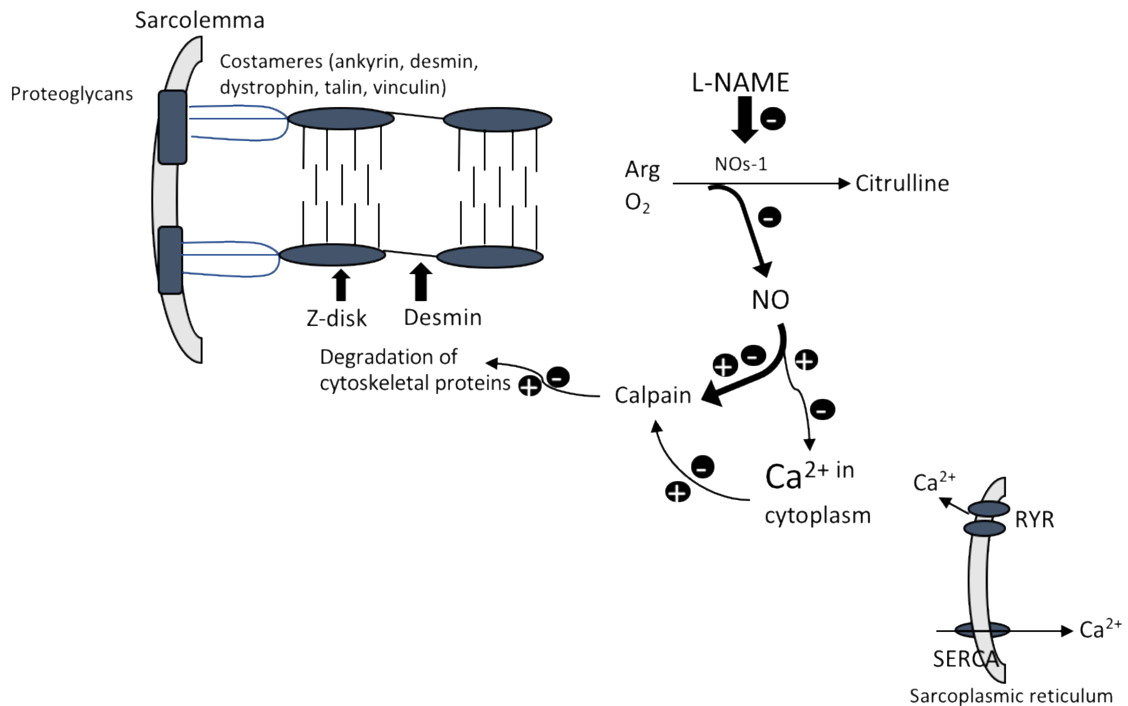

Figure 1.5 Effects of NO on Meat Tenderness (adapted)
Mechanism in sarcolemma and sarcoplasmic reticulum to demonstrate negative effects of NO on meat tenderness by concentration of calcium and negative feedback on calpain activation. L-NAME (NOS inhibitor) positively influences meat tenderness in a counter reaction by removing negative feedback on calcium concentration in the cell and activates calpain. Adapted with permission from "Trends in skeletal muscle biology and the understanding of toughness in beef" by Harper, 1999. *Australian Journal of Agricultural Research*, 50, 1105-1129, Copyright 1999 by CSIRO Publishing. [100]

Muscle redox state in vivo is regulated by NO, which can serve as an antioxidant or combine with other free radicals that also react quickly with lipid membranes. NOS activity is increased during contraction by various stimulation protocols and exercise.[64]

In a study by Melody et al.,[101] *psoas major, longissimus dorsi,* and *semimembranosus* muscles were taken from pigs sampled at 30 min, 45 min, 1 h, 6 hr., 12 h, and 24 h. The *semimembranosus* muscle in pigs had higher levels of calpastatin activity and NOS regulation of calpain activity, affecting shear force and tenderness postmortem.[101]

1.8. Literature Summary

Sodium nitrite has been used for many years as a safe way to cure meat products for a longer shelf life and antimicrobial properties in food storage. The cured "pink" color is characteristic throughout cure meat products and an appealing texture and color to consumers. The myoglobin pigment complexing with nitrite or nitric oxide and protein being denatured by heat and combining with nitrite to forms the desired cured color pigment nitrosohemochromagen. Although current curing methods with sodium nitrite are efficient and safe, the recent concerns of carcinogenic compounds (i.e. nitrosamines)[2, 3] that can be formed in cured meat products has pushed the meats industry to develop alternative curing methods by indirect nitrite sources coupled with antimicrobial compounds and color stabilizing ingredients. However, multiple studies have shown these methods to be less efficient as sodium nitrite and produce less than desirable organoleptic properties.

Focusing on the Nitric Oxide Synthase (NOS) system that is involved within the muscle that uses L-arginine to convert nitrite to nitric oxide can correlate to the same form of nitric oxide used to cure meat products. The NOS system is found in most tissues within the body and is found in inducible NOS (iNOS) forms when said tissues are exposed to hypoxia or ischemia/reperfusion. Other forms such as neuronal

NOS(nNOS) that signals contraction and relaxation of nitric oxide within the nervous system and sarcoplasmic reticulum, endothelial NOS (eNOS) within blood vessel walls in vasculature, and constitutive endothelial NOS (ecNOS) that co-localizes with mitochondrial NOS (mtMOS) within tissue mitochondria and skeletal muscle can also be activated by L-arginine to produce nitric oxide with antimicrobial functions and storage of nitric oxide capabilities. Due to nitric oxide having a short half-life, the NOS system is harder to measure for efficiency in producing nitric oxide by arginine activation. Arginine is an essential amino acid and is mostly synthesized by citrulline that can be found in most tissues with the ability to generate nitric oxide in a biosynthetic pathway that can recycle through the citrulline/NO cycle with the citric acid cycle.

With little research into the activation of the NOS system in muscles after death, the only meat based studies have focused on the effects of nitric oxide on meat quality by the inhibition or promotion of calpains, stress physiology, and calpastatin activity.[93-95, 101]

1.9. Research Objectives

The objective of this study was to investigate the efficacy of the essential amino acid L-arginine in generating nitric oxide through the Nitric Oxide Synthase (NOS) system in pre-rigor *Semimembranosus* pork muscles and determine if the cured pigment nitrosohemochromagen would form in cooked muscle samples.

2. MATERIALS AND METHODS

2.1. Experimental design

Pre-rigor pork *Semimembranosus* muscle samples were collected from four pre-rigor pork carcasses (n=4) harvested at the Rosenthal Meat Science and Technology Center at Texas A&M University at six separate times over a four-day period (N=24). Muscle samples were treated with five concentrations of L-arginine, using water as the control treatment. Samples were either subjected to heat treatment (water bath increasing from room temperature (~24°C) to 62°C for 60 min) or left raw/fresh (uncooked). All treated muscle samples were analyzed for residual nitrite, and cooked samples were analyzed for nitrosylhemochromagen levels to determine curing efficiency. The overall experiment was designed as a 6 (5 L-arginine concentrations plus a control) by 4 (pork carcasses) by 2 (heating treatment (raw and cooked)) factorial replicated 6 times (collection) (N=288).

2.2. Reagent preparation

2.2.1. Immersion/stabilization phase

Solutions were prepared according to Wu to stabilize the muscle samples for enclosed reactions within 50 mL conical tubes. Each 50 mL conical tube contained for the first immersion and stabilization phase 72 mg sodium chloride (NaCl) (granular USP, FCC, Avantor-Macron Fine Chemicals) and 576 ppm sodium erythorbate (NaE) (FCC, Spectrum Chemical) in 8 mL deionized. The NaCl was used to stabilize any reaction and the NaE was used to accelerate any cure reaction that could occur. The L-

arginine was prepared separately so as to be administered at a specific concentration once the five grams of meat was added to the reaction tubes by creating dilutions of millimolar concentrations at 0 mM, 2 mM, 4 mM, 8 mM, 16 mM, and 32 mM.

2.3. Sample collection, preparation, and treatment

Pre-rigor *semimembranosus* muscles were collected from four pork carcasses (left side of each carcass) at six different harvest times across four days approximately one hour after exsanguination. The *semimembranosus* muscle was selected in this study due to its myoglobin and mitochondria content within lean skeletal muscle, providing assurance of the presence of the nitric oxide synthase (NOS) system that generates nitric oxide if it remained viable. Samples were aseptically excised (60-75 g) from the left and right side of the *semimembranosus* muscle, using the aitch bone as the reference point. The muscle samples were transported to the research lab are within the Rosenthal Meat Science Center and any excess connective tissue and fat was removed.

2.4. Treatment of Pre-rigor Pork *Semimembranosus* Muscles

2.4.1. Raw Muscle Samples

The procedures for determining the following sample reactions followed the procedures outlined by Wu.[102] Twenty-four 5g muscle samples were individually placed into 50 mL conical tubes. A total of 24, 5g samples were produced, with 6 tubes for each carcass muscle sample. Each tube received 8 mL of the 0.9% NaCl and 576 ppm sodium erythorbate solution. Next, 2 mL of the deionized, distilled water (control), or one of the five L-arginine solutions was added. The L-arginine solution was used to activate the NOS system to generate NO and residual nitrite.[102]

After two hours of immersion, the samples were reweighed to determine the percent solution pickup and subsequently transferred to new 50 mL centrifuge conical tubes containing 25 mL 0.9% NaCl solution to stabilize and cease any further reactions. Raw (unheated) samples were homogenized immediately for 30 sec at 7000 rpm (Kinematica Polytron Pt-10-35 GT Homogenizer, Kinematica Inc, Bohemia, NY). Samples were centrifuged at 4500 *xg* for ten minutes at 4°C until the supernatant was clear. (Avanti J-25 Centrifuge with JA 17 Rotor, Beckman Coulter Inc., Atlanta, GA). The supernatant was removed by pipetting 10 mL and transferring the supernatant into a 15 mL centrifuge tube. The supernatant was then tested for pH before being stored at -30°C.

2.4.2. Cooked Muscle Samples

Muscle samples subjected to heating followed the same protocol as described for raw (unheated) samples except the sample tubes were placed in a water bath and cooked from 27°C to 62°C internal temperature within one hour to simulate a hot dog cook cycle. Temperature was monitored using a thermocouple probe (HH501BT Type T Thermometer, Omega Engineering Inc., Norwalk, CT) All cooked samples were weighed for weight pickup and then subjected to the same procedures as the raw samples except that both the supernatant and residual pellet of cooked meat samples were subjected to cure efficiency testing later.

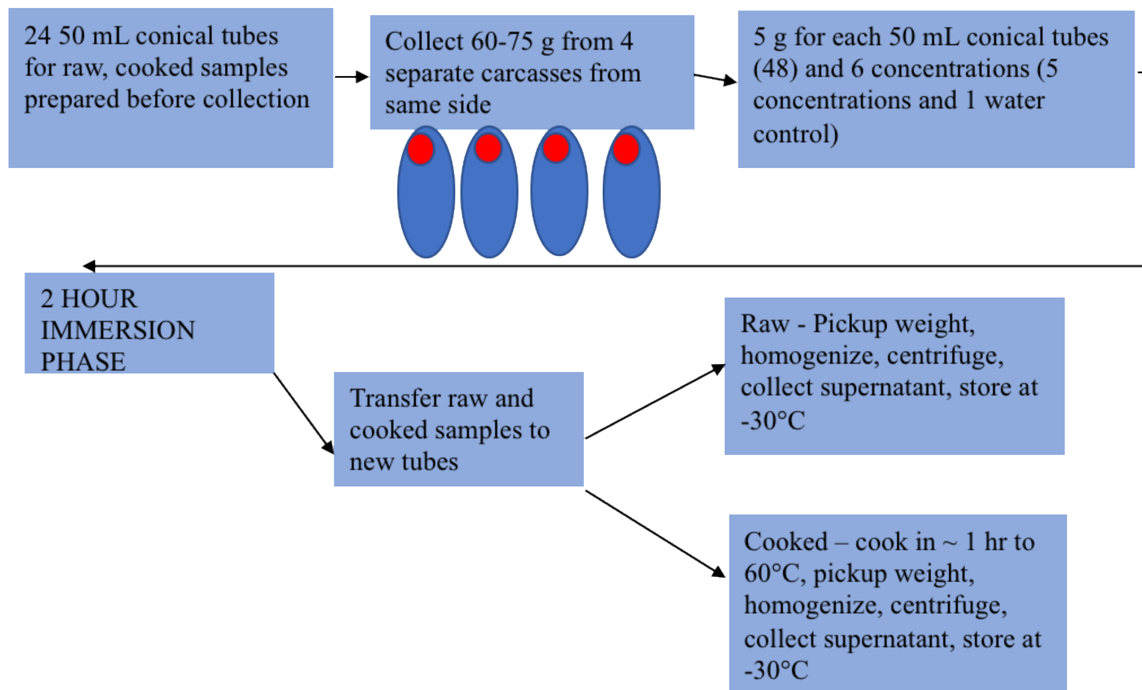

Figure 2.1 Procedure Flow
The procedure applied to six collection times over a four-day period to separate treatments for reps. Due to the sensitivity of pre-rigor meat, the samples were collected, treated with L-arginine concentrations, stabilized, and frozen all in the same day.

2.5. pH assessment

Initial pre-rigor muscle sample pH (approximately one hour after exsanguination) of the muscle sample before muscles were dissected into 5g samples for analysis (5-7 minutes after excision from carcass) was taken via a handheld pH probe. Supernatant were used to determine pH with a glass probe (probe placed into tube directly to not dilute sample further since samples were already diluted by the deionized water). pH of raw and cooked samples was determined using a benchtop pH meter (VWR Symphony 810, VWR International) with a glass probe (VWR Symphony Red Tip Reference Probe, VWR International Radnor, PA) and benchtop pH meter.

2.6. Proximate composition assessment

Samples of untreated pre-rigor meat were comminuted and then subjected to liquid nitrogen freezing and then powdered using a Waring blender (Model 33BL79, Waring Commercial, New Hartford, CT) to determine moisture (AOAC 985.14 oven drying method) and protein (AOAC 992.15) using a nitrogen analyzer (F528, Leco Corp., St. Joseph, MI). Fat content was determined by subtracting moisture and protein from 100% (AOAC 2005, AOAC 2019).

2.7. Residual nitrite assessment

The supernatant for raw and cooked samples were tested for residual nitrite after deproteinization by sample preparation for amino acid analysis by HPLC.[103] Determination of nitrite (UV/VIS Spectrophotometric Method) was used to detect any residual nitrite for best results in detecting minute amounts of nitrite from an enclosed system.[103]

2.8. Cure efficiency assessment

Cooked meat samples were subjected to analysis to determine the degree of nitrosylation of residual nitrite, by the procedure set forth by Pearson and Tauber[104] from a modified Hornsey's [18] procedure for analyzing small samples was used[105] to analyze sample supernatant and pellet. 2 mL or 2 g of the samples were treated with acetone to measure NO-heme concentration and separately 2 mL or 2 g of the samples were treated with acetone and hydrochloric acid to measure total heme concentration in a 1 cm quartz cuvette. NO-heme concentration (ppm acid hematin) was determined using a spectrophotometer (2100 Series Spectrophotometer, Unico, Dayton, NJ) by reading

samples at 540 nm. Total heme concentration (ppm acid hematin) was determined at 640 nm determined nitrosylation, which indicated how well varying concentrations of L-arginine affected the NOS system's ability to cure, known as cure efficiency. This procedure measured how much myoglobin was converted to nitrosohemochromagen, the cured meat color, and the level of conversion compared to all pigmentation within the sample.

2.9. Statistical analysis

Data was analyzed by JMP software for Least Square Means and ANOVA with $P=0.05$ to determine significant main effects (arginine concentration, nitrosylation). Least squares means were calculated to determine significant main effects, and significant differences were determined by Tukey's HSD with $P<0.05$. Analysis for the samples were replicated three times. The experiment was replicated three times. Sample analyses were conducted in triplicate.

3. RESULTS AND DISCUSSION

3.1. Proximate Composition and pH

Initial muscle sample pH (Table 3-1) was slightly lower than sample pH after the immersion phase (5.87) but was not significantly different (5.83-6.05). The increase in muscle sample pH was due to the partially alkaline L-arginine, which under physiological conditions can vary from a pH of 7.2 to 8.2.[106] Moisture (72.29%), protein (20.13%), and fat (1.23%) contents for untreated muscle samples were consistent across all replications (Table 3-1). Initial pH values were important to determine a baseline between all carcasses to ensure there were no outliers or carcasses that would skew end pH values. End pH values were important to determine any effects L-arginine could have on the samples that would affect color change or stability.

Percent sample weight pickup was determined after two hours of immersion in the L-arginine, sodium erythorbate, and NaCl for raw (unheated) samples. Percent for cooked samples was determined after two hours of immersion and after one hour of cooking to an internal temperature of 62°C and cooling to room temperature. The pickup percentages (Table 3-1) across all treatments (0 mM-32 mM) were not significantly different between L-arginine concentrations or heat treatment (raw and cooked) (24.38-28.09%). The importance of sample pickup was to determine the intake of L-arginine for a better immersion and reaction phase to activate the NOS system.

Table 3-1. Least Squares Means of Proximate Composition, Initial pH of Carcass Samples, and Percent Solution Pickup of Raw and Cooked Pre-rigor *Semimembranosus* Pork Muscles

Variable	Carcass	Treatment		SEM[1]
		Raw	Cooked	
n	24	96	96	
Proximate Composition				
Moisture, %	72.29			1.54
Protein, %	20.13			1.79
Fat, %	7.58			1.23
Initial pH	5.87			
SEM[1]	0.05			
pH after 2 hours of sample immersion				
0 mM		5.83[a]	5.88[a]	
2 mM		5.84[a]	5.88[a]	
4 mM		5.85[a]	5.86[a]	
8 mM		5.83[a]	5.87[a]	
16 mM		5.88[a]	5.94[a]	
32 mM		6.01[a]	6.05[a]	
SEM[1]		0.05	0.05	
Sample Pickup, %				
0 mM		25.16[a]	24.38[a]	
2 mM		26.57[a]	25.68[a]	
4 mM		26.89[a]	25.00[a]	
8 mM		26.84[a]	25.68[a]	
16 mM		28.09[a]	25.86[a]	
32 mM		27.25[a]	27.09[a]	
SEM[1]		1.38	1.24	

[1]SEM: Standard error of the mean (largest) of the least squares means
[ab]LSMeans within a column with different superscripts are significantly different ($P < 0.05$)

3.2. Residual Nitrite Values for Supernatant (Raw and Cooked) and Pellet (Cooked) Samples

Residual nitrite was determined using the modified AOAC Official Method 973.31 (Nitrite Analysis in Cured Meats Procedure) and was verified by Wu[102, 103, 107] with sample (plasma or serum) preparation for amino acid analysis by HPLC and determination of nitrite (UV/VIS Spectrophotometric Method).[103] This procedure was used to determine any residual nitrite found in samples. All samples were tested three weeks after being held in a -30°C freezer then thawed in a 0°C cooler.

Residual nitrite (Table 3-2) of raw muscle sample supernatant shows that the 32 mM L-arginine treatment resulted in higher residual nitrite levels (14.34 ppm) than the control (0.08 ppm). The 4 mM (8.00 ppm), 8 mM (9.66 ppm), 16 mM (9.23 ppm) L-arginine treatments were significantly different than the control or the 32 mM treatment, but were not different among the three L-arginine concentrations. The residual nitrite range generated by the NOS system at each L-arginine concentration is shown in Table 3-2 and indicates the variability of the NOS system in generation residual nitrite through the addition of varying concentrations of L-arginine.

The wide variation in residual nitrite at each L-arginine concentration tested may be due to length of storage, which resulted in pigment fading and nitrite converting to nitric oxide (a gas), as evidenced by previous studies done by Cassens et al.[10, 24, 25] and Keeton et al.[22] that showed a loss of 70 to 80% nitrite during storage and would not be measured as residual nitrite but later measured as nitrosohemochromagen. The samples

are better tested when freshly converted and not stored, allowing 50 to 70% nitrite to be analyzed in the product after it is formulated.[13]

Table 3-2. Least Squares Means for Residual Nitrite of Raw Pre-rigor *Semimembranosus* Muscles Supernatant Generated by the Nitric Oxide Synthase System at Various L-Arginine Concentrations

Concentration	Residual Nitrite (ppm)[1]	Range of Residual Nitrite (ppm)
n	144	
0 mM	0.08[b]	0, 0.69
2 mM	4.53[b]	0, 20.22
4 mM	8.00[ab]	0, 33.48
8 mM	9.66[ab]	0, 21.62
16 mM	9.23[ab]	0, 28.95
32 mM	14.34[a]	0, 66.61
SEM[2]	2.37	

[1] ppm = absorbance level x standard curve slope (1.7438) x dilution factor (200),
[ab]LSMeans within a column with different superscripts are significantly different ($P < 0.05$)
[2]SEM: Standard error of the mean (largest) of the least squares means

Residual nitrite means of the supernatant of cooked muscle samples are reported in Table 3-3. The 4 mM treatment was significantly higher for residual nitrite (8.79 ppm) compared to the other treatments and the control (0.07 ppm). The remaining L-arginine treatments were also different from the control. The 2 mM (5.81 ppm), 8 mM (5.85 ppm), 16 mM (3.19 ppm), and 32 mM (7.86 ppm) concentrations, however, were not significantly different between each other.

The lower residual nitrite values for cooked meat supernatant compared to raw supernatant samples (32 mM cooked - 7.86 ppm versus 32 mM - 14.34 ppm) may be attributed to both heating and perhaps length of frozen storage prior to analysis. It has

been reported that thermal processing can result in a 20 to 80% loss of nitrite[13], therefore the amount of residual nitrite in cooked meat samples would expected to be lower than the raw samples tested.

Table 3-3. Least Squares Means for Residual Nitrite of Cooked Pre-rigor *Semimembranosus* Muscles Supernatant Generated by the Nitric Oxide Synthase System at Various L-Arginine Concentrations

Concentration	Residual Nitrite (ppm)[1]	Range of Residual Nitrite (ppm)
n	144	
0 mM	0.07[b]	0, 1.05
2 mM	5.81[ab]	0, 13.24
4 mM	8.79[a]	0, 38.36
8 mM	5.85[ab]	0, 20.93
16 mM	3.19[ab]	0, 11.51
32 mM	7.86[ab]	0, 18.48
SEM[2]	1.93	

[1] ppm = absorbance level x standard curve slope (1.7438) x dilution factor (200), [ab]LSMeans within a column with different superscripts are significantly different ($P < 0.05$)
[2]SEM: Standard error of the mean (largest) of the least squares means

Residual nitrite values of cooked muscle pellet samples are reported in Table 3-4. The 32 mM treatment was significantly higher for residual nitrite (15.69 ppm) compared to the other treatments and the control (0.04 ppm). The remaining L-arginine treatments were also different than the control. The 2 mM (10.46 ppm), 4 mM (9.35 ppm), 8 mM (11.22 ppm), 16 mM (14.06 ppm), and 32 mM (15.69 ppm) concentrations, however,

were not significantly different between each other. Again, this observation may be attributed to both heating and the length of frozen storage prior to analysis. It has been reported that thermal processing can result in a 20 to 80% loss of nitrite[13], therefore the amount of residual nitrite in cooked meat sample pellets would expected to be lower than the raw samples tested.

Table 3-4. Least Squares Means for Residual Nitrite of Cooked Pre-rigor *Semimembranosus* Muscles Pellet Generated by the Nitric Oxide Synthase System at Various L-Arginine Concentrations

Concentration	Residual Nitrite (ppm)[1]	Range of Residual Nitrite (ppm)
n	96	
0 mM	0.04[b]	0, 0.70
2 mM	10.46[a]	1.05, 24.41
4 mM	9.35[a]	0.70, 23.37
8 mM	11.22[a]	1.05, 29.65
16 mM	14.06[a]	2.44, 29.30
32 mM	15.69[a]	0, 36.62
SEM[2]	2.19	

[1] ppm = absorbance level x standard curve slope (1.7438) x dilution factor (200),
[ab]LSMeans within a column with different superscripts are significantly different ($P < 0.05$)
[2]SEM: Standard error of the mean (largest) of the least squares means

3.3. Pigmentation and Curing Efficiency for Cooked Samples

Curing efficiency or nitrosylation is calculated as the percentage conversion of NO-heme to total heme. Cure efficiency is the percentage of total pigment converted to nitroso pigment and indicates the degree of cured color fading.[18] Cured meat pigment is extracted in a solution of 80% acetone and 20% water that extracts cured pigment heme. Total heme pigments are extracted using an acidified acetone solution that extracts heme

from all heme proteins as first used by Hornsey.[18] NO-heme concentration (as ppm acid hematin) = sample $A_{540} \times 290$ determines the specific cure color pink from the rest of the heme pigmentation of the sample. Total heme concentration (ppm acid hematin) = sample $A_{640} \times 680$ determines the total heme pigmentation of the sample. Cure efficiency (%) = (ppm of nitrosoheme ÷ ppm of total pigment) × 100 and indicates how much of the product is cured and holds the cured pink stability once cooked.[18, 105]

Least squares means for the degree of nitrosylation or curing efficiency for cooked sample supernatant is reported in Table 3-5. No concentration had the highest significant difference between sample treatments for NO-hemochrome or nitrosylation in the supernatant samples. The 2 mM (24.22 ppm), 4 mM (34.17 ppm), 16 mM (20.14 ppm) and 32 mM (31.67 ppm) L-arginine treatments had significantly higher levels of the total heme pigment than the control (0.00 ppm) however, they were not significantly different between each other. There is no observed difference between 32 mM, 16 mM, 8 mM, 4mM, and 2mM that shows higher levels of nitrosylation consistently. There is also an uncharacteristically high cure efficiency at all L-arginine concentrations (over 100%) indicating the ability of the NOS system to generate nitrite and nitric oxide under these specific laboratory research conditions total heme pigmentation.

Table 3-5. Least Squares Means for NO-Heme, Total Heme, and Percent Nitrosylation (Cure Efficiency) of Cooked Pre-rigor Pork *Semimembranosus* Muscles Supernatant Generated by the Nitric Oxide Synthase System at Various L-Arginine Concentrations

Concentration	NO-Heme (ppm)[1]	Total Heme (ppm)[2]	Nitrosylation (%)[3]
n	144		
0 mM	0.01[b]	0.00[b]	0.00[a]
2 mM	43.94[a]	24.22[ab]	125.52[a]
4 mM	21.09[a]	34.17[ab]	155.68[a]
8 mM	16.44[a]	70.38[a]	108.99[a]
16 mM	16.73[a]	20.14[ab]	110.11[a]
32 mM	16.86[a]	31.67[ab]	50.48[a]
SEM[2]	13.90	15.04	42.58

[1] NO-heme pigment concentration (ppm acid hematin) = sample $A_{540} \times 290$.
[2]Total heme pigment concentration (ppm acid hematin) = sample $A_{640} \times 680$
[3]Percentage nitrosylation = (ppm NO-hemochrome/ppm total pigment) x 100
[ab]LSMeans within a column with different superscripts are significantly different ($P < 0.05$)
[2]SEM: Standard error of the mean (largest) of the least squares means

Least squares means for the percentage of nitrosylation or curing efficiency in cooked sample pellets are reported in Table 3-6. There was a significant difference ($P<0.05$) in ppm NO-hemochrome levels between all L-arginine treatment combinations compared to the control but there were no differences between treatment concentrations. For total heme pigmentation all L-arginine treatment concentrations were higher than the control while no differences existed among the individual treatment concentrations. The 2 mM treatment had the highest concentration of total heme pigmentation (64.77 ppm). Percent nitrosylation was the greatest at 32 mM (156.72%) and was significantly different from the other treatment concentrations. The curing efficiency for 2 mM

(46.51%), 4 mM (74.27%), 8 mM (70.13%), and 16 mM (97.82%) L-arginine concentrations were not significantly different from each other.

There is an observed difference between 32 mM, 16 mM, 8 mM, 4mM, and 2mM L-arginine concentrations that shows higher levels of nitrosylation in a more consistent manner compared to the cooked muscle supernatant. This suggests that the cooked pellet may be a more suitable source than the cooked supernatant to measure for nitrosylhemochrome and total heme concentrations for a more accurate representation of the fluctuation of color pigments found throughout meat products.

Table 6. Least Squares Means for NO-Heme, Total Heme, and Percent Nitrosylation (Cure Efficiency) of Cooked Pre-rigor Pork *Semimembranosus* Muscles Pellet Generated by the Nitric Oxide Synthase System at Various L-Arginine Concentrations

Concentration	NO-Heme (ppm)[1]	Total Heme (ppm)[2]	Nitrosylation (%)[3]
n	96		
0 mM	0.03[b]	0.12[b]	0.00[b]
2 mM	8.70[a]	64.77[a]	46.51[ab]
4 mM	7.77[a]	35.87[ab]	74.27[ab]
8 mM	9.33[a]	33.44[ab]	70.13[ab]
16 mM	11.69[a]	36.04[ab]	97.82[ab]
32 mM	13.05[a]	54.40[ab]	156.72[a]
SEM[2]	1.82	15.42	33.16

[1] NO-heme pigment concentration (ppm acid hematin) = sample $A_{540} \times 290$.
[2]Total heme pigment concentration (ppm acid hematin) = sample $A_{640} \times 680$
[3]Percentage nitrosylation = (ppm NO-hemochrome/ppm total pigment) x 100
[ab]LSMeans within a column with different superscripts are significantly different ($P < 0.05$)
[2]SEM: Standard error of the mean (largest) of the least squares means

4. CONCLUSIONS

Results of this study indicate that the Nitric Oxide Synthase (NOS) system is viable, functional, and capable of generating NO and residual nitrite from pre-rigor pork *semimembranosus* muscle. Residual nitrite was determined to be present in both raw and cooked samples even after three weeks of storage. Raw samples held more residual nitrite than cooked samples, indicating that a cure reaction used up available nitrite when heated in cooked samples and converted to nitrosylhemochromagen. Cooked samples nitrosylation percentages showed that while the supernatant seemed to hold a better nitrosylation, there was a more thorough distribution of pigmentation within the pellet that held significant concentrations of nitrosylhemochrome and nitrosylation.

The small levels of nitrite produced by the arginine treatments can still be significant enough to color meat and poultry that results in the characteristic cured "pink" color, with the lowest created nitrite levels at 1 ppm enough to cure poultry and 4 ppm to cure pork shoulders[108]. The higher levels of nitrite needed to cure meat for antimicrobial properties would have to reach 50-60 ppm in conjunction with pH, salt concentration, reductants, and iron content to protect against *Staphylococcus aureus* and *Clostridium botulinum*.[28] The control of *C. botulinum* spores and any toxin production would require levels higher than 70 ppm.[29] With the long storage times still producing results, there is an indication that the samples will be shelf stable and storage stable to continue the cure reaction when heated then stored.

Based upon data in these applications of arginine concentrations to pre-rigor samples of *Semimembranosus* pork muscle, there is evidence the Nitric Oxide Synthase system is still producing nitric oxide without being in vivo. The evidence of the NOS system is found in multiple medical studies of the human metabolism[6, 55, 56, 109], and in animal studies as well,[68, 92, 98] suggesting the NOS system is a viable option for nitric oxide generation in tested livestock species beyond pigs.[93, 9999, 102, 110] The evidence of the NOS system in pre-rigor meat suggests future research to test how long the NOS system is viable outside of pre-rigor meat by testing post-rigor meat and how long the meat has been aged (i.e. number of hours post slaughter extended to aged meat used in current cured meat products). To continue testing treatments of L-arginine at higher concentrations beyond the treatments in this study would be imperative to verify the full efficacy and efficiency of the Nitric Oxide Synthase system as an alternative curing method to generate its own nitric oxide from the meat itself.

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APPENDIX A

POWDERING SAMPLES FOR ANALYSIS

1. Previously frozen samples were thawed until meat could be cut.

2. Meat samples were hand cut into small cubes ½ inch or smaller.

3. Sample was placed into a wire straining basket and lowered into a container of liquid nitrogen.

4. Samples were submerged for 30 sec or until liquid nitrogen stopped bubbling.

5. Frozen sample pieces were transferred to a stainless-steel waring blender and blended until a homogenous powder was formed.

6. Powdered samples were transferred to a whirl pack back and stored frozen until analysis.

APPENDIX B

LECO F-528 RAPID NITROGEN/PROTEIN ANALYSIS

Perform Leak Checks prior to running any samples

1. Press “Diagnostics”, then press “Leak Check”, Select either “Oxygen Leak Check” or “Helium Leak Check”. (Both leak checks should be performed).

2. If leak check is ok, continue on to analysis. If leak check does not pass, refer to instrument manual.

Running Blanks

1. Press “Analyze” then press “Start” to open the sample chamber, press “Start” again to close the chamber and begin blank.

2. Run blanks until protein reading is near zero (0.012 or -0.012), approximately 5 blanks

3. Check the S.D. of blanks by pressing “Results”, select at least 3 blanks by highlighting blanks and pressing “Select”, then press “Menu” and select “Statistics”. The S.D. should be ≤ 0.03.

4. Calculate blank by pressing “Calibrate”, select at least 3 blanks by highlighting blanks and pressing “Select”, then press “Menu” and select “Calculate Blank”, press “Exit”

Running Standards (Performed before new project, after bottles are changed, after maintenance)

1. Weigh ~.3500 grams of standard (EDTA) in tin foil cups, record weights (Need at least 5).

2. Press “Analyze”, enter the weight, press “Select” twice, then enter Sample ID.

3. Place standard in opening, then press “Start” to open the sample chamber, press “Start” again to close the chamber and begin analysis.

4. The first few standards will be conditioning standards, do not use for calibration.

5. Check the S.D. (or RSD) of standards by pressing “Results”, select at least 3 standards by highlighting blanks and pressing “Select”, then press “Menu” and select “Statistics”. The target S.D. is on the certificate of analysis with the standard.

6. Calculate calibration by pressing “Calibrate”, select at least 3 standards by highlighting standards and pressing “Select”, then press “Menu” and select “Calculate Calibration”, enter Nitrogen Standard value found on certificate of analysis with the standard, press “Select”, press “Yes”, press “ Exit”.

7. Recalculate by pressing “Menu” on calibration screen, press “Recalculate”, press “Recalculate Today”, press “Exit”.

Running Samples

1. Weigh ~.3000 grams of sample in tin foil cups, record weights (done in triplicate).

2. Press “Analyze”, enter the weight, press “Select” twice, then enter Sample ID.

3. Place sample in opening, then press “Start” to open the sample chamber, press “Start” again to close the chamber and begin analysis.

4. Record % Protein from screen.

APPENDIX C

AOAC 950.46 MOISTURE ANALYSIS

Equipment:
Gloves
Whatman Filter paper: #2 Qualitative Circles, 125 mm
Stapler with staples
#2 pencil
Desiccator with desiccant
Analytical balance/scale
Convection oven

**Gloves should be worn at ALL times

Procedure

1. Construct thimbles from Whatman #2 filter paper folded into a sleeve open at one end and stapled at the other end
2. Label thimbles with #2 pencil
3. Dry thimbles for a minimum of 12 hours at 100°C using an air dry oven. Oven should not be overfilled. Only 1 pan per shelf and not stacked on desiccant. Metal pans should not tough any of the walls of the oven, as air must be able to circulate.
4. Ensure desiccator is properly equipped with functional desiccant, sealant, and is not overfilled with thimbles/samples
5. Desiccator should be opened by sliding lid to remove thimble/sample and then immediately sealed.
6. Transfer dried thimbles to desiccator
7. Cool thimbles in desiccator for 30 minutes

8. Record dried thimble weight and 1 staple to the nearest 0.0001g. This is “initial thimble weight”. See #5 for opening/closing desiccator and place thimble immediately on the scale. **Record 1st weight**.
9. Put 2-3 grams of powdered homogenous sample into thimble and record the weight plus 1 staple to the nearest 0.0001 grams. This is “initial thimble/sample weight”. **Each sample should be performed in triplicate**.
10. Fold over open end of the thimble and seal with a staple.
11. Place thimble on clean metal pan. Samples should be laid flat and not overlapping.
12. Dry in 100°C dry oven for 16-18 hours. Oven should not be overfilled. Only 1 pan per shelf and not stacked on desiccant. Metal pans should not tough any of the walls of the oven, as air must be able to circulate.
13. Coll in desiccator for at least 1 hour. #4 should still be true.
14. Record dried thimble weight and 1 staple to the nearest 0.0001 gram. This is “dried thimble/sample weight”. See #5 for opening/closing desiccator and place thimble immediately on the scale.

APPENDIX D

MEAT PH MEASUREMENT PROCEDURE

Equipment:

Blender

Pint Jars

pH meter with pH electrode

Stir plate

Magnetic stir bars

Reagents:

Distilled water

Buffer, pH 4.0 and pH 7.0

Procedure:

1. Place approximately 10 g of the frozen powdered sample into a pint jar.

2. Add 90 g distilled water to the pint jar, attach blender blade, o-ring, and screw cap. Blend on high speed for 15 to 20 seconds to make a smooth slurry.

3. Place a magnetic stir bar in the bottom of the jar and place on stir plate. Stir plate should be moderately agitating the sample (~200 RPM) when the probe is lowered into the sample jar.

4. Measure the pH of this slurry with a pH meter that has been calibrated with two standard buffer solutions. One buffer at pH = 7.0 and the other (either 4 or 10) having a pH value near that of the final.

5. The electrode should be placed in the stirred slurry for about 30 seconds to allow the electrode to equilibrate.

6. Press read to begin pH measurement. “Stable” will appear when reading is finished. Record the pH of the slurry after the electrode has stabilized.

6. Do NOT leave the pH probe in the meat slurry. Remove the pH probe from the slurry and wash it thoroughly with distilled water. Be sure to gently wipe all fat and connective tissue from the probe.

7. Always store the pH probe in CLEAN distilled water or pH 7 buffer.

NEVER let the bulb dry out.

APPENDIX E

NITRITE ANALYSIS IN CURED MEATS PROCEDURE

(AOAC Official Method 973.31, 2000, 39.1.21, PAGE 8)

EQUIPMENT:

100 ml beakers

Glass rods

1000 ml Volumetric flasks

500 ml Volumetric flasks

50 ml Volumetric flasks

Hot Plate

Spectrophotometer (UV/VIS 540 nm)

Spec cuvettes

5 ml Pipettes

10 ml Pipettes

500 ml Erlenmeyer flasks

Whatman® No. 2 Filter paper

Heated Water Bath

Analytical balance

Homogenizer or food processor

REAGENTS:

NED Reagent: Dissolve 0.2 g N-(1-naphthyl)ethylene diamine • 2HCl in 150 ml 15% (v/v) acetic acid. Store in a glass-stoppered brown glass bottle. If necessary, filter before use.

Sulfanilamide Reagent*: Dissolve 0.5 g sulfanilamide in 150 ml 15% (v/v) acetic acid. *Store in dark or brown glass bottle. If necessary, filter before use.

Standard Curve Preparation:

Nitrite Standard Solution

Stock solution (1,000 ppm $NaNO_2$): Dissolve 1 g (± 0.0001) $NaNO_2$ in distilled water and dilute to 1 L.

Intermediate Solution (100 ppm $NaNO_2$): Dilute 100 ml of Stock Solution to 1,000 ml with distilled water.

Working Solution (1 ppm $NaNO_2$): Dilute 10 ml of Intermediate Solution to 1,000 ml with distilled water.

Filter Paper:

Randomly select 3 to 4 sheets per box. Filter 40 ml water through each sheet.

Add 4 ml sulfanilamide reagent, mix and wait 15 min.

If any sheets are positive, discard entire box.

PROCEDURE:

1. Weigh 5 g (±0.01) of finely comminuted and thoroughly mixed sample into a 100 ml beaker.
2. Add approximately 40 ml distilled water and heat to 80°C. Use a glass rod to break up all lumps and mix thoroughly.
3. Transfer the heated solution to a 500 ml volumetric flask. Quantitatively wash the beaker and rod with successive portions of the hot distilled water, adding all washings to the flask (approximately 300 ml).
4. Transfer the flask to a steam bath (~100°C) and shake occasionally for 2 hours. After cooling to room temperature, bring the volume to 500 ml with distilled water and remix. Filter through two Whatman No. 2 filter papers into flask and mix solution thoroughly (discard the residue). Then transfer 25 ml of the filtrate

into a 50 ml volumetric flask then add 2.5 ml sulfanilamide reagent, mix thoroughly.

5. After setting for 5 min, add 2.5 ml NED reagent, mix. Dilute to volume with distilled water, mix and set for another 15 min to let the color develop.
6. Transfer a portion of the solution to the cuvette and read absorbance at 540 nm against a blank of 45 ml distilled water + 2.5 ml sulfanilamide reagent + 2.5 ml NED reagent.

Standard Curve Preparation:

Add 10, 20, 30 and 40 ml of nitrite working solution to individual 50 ml volumetric flasks. The nitrite concentration in each flask is 0.2, 0.4, 0.6 and 0.8 ppm, respectively. Add 2.5 ml of sulfanilamide reagent, mix and proceed as in steps 5 and 6. The standard curve is straight line to 1 μg/ml $NaNO_2$ in final solution.

CALCULATION:

Nitrite Residual (ppm or μg/g) = Absorbance x K x F

Where: K = Standard Curve Slope = 1.7438

F = Dilution Factor = 500 x 2 x 1/5 = 200

OR

The concentration may be read directly off of the spectrophotometer. Thus, K, Abs nor F are required in this case.

APPENDIX F

SAMPLE (PLASMA OR SERUM) PREPARATION FOR AMINO ACID ANALYSIS BY HPLC

A. Materials and Chemicals

$HClO_4$ (70%), HPLC-grade H_2O, K_2CO_3

Polypropylene tubes (12 x 75 mm)

Microcentrifuge tubes (1.5 mL)

B. Preparing 1.5 M $HClO_4$ and 2 M K_2CO_3 solutions

1.5 M $HClO_4$: Add slowly 32.2 mL of 70% $HClO_4$ to 150 mL HPLC-grade H_2O. Make up to final volume of 250 mL with HPLC-grade H_2O. Mix thoroughly.

2 M K_2CO_3: Dissolve 69.11 g of K_2CO_3 in 150 mL HPLC-grade H_2O. Make up to a final volume of 250 mL with HPLC-grade H_2O. Mix thoroughly.

C. Procedure for Processing Plasma or Serum Samples for Amino Acid Analysis

1. Pipette 0.5 mL of plasma (or serum) to a 12x75 mm polypropylene tube. Place the tube in ice.
2. Add 0.5 mL of 1.5 $HClO_4$ to the tube (the tube remains in ice).
3. Mix the tube well (using a vortex).
4. After 2 min, add 0.25 mL of 2 M K_2CO_3 to the tube (the tube remains in ice).
5. After 3 min, mix the tube well. Then place the tube in ice for 3 min.
6. Centrifuge the tube at 2000 g and 4°C for 15 min.
7. Store the supernatant in 1.5 mL Microcentrifuge tube at -80°C or test immediately.

APPENDIX G

DETERMINATION OF NITRITE (UV/VIS SPECTROPHOTOMETRIC METHOD)

A. Chemicals

1. Use deionized H_2O or double glass distilled H_2O.
2. S-Reagent: Mix 50 mL of 37% HCl with 300 mL of H_2O. Add 5 g sulfanilamide. Make up to 500 mL with H_2O. Store in a brown bottle at 4°C.
3. N-Reagent: Dissolve 0.5 g N-(1-Naphthyl-)ethylenediamine dihydrochloride in 500 mL of H_2O. Store in a brown bottle at 4°C.
4. 125 mM $NaNO_2$ standard: Dissolve 86.3 mg of $NaNO_2$ in 10 mL H_2O.
    a. 1.25 mM $NaNO_2$ standard: Dilute 1 mL of 125 $NaNO_2$ in 100 mL of H_2O. (Stock solution)
    b. 80 µM $NaNO_2$: Dilute µl of 1.25 mM $NaNO_2$ in 3 mL of H_2O.

B. Assay Procedures

1. To $NaNO_2$ Standard, add the following: 0.5 mL of $NaNO_2$ standard and 0.5 mL of blank cell-culture medium

   To sample tube, add the following: 0.5 mL of sample (cell-culture medium) and 0.5 mL of H_2O
2. Add 100 µl of S-Reagent. Mix. Wait for 2 min.
3. Add 100 µl of N-Reagent. Wait for 10 min. Measure absorbance at 543 nm.

The colorimetric reaction is completed in 10 min, and color is stable for at least 1 hour.

This assay is not interfered by glucosamine, aminoguanidine, or N^G-nitro-L-arginine (NNA)

APPENDIX H

NITROSOHEME AND TOTAL HEME CONTENT OF SMALL SAMPLES

Reagents

1. Acetone-a (aqueous acetone): Place 90 mL distilled water in a 1 L volume flask; add spectrophotometric grade acetone, mix and bring to volume.

2. Acetone-b (acidic acetone): Slowly add 20 mL of concentrated HCl to 80 mL of water. Transfer the dilute HCl solution to a 1 L volumetric flask, mix, and bring to volume with additional spectrophotometric grade acetone.

Procedures

1. Do all procedures in subdued light to reduce fading of pigment.

2. Weigh out 2.0 g minced lean meat sample in a 50-mL polypropylene centrifuge tube.

3. Pipette 9.0 mL acetone-a into 50-mL tube, to obtain acetone concentration of 80%.

4. Mix thoroughly with a probe-type homogenizer or a glass rod.

5. Cap the tube to minimize evaporation of acetone, and mix by gentle swirling.

6. Let stand 10 minutes in the dark, then filter through medium-fast filter paper into a glass test tube.

7. Transfer filtrate into a 1-cm quartz cuvette and read absorbance at 540 nm. (Avoid use of disposable plastic cuvettes. They become opaque upon exposure to acetone). Calculate nitroso pigment concentration as previously described.

8. Prepare another 2.0-g sample, using acetone-b.

9. Macerate and hold 1 hour in the dark before filtering.

10. Filter the extract as before, and read absorbance at 640 nm. Calculate total pigment and cure efficiency as previously described.