EFFECTS OF 4-HYDROXY-2-NONENAL AND 4-OXO-2-NONENAL ON BIOCHEMICAL PROPERTIES OF BOVINE HEART MITOCHONDRIA

by

DEEPAK KAFLE

(Under the Direction of ANAND MOHAN)

ABSTRACT

Lipid oxidation in post mortem muscle is characterized by a series of complex reactions, which are closely interlinked and are responsible for the loss of meat quality. The aim of this study was to examine the impact of lipid oxidation products 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE) on bovine heart mitochondria, which are important organelles responsible for determining meat quality. For this, mitochondria were isolated from beef heart and incubated with HNE, ONE or ethanol to examine induced changes in their structure and functions. The electron micrographs and membrane permeability assay showed that the HNE and ONE incubated mitochondria were larger ($P < 0.05$) in area and had increased permeability compared with ethanol control. Similarly, oxygen consumption rate and metmyoglobin reducing activity were also decreased ($P < 0.05$) due to HNE or ONE. Hence, the result of this study indicated that the HNE and ONE could lead to mitochondria mediated meat quality loss.

INDEX WORDS: 4-hydroxy-2-nonenal, 4-oxo-2-nonenal, mitochondria, lipid peroxidation

EFFECTS OF 4-HYDROXY-2-NONENAL AND 4-OXO-2-NONENAL ON BIOCHEMICAL PROPERTIES OF BOVINE HEART MITOCHONDRIA

by

DEEPAK KAFLE

B.V.SC. & A.H., TRIBHUVAN UNIVERSITY, NEPAL, 2005

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2020

EFFECTS OF 4-HYDROXY-2-NONENAL AND 4-OXO-2-NONENAL ON BIOCHEMICAL PROPERTIES OF BOVINE HEART MITOCHONDRIA

by

DEEPAK KAFLE

Major Professor:	Anand Mohan
Committee:	Rakesh K. Singh
	Yen-Con Hung

Electronic Version Approved:

Ron Walcott
Interim Dean of the Graduate School
The University of Georgia
August 2020

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

In the U.S., retail stores discard about 2.4 million pounds (worth $ 5 million) of ground beef annually due to turning brown prematurely (Smith et al., 2000). Discoloration of meat impacts negatively on the purchasing decision of consumers (Troy & Kerry, 2010). Consumers decide to purchase meat based on the characteristics of color, texture, and juiciness as well as freshness of meat (Materneh, 2017). Different biochemical, physical, and structural changes occur in postmortem muscle after the animal slaughter. Oxidation of myoglobin results in accumulation of metmyoglobin, which results in brownish discoloration of meat. During conversion of muscle to meat, the stress-related signaling pathways and stress mediators exert effects on postmortem energy metabolism, protein modification, and proteolysis leading to change of muscle structure (Xing et al., 2019). There are various physiological changes, followed by physical and chemical changes in the muscle after animal harvesting. The pH of muscle starts to decline from 7.4 and reaches up to ultimate pH of 5.6 due to hydrolysis of glycogen reserve to glucose and anaerobic breakdown thereafter. Inadequate oxygen supply switches breakdown of pyruvate to lactic acid leading to rapid decline of postmortem muscle pH. The rate and extent of pH decline in post-mortem pH determines the meat quality (Hudson, 2012). The rate of pH as well as temperature decline is two important factors affecting meat quality such as color, water holding capacity (WHC), and tenderness (Kim, Warner, & Rosenvold, 2014). For example, pale, soft and exudative (PSE) is a characteristic of rapid pH fall and high temperature pre-rigor. Post-mortem tenderization is caused by enzymatic degradation of

key myofibrillar and associated proteins. The function of these proteins is to maintain the structural integrity of myofibrils. Current data indicates that μ- calpain is responsible for degradation of these proteins.

Mitochondria are first and foremost organelle affected by post mortem changes in meat. They play an important role in cellular response pathway leading to cell death and final meat quality (Veronica & Mammen, 2013). In living tissues, they metabolize energy by utilizing substrates and oxygen. In postmortem muscle, they continue to consume oxygen and influence the meat color (Ramanathan et al., 2012). Mitochondrial respiration in post-mortem muscle has implication in meat industry because it has a close association with myoglobin in determining meat color. Myoglobin serves as a reservoir and transporter of oxygen to the mitochondria and it exists in three red-ox forms: -deoxy, -oxy and -metmyoglobin. These functional and structural characteristics of mitochondria in postmortem muscle make the beef susceptible to color change during the extended period of aging (Ramanathan & Mancini, 2014). Moreover, high mitochondrial content is responsible for rapid decline of pH in postmortem muscle (Hudson, 2012). Acidification of muscle decreases protein charges, which increases hydrophobic nature of the protein, thereby reducing water retention (Luciano, Anton, & Rosa, 2006). Oxidative stress condition arises when ROS generated exceeds the antioxidative potential of the system neutralizing free radicals, which leads to damage of internal components. Superoxide is generated at seven different sites of inner mitochondrial membrane and these superoxides are generated mainly on the matrix side of the membrane (Kotiadis, Duchen, & Osellame, 2014). These superoxides (free radicals) degrade polyunsaturated fatty acids, which then leads to formation of stable secondary aldehyde compounds such as 4-hydroxy-2-nonenal and (HNE) and

4-oxo-2-nonenal (ONE). So formed aldehydes are very reactive to components of cells. These compounds can even diffuse out of their site of production to modify proteins, lipids, and nucleic acids (Doorn & Petersen, 2002).

Lipid and protein oxidation have their unique characteristics in terms of deterioration of meat quality. Lipid peroxidation is associated with discoloration, impaired WHC, rancid odor, off-flavor, loss of nutrients, and production of toxic compounds, whereas protein oxidation leads to proteolysis affecting negatively meat quality traits (Xing et al., 2019). Color, flavor, aroma, juiciness, and tenderness are all essential attributes of meat palatability from the point of view of consumer satisfaction, with tenderness being the most important drive for consumers (Wu et al., 2015). Maximum toughness is observed between 12 to 24 h post mortem (Koohmaraie, 1996). ATP depletion under postmortem anoxic conditions causes excessive Ca^{++} overload in mitochondria increases the formation of ROS, which induces oxidative stress leading to mitochondrial transition pore opening (Materneh, 2017). Due to increased Ca^{++} in postmortem muscle, induced swelling and extensive alteration of mitochondrial structure with release of cytochrome c activates caspase 9 (Luciano, Anton, & Rosa, 2006). These proteins are apoptosis inducing proteins released into cytosol leading to cell death.

Therefore, we hypothesize that ONE as compared to HNE, is a potent toxic product of the lipid oxidation and affects the structural and functional properties of bovine heart mitochondria. The objectives were designed to compare the effects of HNE and ONE on structure, function, and antioxidative capacity of the bovine heart mitochondria.

References

Doorn, J. A. & Petersen, D. R. (2002). Covalent modification of amino acid nucleophiles by the lipid peroxidation products 4- Hydroxy-2-nonenal and 4-Oxo-2-nonenal. *Chemical Research in Toxicology*, 15, 11, 1445-1450.

Hudson, N. J. (2012). Mitochondrial treason: a driver of pH decline rate in post-mortem muscle? *Animal Production Science*, 52, 1107-1110.

Kim, Y. H. B., Warner, R. D., & Rosenvold, K. (2014). Influence of high pre-rigor temperature and fast pH fall on muscle proteins and meat quality: a review. *Animal Production Science,* 54, 375-395. http://dx.doi.org/10.1071/AN13329.

Koohmaraie, M. (1996). Biochemical factors regulating the toughening and tenderization processes of meat. *Meat Science,* 43, 193-201.

Kotiadis, V. N., Duchen, M. R., & Osellame, L. D. (2014). Mitochondrial quality control and communications with the nucleus are important in maintaining mitochondrial function and cell health. *Biochimica et Biophysica Acta,* 1840, 1254-1265.

Lana, A., & Zolla, L. (2015). Apoptosis or autophagy, that is the question: Two ways for muscle sacrifice towards meat. *Trends in Food Science & Technology*, 46, 231-241.

Luciano, F. B., Anton, F. B., & Rosa, C. F. (2006). Biochemical aspects of meat tenderness: A brief review. *Archivos de Zootecnia*, 55, 1-8.

Materneh, S. K. (2017). Defining the role of mitochondria in fresh meat quality development. Dissertation.

Ramanathan, R., Mancini, R. A., Suman, S. P., & Cantino, M. E. (2012). Effects of 4-hydroxy-2-nonenal on beef heart mitochondrial ultrastructure, oxygen consumption, and metmyoglobin reduction. *Meat Science*, 90, 564-571.

Ramanathan, R., & Mancini, R. A. (2018). Role of mitochondria in beef color: A review. *Meat and Muscle Biology*, 2, 309-320.

Smith, G., Belk, K. E., Sofos, J. N., Tatum, J. D., Williams, S. N., Decker, E., Faustman, C., & Lopez-Bote, C. J. Economic implications of improved color stability in beef, John Wiley & Sons, Inc.: Hoboken, NJ: 2000; pp 397-426.

Troy, D. J., & Kerri, J. P. (2010). Consumer perception and the role of science in the meat industry. *Meat Science*, 86, 214-226.

Veronica, S., & Mammen, O. (2013). Role of mitochondria on muscle cell death and meat tenderization. *Endocrine, Metabolic & Immune Drug Discovery*, 7, 120-129.

Wu, W., Fu, Y., Therkildsen, M., Li, X.- M., & Dai, R.-T. (2015). Molecular understanding of meat quality through application of proteomics. *Food Reviews International,* 31, 13-28.

Xing, T., Gao, F., Tume, R. K., Zhou, G., & Xu, Xinglian. (2019). Stress effects on meat quality: A mechanistic perspective. *Comprehensive Reviews in Food Science and Food Safety,* 18, 380-401.

Xing, T., Gao, F., Tume, R. K., Zhou, G., & Xu, Xinglian. (2019). Stress effects on meat quality: A mechanistic perspective. *Comprehensive Reviews in Food Science and Food Safety,* 18, 380-401.

CHAPTER 2

LITERATURE REVIEW

Conversion of muscle into meat

The process of conversion of muscle into meat is a complex biochemical process in which all the mechanisms are interdependent and responsible for development of meat quality (Ahmed et al., 2006). As soon as an animal is slaughtered and bled, tissues are deprived of oxygen and live muscle starts to change into meat. Even though the anaerobic glycolysis predominates, mitochondria modify post mortem energy metabolism and may contribute to pH decline and transform muscle into meat (England et al., 2013). The lactic acid accumulation is termed as muscle acidification, which causes loss of water holding capacity (WHC) and calcium release leading to cross bridging between actin and myosin filaments (Paredi et al., 2012). The muscle cell has to undergo different catabolic mechanisms such as apoptosis, autophagy etc. The biochemical changes that follow conversion of muscle to meat favors the environment in which the oxidation of unsaturated fatty acids present in subcellular membrane is no longer strictly controlled (Grotta et al., 2017). The cells undergo apoptosis, which is also called programmed cell death. The lack of oxygen in muscle leads to cessation of electron transport chain. This makes shifting of ATP production to anaerobic glycolysis. Cellular pH decreases and this gradually decreases mitochondrial permeability. It can trigger mitochondrial matrix swelling and collapse of mitochondrial permeability. Large increase of Ca^{++} overload in mitochondrial matrix contributes to permeability transition that makes inner membrane to become more permeable to solutes (England et al., 2013). Due to the complex reactions in meat after its harvest, it becomes

extremely susceptible to deterioration. Two main problems of meat industry during processing and subsequent storage are lipid oxidation and deterioration due to microorganism growth (Dominguez et al., 2018).

Lipid oxidation in meat

Lipid peroxidation starts immediately after slaughter and during the post slaughter period (Min & Anh, 2005). It leads to production of a large number of compounds with different chemical functions. The factors affecting lipid peroxidation after slaughtering of animal are as follows: a) species b) diet c) anatomical location c) environmental temperature d) sex & age f) phospholipid composition and content and during processing other factors such as g) composition and freshness of raw meat components h) cooking i) chopping, deboning j) adding exogenous salts and antioxidants (Kanner, 1994).

Lipid oxidation also mediates protein dysfunction through its products such as HNE and ONE. The scope of these products ranges from food quality research to various aspects of health concerns of people (Gueraud et al., 2010). The adduct formation of these compounds with proteins is associated with cytotoxic effects such as inhibition of enzyme activity, mitochondrial dysfunction, impaired energy metabolisms, disruption of cell signaling etc. (Gueraud et al., 2010). Even though the lipid oxidation in meat has been widely investigated topic, many of the factors and mechanisms involved in this reaction have not yet been completely clear (Amaral, Silva, & Lannes, 2018). Fresh meat is normally stable from oxidative stress, which otherwise will lead to different forms of quality deterioration such as discoloration, development of rancidity, loss of nutrients and toxic oxidation of products. Generally, an anaerobic breakdown of

glycogen into lactate and H^+ is considered primary metabolism in post mortem muscle; however, there are increasing evidence that mitochondrial respiratory activity also affects postmortem metabolism and meat quality development (Ramos et al., 2020). Meat processing practices such as freezing, thawing, mincing, salt addition, cooking, pressure treatments are all conditions favorable for giving rise to oxidative stress in meat. When animal is slaughtered, different metabolic processes are stimulated due to ischemia and accumulation of sarcoplasmic calcium. The important changes are the contraction of myofibrils, the transition to glycolysis and the production of lactate. This condition favors acidification of the tissue accompanied by shrinkage of the interfibrillar space and denaturation of the proteins (Werner et al., 2010). Since, pH is a function of concentration of hydrogen ions (H^+); the accumulation of H^+ is the net effect of cellular reactions that utilize and reduce H^+ (Scheffler et al., 2015). The pH in post mortem muscle contributes to water-holding capacity, texture, and color also.

Post mortem muscle with high mitochondrial oxygen consumption rate results in more tender meat (Grabez et al., 2015). Moreover, the tenderness of meat is also attributed to higher prevalent of antioxidants that reduce reactive oxygen species that prolongs the oxygen removal by the electron transport system. Several enzymes such as electron transport system enzymes, Kreb's cycle, and glycolytic enzymes are abundantly present in tender meat (Grabez et al., 2015). Some of the potential mechanisms by which mitochondria play a significant role in preserving meat quality are: 1) by reducing oxygen partial pressure. This will darken meat color by maintaining myoglobin in a deoxy state. 2) By conversion of oxy- to deoxymyoglobin (Ramanathan et al., 2014).

Lipid oxidation also has positive effect in relation to enhancement of quality of meat especially during ageing of beef. There are different quality attributes, which depend on lipid oxidation for enhancement of flavor, tenderness, color & color stability (Kim et al., 2018). It has been reported that dry aging is a condition where meat is stored in a low temperature for an extended period of time in order to develop palatable characteristics. Lipid and protein oxidation are attributed to enhance flavor in dry aged meat without development of rancidity or off-flavor. Thus oxidation is also associated with favorable sensory perception in dry aged meat. In an experiment, the combination of freezing/ thawing and aging condition of pork indicated that aging prior to freezing/thawing increased lipid oxidation compared to freezing/thawing and aging of meat on eighth day of display (Kim et al., 2018).

HNE and ONE: Two major products of lipid peroxidation

It has been shown by different researchers that lipid peroxidation leads to formation of different compounds with potent biological activities. Among these compounds α, β – unsaturated aldehydes have received a lot of attention in the recent years. This is due to their ability to interact and modify proteins and lipids. These aldehydes are mainly derived from n-6 polyunsaturated fatty acids (PUFA) like linoleic acid, linolenic acid, and arachidonic acid containing a C=C double bond after oxidative damage of bio-membranes of the cells by reactive species (Chen et al., 1998).

The relationship between HNE, 2-thiobarbituric acid reactive substance (2-TBARS), and n-6 polyunsaturated fatty acids were investigated in pork stored at different temperatures (Sakai, et al., 1998). Their results suggested that significant correlation exists between HNE and TBARS

for the pork samples stored at 0^0C, -20^0C, and -80^0C. The n-6 PUFA content remained unchanged during these conditions. The samples did not show any significant changes during 7 days of storage, however during 7 through 12 days, the HNE content and TBARS values increased significantly due to consumption of antioxidant such as α – Tocopherol.

The two aldehydes HNE (4-Hydroxy-2- nonenal) and ONE (4-oxo-2-nonenal) can oxidize lipids and modify proteins and influence meat quality. The relationship between HNE, 2-thiobarbituric acid reactive substances and n-6 polyunsaturated fatty acids in pork was studied (Sakai et al., 1995). The result demonstrated that there is about 14.0-150 nmol/g of HNE content in beef (Sakai et al., 1995) There is a linear correlation between the content of HNE and the content of total ω-6 fatty acids, linoleic acid, or arachidonic acid in meat. ONE is a structural analogue of HNE and is more reactive than HNE towards protein nucleophiles.

Role of myoglobin in meat quality determination

Myoglobin is an essential component of a cell, and it has highly been studied for its association with meat color. Meat color is a single important quality attribute that is responsible for huge economic loss in the United States. The purchasing decision of customers depends on how the consumers see the product on retail display. They relate wholesomeness and freshness of meat to bright cherry-red color (Suman, 2013). In meats, there are other pigments such as hemoglobin and cytochrome, but they control color characteristic in very less extent. In a study conducted on pork has also demonstrated that myoglobin content was responsible for contributing its color. The range varied from 1.2 mg/g to 2.1 mg/g of the pork. The higher the myoglobin content, the more red was the pork (Kim et al., 2010).

Mainly four major chemical states of myoglobin govern meat color (Mancini & Hunt, 2005). Reduced (or deoxy) ferrous myoglobin is the purple pigment that can been seen on meat surfaces under vacuum. On exposure to air, myoglobin combines with oxygen to form (MbO_2) bright red-cherry color, which is considered fresh and attractive by consumers. With extended period of time the red color is lost and the meat becomes completely brown due to conversion of oxymyoglobin to metmyoglobin (Renerre, 1990). Carboxy myoglobin is formed when deoxymyoglobin reacts with carbon monoxide resulting meat very bright-red color, which is relatively stable. There is a current trend of packaging meat with low levels of carbon monoxide. The bound carbon monoxide is slowly released into the air on exposure to air after significant period of time (Mancini & Hunt, 2005). After slaughter, the rate of metmyoglobin tends to accumulate on the surface during storage these are governed by many intrinsic factors such as pH, muscle type, animal, breed, age, sex and diet. Similarly, extrinsic factors are temperature, oxygen availability, lighting, surface microbial growth, storage conditions (air, modified atmosphere, vacuum etc.). Discoloration during display conditions of retail packaged meat is another important factor favoring muscle pigment oxidation (oxymyoglobin to metmyoglobin) and lipid oxidation of membrane phospholipids (Troy & Kerry, 2010). Modified oxygen packaging has been practiced in order to prolong the fresh meat color and freshness by incorporating high oxygen concentration within retail packs, which promotes the development of oxymyoglobin (Troy & Kerry, 2010). Generally, the composition of gas in modified atmosphere packaging (MAP) used to contain meat consists of oxygen 80%, and carbon dioxide (20%). The higher oxygen percentage develops brighter red color of meat making it visually attractive to consumers (Kim et al., 2010). Even though the high-oxygen atmosphere packaging is considered favorable for developing brighter red meat color, there are evidences that degrade the quality of

meat by lowering tenderness, juiciness and increasing off-flavor (Kim et al., 2010). They found that steaks packed in high oxygen percentage showed more lipid oxidation and lower tenderness after 9 days of display than those packed and stored in vacuum. In their experiment, semimembranosus and adductor muscles were found to be more susceptible to lipid oxidation compared to longissimus lumborum when exposed to the same condition, whereas longissimus lumborum had higher tenderness compared to adductor and semimembranosus. Regarding surface redness, semimembranosus and adductor had greater decrease in color than longissimus lumborum after 9 days of display. This result suggested that physiologically different bovine muscles have different myoglobin oxidation stability under the high oxygen-MAP condition. The heme prosthetic group (the porphyrin) in the myoglobin serves to stabilize the configuration of the molecule and is responsible for oxygen binding and confers specific colors such as red and brown on the protein due to the cyclic conjugated tetrapyrole structure.

Lipid oxidation and myoglobin oxidation are related

Pigment and lipid oxidation are interrelated and is promoted by ferric hemes (Love & Pearson, 1970). Lipid oxidation is characterized by off flavor development, whereas myoglobin oxidation as a discoloration. These two reactions are linked to each other i.e. oxidation of one compound generate a product, which will lead to oxidation of another, and both of these are responsible for exacerbating fresh quality of meat (Faustman et al., 2010). It is noted that decreasing metmyoglobin reducing system along with depletion of co-factors and alteration of mitochondrial structure or functionality are responsible for rapid discoloration of post mortem aging meat. For instance, the study on dark cutting condition of beef carcass (McKeith et al., 2016) revealed that increasing dark cutting condition is associated with greater oxidative

metabolism with less efficient mitochondria resulting in depletion of glycogen during stress. Hence, their data suggested that dark cutting severity is increased with increasing oxygen consumption and reducing ability coincided with greater myoglobin concentration and greater number of less efficient mitochondria. Similarly, in another experiment, increased oxygen consumption of mitochondria was found to affect myoglobin redox state, and this effect was associated with darkening effect of lactate enhanced beef (Ramanathan et al., 2013).

Few works have been done on the effect of secondary aldehydes on meat quality. The hydroperoxide breakdowns of lipids mainly consist of aldehydes, hexanal, HNE, ONE and acrolein (Gueraud et al., 2010). The researchers have demonstrated that HNE adduct formation with enzymes have reduced their activities. In a study, HNE was shown to bind with lactate dehydrogenase; an important enzyme responsible for NADH mediated metmyoglobin reduction activity. The covalent binding of HNE with cysteine and histidine residues of LDH decreased beef color (Ramanathan et al., 2014). Similarly, protein responsible for meat color development has been shown to be modified by HNE. The histidine residue of bovine myoglobin is covalently modified by HNE to oxidize oxymyoglobin (Alderton et al., 2003).

Bovine heart mitochondria: characteristics & nature

Mitochondria are key organelles to synthesize most of the cell's ATP via the oxidative phosphorylation system (Fernandez-Vizzara et al., 2010). Fleischer et al. (1967) studied about the composition of mitochondria from bovine heart, liver and kidney. They found that heart mitochondria contained highest amount of lipid content, and liver mitochondria the lowest. Lipids are one of the essential components of mitochondria for function. Heart mitochondria also

have higher amount of cytochrome system and a greater ATPase activity. The higher respiratory activity of isolated mitochondria is due to their difference in physiological characteristic (Holton et al., 1957). Liver mitochondria from rats are best suited for the studies related to biochemical assays on the pharmacological effects of different drugs or on effects of specific diets on mitochondrial membrane composition (Palloti & Lenaz, 2007). Mitochondrial research in the past as well as in the current days are performed mainly from the mitochondria prepared from rat liver, rat heart or beef heart (Pallotti & Lenaz, 2007). Stoner & Sirak (1969) studied about the properties of mitochondria isolated from rat liver and bovine heart mitochondria. They found that liver and heart mitochondria do not differ in size but the amount of inner membrane and the matrix materials content. Due to higher content of inner membrane and matrix material heart mitochondria are relatively more susceptible to inner compartment swelling. The appearance of inner mitochondrial membrane morphological appearance depends on osmotic changes created by experimental procedure and metabolism under steady state condition (Weber & Blair, 1969). There are some advantages of mitochondria prepared from heart muscle over those from other mammalian tissues because of their stability with respect to oxidation and phosphorylation for up to a week when stored at 4^0C, and for up to a year at -20^0C (Pallotti & Lenaz, 2007). Generally, fresh tissues are preferred for the isolation of mitochondria for most biochemical assays and also when further modifications of mitochondria are required in certain assays (Pallotti & Lenaz, 2007).

Mitochondria & secondary aldehydes interaction influence meat quality

While many researches were focused on evaluating the role of secondary aldehydes in pathophysiology of human diseases, few studies tried to show the ability of HNE & ONE in

modifying subcellular structures such as mitochondria in muscle foods. In a study using beef heart mitochondria, it was found that HNE was effective in modifying ultrastructure, oxygen consumption, and metmyoglobin reduction of beef heart mitochondria at pH 5.6 and 7.4 (Ramanathan et al., 2012). Their result has demonstrated that in addition to covalent binding with myoglobin, HNE influenced beef color stability by interacting with mitochondria. Similarly, Gonzalez & Mohan (2016) showed that ONE was a potent reactive species than HNE in modifying different aspects of meat qualities. Mitochondria contain their own genome and can control many cellular functions e.g. energy production, heat generation, apoptosis, calcium homeostasis etc. Major ATPs produced in cells are generated by mitochondria via oxidative phosphorylation, and mitochondrial oxidative phosphorylation is coupled incompletely due to proton leakage across the inner membrane, and the efficiency can vary according to cellular energy demand (Masaaki et al., 2011). The majority of ROS is produced during mitochondrial respiration, and out of total molecular oxygen consumed during normal respiration, 1 % - 2% are converted to superoxide radicals (Orrenius, Vladimir, & Zhivotovsky, 2007). Mitochondria in post mortem muscle continue to remain active and can influence beef color by oxygen consumption and metmyoglobin reduction (Ramanathan & Mancini, 2018). Increased storage time has several effects on mitochondria such as decreased oxygen consumption rate, swelling and breakage of morphological structure as well as decreased pH and decreased oxygen consumption rate (Tang et al., 2005). They are producer as well as targets of reactive oxygen species (ROS) under normal physiological and pathological conditions (Reinheckel et al., 1998). The study (Galam et al., 2015) showed direct interaction of HNE with mitochondrial respiratory chain using rotenone. Furthermore, 25 μM of HNE was sufficient to induce reduction of total thioredoxin (Trx) activity. Their study confirmed that HNE had a potential to induce cellular and

mitochondrial dysfunction with inactivation of endogenous antioxidant response. They constant protection from the damage of toxic effects. This protection is due to the counter effect of various antioxidants and multiple enzymatic defenses against them (Orrenius, Vladimir, & Zhivotovsky, 2007). Mitochondrial proteins are vulnerable to modification by electrophiles at alkaline pH environment. Thus, thiols are more ionized at higher pH values and they have cysteine residues. The ionized form of cysteine is more reactive. The two-thiol antioxidant systems are present in mitochondria and depend mainly on GSH and thioredoxin -2 (Roede & Jones, 2010).

Mitochondrial functional integrity is lost with the change of permeability of mitochondrial membrane and consequently decreases mitochondrial respiratory activity. This is associated with general decreased antioxidant defense system efficiency due to reduced enzyme activity level (Meng et al., 2007). The two membranes of mitochondria differ in their permeability characteristics; the outer membrane is permeable to all low molecular weight compounds, whereas the inner membrane is permeable through specific transporters only (Lemasters, 2007). Mitochondrial membrane permeability is a calcium-dependent increase in the permeability of the mitochondrial membrane, which leads to increased potential, mitochondrial swelling, and rupture of the outer mitochondrial membrane (Tsujimoto, Nakagawa, & Shimizu, 2006). Prolonged opening of mitochondrial permeability transition pore leads to dissipation of inner membrane potential, cessation of ATP synthesis, bioenergetics crisis, and cell death. All these phenomenon are characterized as a primary characteristic of mitochondrial disorder (Sileikyte & Forte, 2019). Mitochondrial lipids have following functions: 1) respiratory activity (energy production), 2) permeability and transport processes across the membrane 3)

mitochondrial protein synthesis 4) maintenance of membrane structure 5) the activity of mitochondrial enzymes *in vivo* and *in vitro;* 6) stability and osmotic behavior of mitochondria (Daum, 1985).

Preservation of fresh meat

There are a lot of works done on extending shelf life of meat. As meat is highly perishable food commodity due to its chemical composition, meat researchers have developed a number of new preservation techniques to extend its shelf-life. Examples of some of the modern technologies are high hydrostatic pressure (HHP), modified atmosphere packaging (MAP) & active packaging (AP), natural antimicrobial compounds and bio-preservation (Zhou, Xu, & Liu, 2010). Different chemical compounds have been tried by different researchers to preserve meat quality for a long time. Nicotinamide (Nam) in combination with ascorbic acid reduced metmyoglobin formation and reduced oxygen consumption in ground beef during refrigeration or freezer storage (Kendrick & Watts, 1969). Meat spoilage due to growth of microorganisms has been prevented with the use of essential oils. The chemical compound present in plant derived essential oils are carvacrol, eugenol, thymol etc (Jayasena & Jo, 2013). Since lipid oxidation are best inhibited by antioxidants and chelators, several researchers have tried polyphosphate, propyl gallate and butylated hydroxyanisole to preserve meat free from lipid oxidation (Love & Pearson, 1970). Although natural antioxidants are in most demand today for increasing oxidative stability of meat, they might be associated with negative effects such as toxins and anti-nutritional factors (Amaral, Silva, & Lannes, 2018). Antioxidants from herbal extracts have seemed to be studied well. The combined application of antioxidants with modified atmosphere packaging of beef patties showed that the oxidation of lipid does not seem to follow

the same pattern as protein oxidation. A high level of oxygen in the packaging atmosphere favored both lipid and protein oxidation during storage of beef patties at 4^0C for 6 days. However, the antioxidants such as rosemary extract and ascorbate/citrate combination inhibited lipid oxidation but not the protein oxidation (Lund, Hviid, & Skibsted, 2007).

Future Research

This review has compiled all the major works of lipid oxidation in meat quality aspects. Major findings of current research work related to this topic have been summarized in the table 1. Lipid oxidation of meat, its prevention and impact has been subject of interest of past researchers as early as 19th century. Every component of meat system has been studied well by the past researchers. This work has sought to understand the endogenous mechanism that brings about quality degradation of postmortem muscle. The consumers always demand healthy foods with high eating quality. The future challenge for the meat industry would be to develop low fat-products with healthy fatty acid composition.

References

Ahmed, O., Herrera-Mendez C. H., Coulis, G., Becila, S., Boudjellal, A., Aubry, L., & Sentandreu, M. A. (2006). Revisiting the conversion of muscle into meat and the underlying mechanisms. *Meat Science*, 74, 44-58.

Alderton, A. L., Faustman C., Liebler D. C., & Hill, D.W. (2003). Induction of redox instability of bovine myoglobin by adduction with 4-hydroxy-2-nonenal. *Biochemistry*, 42, 4398-4305.

Amaral, A. B., Silva M. V., & Lannes. S. C. S. (2018). Lipid oxidation in meat: mechanisms and protective factors – a review. *Food Science and Technology*: 38, 1-15.

Campo, M.M., Nute, G.R., Hughes, S.I., Enser, M., Wood, J.D., & Richardson, R.I. (2006). Flavor perception of oxidation in beef." *Meat Science*, 72, 303-311.

Cheah, K.S. & Cheah, A.M. (1971). Post-mortem changes in structure and function of ox muscle mitochondria. 1. Electron microscopic and polarographic investigation. *Journal of Bioenergetics*, 2, 85-92.

Chen, J., Schenker, S., Frosto, T. A., & Henderson, G.I. (1998). Inhibition of cytochrome c oxidase activity by 4-hydroxynonenal (HNE) Role of HNE adduct formation with the enzyme subunits. *Biochimica et Biophysica Acta*, 1380, 336-344.

Daum, G. (1985). Lipids of mitochondria. *Biochimica et. Biophysica Acta*, 822, 1-42.

Dominguez, R., Barba, F. J., Gomez, B., Putnik, P., Kovacevic, D. B., Pateiro, M., Santos, E. M., & Lorenzo, J. M. (2018). Active packaging films with natural antioxidants to be used in meat industry: A review. *Food Research International*, 113, 93-1010.

England, E. M., Matarneh, S. K., Mitacek, R. M., Abraham A., Ramanathan, R., Jordan C. W., Shi H., Scheffler, T. L., Oliver E. M., Helm E. T., & Gerrard D. E. (2018). Presence of oxygen and mitochondria in skeletal muscle early postmortem. *Meat* Science, 139, 97-106.

England, E.M., Scheffler, T. L., Kasten S. C., Matarneh S. K., & Gerrard D. E. (2013). Exploring the unknowns involved in the transformation of muscle to meat. *Meat Science,* 95, 837-843.

Faustman, C., Sun Q., Mancini R., & Suman S. P. (2010). Myoglobin and lipid oxidation interaction: mechanistic bases and control." *Meat Science,* 86, 86-94.

Fernandez-Vizzarra, E., Ferrin, G., Perez-Martos, A., Fernandez-Silva, P., Zeivani, M., & Enriquez, J. A. (2010). Isolation of mitochondria for biogenetical studies: An update. *Mitochondrion,* 10, 253-22.

Fleischer, S., Rouser, G., Fleischer, B., Casu, A., & Kritchevsky, G. (1967). Lipid composition of mitochondria from bovine heart, liver, and kidney. *Journal of Lipid Research,* 8, 170-180.

Galam, L., Failila, A., Soundararajan, R., Lockey, R. F., & Kolliputi, N. (2015). 4-Hydroxynonenal regulates mitochondrial function in human small airway epithelial cells. *Oncotarget,* 6, 41508-41521.

Gasc, N., Tache, S., Rathahao, E., Bertrand-Michel, J., Roques, V., & Gueraud, F. (2007). 4-Hydroxynonenal in foodstuffs: heme concentration, fatty acid composition and freeze-drying are determining factor. *Communication in Free Radical Research,* 12, 1-2.

Gonzalez, S. A., & Mohan, A. (2016). The effects of lipid peroxidation products 4-HNE and 4-ONE on lactate dehydrogenase activity, myoglobin redox stability, and mitochondria structural integrity. MS Thesis.

Grabez, V., M. Kathri, M., Phung, V., Moe, K. M., Slinde, E., Skaugen, M., Saarem, K., & Egelandsdal, B. (2015). Protein expression and oxygen consumption rate of early postmortem mitochondria related to meat tenderness. *Journal of Animal Science,* 93, 1967-1979.

Grotta, L., Catellani, F., Palazzo, F., Haouet, M. N., & Martino, G. (2017). Treatment optimization and sample preparation for the evaluation of lipid oxidation in various meats through TBARs assays before analysis. *Food Analytical Methods,* 10, 1870-1880.

Gueraud F., Atalay M., Bresgen, N., Cipak, Eckl, P.M., Huc, L., Jouanin, I., Siems, W., Uchida, K. (2010). Chemistry and biochemistry of lipid peroxidation products. *Free Radical Research,* 44, 1098 – 1124.

Holton, F. A., Hulsmann, W.C., Myers, D. K., and Slater, E. C. (1957). A comparison of the properties of mitochondria isolated from liver and heart. *The Biochemical Journal 67,* 579-594.

Huff-Lonergagn E., & Lonergan, S.M. (2005). Mechanisms of water-holding capacity of meat: The role of postmortem biochemical and structural changes. *Meat Science,* 71, 194-204.

Isom, A. L., Barnes, S., Wilson, L., Kirk, M., Coward, L., & Darley-Usmar, V. (2004). Modification of cytochrome c by 4-Hydroxy-2-nonenal: evidence for histidine, lysine and arginine-aldehyde adduct. *Journal of American Society of Mass Spectrom*, 15, 1136-1147.

Jayasena, D. D. & Jo, C. (2013). Essential oils as potential antimicrobial agents in meat and meat products: A review. *Trends in Food Science & Technology*, 34, 96-108.

Kanner, J. (1994). Oxidative processes in meat and meat products: quality implications. *Meat Science*, 36,169-189.

Kendrick, J. L. & Watts, B. M. (1969). Nicotinamide and nicotinic acid in color preservation of fresh meat. *Journal of Food Science*, 34, 292-294.

Kim, G-D., Jeong, J., Hur, S-J., Yang, H.S., Jeon, J-T., & Joo, S-T. (2010). The relationship between meat color (CIE L* and a*), myoglobin content, and their influence on muscle fiber characteristics and pork quality. *Food Science of Animal Resources*, 30, 626-633.

Kim, H-W., Kim, J-H., Seo, J-K., Setyabrata, D., & Kim, Y.H.B. (2018). Effects of aging/freezing sequence and freezing rate on meat quality and oxidative stability of pork loins. *Meat Science*, 139, 162-170.

Kim, Y. H. B., Ma, D., Setyabrata, D., Farouk, M. M., Lonergan, S. M., Huff-Lonergan, E., Hunt, M. C. (2018). Understanding postmortem biochemical processes and post-harvest aging factors to develop novel smart-aging strategies *Meat Science,* 144, 74-90.

Kim, Y.H., Huff-Lonergan, E., Sebranek, J.G., Lonergan, S. M. (2010). High-oxygen modified atmosphere packaging system induces lipid and myoglobin oxidation and protein polymerization. *Meat Science,* 85, 759-767.

Lee, J.H., Min, B.R., & Lemma B.B. (2017). Quality characteristics of goat meat as influenced by condensed tannins-containing pine bark. *Small Ruminant Research* 146, 28-32.

Lemasters, J.J. (2007). Modulation of mitochondrial membrane permeability in pathogenesis, autophagy and control of metabolism. *Journal of Gastroenterology and Hepatology,* 22, S31-S37.

Li, P., Wang, T., Mao, Y., Zhang, Y., Niu, L., Liang, R., Zhu, L., Luo, X. (2014). Effect of ultimate pH on postmortem myofibrillar protein degradation and meat quality characteristics of Chinese yellow crossbreed cattle. *The Scientific World Journa*l, 8, 1-8.

Love, J. D. & Pearson, A. M. (1970). Lipid oxidation in meat and meat products - A review. *Journal of The American Oil Chemist's Society,* 48, 547-549.

Lund, M. N., Hviid, M. S., & Skibsted, L. H. (2007). The combined effect of antioxidants and modified atmosphere packaging on protein and lipid oxidation in beef patties during chill storage. *Meat Science*, 76, 226-233.

Mancini, R. A., & Hunt, M.C. (2005). Current research in meat color. *Meat Science*, 71, 100-121.

Masaaki, T., Kikusato, M., Kawabata, Y., Azad, M.A.K., Inui E., & Amo, T. (2011). Meat-type chickens have a higher efficiency of mitochondrial oxidative phosphorylation than laying-type chickens. *Comparative Biochemistry and Physiology*, 159, 75-81.

McKeith, R. O., King, D. A., Grayson, A. L., Shackelford, S. D., Gehring, K. B., Savell, J. W., & Wheeler, T. L. (2016). Mitochondrial abundance and efficiency contribute to lean color of dark cutting beef. *Meat Science*, 116, 165-173.

Meng, Q., Wong, Y.T., Chen, J., & Ruan, R. (2007). Age-related changes in mitochondrial function and antioxidative enzyme activity in fischer 344 rats. *Mechanisms of Ageing and Development*, 128, 286-292.

Min, B., & Anh, D. U. (2005). Mechanism of lipid peroxidation in meat and meat products – a review. *Food Science and Biotechnology*, 14, 152-163.

Orrenius, S., Vladimir, G., & Zhivotovsky, B. (2007). Mitochondrial oxidative stress: implications for cell death. *The Annual Review of Pharmacology and Toxicology*, 47, 143-183.

Pallotti, F. & Lenaz, G. (2007). Isolation and subfractionations of mitochondria from animal cells and tissue culture lines. *Methods in Cell Biology*, 80, 3-44.

Papuc, C., Goran, V. G., Predescu C. N., & Nicorescu. V. (2016). Mechanisms of oxidative processes in meat and toxicity induced by postprandial degradation products: a review. *Comprehensive Reviews in Food Science and Food Safety*, 16, 96-123.

Paredi, G., Raboni, S., Bendixen, E., Almeida, A. M., & Mozzarelli, A. (2012). "Muscle to meat" molecular events and technological transformations: The proteomic insight. *Journal of Proteomics*, 75, 4275-4289.

Picklo, M. J., Amarnath, V., McIntyre, J.O., Graham, D.G., & Montine, T.J. (1999). 4-Hydrox-2(E)-nonenal inhibits CNS mitochondrial respiration at multiple sites. *Journal of Neurochemistry*, 72, 1617-1624.

Poli, G., & Schaur, R.J. (2000). 4-Hydroxynonenal in the pathomechansms of oxidative stress. *IUBMB Life*, 50, 315-321.

Purchas, R. (2004). Tenderness of beef *M. longissimus lumborum* at normal and intermediate ultimate pH before and after a period of ageing. *New Zealand Journal of Agricultural Research*, 47, 299-305.

Ramanathan, R., Mancini, R. A., Suman, S. P., & Cantino M. E. (2012). Effects of 4-hydroxy-2-nonenal on beef heart mitochondrial ultrastructure, oxygen consumption, and metmyoglobin reduction. *Meat Science*, 90, 564-571.

Ramanathan, R., Mancini, R. A., Joseph, P., & Suman, S. P. (2013). Bovine mitochondrial oxygen consumption effects on oxymyoglobin in the presence of lactate as a substrate for respiration. *Meat Science*, 93, 893-897.

Ramanathan, R., Mancini, R. A., Suman, S.P., Beach, C. M. (2014). Covalent binding of 4-hydroxy-2-nonenal to lactate dehydrogenase decreases NADH formation and metmyoglobin reducing activity. *Journal of Agricultural and Food Chemistry*, 62, 2112-2117.

Ramanathan, R., & Mancini, R.A. (2018). Role of mitochondria in beef color: a review. *Meat and Muscle Biology*, 2, 309-320.

Ramos, P.M., Li, C., Elzo, M. A., Wohlgemuth, S.E., & Scheffler, T.L. (2020). Mitochondrial oxygen consumption in early postmortem permeabilized skeletal muscle fibers is influenced by cattle breed. *Journal of Animal Science* 98: 1-10.

Reinheckel, T., Noack, H., Lorenz, S., Wiswedel, I., & Augustin, W. (1998). Comparison of protein oxidation and aldehyde formation during oxidative stress in isolated mitochondria. *Free Radical Research*, 29, 297-305.

Renerre, M. (1990). Review: factors involved in the discoloration of beef meat. *International Journal of Food Science and Technology,* 25, 613-630.

Roede, J. R., & Jones D. P. (2010). Reactive species and mitochondrial dysfunction: mechanistic significance of 4-Hydroxynonenal. *Environmental and Molecular Mutagenesis,* 51, 380-390.

Sakai, T., Yamauchi, K., Kuwazuru, S., & Gotoh, N. (1998). Relationships between 4-Hydroxy-2-nonenal, 2- thiobarbituric acid reactive substances and n-6 polyunsaturated fatty acids in refrigerated and frozen pork. *Bioscience Biotechnology, and Biochemistry,* 62, 2028-2029.

Scheffler, T.L., Matarneh, S. K., England, E. M., & Gerrard, D. E. (2015). Mitochondriainfluence postmortem metabolism and pH in an *in vitro* model. *Meat Science,* 110, 118-125.

Sierra, V., & Olivan, M. (2013). Role of mitochondrion on muscle cell death and meat tenderization. *Recent Patents on Endocrine, Metabolic & immune Drug Discovery,* 7, 120-129.

Sileikyte, J., & Forte M. (2019). The mitochondrial permeability transition in mitochondrial disorders. *Oxidative Medicine and Cellular Longevity,* 19, 1-11.

Silva, J.A., Patarata, L., & Martins, C. (1999). Influence of ultimate pH on bovine meat tenderness during ageing. *Meat Science,* 52, 453-459.

Spickett C. M., & Pitt, A. R. (2019). Modification of proteins by reactive lipid oxidation products and biochemical effects of lipoxidation. *Essays in Biochemistry,* 64, 19-31.

Stoner, C. D. & Sirak, H. D. (1969). Osmotically-induced alterations in volume and ultrastructure of mitochondria isolated from rat liver and bovine heart. *The Journal of Cell Biology*, 43, 521-538.

Suman, S. P., & Joseph P. (2013). Myoglobin chemistry and meat color. *The Annual Review of Food Science and Technology*, 4, 79-99.

Tang, J., Faustman, C., Hoagland, T.A., Mancini, R.A., Seyfert, M. & Hunt, M.C. (2005). Postmortem oxygen consumption by mitochondria and its effects on myoglobin form and stability. *Journal of Agricultural and Food Chemistry*, 53, 1223-1230.

Troy D. J., & Kerry J. P. (2010). Consumer perception and the role of science in the meat industry. *Meat Science*, 86, 214-226.

Tsujimoto, Y., Nakagawa, T., & Shimizu, S. (2006). Mitochondrial membrane permeability transition and cell death. *Biochimica et Biophysica Acta (BBA)- Bioenergetics*, 1757, 1297-1300.

Weber, N. E., & Blair, P. V. (1969). Ultrastructural studies of beef heart mitochondria. I. Effects of adenosine diphosphate on mitochondrial morphology. *Biochemical And Biophyscial Research Communications 36*, 987-993.

Werner, C., Natter, R., Schellander, K., & Wicke, M. (2010). Mitochondrial respiratory activity in porcine longissimus muscle fibers of different pig genetics in relation to their meat quality. *Meat Science,* 85, 127-133.

Zhou, G. H., Xu, X. L., & Liu, Y. (2010). Preservation technologies for fresh meat – A review. *Meat Science,* 86, 119-28.

Table. 1. Recent findings of lipid peroxidation, mitochondrial integrity and meat quality.

Author and Year	Findings
Ramanathan et al. (2012)	In addition to covalent binding with myoglobin, HNE may influence beef color stability by interacting with mitochondria.
Ramanathan et al. (2013)	Increased oxygen consumption can influence myoglobin redox state and this might effect in the darkening of lactate-enhanced beef.
Chen et al. (1998)	Mitochondrial GSH protects mitochondria from the deleterious effects of HNE.
Doorn et al. (2006)	4-ONE is a potent irreversible inhibitor of mitochondrial enzymes.
Tang et al. (2005)	Mitochondrial respiration facilitates a shift in myoglobin from oxymyoglobin to deooxymyoglobin or metmyoglobin, and this is dependent on pH, oxygen availability, and mitochondrial density.
Meng et al. (2007)	Dysfunction of mitochondria is associated with decrease of antioxidant enzyme efficiency.
Ramanathan et al. (2009)	Understanding mitochondrial function and myoglobin reduction help pre- and post-harvest strategies to maximize economic benefits associated with improving the appearance of muscle foods.
Ramanathan and Mancini (2008)	Postmortem oxygen consumption and metmyoglobin reduction are essential in beef color development and stability
Picklo et al. (1999)	Reactive oxygen species cause mitochondrial damage directly or indirectly through secondary aldehydes such as HNE.
Chen and Yu (1994)	Reactive lipid peroxidation products, HNE can play important role in decreasing mitochondrial membrane fluidity
Mancini and Ramanathan (2014)	Decreased mitochondrial oxygen consumption associated with longer aging times increases color stability in beef steak.
Lin et al. (2005)	ONE is 6-31 times more reactive than HNE towards proteins such as β-lactaglobulin and ribonuclease A.
Saito et al. (2011)	Pro-oxidative effect of ONE is due to its ability to reduce transition metals and to chelate the reduced form of metal ions

CHAPTER 3

EFFECTS OF 4-HYDROXY-2-NONENAL AND 4-OXO-2-NONENAL ON BIOCHEMICAL PROPERTIES OF BOVINE HEART MITOCHONDRIA

Kafle, D., Mohan, A., Singh, R. K., & Hung, Y- C. To be submitted to Food Chemistry

Abstract

The effects of 4-oxo-2-nonenal (ONE) and 4-hydroxy-2-nonenal (HNE) on mitochondria isolated from bovine heart were assessed using different assays such as antioxidant activity, oxygen consumption, ultrastructure, metmyoglobin reduction, and membrane permeability. Incubation of bovine heart mitochondria with ONE exhibited a drastic decrease in oxygen consumption compared with HNE and control (without ONE and HNE) ($P < 0.05$). The hydrogen peroxide assay (H_2O_2), ferric reducing antioxidant properties (FRAP) and 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) assays showed that ONE was more potent inhibitor ($P < 0.05$) of endogenous antioxidant system of mitochondria compared to HNE. Furthermore, electron microscopy revealed that ONE incubation resulted in more severe swollen ($P < 0.05$) mitochondria compared with HNE and had increased membrane permeability ($P < 0.05$) at both pH 5.6 and 7.4, compared with controls. However, mitochondria incubated with ONE at pH 5.6 had both decreased oxygen consumption rate and volume ($P < 0.05$). ONE interaction with isolated bovine heart mitochondria at both pH 5.6 and 7.4 resulted in lower metmyoglobin reduction and NADH-dependent metmyoglobin reductase activity compared with HNE and control ($P < 0.05$).

Keywords: HNE, ONE, Ultrastructure, Oxygen consumption rate, Beef heart mitochondria.

Introduction

Meat and meat products during the cold management chain undergo oxidative process that in consequence initiates lipid oxidation, color deterioration, and undesirable flavor development. Deterioration of meat color and development of rancidity and undesirable flavor in meat influences consumer appeal and purchasing decisions. The nutrient composition,

processing, intrinsic and extrinsic factors including storage conditions influences the biochemical composition and oxidative process in meat. The process of lipid oxidation reactions generates toxic chemical species that accelerate deteriorative reactions in meat.

Meat is a complex food matrix and biochemical interactions of lipid oxidation process are likely to generate primary and secondary oxidation products including aldehydes, ketones and epoxides (Sakai et al., 1995; Sakai et al., 1998). HNE and ONE are secondary products of lipid peroxidation. Both of them are derived from polyunsaturated fatty acids (PUFA) like linoleic acid, linolenic acid and arachidonic acid (Poli & Schaur, 2000), and ONE is the keto-form of HNE (Zhu & Sayre, 2007).

HNE is a unique compound with three functional groups, the C=C double bond, the –OH group and the –CHO (aldehyde group), which determine its reactivity with many molecular species especially proteins (Chen & Yu, 1994). The reactivity of these aldehydes towards the amino acids is in the order Cys >> His > Lys > (> Arg for 4 ONE) (Doorn & Peterson, 2002). HNE can modify the phosphatidylethanolamine, glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, glutathione S- transferase, glutathione reductase, and aldose reductase, $Na^{+}K^{+}$ ATPase, GLUT 3 (Uchida, 2003); aminopeptidase (Lee et al., 2010), whereas ONE can modify proteins ribonuclease A and beta-lactaglobulin (Zhang et al., 2003).

Approximately, 50% of mitochondrial membrane fatty acids are unsaturated (Gutierrez, Reboredo, & Catala, 2002). These membranes are sites of production of secondary aldehydes e.g. HNE and ONE due to lipid peroxidation, and these products can diffuse out of the membranes to modify nucleic acid and proteins (Esterbauer, Schaur, & Zollner, 1991). For instance, Oxidation decomposition of n-6 polyunsaturated fatty acids result in lipid

hydroperoxides leading to the formation of HNE and ONE that are protein reactive and cytotoxic (Saito, Iwamoto & Yamauchi, 2011). HNE when incubated with mitochondria isolated from rat brain can decrease membrane fluidity by directly interacting with membrane phospholipids, which can be confirmed by the generation of fluorescent complex (Chen & Yu, 1994). This can affect mitochondrial morphology and function (Humphries, Yoo, & Szweda, 1998). HNE inhibits complex I-linked and complex II-linked state 3 respirations in rat brain (Picklo et al., 1999).

Mitochondrial matrix has a high concentration of antioxidant defense enzymes, which have a capacity to serve as a cellular H_2O_2 stabilizing device (Mailloux, 2018) including the integrated thiol system, which protect mitochondria from the oxidative damages (Murphy, 2012). Research in human medicine has shown 4-ONE to be highly cytotoxic and more potent neurotoxic than HNE (Lin et al., 2005). Most of the earlier works related to 4-ONE were emphasized in understanding its role as a mediator in human pathophysiology. Lee and Blair (2000) showed that 4-ONE is a significant product of the breakdown of lipid hydroperoxides. Although, there are numerous studies available on HNE effects on mitochondria structure and function with respect to meat color stability, and their results have shown that it can bind covalently with histidine residue within myoglobin and can decrease color stability (Alderton et al., 2003).

Practically, no work has been done to assess the extent of mitochondrial structural and functional damage due to the effect of ONE on meat. Our hypothesis is that the reactivity of HNE and ONE towards proteins can influence structure, function as well as defensive capacity of mitochondria. Therefore, the objectives of the present study were to compare the effects of HNE and ONE on structure, function and antioxidative capacity of bovine heart mitochondria. In

this study, electron microscopy and permeability were used to characterize structural changes, whereas oxygen consumption was used to characterize mitochondrial function. Similarly, radical scavenging assay of mitochondrial extract was performed to understand the effect on detoxification capacity of endogenous defense system to these aldehydes i.e. HNE and ONE.

Materials and Methods

Materials and Chemicals

Following materials and chemicals were purchased for the experiment. Fresh beef heart from local slaughterhouse, bovine serum albumin (BSA), sucrose, tris hydroxymethyl aminomethane hydroxchloride (Tris-HCl), potassium phosphate monobasic (KH2PO4), potassium phosphate dibasic (K2HPO4), ethylene glycol-bis (β - aminoethyl ether) – N,N,N',N'-tetracacetic aci (EGTA), N-[2-hydroxyethyl], piperazine-N'-[2-ethanesulfonic acid] , adenosine 5'-diphosphate (ADP), proteinase K, $CaCl_2$, Succinic acid, EDTA and Bicinchoninic Acid Protein Assay Kit were purchased from Sigma Chemical Co. (St, Louis, MO); sodium succinate was purchased from Fisher Scientific (Fair Lawn, NJ). HNE and ONE were purchased from Cayman Chemical Co. (Ann Arbor, MI). Reagents such as glutaraldehyde, osmium tetroxide, for Electron Microscopy were also purchased from Sigma Chemical Co. (St. Louis, MO). The ABTS diammonium salt, horseradish peroxidase ($\geq$ 250 units /mg), phosphate buffer solution (PBS) and ferrous sulfate heptahydrate ($FeSO4.7H_2O$) were purchased from Sigma-Aldrich (St. Louis, MO), H_2O_2 solution (30% w/w solution), and 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ) were purchased from ACROS Organic (Morris, NJ, USA). Ferric chloride anhydrous ($FeCl_3$) was purchased from Fisher Chemical (Fair Lawn, NJ, USA).

Isolation of mitochondria

Mitochondria were isolated from bovine heart according to the briefly modified method by Ramanathan et al. (2012). A hundred gram of heart tissue devoid of connective tissues and fat was obtained and finely minced with stainless steel scissors. Minced heart muscle was washed twice with 250 mM sucrose and suspended in 200 mL of mitochondrial isolation buffer (1 mM EGTA, 10 mM HEPES, 250 mM sucrose, pH 7.4). This suspension was stirred and treated with 0.5-mg /g protease. The bacterial protease was allowed to react for 20 min while maintaining the pH at 7.4. After 20 minutes, 800 mL of suspension buffer was added. The suspension was homogenized by three passes with a Kontes Duall tissue grinder by Kimbal Chase (Vineland, NJ, USA), followed by three passes with a Wheaton Potter-Elvehjem tissue grinder (Milville, NJ, USA). The homogenate was centrifuged at 4^0C (1200 × g) for 20 min with a Sorval RC-6-Plus centrifuge (Thermo Electron Corporation, Waltham, MA, USA). Resulting supernatant was filtered through double-layered cheese cloth and centrifuged it again at 26,000 × g for 20 min at 4^0C. The recovered mitochondrial pellet was washed twice with isolation buffer with further centrifugation and suspended in mitochondrial suspension buffer (250 mM sucrose, 10 mM HEPES, pH 7.4).

Isolation of bovine myoglobin and purification

Bovine myoglobin isolation was performed based on a briefly modified method of Ramanathan et al., (2012). Accordingly, bovine cardiac muscle was rinsed with 2 mM phosphate buffer (pH 7.4) and excess fat and connective tissue was removed. Cardiac muscle was homogenized in buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0, 4 °C) and centrifuged for 15 min at 5000 *g*. The supernatant was saturated with 70% ammonium sulfate and further

centrifuged at 18,000 *g* for 15 min. The resulting supernatant was saturated with 100% ammonium sulfate and further centrifuged at 20,000 *g* for 30 min. The precipitate was collected and resuspended in homogenization buffer and dialyzed (3 volumes) against 10 mM Tris–HCl, 1 mM EDTA, at pH 8.0, 4 °C for 24 h. Myoglobin was separated from hemoglobin using a Sephacryl 200-HR gel filtration column (2.5×100 cm). The elution buffer contained 5 mM Tris–HCl, 1 mM EDTA at pH 8.0 and the flow rate was 60 mL/h. Bovine myoglobin was stored at -80 °C until further use.

Effect of HNE and ONE on morphology of mitochondria

The method described in a previous study (Ramanathan et al., 2012) was used to compare the effect of HNE and ONE on morphology of mitochondria. Isolated mitochondria were incubated with either ethanol or HNE or ONE at pH 7.4 (120 mM KCl, 5 mM KH2PO4, 30 mM tris –HCl) or 5.6 (120 mM KCl, 5 mm KH2PO4, 30 mM maleic acid) in an Eppendorf tube for 8 min at 25^0C. The final concentration of mitochondria and HNE or ONE in the reaction tube was 10 mg/mL and 1 mM respectively.

Preparation of samples for transmission electron microscopy

Samples were fixed by addition of the primary fixative chemicals composed of 1.5% glutaraldehyde and 1.5% formaldehyde. Following fixation for 20 min, the samples were then washed with 3x PBS to remove excess fixative and immersed in 1% osmium tetroxide (OsO_4), 0.8% potassium ferricyanide, 0.1 M HEPES, and 0.08 M NaCl) for 1 hour at 4^0C. Samples were then washed three times for 10 minutes with distilled water. After washing, dehydration was performed by a graded series of ethanol to 100% (25, 50, 75, 85, 95 and 100%) with 10 minutes

at each step. Infiltration of Spurr's resin (Low viscosity resin, Electron microscopy science, Hatfield PA) was done in 25% steps of ethanol/resin for 1 hr at each step until three changes of 100% resin. The last change of resin was held for three hours. Each sample was placed in a 0.5 mL microcentrifuge tube and filled with fresh resin. The samples were then placed in a 70^0C oven for 18 hr. to polymerize. They were ultrathin sectioned (50-70nm) with a RMC MTX ultramicrotome. Sections were post-stained in 4% aqueous uranyl acetate for 30 minutes before imaging. Images were obtained on a JEOL 1011 TEM (JEOL USA, Dearborn MA).

Effects of HNE and ONE on oxygen consumption

The procedure previously described in a research (Ramanathan et al., 2012) was used to measure the rate of oxygen consumption of isolated mitochondria. Mitochondria (2.5 mg/ mL) were pre-incubated with HNE or ONE (0.20 mM) in a Clark electrode chamber at 25^0C. Control samples were incubated only with ethanol at a volume equal to that used to deliver HNE or ONE for the same time interval. Following pre-incubation with HNE, ONE or ethanol for 3 min, oxygen consumption was measured after addition of succinate 8 (mM). The incubation chamber was maintained at either pH 5.5 or 7.4 and the temperature of reaction was kept at 25^0C by a circulating water bath. The reaction chamber was stirred with a 10 mm Teflon covered bar at 600 rpm. The electrode was attached to a Rank Brothers digital model 20 oxygen controller (Cambridge, England) and connected to a personal computer and data logger. Oxygen consumption was recorded over time by suspending mitochondria at either pH 5.6 (250 mM sucrose, 5 mM KH2 PO4, 5 mM MgCl2, 0.1 mM EDTA, 0.1% BSA, and 20 mM maleic acid) or 7.4 (250 mM sucrose, 5 mM KH2PO4, 5mM MgCl2, 0.1 mM EDTA, 0.1% BSA, and 20 mM HEPES). All treatments were added to mitochondria (0.25 mg protein/mL in respiration buffer)

through 1 mm port in the incubation chamber of Clark cell oxygen electrode. State IV respiration (absence of ADP) was measured using succinate (5 mM) as substrates. State III respiration was measured by adding ADP (500 μM). The ratio of [state III] to [state IV] was also calculated to determine respiratory control index (RCR), which indicates the tightness of the coupling between respiration and phosphorylation.

Estimation of mitochondrial permeability

Mitochondrial membrane permeability was determined after incubating it with HNE or ONE according to the study (Ramanathan et al., 2012) with modification. Mitochondria (0.5 mg protein/mL) were incubated with either HNE (0.05 mM), ONE (0.05 mM) or ethanol control at pH 5.6 (0.1 M MES, 250 mM sucrose) or 7.4 (0.1 M Tris-HCl, 250 mm sucrose) at 25^0C for 50 min. Mitochondria with ethanol and either $CaCl_2$ (0.025 mM) or ATP (3 mM) plus EDTA (0.3 mm) were used as positive controls for swelling and contraction respectively. Change in absorbance was measured by using UV- 1800 UV-Vis spectrophotometer by Shimadzu Scientific Instruments Inc. at 10 min interval for 60 min (Goodell & Cortopassi, 1998). A decrease in absorbance at 520 nm indicates mitochondrial swelling and an increased absorbance indicates contraction.

Metmyoglobin reducing activity

Metmyoglobin reductase activity was used for evaluating the influence of HNE and ONE on mitochondria mediated metmyoglobin reduction. Isolated bovine cardiac mitochondria (5 mg/mL) was incubated with either (0.40 mM) of HNE or ONE at both pH 5.6 and 7.4, for 10 min at 25 °C. Control samples received an equal volume of ethanol similar to that used for HNE or ONE. Following incubation with HNE or ONE, mitochondria were incubated with antimycin

(0.01 mM) and rotenone (0.02 mM) for 10 min at 25 °C in with either pH 7.4 buffer (120 mM KCl, 5 mM KH_2PO_4, 30 mM K_2HPO_4), or pH 5.6 (120 mM KCl, 5 mM KH_2PO_4, 30 mM maleic acid) in order to inhibit ETC-mediated metmyoglobin reduction. Mitochondria were then combined with bovine metmyoglobin (0.15 mM), potassium ferrocyanide (3 mM), and EDTA (5 mM) at pH 5.6 and 7.4. The reaction was initiated with the addition of NADH (0.02 mM). Metmyoglobin reductase activity was calculated as nanomoles of metmyoglobin reduced (equal to nanomoles of oxymyoglobin formed) per min per mg mitochondria used during the initial linear phase of the assay (using a difference in molar absorptive coefficient of 12,000 $Lmol^{-1}$ cm^{-1} at 580 nm). The difference in absorbance for oxymyoglobin and metmyoglobin was observed at absorbance 580 nm.

Inhibition of radical scavenging activity of mitochondria

The inhibition of radical scavenging activity of mitochondria was measured by ABTS, H_2O_2 and FRAP assay by using HNE and ONE. After disruption of mitochondria by freezing and thawing and diluting in buffer solution, the enzymes were recovered to test the radical scavenging ability (Tonkonogi et al., 2000). Freezing and thawing cycle of 0.5 g of mitochondria was repeated 2-3 times. The sample was mixed with 10 mL of PBS and vortexed sufficiently to allow the sample to mix uniformly. The mixture was filtered through the microfilter paper to collect the filtrate.

The collected filtrate was incubated with 0 μM, 15μM, 30 μM, 45 μM and 60 μM HNE or ONE for 8 minutes at room temperature. These samples were assayed spectrophotometrically for radical scavenging ability by FRAP, $ABTS^{\cdot+}$, and H_2O_2 assay.

FRAP assay

The FRAP assay was conducted according to the procedure described by (Benzie & Strain, 1996) with modification. The FRAP reagent was prepared by mixing solutions of 10 mM TPTZ (in 40 mM HCl), 20 mM FeCl3, and 300 mM acetate buffer (pH 3.6) at a volumetric ratio of 1:1:10. Aqueous solutions of FeSO4.7H2O were prepared at concentration of 10, 200, 500, and 1000 μM for calibration. Water was used as the blank. Freshly prepared FRAP reagent (900 μL) was mixed with 30 μL of the sample solution, then with 90 μL water making the final dilution of the sample in the reaction mixture to be 34:1. The reaction mixture was then incubated in the dark at 37^0C for 4 min. The absorbance of the mixture was monitored at 593 nm.

$ABTS^{\cdot+}$ scavenging assay

$ABTS^{\cdot+}$ radical cation decolorization assay was performed with modifications (Re et al., 1999; Phonsatta et al., 2017). A 2.45 mM potassium persulfate was mixed with 7 mM ABTS water solution in the dark at room temperature for 16 hr. The $ABTS^{\cdot+}$ solution was then diluted with ethanol to obtain an absorbance of 0.70 ($\pm$ 0.01) at a wavelength of 734 nm. Ethanol was used for the control groups. A 100 μL sample was mixed with 900 μL of the $ABTS^{+}$ solution, then the mixture was incubated in the dark at 30^0C for 6 min. The absorbance of was measured at 734 nm with the spectrophotometer. All experiments were performed in triplicate. All the samples and reagents were freshly prepared daily.

H_2O_2 scavenging assay

The H_2O_2 scavenging assay was performed (Pick & Keisari, 1980; Sroka & Cisowski, 2003) with modifications. Equal amounts of the sample and 0.002% (w/w) H2O2 solution were mixed with 0.8 mL PBS an incubated in the dark at 37^0C for 10 min. 1 mL assay reagent,

containing 0.2 mg/mL phenol red and 0.1 mg/mL horseradish peroxidase in PBS, was added and incubated under the same condition for 15 min. After incubation 50 μL of 1 M sodium hydroxide was added and the absorbance of the mixture was measured immediately at 610 nm using spectrophotometer. H2O2 solutions at different concentrations (0.0002, 0.0005, 0.001, and 0.002% w/w) were used as the calibration curve.

Statistical analysis

The data were analyzed using regression analysis and ANOVA by JMP ® software (Version 13.2.0, SAS Institute, Inc., Cary, NC, USA), and presented as means followed by standard errors.

Results and discussion

Effect of HNE and ONE on morphology of mitochondria

The fig 1 shows the electron micrograph of mitochondria treated with aldehydes. Changes in morphological structures are obvious with different treatments. HNE and ONE treated mitochondria responded differently ($P < 0.05$) to different pH 7.4 & 5.6. The areas of mitochondria were measured using image J software and these areas in sq. micrometer were presented as a bar graph (Fig 2). These data suggest that the mitochondria incubated at pH 7.4 had greater area ($P < 0.05$) (swelling) compared with those incubated at pH 5.6. The control mitochondria had more intact and consistent structure, whereas those incubated at pH 5.6 with ONE exhibited disruption of morphological structure. This phenomenon could be explained on the basis of relative toxic nature of these aldehydes (Picklo, Azenkeng, & Hoffman, 2011; Lin et al., 2005; Doorn & Petersen, 2002). (Ramanathan et al., 2012) also conducted image analysis of mitochondria isolated from beef heart. Their findings were in agreement with the result of this

study. They found that the mitochondria incubated with HNE at pH 7.4 had greater ($P < 0.05$) area compared to those that were incubated with either HNE at pH 5.6 or ethanol controls. The highest area of mitochondria was found to be 0.95 sq. micrometers with ONE at pH 7.4 compared with HNE incubated at pH 5.6 i.e. 0.375 square micrometer area. Mitochondria can undergo structural changes in response to external stimuli (Pivovarova & Andrews, 2010). Lipid peroxidation product such as HNE can modify membrane fluidity by interacting with phospholipids present in mitochondrial membrane (Chen & Yu, 1999). HNE can induce calcium-dependent mitochondrial permeability transition, thereby opening the pore and causing swelling of the structure (Kristal, Park, & Yu, 1996). Despite the potential of aldehydes to alter the membrane fluidity, only few researches have assessed their effects on mitochondrial structure alterations. In a comparative study, HNE and ONE both disrupted the normal morphological structure of mitochondria (Gonzalez, 2013). The mitochondria incubated with ONE presented the largest swelling (area) compared to those with other two treatments (control and HNE). Different researchers have reported the electrophilic characteristics of HNE and ONE differently. Although both of these are diffusible electrophiles that can reach protein targets from their site of generation (Zhong et al., 2014), ONE is more reactive towards thiols and amines by making itself a suicide substrate for metabolic enzymes, which interact with HNE, thus increasing the half-life of HNE (Doorn, Hurley, & Petersen, 2006). However, ONE is less reactive than HNE towards Lys/amine conjugate adduction (Sayre et al., 2006).

Effect of HNE and ONE on oxygen consumption

A significant effect of HNE and ONE on mitochondrial oxygen consumption was observed at both pH 5.6 and 7.4 (Table 2). Pre-incubation of mitochondria with these aldehydes

decreased RCR, state III, and state IV oxygen consumption compared with control samples without aldehydes ($P < 0.05$). The effects of HNE at pH 5.6 and 7.4 in this study are consistent with findings of previous research (Ramanathan et al., 2012), which reported decrease of RCR, state III, state IV respiration due to the effect of HNE on mitochondria. However, in this experiment, ONE decreased oxygen consumption rate more than HNE ($P < 0.05$). This characteristic of ONE was also previously observed in rat brain mitochondria (Picklo, Azenkeng, & Hoffman, 2011), where ONE was sensitive enough to inhibit succinate-linked respiration. Although different researchers have reported the reactivity of ONE differently, when it comes to modifying mitochondrial proteins, it is about 100 times more reactive than HNE during the condition of oxidative stress (Long, Olson, & Bernlohr, 2013).

It appears that the main effect of HNE are on complex I, complex II, cytochrome c, and cytochrome c oxidase in reducing oxygen consumption rate by mitochondria (Ramanathan et al., 2012). (Humphries, Yoo & Szweda, 1998) attributed decreased oxygen consumption by rat brain mitochondria to be NADH linked state 3 respiration by inactivating complex I by HNE.

Mitochondria of different tissues respond differently to the effect of HNE (Picklo et al., 1999). The difference in mitochondrial oxygen consumption rate could be attributed to the relative reactivity of 4-HNE and 4-ONE towards various amino acids.

Effects of HNE and ONE on mitochondrial membrane permeability and metmyoglobin reduction

There are several factors affecting permeability of mitochondria. One of the factors responsible for increased permeability could be due to either mitochondrial damage or induction of mitochondrial permeability transition (MPT) (Goodell & Cortopassi, 1998). Soon after the

isolation of mitochondria, they begin to swell, lose their ability to phosphorylate and release matrix contents if Ca^{2+} ions are not excluded from the suspension (Zoratti & Szabo, 1995).

At pH 7.4, ONE treated mitochondria were swollen compared to ethanol controls whereas at pH 5.6 mitochondria treated with ONE contracted. This phenomenon was supported by measurement of area as well as membrane permeability (Figs 2, 3A & 3B). ONE-treated samples at pH 7.4 had decreased absorbance at 520 nm, this indicates mitochondrial swelling compared with control samples ($P < 0.05$). Conversely, mitochondria incubated with ONE at pH 5.6 had decreased area (contraction) and permeability, which had increased absorbance at 520 nm. Similarly, HNE at pH 7.4 also had influence on mitochondrial membrane pore opening, which also resulted swelling of mitochondria (decreased absorbance 520 nm) (Ramanathan et al., 2012). Prooxidants are generally supposed to trigger the mitochondrial permeability transition (MPT) (Kushnareva & Sokolove, 2000), which is induced by several cellular toxins including oxidants such as hydroperoxides, Ca^{+2}, and neurotoxins etc. (Yang & Cortopassi, 1998).

Bovine cardiac mitochondria incubated with either HNE or ONE reduced metmyoglobin reduction at both pH 5.6 and 7.4 (Fig 3C). Mitochondria are known for generating reducing equivalents NADH, necessary for metmyoglobin reduction. They reduce metmyoglobin through several mechanisms including electrons transfer pathways between complexes III and IV via the cytochrome c: cytochrome b5 complex, and NADH-dependent cytochrome b_5 reductase (Arihara et al., 1995; Tang et al., 2005; Watts et al., 1966). Present findings suggested both HNE and ONE affected this phenomenon. Specifically, ONE exhibited significant decrease ($P < 0.05$) in mitochondrial metmyoglobin reduction as compared to HNE and respective controls at both pH 7.4 compared with pH 5.6. Alderton, et al. (2003) reported that covalent interactions of myoglobin with HNE were responsible for redox instability of myoglobin. More specifically,

covalent binding of HNE to histidine can destabilize heme iron within myoglobin and promote metmyoglobin formation (Faustman, et al., 1999; Naveena, et al., 2010; Suman et al., 2007).

Effects of HNE and ONE on antioxidant activity

Mitochondria contain antioxidant defense mechanism to quench ROS, and not all the defense system is used up to clear these free radical species (Mailloux, 2018). The mitochondrial antioxidant defense system includes both enzymes e.g. SOD, glutathione peroxidase and gluthatione reductase, and non-enzymatic substances e.g. glutathione and α- tocopherol and coenzyme Q (Paradies et al., 2001). The $ABTS^{\cdot+}$ radical scavenging, FRAP, and H_2O_2 scavenging assays were conducted to understand the inhibition effect of HNE and ONE on it (Fig 4). All these assays indicated that ONE had a greater capacity of inhibiting the antioxidant defense system than HNE ($P < 0.05$).

The antioxidant activity of compounds can be expressed as the effective concentration for obtaining a 50% response (EC_{50}) or the inhibitory concentration at 50% response (IC_{50}) (Zhang & Akoh, 2019). In the present study, IC_{50} (the concentration of tested aldehydes in the antioxidant system to reduce the free radical to 50% of initial concentration) was used to quantify the inhibition of antioxidant activity.

Some researchers (Reinheckel et al., 1998) suggested that mitochondria could generate sufficient amount of secondary aldehydes, which can deplete GSH levels. (Chen & Yu, 1994) reported that incubation of 10-400 μM HNE results in HNE adduct formation with cytochrome oxidase which in turn inhibit the enzyme activity. Although HNE was already established as a modifier of mitochondrial enzymes; no research has assessed the effect of ONE on this antioxidant system.

Although HNE and ONE can modify amino acid nucleophiles, their reactivity differ both qualitatively and quantitatively (Doorn & Petersen, 2002). ONE used in this experiment exhibited more pronounced effect in inhibiting antioxidants than HNE. Partial explanation on this could be due to higher reactivity of ONE towards thiol nucleophiles, which is an essential component of glutathione (GSH) (Lin et al., 2005). The research conducted to determine inhibition potential of HNE and ONE to human mitochondrial aldehyde dehydrogenase suggested that in a fairly large diluted condition (1:40), the enzyme lost 10% activity with HNE whereas it lost more than 90% activity with 4 ONE. This characteristic indicated that enzyme inhibition by HNE was reversible whereas that of ONE was primarily irreversible (Doorn et al., 2006).

The study conducted on yak muscle suggested that the ROS increased mitochondrial lipid peroxidation and suppress the activities of SOD, CAT, and GSH-Px (Wang et al., 2018). Prooxidants are detoxified by endogenous defense system to balance generated toxic compounds due to metabolic activity in the tissues.

Conclusion

Both HNE and ONE can influence the structure and function of beef heart mitochondria. In comparison to HNE, ONE exhibited more disruption of morphological structure of mitochondria at pH 5.6. However, the ONE treated mitochondria were more swollen at pH 7.4, and this effect was supported by the greater permeability achieved when incubated with ONE at this condition. Similarly, pre-incubation of these aldehydes with mitochondrial extracts showed greater inhibition of antioxidant activity by ONE than HNE. The result of this study confirms that ONE is more toxic biomarker of lipid peroxidation product than HNE.

References

Alderton, A.L., Faustman C., Liebler D.C., & Hill, D.W. (2003). Induction of redox instability of bovine myoglobin by adduction with 4-hydroxy-2-nonenal. Biochemistry, 42, 4398-4305.

Arihara, K., Cassens, R. G., Greaser, M. L., Luchansky, J. B., & Mozdiak, P. E. (1995). Localization of metmyoglobin-reducing enzyme (NADH-cytochrome b5 reductase) system components in bovine skeletal muscle. *Meat Science*, 39, 205-213.

Watts, B. M., Kendrick, J., Zipser, M. W., Hutchins, B., & Saleh, B. (1966). Enzymatic reducing pathways in meat. *Journal of Food Science,* 31, 855-862.

Benzie, I. F. F. & Strain J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power." The FRAP assay. *Analytical Biochemistry,* 239, 70-76.

Chen, J. J. & Yu B. P. (1994). Alterations in mitochondrial membrane fluidity by lipid peroxidation products. *Free Radical Biology and Medicine,* 17, 411-418.

Doorn, J. A., & Petersen D. R. (2002). Covalent modification of amino acid nucleohiles by the lipid peroxidation products 4-Hydroxy-2-nonenal and 4-oxo-2-nonenal. *Chemical Research in Toxicol*ogy, 15, 1445-1450.

Doorn, J. A., Hurley, T. D., & Petersen, D. R. (2006). Inhibition of human mitochondrial aldehyde dehydrogenase by 4-Hydroxynon-2-enal and 4-Oxonon-2-enal. *Chemical Research Toxicology,* 19: 102-110.

Esterbauer, H., Schaur R.J., & Zollner H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology & Medicine,* 11, 81-128.

Faustman, C., Liebler, D. C., & Burr, J. A. (1999). Alpha tocopherol oxidation in beef and in bovine muscle microsomes. *Journal of Agricultural and Food Chemistry,* 47, 1396-1399.

Gonzalez, S. (2013). The effects of lipid peroxidation products 4-HNE and 4-ONE on lactate dehydrogenase activity, myoglobin redox stability, and mitochondrial structural integrity. MS thesis. University of Georgia, Athens.

Goodell, S., & Cortopassi, G. (1998). Analysis of oxygen consumption and mitochondrial permeability with age in mice. *Mechanism of Ageing and Development,* 101, 245-256.

Gutierrez, A.M., Reboredo G.R., & Catala A. (2002). Fatty acid profiles and lipid peroxidation of microsomes and mitochondria from liver, heart and brain of Cairina moschata. *The International Journal of Biochemistry & Cell Biology,* 34, 605-612.

Humphries, K. M., Yoo Y., & L. I. Szweda. (1998). Inhibition of NADH-linked mitochondrial respiration by 4-hydroxy-2-nonenal. *Biochemistry,* 37, 552-557.

Kristal, B.S., Park B. K., and Yu, B.P. (1996). 4-Hydroxyhexenal is a potent inducer of the mitochondrial permeability transition. *Journal of Biological Chemistry,* 271, 6033-6038.

Kushnareva, Y. E & Sokolove, P. M. (2000). Prooxidants open both the mitochondrial permeability transition pore and a low-conductance channel in the inner mitochondrial membrane. *Archives of Biochemistry and Biophysics,* 376, 377-388.

Lee, S. H., & Blair, I. A. (2000). Charecterization of 4-Oxo-2-nonenal as a novel product of lipid peroxidation. *Chemical Research in Toxicology,* 13, 698 -602.

Lee, S.H., Takahashi R., Goto T., & T. Oe. (2010). Mass spectrometric characterization of modifications to angiotensin II by lipid peroxidation products, 4-ONE and 4-HNE. *Chemical Research in Toxicology,* 23, 1771-1785.

Lin, D., Lee, H-G., Liu, Q., Perry, G., Smith, M. A., and Sayre, L. M. (2005). 4-Oxo-2-nonenal is both more neurotoxic and more protein reactive than 4-Hydroxy-2-nonenal. *Chemical Research in Toxicology,* 18, 1219-1231.

Long, E.K., Olson D. M., & Bernlohr D. A. (2013). High-fat diet induces changes in adipose tissue trans-4-oxo-2-nonenal and trans-4-hydroxy-2-nonenal levels in a depot-specific manner. *Free Radical Biology and Medicine,* 63, 390-398.

M.P. Murphy. (2012). Mitochondrial thiols in antioxidant protection and redox signaling: distinct roles for glutathionylation and other thiol modifications. *Antioxidants & Redox Signaling,* 16, 476-495.

Mailloux, R. J. (2018). Mitochondrial antioxidants and the maintenance of cellular hydrogen peroxide levels. *Oxidative Medicine and Cellular Longevity,* 2018, 1-10.

Naveena, B. M., Faustman, C., Tatiyaborworntham, N., Yin, S., Ramanathan, R., & Mancini, R. A. (2010). Detection of 4-hydroxy-2-nonenal adducts of turkey and chicken myoglobins using mass spectrometry. *Food Chemistry,* 122, 836–840.

Paradies, G., Petrosillo, G., Pistolese, M., and Ruggiero, F.M. (2001). Reactive oxygen species generated by the mitochondrial respiratory chain affect the complex III activity via cardiolipin peroxidation in beef-heart submitochondrial particles. *Mitochondrion,* 1, 151-159.

Phonsatta, N., Deetae, P. L, Grajeda-Iglesias, C., M. C. Figueroa-Espinoza, Le, C. J., & Panya, A. (2017). Comparison of antioxidant evaluation assays for investigating antioxidative activity of

gallic acid and its alkyl esters in different food matrices. *Journal of Agricultural and Food Chemistry,* 65, 7509 -7518.

Pick, E., & Keisari Y. (1980). A simple colorimetric method for the measurements of hydrogen peroxide produced by cells in culture. *Journal of Immunolgical Methods,* 38, 161-170.

Picklo, M. J., Amarnath, V., McIntyre, J. O., Graham, D.G. & Montine, T. J. (1999). 4-Hydroxy-2 (E)-Nonenal inhibits CNS mitochondrial respiration at multiple sites. *Journal of Neurochemistry,* 72, 1617-1624.

Picklo, M. J., Azenkeng, A., and Hoffman, M. R. (2011). Trans-4-oxo-2-nonenal potently alters mitochondrial function. *Free Radical Biology and Medicine,* 50, 400-407.

Pivarova N. B. & Andrews S. B. (2010). Calcium-dependent mitochondrial function and dysfunction in neurons. *FEBS Journal*, 277, 3622-3636.

Poli, G., & Schaur R. J. (2000). 4-Hydroxynonenal in the pathomechanisms of oxidative stress. *IUBMB Life* 50, 315-321.

Ramanathan, R. Mancini R. A., Suman S. P., & Cantino M. E. (2012). Effects of 4-hyroxy-2-nonenal on beef heart mitonchondrial ultrastructure, oxygen consumption, and metmyoglobin reduction. *Meat Science,* 90, 564-571.

Re, R., Pellegrini N., Proteggente A., Pannala A., Yang M., & Rice-Evans C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine,* 26, 1231-1237.

Reinheckel, T., Noack, H., Lorenz S., Wiswedel I., & Augustin, W. 1998. Comparison of protein oxidation and aldehyde formation during oxidative stress in isolated mitochondria. *Free Radical Research,* 29, 297-305.

Saito, F., Iwamoto S., & Yamauchi R. (2011). 4-oxo-2- nonenal as a prooxidant during the autoxidation of methyl linoleate in bulk phase. *Food Chemistry,* 124, 1496-1499.

Sakai, T., Kuwazuru, S., Yamauchi, K., & Uchida, K. (1995). A lipid peroxidation-derived aldehyde, 4-hydroxy-2-nonenal and omega 6 fatty acids contents in meats. *Bioscience Biotechnology, and Biochemistry,* 59, 1379-1380.

Sakai, T., Yamauchi, K., Kuwazuru, S., & Gotoh, N. (1998). Relationships between 4-Hydroxy-2-nonenal, 2- thiobarbituric acid reactive substances and n-6 polyunsaturated fatty acids in refrigerated and frozen pork. *Bioscience Biotechnology, and Biochemistry,* 62, 2028-2029.

Sayre, L.M., Lin D.,Yuan Q., Zhu X., & Tang X. (2006). Protein adducts generated from products of lipid oxidation: focus on HNE and ONE. *Drug Metabolism Reviews,* 38, 651-675.

Sroka, Z. & Cisowski W. (2003). Hydrogen peroxide scavenging, antioxidant and antiradical activity of some phenolic acids. *Food and Chemical Toxicology,* 41, 753-758.

Suman, S. P., Faustman, C., Stamer, S. L., & Liebler, D. C. (2007). Proteomics of lipid oxidation-induced oxidation of porcine and bovine oxymyoglobins. *Proteomics, 7*, 628 -640.

Tang, J., Faustman, C., Hoagland, T.A., Mancini, R.A., Seyfert, M. & Hunt, M.C. (2005). Postmortem oxygen consumption by mitochondria and its effects on myoglobin form and stability. *Journal of Agricultural and Food Chemistry*, 53, 1223-1230.

Tonkonogi, M., Walsh B., Svensson M., & Sahlin K. (2000). Mitochondrial function and antioxidative defence in human muscle: effects of endurance training and oxidative stress. *Journal of Applied Physiology*, 528, 379-388.

Uchida, K. (2003). 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Progress in Lipid Research*, 42, 318-343.

Wang, L-L., Yu, Q-L., Han, L., Ma, X-L., Song, R.-D., Zhao, S.-N., and Zhang, W-H. (2018). Study on the effect of reactive oxygen species-mediated oxidative stress on the activation of mitochondrial apoptosis and tenderness of yak meat. *Food Chemistry,* 244, 394-402.

Yang, J. C. & Cortopassi. G. A. (1998). Induction of the mitochondrial permeability transition causes release of the apoptogenic factor Cytochrome C. *Free Radical Biology & Medicine,* 24, 624-631.

Zhang, S., & Akoh, C. C. (2019). Enzymatic synthesis of 1-*o*- galloylglycerol: characterization and determination of its antioxidant properties. *Food Chemistry,* 305, 1-9.

Zhang, W-H., Liu, J., Xu, G., Yuan, Q., & Sayre, L.M. (2003). Model studies on protein side chain modification by 4-oxo-2- nonenal. *Chemical Research in Toxicology,* 16, 512-523.

Zhong, H., Lu J., Xia L., Zhu M., & H. Yin. (2014). Formation of electrophilic oxidation products from mitochondrial cardiolipin in vitro and in vivo in the context of apoptosis and atherosclerosis. *Redox Biology*, 2, 878-883.

Zhu, X., & Sayre, L. M. (2007). Long-lived 4-Oxo-2-enal-derived apparent lysine Michael adducts are acturally the isomeric 4-ketoamides. *Chemical Research in Toxicology*, 20, 165-170.

Zoratti, M., & Szabo, I. (1995). The mitochondrial permeability transition. *Reviews on Biomembranes*, 1241, 139-176.

List of Tables

List of Figures

Table. 2. Effect of HNE and ONE on oxygen consumption of bovine heart mitochondria

Treatment	pH	State III	State IV	RCR
Succinate	7.4	274.64 [a]	95.4 [a]	2.88 [a]
Succinate +HNE	7.4	124.166 [b]	73.2 [b]	1.696 [b]
Succinate + ONE	7.4	91.453 [c]	65.63 [c]	1.394 [c]
Succinate	5.6	203.653 [a]	84.533 [a]	2.412 [a]
Succinate + HNE	5.6	93.346 [b]	71.933 [b]	1.297 [b]
Succinate + ONE	5.6	70.73 [c]	63.933 [b]	1.106 [c]

[a,b,c] letters indicate significant statistical difference at $p < 0.05$.

State III: Oxygen consumption rate (nmol O/min mg mitochondrial protein) of isolated mitochondria in the presence of substrate and ADP.

State IV: Oxygen consumption rate (nmol O/min mg mitochondrial protein) of isolated mitochondria in the presence of only added substrate.

RCR: ratio between state III and state IV respiration

Table. 3. Inhibition of antioxidant activities of bovine heart mitochondria by HNE and ONE

Aldehydes	$ABTS^{\cdot+}$	H_2O_2	FRAP
	IC_{50} (μM)	IC_{50} (μM)	$IC_{0.25}$ (μM)
HNE	29.516 ± 1.05^{a}	25.693 ± 1.253^{a}	42.35 ± 3.71^{a}
ONE	26.67 ± 0.635^{b}	20.131 ± 1.539^{b}	34.39 ± 3.1^{b}

[a,b] Letters indicate significant statistical difference at $p < 0.05$. All the results are expressed as means (n=3) $\pm$ standard error.

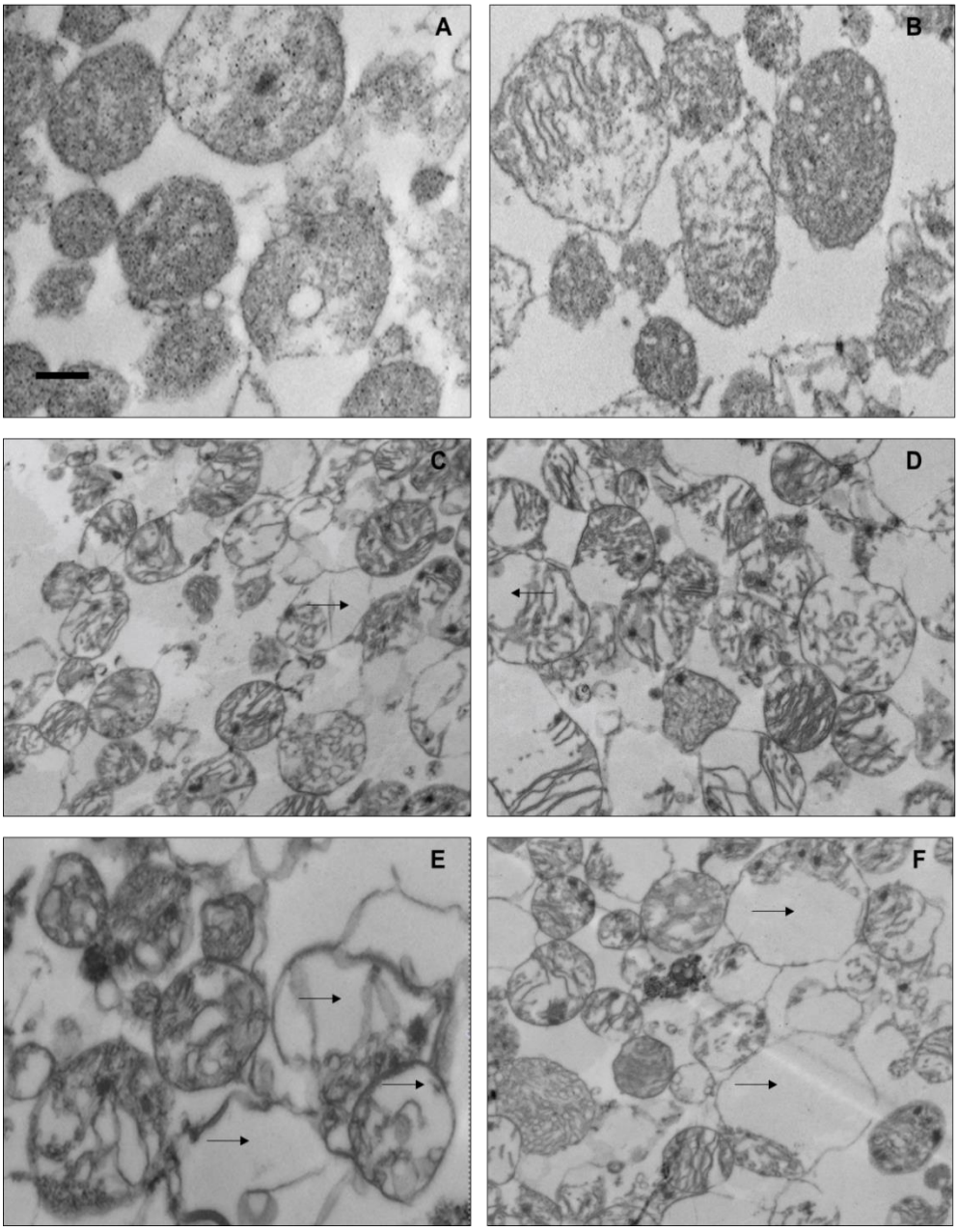

Fig. 1. Electron micrographs of mitochondria incubated in different buffer A) pH 7.4, B) pH 5.6, C) pH 7.4 with 0.4 mM 4-HNE, D) pH 7.4 with 0.4 mM 4-ONE, E) pH 5.6 with 0.4 mM 4-HNE, and F) pH 5.6 with 4-ONE. The scale bar in panel A corresponds to 500 nm and is the same for panels A-F.

Magnification = 10,000×.

Arrows in panels C through F indicate mitochondrial vacuolization.

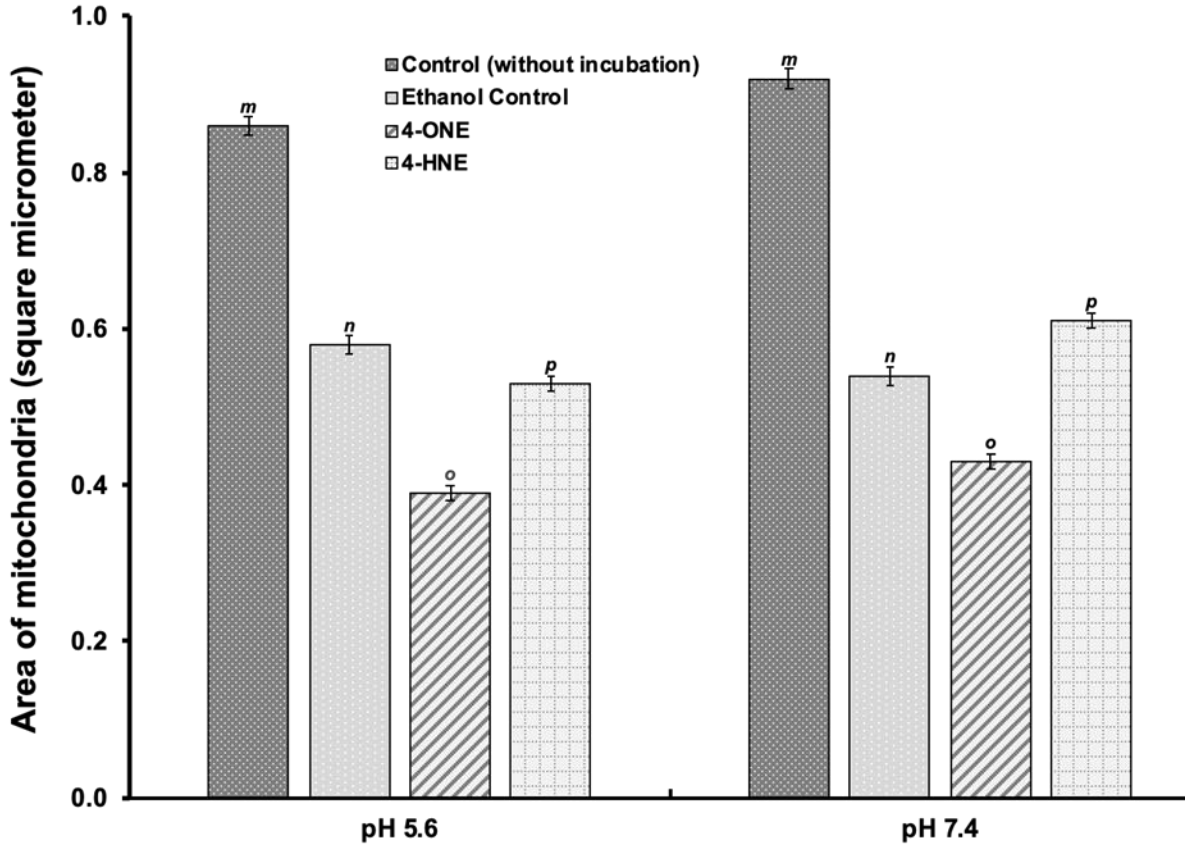

Fig. 2. The areas of mitochondria incubated with HNE and ONE at pH 5.6 and 7.4 are represented by the length of bars. (The areas were measured by the image J software)

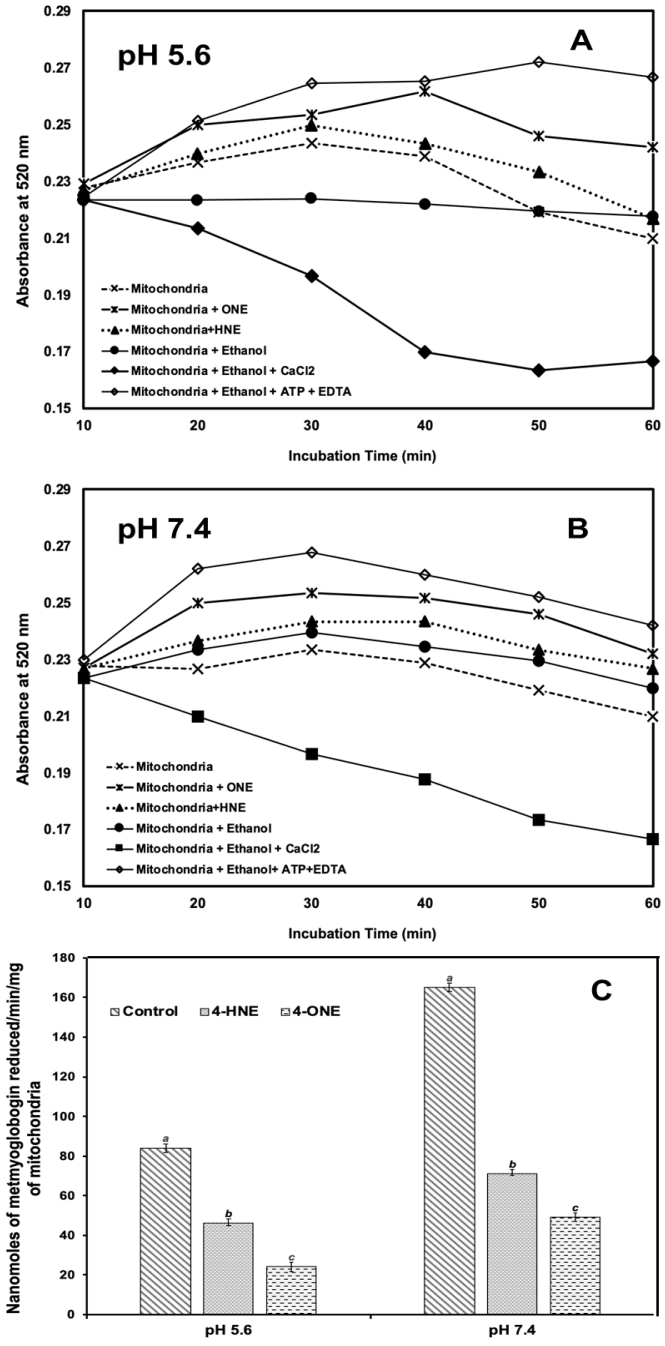

Fig. 3. Effect of HNE and ONE on membrane permeability (3A and 3B) at pH 5.6 and 7.4, and metmyoglobin reduction (3C) of beef heart mitochondria.

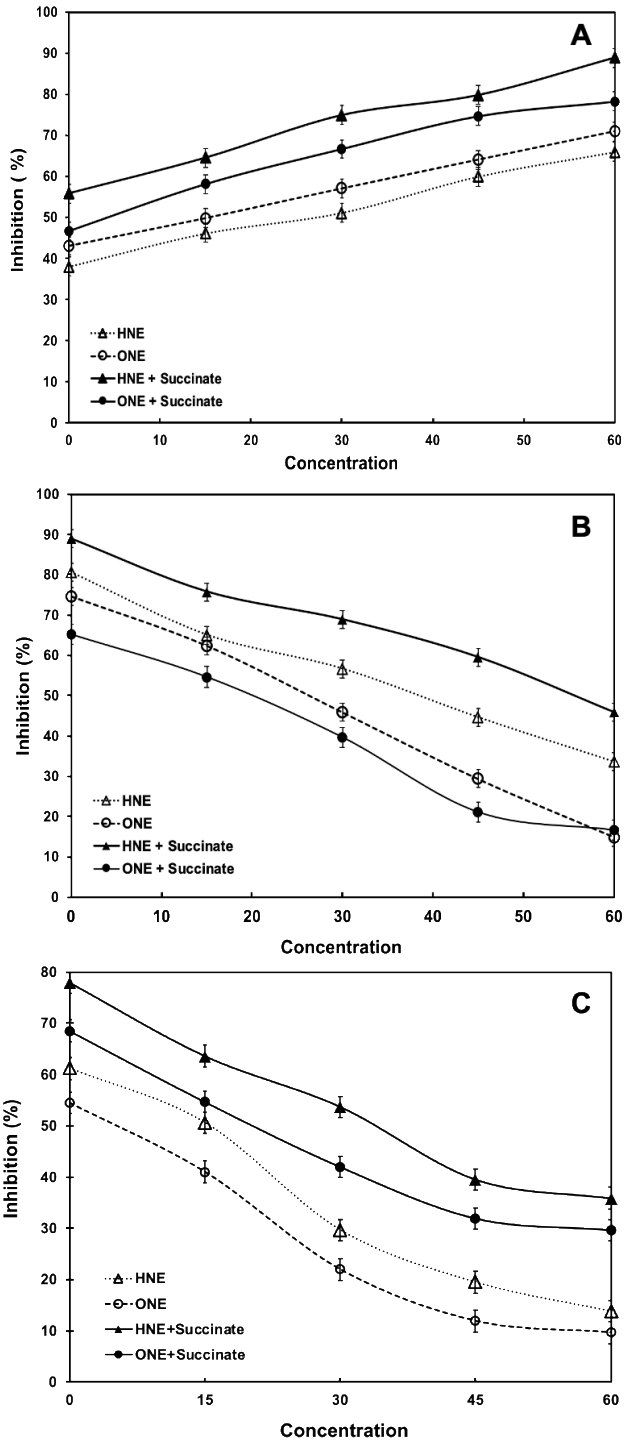

Fig. 4. Inhibition of radical scavenging activity of bovine heart mitochondria by HNE and ONE (A) H_2O_2 Scavenging assay (B) FRAP Assay (C) $ABTS^{\cdot+}$ scavenging assay

CHAPTER 4

CONCLUSIONS

The electron micrograph revealed that 4-HNE and 4-ONE were both reactive aldehydes, but ONE had more influence on increasing area of mitochondria at pH 7.4. This nature of ONE was also further supported by increased permeability observed in permeability assay. Moreover, ONE decreased mitochondrial metmyoglobin reduction more than HNE. These data suggested that 4-ONE has more detrimental effect on meat color stability due to increased tendency of mitochondrial damage.

Although, 4-ONE and 4-HNE both decreased oxygen consumption rate of mitochondria at pH 5.6 and 7.4, the state III respiration was more pronounced due to the effect of 4-ONE at pH 5.6. It appears that complex II linked mitochondrial activity can affect more in beef color stability due to the induced effect of ONE at both the pH conditions. However, no difference was observed between ONE and HNE in state IV respiration at pH 5.6.

4-ONE and 4-HNE both inhibited endogenous defense mechanism. Apart from destroying the structure and function of mitochondria, they also inhibited the antioxidant capacity.