A HISTOCHEMICAL ANALYSIS OF MITOCHONDRIAL ABNORMALITIES IN THE TYPE I FIBERS OF HUMAN POSTERIOR CRICOARYTENOID MUSCLE

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A Histochemical Analysis of Mitochondrial Abnormalities in the Type I Fibers of Human Posterior Cricoarytenoid Muscle

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Mitochondrial deficiencies are present in limb skeletal muscle fibers throughout normal aging and can increase with accumulated exposure to reactive oxide species. Exogenous sources of reactive oxide species include cigarette smoke and other environmental toxins. Intrinsic laryngeal muscles are directly exposed to inhaled toxins which may increase the percentage of cytochrome c oxidase deficient fibers over time. Serial sections of posterior cricoarytenoid and control strap muscle were harvested post laryngectomy from 10 males age 54-78 years old. Cytochrome c oxidase, succinate dehydrogenase, and modified Gomori trichrome stains were used to determine the percentage of type I fibers with cytochrome c oxidase deficiency. Staining with myosin antibodies was used to determine fiber type. The posterior cricoarytenoid muscle contained a significantly higher percentage of type I fibers with cytochrome c oxidase deficiency ($p=.002$, $t = 4.939$) compared to the control strap muscle. The percentage of cytochrome c oxidase deficient fibers was also significantly correlated ($r = +.851$; $p < .01$) with age in posterior cricoarytenoid muscle. The percentage of type I fibers with cytochrome c oxidase deficiency increases with age in the posterior cricoarytenoid muscle and may be due to the accumulated exposure to reactive oxide species over time.

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PREFACE

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1. CHAPTER I – INTRODUCTION

Laryngeal physiology involves the complex coordination of several different muscles that, when activated, are responsible for the vegetative functions of airway protection, pushing and pulling, deglutition, and respiration, as well as the production of sound during phonation. Although there is an abundance of literature on the physiology and histology of normal functioning intrinsic laryngeal muscles, there is scant literature on how the biochemical and metabolic aspects of these muscles change when exposed to a pathology or disorder. Not only is it necessary to know how a muscle normally functions, it is also important to understand why it functions in a particular way and under what circumstances would changes in muscle structure and composition negatively affect muscle function. Understanding the potential changes in histology and metabolic capacity of the muscles fibers of the intrinsic laryngeal muscles will enable voice science researchers and voice care providers to base the design and development of behavioral voice diagnosis and treatment in biological voice science.

One change that can occur to limb skeletal muscle fibers throughout the normal aging process is an increase in mitochondrial deficiency. Although mitochondrial deficiencies are normal to aging, accumulated exposure to reactive oxide species can cause an increase in mitochondrial abnormalities. These abnormalities are identified by an increase in cytochrome c oxidase deficient fibers. As the percentage of cytochrome c oxidase deficient fibers increases, oxidative capacity of the muscle fibers decreases and in turn increases the risk for muscle fatigue (Pesce, Cormio, Francasso, Vecchiet, Felanzi, et al., 2001). Exogenous sources of reactive oxide species include cigarette smoke and other environmental toxins (Cross, Halliwell, Borish, Pryor, Ames et al., 1987). Because of their close proximity to the epithelial lining in the larynx, intrinsic laryngeal muscles are directly exposed to the toxins in cigarette smoke and other environmental

toxins which may increase the percentage of cytochrome c oxidase deficient fibers in these muscles. Individuals with a smoking history, therefore, may have an increased risk for a greater accumulation of reactive oxide species and cytochrome c oxidase deficiency.

Much of the literature regarding the basic biologic properties of the intrinsic laryngeal muscles has focused on human and canine thyroarytenoid and posterior cricoarytenoid muscles. Posterior cricoarytenoid was chosen as a starting point for this research because of its primary role in the abduction of the vocal folds. Although all of the intrinsic laryngeal muscles work in a synergistic manner, the PCA muscle is the only intrinsic laryngeal muscle that functions in a specific, unique way to induce abduction. Furthermore, the PCA contains the highest proportion of type I fibers (i.e., fibers most affected by mitochondrial deficiency) compared to the other intrinsic laryngeal muscles and may therefore be at an increased risk for muscle fatigue in individuals with a smoking history. The purpose of this dissertation, therefore, is to determine if there is a significant increase in type I fiber with cytochrome c oxidase deficiency in the posterior cricoarytenoid muscle of individuals with a smoking history.

The following chapters will provide background information regarding general limb skeletal muscle histology, morphology, and metabolic capacity. Chapters two and three will discuss in detail the literature on normal intrinsic laryngeal muscle histology and metabolic capacity, focusing particularly on the literature regarding posterior cricoarytenoid muscle. The fourth chapter will address the most important research available on how the accumulation of reactive oxide species affects mitochondrial abnormalities and cytochrome c oxidase deficiency in muscle. The potential effects of cytochrome c oxidase deficiency on the posterior cricoarytenoid muscle will also be elaborated in this section.

2. CHAPTER II

2.1. Posterior Cricoarytenoid Muscle Anatomy, Morphology and Histology

2.1.1. Overview of Intrinsic Laryngeal Muscles

The larynx is composed of intrinsic and extrinsic laryngeal muscles used during deglutition, respiration, and phonation (Zemlin, 1988). The extrinsic muscles have attachments outside of the larynx and are involved in the support and positioning of the larynx. There are five intrinsic laryngeal muscles: interarytenoid, thyroarytenoid, lateral cricoarytenoid, cricothyroid, and posterior cricoarytenoid. These muscles have attachments within the larynx and are responsible for the airway regulation and sound. Intrinsic laryngeal muscles have traditionally been categorized into abductors (i.e., muscle that open the glottis), adductors (i.e., muscles that close the larynx), tensors (i.e., muscles that shorten the vocal folds), and elongators (i.e., muscle that lengthen the vocal folds).

2.1.2. Anatomy and Physiology of Posterior Cricoarytenoid Muscle

The posterior cricoarytenoid (PCA) muscle is considered the primary muscle of abduction; however, it can also be used in other tasks such as vocal fold elevation, elongation, and thinning (Sataloff, 1998). Hillel (2001) also noted PCA activation in both respiration and phonation. Originally thought to be one muscle, the PCA is now believed to be divided into more than one compartment. Sanders and colleagues (1993) have noted three subcompartments in canine larynges (Sanders, Jacobs, Wu, & Biller, 1993). A recent study, however, demonstrated that human PCA only has two subcompartments. (Bryant, Woodson, Koufman, Rosen, Hengesteg et al., 1996). Several studies confirm that there are only two subcompartments in human PCA muscle (Sanders, Wu, Mu, & Biller, 1994; Sellars & Sellars, 1983; Zemlin, Davis, & Gaza,

1984). These subcompartments have been termed lateral-oblique or vertical and medial-horizontal or horizontal.

As well as being distinct subcompartments of the muscle, the vertical and horizontal bellies may also have differential mechanical actions on the cricoarytenoid joint (Bryant et al., 1996). Although anatomically, different compartments suggest different function, it is uncertain whether individual bellies of the PCA actually perform different actions (Bryant et al., 1996). If the muscle compartments do perform different functions, then there are clinical implications to voice treatment and diagnosis. Voice care providers have to be aware that injury to PCA may affect both abductory and adductory tasks.

There are several ways to determine if the different muscle bellies of the PCA have different functions. Four methods that have been reported in the literature are simulation and electrical stimulation of muscle contraction, identification of muscle spindles, characterization of nerve supply to the muscle bellies, and the analysis of the histologic and metabolic properties of the muscle bellies.

Differentiation of subcompartment function can be identified through simulated contraction and electrical stimulation. Bryant and colleagues (1996) used a simulated three-dimensional model of PCA contraction in 12 fresh cadaveric larynges to explore the differential functioning of the two subcompartments. Results indicated that the lateral-oblique compartment caused the arytenoids to rock backward more than swivel horizontally while the medial-horizontal compartment is involved in swiveling the arytenoids. Sanders, Rao, and Biller (1994) conducted laryngeal electromyographic (EMG) analyses on the different compartments of canine larynges the results of which are similar to those from Bryant and colleagues' study. Results indicated that when simultaneously stimulated, both bellies caused the arytenoids to slide

laterally and rock backward. Stimulation of just the lateral-oblique compartment, however, caused the arytenoids to rock backward and slide laterally which is likely implicated in abducting the vocal folds during respiration. Stimulation of just the medial-horizontal subcompartment caused only the sliding or swiveling action of the arytenoids. Sanders and colleagues (1994) noted that this swiveling action did not abduct the vocal folds but rather moved the vocal processes in a lateral direction implicating this subcompartment in the positioning of the vocal folds during phonation. The possibility of a large number of muscle spindles[1] in the horizontal compartment of the PCA gives credence to the positioning role of this compartment (Sanders, Jacobs, Wu, & Biller, 1993).

Muscle spindles in the horizontal compartment would suggest that this subcompartment functions differently than the vertical compartment, however, the presence of spindles in the PCA is still uncertain. Grim (1967) reported muscle spindles in the PCA but used hemotoxylin and eosin staining to identify the structures. Muscle spindle structures, however, are difficult to identify with this type of staining (Brandon, Rosen, Georgelis, Horton, Mooney et al., 2003a). Spindles are more accurately identified using tonic and neonatal myosin heavy chain antibody staining which stain the bag_1 and bag_2 fibers respectively within the spindle. Okamura and Katto (1988) also found muscle spindles in the PCA using electron microscopy. The authors, however, noted that the spindles were located in the medial-oblique portion of the PCA and not the horizontal belly as was reported by Sanders and colleagues (1993). Although identification of spindles was not a major goal in their study, Brandon and colleagues (2003a) were unable to

[1] Muscle spindles are stretch receptors that provide afferent information to the central nervous system regarding muscle stretch and are essential in reflex activity, load compensation, motor control, and muscle kinesthesia. These sensory organs contain intrafusal fibers which are associated with a unique MHC isoform. Intrafusal fibers are classified into three categories, bag_1 which contain high levels of tonic MHC, bag_2 which contain neonatal myosin, and chain fibers which also contain neonatal myosin (Rowlerson, Mascarello, Barker, & Saed, 1988). These fibers are encapsulated in connective tissue and are innervated by both motor and sensory afferent nerves. Spindles can either provide "static sensitivity" about the postural activity and resting length of the muscle or "dynamic sensitivity" about the rate of shortening during contraction (Bakker & Richmond, 1981).

confirm the presence of muscle spindles in the PCA using neonatal and tonic MHC antibody staining.

Although muscle spindle presence in each subcompartment is debatable, reports of nerve supply to the PCA indicate that each muscle belly has its own distinct innervation and is, therefore, thought to be controlled separately by the central nervous system (Sanders et al., 1994). There are two branches of the vagus nerve that innervate the intrinsic laryngeal muscles; the superior laryngeal nerve and the recurrent laryngeal nerve. Traditionally, the PCA muscle was thought to be innervated by a single branch of the recurrent laryngeal nerve. Sanders and colleagues (1994), however, reported that in the majority of specimens they studied (9 of 15), the vertical and horizontal compartments were innervated by separate, distinct branches of the recurrent laryngeal nerve without any overlap. The results of this study support the notion that the two muscle bellies have different functions; however, more research is needed to confirm the roles of individual subcompartment function.

Analysis of the histologic and morphologic properties of the PCA muscle is the final method that has been used to differentiate subcompartments. Understanding the histologic and metabolic properties of the muscle will help to determine if these subcompartments function in different capacities. For example, if the horizontal belly contains a substantially higher proportion of type I fibers to type II fibers compared to the vertical belly then it can be inferred that the horizontal belly is probably involved in more low intensity, longer duration tasks (specifics of which will be described in the "General Human Skeletal Muscle" subsection). Differences in the histologic and metabolic properties between the two subcompartments can also help to determine how biochemical changes with a disorder or pathology can affect the functioning of each subcompartment.

A number of animal studies have been conducted since the 1960s to determine fiber type composition, distribution, and metabolic capacity of the intrinsic laryngeal muscles, as well as the presence of muscle spindles and other unique features. These studies include but are not limited to horse (Lopez-Plana, Sautet, Ruberte, & Sabate, 1994), cat (Yokoyama, Nonaka, & Mori, 1995), rat (Wu, Baker, Crumley, & Caiozzo, 2000), sheep (Happak, Zrunek, Pechmann, & Streinzer, 1989), and dogs, which have been the most widely studied animal (Braund, Steiss, Marshall, Mehta, & Amling, 1988; Sanders et al., 1993). Biomechanical studies of laryngeal muscles use dog models most often because the laryngeal structures of the dog are similar in size and proportion to human laryngeal muscles, more so than any other laboratory species available (Berke & Gerratt, 1993; Ludlow, Bielamowicz, Daniels Rosenberg, Ambalavanar et al., 2000). To appreciate the biologic properties of the PCA muscle, however, it is important to understand the basic histology of human skeletal muscle.

2.1.3. General Human Skeletal Muscle Histology

Skeletal muscle fibers are composed of striated myofibrils that contain a specific organization of contractile proteins arranged within the sarcomere (Schiaffino & Reggiani, 1996). Each myofibrillar component contains multiple myofibrillar protein isoforms. These isoforms are proteins with homologous functions and/or sequences but are usually the product of alternative splicing (i.e., generation of multiple protein isoforms from a single gene). The contractile properties of skeletal muscle fibers are determined primarily by this variability in myofibrillar protein isoform expression. The main components found within the sarcomere are (1) thin filaments, (2) Z-disks, and (3) thick filaments. Nebulin and titin are also found within the sarcomere. Nebulin is a protein associated with myosin and titin is a protein associated with actin molecules.

The thin filament participates in muscle contraction secondary to the actions of its constituents: actin, troponin, and tropomyosin. Descriptions of Z-disks, nebulin, and titin are beyond the scope of this paper. The remainder of this section will focus on components of the thick filaments, especially myosin, and their relationship to muscle contraction.

Myosin is a family of motor proteins that are part of the thick filaments in the sarcomere. Each myosin molecule is composed of a motor domain that consists of two heads which are sites for adenosine triphosphate (ATP) and actin-binding during the contraction process (Figure 1). The myosin head is connected to a long tail by a region of the molecule called the neck. Two essential myosin light chains (MLCs) and two regulatory MLCs are found on the globular portion of the molecule. Essential MLCs are located closer to the motor domain of the molecule than the regulatory MLCs. During muscle contraction it was previously thought that MLCs did not regulate the physiological processes of muscle contraction in humans (Barany, 1967). Recent research, however, has reported that MLC isoforms have the capacity to modify or regulate unusually fast unloaded shortening velocities in human masseter muscle (Sciote, Horton, Rowlerson, & Link, 2003) and fast type IIA fibers in human thyroarytenoid muscle (D'Antona, Megighian, Bortlotto, Pellegrino, Ragona et al., 2002). Myosin molecules also contain two myosin heavy chains (MHCs) that reside at the level of the tail. These MHCs intertwine to give the tail its α-helical coiled-coil structure (Figure 1).

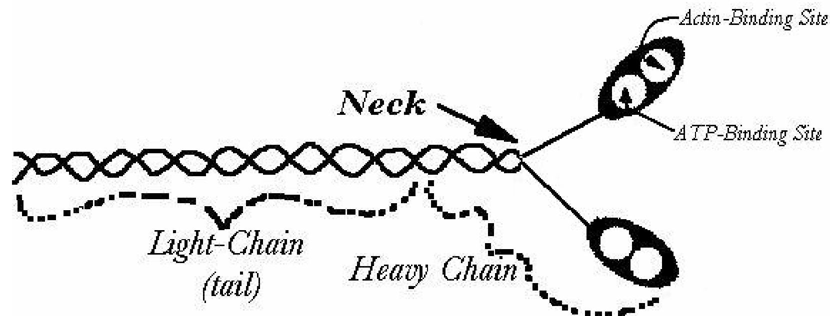

Figure 1. Myosin Molecule

A myosin heavy chain is a multigene family that consists of MHC isoforms which are all encoded by separate genes (Schiaffino & Reggiani, 1996). There are a number of types of MHC isoforms found in mammalian skeletal muscle (i.e., I, IIA, IIB, and IIX, IIM, α, perinatal, and embryonic). These isoforms are the principal regulators of contraction speed in muscle and are expressed in different muscle fibers (Barany, 1967). When fiber type classifications were organized and accepted by their contraction speed, they were called either fast-twitch or slow-twitch (Barnard, Edgerton, Furukawa, & Peter, 1971). Nomenclature, however, has changed over the years to accommodate subdivisions in fiber types. Currently, mammalian skeletal muscle fibers are divided into two subtypes: slow-contracting type I fibers and fast-contracting type II fibers with type II fibers further subdivided into types IIA, IIX, and IIB (Gorza, 1990).

An important distinction for larger mammals, including humans, is that the IIB fiber type (found in small mammals) is not present (Smerdu, Karsch-Mizrachi, Campione, Leinwand, & Schiaffino, 1994). On a continuum for fast contraction speed and increasing fatigability, the type

II fibers are displayed IIA>IIX>IIB; type IIB fibers being the fastest contracting and most highly fatigable of the fast fiber types (Larson, Edström, Lindegren, Gorza, & Schiaffino, 1991). The tension cost for type IIB fibers (i.e., the total amount of ATP that must be used per unit force); however, is dramatically higher than the tension cost for IIA and IIX fiber types (Stienen, Kiers, Bottinelli, & Reggiani, 1996). During evolution to conserve energy large mammals have probably lost the IIB fiber type. In adult human skeletal muscle, fiber types I, IIA, and IIX, therefore, predominate but vary with differences in individual muscle function (Schiaffino & Reggiani, 1996).

MHC isoforms can be used to distinguish different fiber types in skeletal muscle (e.g., presence of a IIA MHC isoform would indicate a IIA fiber type) (Gorza, 1990; Schiaffino, Gorza, Ausoni, Bottinelli, Reggiani et al., 1990). Typically, fiber type represents, physiologically, the functioning of skeletal muscle fibers (i.e., slow or fast-contracting), as well as the metabolic property (i.e., oxidative or glycolytic) of the fibers. Specifics of cellular metabolism will be elaborated in section two. Characterization of skeletal muscle fibers, therefore, runs along a continuum from slow-contracting, fatigue resistant to fast-contracting, fatigable (Brooks, Fahey, White, & Baldwin, 2000; Colliander, Dudley, & Tesch, 1988).

Type I fibers are slow-contracting, fatigue resistant. They contain a large number of mitochondria and are associated with a large capillary network (Kubínová, Janáček, Ribarič, Čebašek, & Eržen, 2001; Leary, Lyons, Rosenberger, Ballantyne, Stillman et al., 2003). Their time to peak tension is long; therefore, during muscle function type I fibers are responsible for low intensity, repetitive tasks (e.g., walking or talking). Type IIA fibers are fast-contracting, fatigue resistant. These fibers take a shorter amount of time than type I fibers to reach peak tension, have a smaller number of mitochondria, and have a less dense capillary network

(Kubínová et al., 2001; Leary et al., 2003). The composition of IIA fibers, therefore, makes them ideal for higher intensity tasks that need to be sustained for longer durations (e.g., running or singing). Muscle fibers during slow to modestly fast contractions do not require an extremely rapid production of energy because their ATP splitting rate is slow and, therefore, take more time to consume ATP during contraction (Bottinelli, Canepari, Reggiani, & Stienen, 1994). These fibers, in turn, rely on the citric acid cycle and oxidative phosphorylation to produce relatively large amounts of ATP. This metabolic pathway avoids lactic acid build up and provides adequate energy for sustained excitation – contraction coupling (Figure 4).

Type IIX fibers are the fastest and most fatigable fibers in human skeletal muscle. They have the least mitochondria, as well as the smallest capillary network (Kubínová et al., 2001; Leary et al., 2003). Tasks (e.g., sprinting or screaming) that depend on the highest intensity over the shortest duration will recruit these fiber types due to their short time to peak tension. During rapid contraction the ATP splitting rate is fast; therefore, type IIX muscle fibers use the glycolytic pathway because ATP production must also be rapid (Bottinelli et al., 1994). This process quickly produces a limited amount of ATP for energy utilization along with the additional metabolic by-product, lactic acid. With rapid contraction at maximal force levels, therefore, ATP stores diminish rapidly, and lactic acid and acidic pH levels increase which may contribute to muscle fiber fatigue.

Figure 2 provides a clear depiction of the association of fiber type composition to task or exercise. Muscle taken from the marathoner (panel b) shows a larger amount of type I (pale) fibers compared to muscle taken from the high jumper (panel a). The fibers in the leg muscle of the marathoner have adapted to the endurance type of exercise performed by the marathoner over time. The type I fibers have a slower rise to peak tension and will therefore fatigue less quickly

allowing the marathoner to run for long amounts of time. The high jumper needs short bursts of energy and this is afforded by the larger number of type II (dark) fibers. Although skeletal muscle fiber type characterizations appear straightforward, there are some skeletal muscle fibers that contain a more dynamic isoform composition. These muscle fibers are called hybrid fibers.

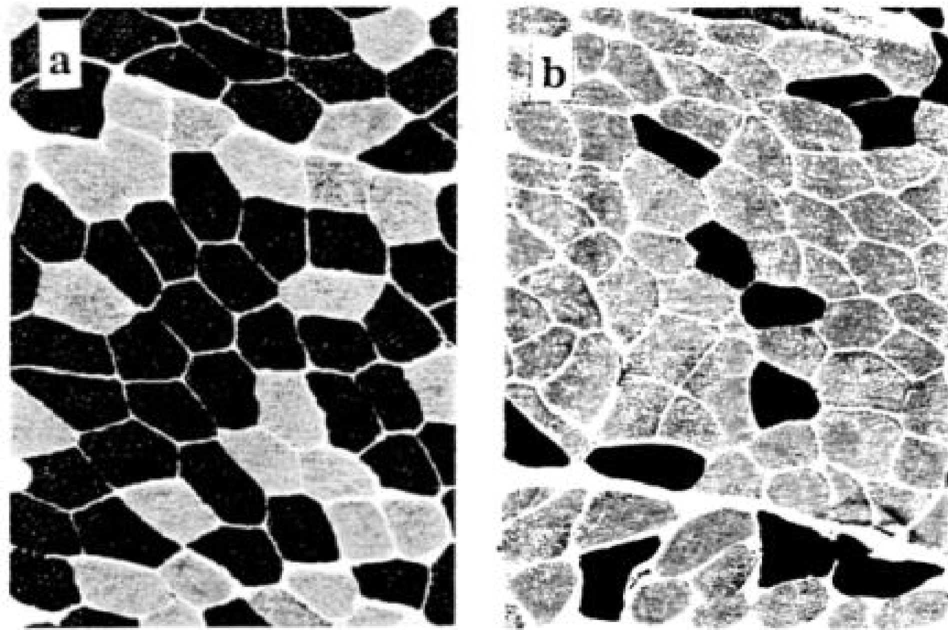

Figure 2. Differences in Fiber Type Composition through Exercise

Represents the difference in fiber type composition seen in the vastus lateralis from (**A**) a high jumper and (**B**) a marathon runner. Dark fibers indicate type II fibers. [Taken from Jones & Round, 1990]

Hybrid fibers that show coexpression of MHC isoforms also exist in human skeletal muscle (Billeter, Weber, Lutz, Howald, Eppenberger et al., 1980; Brooke & Kaiser, 1970). Once thought to be an exception, hybrid fibers that contain different myofibrillar proteins have been noted throughout muscle development (DeNardi, Ausoni, Moretti, Gorza, Velleca et al., 1989) and during physical training (Campos, Luecke, Wendeln, Toma, Hagerman et al., 2002) (Figure

3). These hybrid fibers follow specific patterns of coexpression. Myosin transitions and/or coexpressions usually adhere to a specific continuum: I > I + IIA > IIA > IIA + IIX > IIX (DeNardi et al., 1989; Schiaffino et al., 1990). Coexpressions of type I and IIX MHC isoforms would, therefore, be exceedingly rare. Recent research, however, has identified the I-IIX fiber type in human intrinsic laryngeal muscles (Brandon, Rosen, Georgelis, Horton, Mooney et al., 2003b; Wu et al., 2000). The frequent occurrence of these hybrid fibers supports the notion that there is a dynamic continuum of skeletal muscle performance from isoform diversity to functional diversity (Schiaffino & Reggiani, 1996). Future classifications of fiber types may become more genomically-based due to the increasing number of fibers containing variations in isoform expression (Spangenburg & Booth, 2001; Leary et al., 2003).

Motor Unit Type	**Slow**	**Fast**		
Axon Conduction Velocity	slow	fast		
Recruitment order	early	middle to late		
Tension	small	large		
Activity Pattern	tonic	phasic		
Fatigue Resistance	high	high	low	intermediate
Muscle Fiber Types	**I**	**IIA**	**IIX**	**IIC**
ATPase after:				
Alkaline preincubation	○	●	●	●
Acid (pH 4.6) preincubation	●	○	◍	●
Acid (pH 4.3) preincubation	●	○	○	◒
Antibody Reaction:				
Anti-Type I	●	○	○	●
Anti-Fast Type	○	●	●	●
Anti-Type IIA	○	●	○	●
Metabolic Capacity				
Oxidative	●	◐	○	◉
Glycolytic	○	◐	●	◍

Figure 3. Motor Unit and Muscle Fiber Types

Chart depicts the motoneuron, motor unit and muscle fiber types typically seen in human skeletal muscle. IIC indicates the hybrid fiber (I-IIA coexpression). ● indicates high stain; ◉ indicates moderate stain; ◍ indicates low stain; ◒ indicates that sample can either stain low or moderate; ◐ indicates that stain can appear low, moderate, or high.

2.1.4. Histology and Morphology of Intrinsic Laryngeal Muscles

Most of the studies on the physiologic properties of intrinsic laryngeal muscles have not addressed basic biologic, histologic, and metabolic properties of the muscles. A comprehensive biologic view of human intrinsic laryngeal muscle is necessary to understand the functioning of normal voice and pathologic voice (Brandon et al., 2003a). Differentiation and distribution of fiber types can allow inferences to be made about differences in muscle function. For, example, if a muscle contains a high percentage of type I fibers, then it can be inferred that the muscle is used in tasks of prolonged duration and is probably less prone to fatigue. If a muscle contains a high percentage of type IIX fibers, then the opposite can be assumed; the muscle is probably responsible for rapid, high intensity activities and will likely fatigue. Biologic information can also help to distinguish the functioning of different muscle bellies within a single muscle (e.g., the two compartments of the PCA). Knowledge about the histochemical processes of the muscle also makes it possible to infer relationships between muscle function, training, endurance, and fatigue. This section will review the current biologic view of the intrinsic laryngeal muscles pertaining to the specifics about each individual muscle.

2.1.4.1. Animal Models

Braund and colleagues (1988) conducted one of the first fiber typing studies on intrinsic laryngeal muscle taken from canines. The authors performed hemotoxylin and eosin staining as well as an ATP-ase histochemical analysis on the cricothyroid, cricoarytenoid lateralis (LCA), and the cricoarytenoid dorsalis (PCA) of five dogs. The histochemical stain was completed after preincubation of the sections in pHs of 10.2 and 4.3. Results indicated that there was a high percentage of type II fibers compared to type I fibers in all muscles. Sanders and colleagues (1993) attempted to identify the fiber type composition of the PCA in five dogs after

preincubation in pHs of 9.4. and 4.3. Results were different from those reported by Braund and colleagues (1988). A high percentage of type I fibers were reported from this ATP-ase histochemical analysis. ATP-ase histochemistry identifies fiber type based on the isoform's distinct reactivity to ATP which varies based on intracellular pH levels. The original histochemical protocol for determining fiber types in humans after preincubation in pH buffers of different levels was developed by Brooke and Kaiser (1970). The pH levels normally used to identify fiber types is 10.2 (alkali pH) reactive for both fast fiber types (IIA and IIX), 4.3 (acidic pH) reactive for type I, and 4.6 used to distinguish all three fiber types (see Figure 3).

The discrepancy in the results for the PCA muscle reported by Braund and colleagues (1988) and Sanders and colleagues (1993) may be due to the difference in pH buffers. The lower pH used by Sanders and colleagues (i.e., 9.4) may have also stained some of the type II fibers as well as type I fibers because it was slightly more acidic than 10.2. The inclusion of type II fibers may have artificially increased the total number of type I fibers in the PCA.

A study conducted on rat PCA and TA muscle concurred with the results by Braund and colleagues (1988) reporting a larger percentage of fast type II fibers than type I fibers (DelGaudio, Sciote, Carroll, & Escalmado, 1995). These results were determined using sodium dodecyl sulfate polyacrylamide gel eletrophoresis (SDS-PAGE), myofibrillar ATP-ase histochemical stain, and an immunohistochemical stain. The authors reported that the TA had a small amount of type IIA fibers and a large portion of type I fibers. An additional MHC isoform was also observed after protein separation using (SDS-PAGE). DelGaudio and colleagues (1998) called this MHC isoform, IIL, based on its presence in laryngeal muscle, but with the additional caveat that this band comigrated with the protein species known as extraocular MHC in rat rectus muscle. Other laboratories have attempted to identify the presence of a novel MHC isoform in

human laryngeal muscle, however, results have been variable (D'Antona et al., 2002; Shiotani, Westra, & Flint, 1999; Périé, Agbulut, St Guily, & Butler-Browne, 2000; Wu et al., 2000).

Han and colleagues (1999) have recently attempted to identify the presence of a rare muscle fiber type in human thyroarytenoid muscle, called slow tonic fiber (Han, Wang, Fischman, Biller, & Sanders, 1999). The rationale was that if human TA contained this fiber type, not found in other mammals, then maybe the slow tonic fibers would imply a specialization for speech. Han and colleagues (1999) performed immunohistochemical staining which uses antibodies to identify the MHC isoform composition of muscle fibers. The authors utilized particular myosin antibodies that stained for slow tonic fibers, ALD-19, or slow tonic and type I fibers, ALD-58. A high percentage of slow tonic fibers was found in the TA muscle.

Recent research on the TA muscle, however, has not supported the results by Han and colleagues (1999) (Brandon et al., 2003b). In the study by Brandon and colleagues, a tonic MHC specific antibody was used to determine the presence of bag_1 fibers in muscle spindles. This antibody would also identify slow tonic fibers in the muscle. There was no evidence of muscle spindles and only rarely did the authors note small, isolated tonic fibers in the TA. Due to the controversy surrounding the presence of a novel MHC isoform in laryngeal muscles and the inconclusive evidence of slow tonic fibers in the TA, human laryngeal muscle, therefore, is currently thought to only express the typical MHC isoforms found in limb skeletal muscle, types I, IIA, and IIX.

2.1.4.2. Research on Human intrinsic Laryngeal Muscle Histology

There are several studies that have evaluated the fiber type composition and distribution of *human* intrinsic laryngeal muscle. The majority of the studies have concentrated on PCA and TA. The lateral cricoarytenoid and the IA have received the least attention in the literature. A

comparison study of human and sheep PCA and TA was conducted using hemotoxylin and eosin staining to determine morphology and ATP-ase histochemistry at pH buffers of 10.4 and 4.3 to identify fiber types (Happak et al., 1989). Results indicated that fiber diameters in PCA and TA were larger in human males than females. The PCA muscle contained a high percentage of type I fibers (65%) in humans. Langkau and colleagues (1993) also noted that the human PCA muscle contained a high percentage of type I fibers (63.3 ±12.7%), more than TA, LCA, or CT (Langkau, Martin, & Klingholz, 1993). The authors indicated, however, that the fiber diameter of PCA muscle fibers is similar to the fiber diameter of the other muscles.

Shiotani and colleagues (1999) used SDS-PAGE and a Western Blot Analysis to determine the fiber type composition and distribution of the PCA. Results from this study were similar to those reported by Happak and colleagues (1989) in that a high percentage of type I fibers were found in the PCA. The authors also noted a small percentage of type IIB fibers. As stated previously, the IIB fiber type does not appear in humans, therefore, the authors were probably noting the IIX MHC isoform. One criticism of all of these studies is that no differentiation was made between oblique and horizontal muscle bellies. This information is necessary to further differentiate subcompartments in terms of function.

Wu and colleagues (2000) also performed SDS-PAGE and Western Blot on the PCA. Instead of a whole muscle analysis, the authors dissected single fibers (~40) from the muscles and performed the analyses on the single fibers. Wu and colleagues separated the muscle into two bellies, oblique and horizontal, and identified fiber types specific to each belly. The horizontal belly had a higher percentage of type I fibers (70%) than IIA fibers (10%) and also showed hybrid coexpression of types I and IIA. The oblique PCA, however, had a higher percentage of type IIA than type I fibers though the percentage of type I fibers was higher than

type IIX. There was a discrepancy between the written text and one of the graphs in the article. The text stated that the percentage of IIA fiber types was higher in the oblique belly but the graph showed a higher percentage of type I fibers. Clarification of this discrepancy is needed.

Brandon and colleagues (2003a) stated that problems exist when using single fiber analyses. The authors indicated that results from those analyses may be biased to larger diameter fibers because they are easier to dissect and are, therefore, more frequently extracted from the muscle. Another limitation to the single fiber analysis conducted by Wu and colleagues (2000) is that the authors cannot confirm that they are actually dissecting single fibers because the muscle fibers in the laryngeal muscles are so small. What appears to be a coexpression of fiber types, therefore, may actually be more than one single fiber dissected from the muscle section. Brandon and colleagues suggested using immunohistochemical and ATP-ase histochemical analyses because these analyses provide a complete representation of the muscle fibers that are present along the entire length of the muscle.

Brandon and colleagues (2003a) used ATP-ase histochemistry at pH buffers of 10.2, 4.6, and 4.3 and immunohistochemical analyses to determine fiber type composition of the vertical (i.e., oblique) and horizontal bellies of the PCA. Results indicated that the vertical belly had almost equal amounts of type I (45%) and type IIA fibers (55%). The horizontal belly had a substantially higher amount of type I (80%) than type II fibers (20%). These results are in line with those from Wu and colleagues (2000). Occasionally, exceedingly rare hybrid coexpressions of type I and IIX fibers were found in the vertical belly. The authors also reported coexpressions of I and IIA fiber types in the horizontal belly.

A recent study used a new primary monospecific monoclonal antibody (i.e., 6H1) to identify the IIX MHC isoforms in human PCA, TA, CT, and LCA muscles (Li, Lehar,

Nakagawa, Hoh, & Flint, 2004). Although fiber types I, IIA, and IIX were identified in TA, CT, and LCA, the IIX isoform was not found in the PCA or CT muscles. The authors indicated that expression of the IIX MHC isoform was therefore associated with the muscles that close the glottis. One limitation to this study is that the authors did not differentiate fiber type distribution by subcompartment in the PCA. This limitation causes difficulty with the authors' interpretation of the data especially because the medial-horizontal compartment of the PCA is thought to participate in vocal fold phonation and not abduction. Clear differentiation of subcompartments is needed in future studies.

The fiber type composition and distribution of human thyroarytenoid (TA) muscle has also been studied. Happak and colleagues (1989) indicated that both humans and sheep had a higher concentration of type II fiber than type I with a ratio of approximately 80:20. From these results, the authors concluded that the TA is probably a faster contracting muscle than the PCA. Shiotani and colleagues (1999) noted a higher percentage of type IIB fibers than type I fibers in the LCA and TA muscles of humans with the TA having an even greater number than LCA muscle. Through SDS-PAGE and Western Blot of single fibers, Wu and colleagues (2000) reported that the medial aspect of the TA has a high percentage of type IIA and IIX fibers while the horizontal aspect has a high percentage of type IIA fibers and even percentages of type I and IIX fibers. These results indicated that TA muscle fibers are able to contract at a rapid pace but are fairly resistant to fatigue.

Brandon and colleagues' (2003b) study was also designed to allow for identification of the fiber types in the human thyroarytenoid muscle. Their main goal, however, was to determine the presence of muscle spindles in the TA. The authors conducted an immunohistochemical analysis designed to look for neonatal and tonic MHC antibody reactivity in the intrafusal fibers

of muscle spindles. As reported previously, the authors were unable to identify muscle spindles in the TA; however, they did identify rare, isolated neonatal and tonic fibers within the muscle. Results also indicated the presence of types I, IIA, and IIX fibers in the TA with the highest percentage of fibers being type IIA. Coexpression of I and IIX fiber types, as well as coexpression of neonatal MHC with I and IIA and occasionally I and IIX fiber types were noted. A similar study confirmed the coexpression of fibers in the TA as well as the high percentage of IIA fibers (D'Antona et al., 2002)

Sciote and colleagues (2002) were among the first to conduct a physiologic study on the contraction speed of human intrinsic laryngeal muscles (Sciote, Morris, Brandon, Horton, & Rosen, 2002). The purpose of the study was to identify a structure-function relationship between the myosin isoform content in the PCA and TA muscles and the contraction speed measured through unloaded shortening velocity. In previous animal studies on cats and dogs, TA muscle showed a faster contraction speed (6.5 to 14 ms) than PCA muscle (22 to 40 ms) (Hirose, Ushijima, Kobayashi, & Sawashima, 1969; Martensson & Sklogund, 1964). Sciote and colleagues (2002) performed SDS-PAGE and immunoblotting on single fibers to determine the MHC isoform content in the PCA and TA and then conducted a slack test to identify the unloaded shortening velocity of the fibers. Results indicated that individual MHC isoforms (I, IIA, and IIX) had similar contraction speeds to those found in limb skeletal muscle. Some of the coexpressed, hybrid fibers in the PCA and TA, however, had unloaded shortening velocities that were sometimes twice as fast as limb skeletal muscle.

A study conducted simultaneously to work by Sciote and colleagues (2002), but in different laboratories, assessed the unloaded shortening velocity of 131 individual muscle fibers types taken from human TA muscle (D'Antona et al., 2002). Results were similar to those from

Sciote and colleagues (2002) in that specific MHC isoforms showed comparable contraction speeds to their limb skeletal muscle counterparts. The results also indicated that hybrid fibers coexpressing MHC isoforms sometimes showed extremely rapid unloaded shortening velocities similar to what was found in Sciote and colleagues' study (2002). Rapid contraction of hybrid fibers in the TA and PCA appear to be one characteristic that set the laryngeal muscles apart from limb skeletal muscle.

Studies on cricothyroid and interarytenoid muscles have been reported infrequently in the literature. Happak and colleagues (1989) reported that the CT muscle from sheep showed a higher percentage of type I fibers (~58%) compared to humans (43%). Results from another study concluded that the CT muscle has a high percentage of type I fibers but not as high as the percentage of type I fibers in the PCA (Shiotani et al., 1999).

Tellis and colleagues (2004) is the only study to date conducted on the interarytenoid muscle using immunohistochemical and histochemical analyses (Tellis, Rosen, Thekdi, & Sciote, 2004). Shiotani and colleagues (1999) determined that the IA contained fiber types I, IIA, and IIB with a higher percentage of type I than II using SDS-PAGE and Western Blot; however, the results are questionable because the IIB fiber type does not appear in humans. Tellis and colleagues reported that interarytenoid muscle was phenotypically similar to limb skeletal muscle with larger diameter fibers than the PCA and TA. The IA also contained a higher combined proportion of type II fibers (~60%) than type I fibers (~35%), with type IIA fibers in the greatest proportion (~45%). Different from PCA and TA, the interarytenoid muscle did not contain an abundance of myosin coexpression, the IA muscle only occasionally coexpressed type I and IIX fibers (which is rare), the IA did not contain unusual MHC isoforms (e.g., neonatal, α-cardiac), and the IA showed a substantial presence of muscle spindles.

In summary, there have been several animal studies since the mid 1900s that have attempted to identify the morphologic and histochemical properties of the intrinsic laryngeal muscles. Investigations of these properties in humans began later, but are still increasing. All intrinsic laryngeal muscles contain similar MHC isoforms to the ones found in human limb skeletal muscle (types I, IIA, IIX). Most of the research in humans has focused on PCA and TA muscles. Overall, the TA has been found to be faster-contracting, contain a higher proportion of type II fibers, and show coexpressions of myosins. The posterior cricoarytenoid muscle is slow-contracting and contains more type I fibers than the TA. Some novel MHC isoforms (e.g., neonatal and tonic) are contained in small amounts in the PCA and TA. The cricothyroid and interarytenoids are the least studied muscles of the intrinsic laryngeal muscle group. The cricothyroid has a high percentage of type I fibers but not as high as the PCA. The histology and morphology of the IA muscle is similar to limb skeletal muscle and differs from PCA and TA in several ways. IA fiber diameter is larger than in PCA and TA, the IA contains a large portion of IIA fibers and seldomly expresses hybrid fiber types and novel MHC isoforms. Finally, unloaded shortening velocity for specific MHC isoforms in human intrinsic laryngeal muscles is comparable to velocities for limb skeletal muscle; however, hybrid fibers with MHC coexpression in laryngeal muscles appear to have faster contraction speeds than found in limb muscle.

Research in the area of human intrinsic laryngeal muscle is far from complete. More morphologic and histologic investigations are needed with larger patient samples so that statistical analyses can be conducted to assess whether there is a significant difference between fiber type composition, distribution, and overall morphology in normal and pathologic larynges. Future work is also needed on structure-to-function relationships in these muscles. Continuation

of biochemical research will hopefully begin to bridge the gap between the biologic processes of the intrinsic laryngeal muscles and clinical diagnosis and treatment of voice disorders related to laryngeal muscle function.

3. CHAPTER III

3.1. Metabolic Capacity of Intrinsic Laryngeal Muscles

3.1.1. Overview

Vocal fatigue is often a symptom expressed by individuals with muscle tension voice disorders, vocal fold paresis, vocal fold paralysis, functional voice disorders, and even some hyperfunctional voice disorders (Koufman & Isaacson, 1991). Traditionally it has been thought that vocal fatigue is more a symptom, a "feeling," rather than a biological occurrence in the laryngeal muscles that can be clinically recorded. This view persists today because there is currently no objective, noninvasive way to measure muscle fatigue in the larynx. Furthermore, previous literature has indicated that intrinsic laryngeal muscles are fatigue resistant (Sataloff, 1998; Welham & Maclagan, 2002). The biologic fatigue resistant properties (i.e., metabolic capacity) of the intrinsic laryngeal muscles, however, are not known. It is, therefore, necessary to determine biochemically if the intrinsic laryngeal muscles have the capacity to fatigue.

Current histologic research in the interarytenoid, thyroarytenoid, and posterior cricoarytenoid muscles has indicated that these muscles contain similar fiber phenotypes to human limb skeletal muscle (i.e., I, IIA, and IIX) (Brandon et al., 2003ab; Tellis et al., 2004). A recent study on the interarytenoid muscle has shown a high glycolytic capacity indicating that if the interarytenoid muscle is recruited past maximal force and fiber fatigue levels, it could fatigue (Tellis et al., 2004). Further research is needed to determine the metabolic capacity of the intrinsic laryngeal muscles. This continued research will provide the foundation for fatigability measures in these muscles and facilitate the development of a noninvasive method to measure muscle fatigue in the larynx.

3.1.2. General Limb Skeletal Muscle Metabolism

When activated, all skeletal muscle fibers individually work to maintain an appropriate amount of energy for the muscle to perform an action. Fuels for contraction include adenosine triphosphate (ATP) and phosocreatine. ATP is an adenine nucleotide bound to three phosphates. A large amount of energy is located between the second and third phosphate groups. This energy can be used in many different chemical reactions.

There are two main metabolic pathways in all muscle fibers: glycolysis and oxidative phosphorylation; though which process predominates depends on the metabolic capacity of the fiber (e.g., type I fibers rely more on oxidative metabolism) (Schiaffino & Reggiani, 1996).

A brief summary of the metabolic process of muscle fibers is outlined below starting with the initiation of muscle contraction (Schiaffino & Reggiani, 1996).

1. Muscle begins activation.
2. ATP already present in the muscle fiber is consumed for force, generation of movement, and ionic transport.
3. Creatine phosphokinase yields creatine and inorganic phosphate (P_i) from the hydrolysis of creatine phosphate.
4. ATP is immediately replenished by joining ADP and P_i through the process of glycolysis. This occurs first within seconds of creatine phosphate consumption.
5. Oxidative phosphorylation occurs in the mitochondria within minutes once the oxygen supply increases to the muscle. This process produces large amounts of ATP (12 times more than glycolysis) but is slower than glycolysis.
6. ATP produced by glycolysis and oxidative phosphorylation resynthesizes the creatine phosphate consumed during creatine phosphokinase.
7. Muscle fibers can supply needed ATP as long as the muscle does not exceed maximal force capacity.

The following paragraphs will provide more detail about each stage of the metabolic process.

Muscle fibers store more cellular energy in the form of creatine phosphate (i.e., phosphocreatine) than ATP (Brooks et al., 2000). This is because creatine phosphate can be used

immediately to regenerate ATP at the initiation of contraction through the hydrolysis of phosphocreatine which yields creatine and inorganic phosphate (P_i). This process supplies the muscle fiber with P_i that will be used to create ATP during glycolysis.

The hydrolysis of phosphocreatine is invaluable to muscle fibers because at rest the ATP stores in muscle fibers are low. Muscles use existing ATP as an immediate source of energy. Minimal muscle activity, however, is needed to change the balance in the muscle fiber to allow the hydrolysis of phosphocreatine to begin. The inorganic phosphate yielded from the hydrolysis of phosphocreatine is used to create ATP from glycolysis. ATP stores are quickly replenished and the muscle does not have to use too much of its already low store of ATP at the initiation of exercise (Cain & Davies, 1962).

3.1.2.1. Glycolysis

The body does not store large amounts of oxygen; therefore, the oxygen transport system does not immediately provide needed oxygen to skeletal muscles at the onset of exercise (Willmore & Costill, 1994). Several minutes elapse before oxygen consumption has reached a steady state for oxidative phosphorylation to occur especially since contraction precludes arterial blood supply. Consequently, the muscle is in a state of oxygen debt from the point of rest to initiation of muscle contraction. Glycolysis produces the ATP needed to maintain contraction before oxidative phosphorylation begins.

Glycolysis occurs in the cytosol and has traditionally been thought of as either aerobic (i.e., with oxygen) or anaerobic (i.e., without oxygen) (Brooks et al., 2000). A common misconception is to associate these two forms of glycolysis with the presence or absence of oxygen because glycolysis actually always occurs without oxygen. The terms aerobic and anaerobic identify the byproducts of glycolysis not the presence of oxygen and do not necessarily

occur separately during muscle metabolism. In both types of glycolysis, ADP is joined with inorganic phosphate (P_i) to form ATP. Aerobic glycolysis is slow, produces pyruvic acid, and releases more energy. A small amount of ATP is initially formed in the cytosol. Additional ATP can be produced in the mitochondria if pyruvic acid enters the mitochondria (i.e., place where oxidative phosphorylation occurs) (Figure 4). If oxygen is present, pyruvic acid within the mitochondria then participates in the Krebs Cycle (i.e., Citric Acid Cycle) producing more ATP.

Anaerobic glycolysis occurs quickly and produces a small amount of ATP in the cytosol. This type of glycolysis also produces high amounts of lactic acid. In muscle fiber types that do not contain a large amount of mitochondria (e.g., type IIX fibers), pyruvic acid does not enter the mitochondria and is subsequently reduced to lactic acid. The accumulation of lactic acid can ultimately contribute to muscle fatigue.

3.1.2.2. Oxidative Phosphorylation

The last stage of cellular metabolism is called oxidative phosphorylation. As with most subjects in biochemistry, oxidative phosphorylation has a vast, complex literature base (Balaban, 1990; Boyer, Chance, Ernster, Mitchell, Racker et al., 1977; Korzeniewski, 2001; Senior, 1988).

Oxidative phosphorylation relies on the kinetic interactions between ATP, ADP (adenosine diphosphate), P_i, proton electrochemical gradient (Δp), NADH (nicotinamide adenine dinucleotide), oxygen, and pH (Balaban, 1990). Some of these interactions occur within the mitochondria and some occur between the mitochondria and the cytosol in the cell. Part of the oxidative process involves the introduction of pyruvate to the mitochondria via aerobic glycolysis. Pyruvate contributes to the production of ATP through the Krebs Cycle. Balaban (1990) separated the other control sites of oxidative phosphorylation into three main categories: (1) introduction of nicotinamide adenine dinucleotide (NAD^+) and flavin-adenine dinucleotide

(FAD^+) into the cytochrome chain in their reduced forms, NADH and FADH. These enzymes carry electrons and hydrogen ions into the mitochondria, (2) synthesis of ATP into ADP and P_i, and (3) the transformation of oxygen and reduced cytochrome aa_3 (i.e., cytochrome c) into water and carbon dioxide. Figure 4 provides brief schematic illustrating how glycolysis contributes to oxidative phosphorylation, as well as how ATP hydrolysis in the cytosol breaks down to form the ADP and P_i eventually used to produce ATP in the mitochondria.

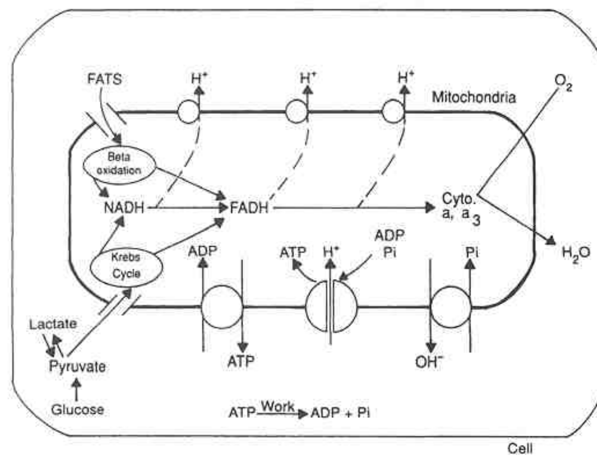

Figure 4. Oxidative Phosphorylation

Brief schematic of oxidative phosphorylation in the cell. [From Balaban (1990)]

Cytosolic ATP is hydrolyzed into ADP and P_i. ADP and phosphate carriers transport ADP and P_i into the mitochondria (Figure 4). Once in the mitochondria, ADP and P_i are resynthesized back into ATP which is then released back into the cytosol via an ATP carrier. The rate of ATP hydrolysis and cellular work relies in part on the concentration of ADP and P_i in the cytosol and in part on the redox state of NAD^+ and oxygen delivery (Balaban, 1990). The energy used to make ATP in the mitochondria comes in the form of NADH and FADH, enzymes that

are reduced through other metabolic processes fueled by fats, lactate, and other metabolic substrates. NADH and FADH transport the energy to the mitochondrial respiratory chain. An increase in NADH and FADH, therefore, increases the rate of ATP hydrolysis and the rate of overall oxidative phosphorylation. All of these processes occur in cells during oxidative metabolism; however, they cannot occur without an adequate concentration of oxygen.

The maximal capacity of a muscle to use oxygen is called oxidative capacity (Willmore & Costill, 1994). Adequate concentrations of oxygen in the muscle are necessary for all stages of oxidative phosphorylation. Oxygen is consumed by the mitochondria in a process called oxygen consumption (Balaban, 1990). Part of the process of oxygen consumption is that cytochrome aa_3 (i.e., reduced form of the protein, cytochrome c) uses energy from NADH and FADH to convert oxygen into water. This conversion of oxygen into water is the culmination of a series of steps that occurs during the process of oxidative phosphorylation called the electron transport system (ETS) (Caprette, 1996). This system conserves energy to be used in the phosphorylation of ADP to ATP. Oxygen, however, is not truly an end point in this process but rather an electron acceptor that allows the electron transport system to continue functioning.

In resting muscle, the oxygen concentration needed to run oxidative phosphorylation is low (Gneiger, Steinlechner-Maran, Méndez, Eberl, & Margreiter, 1995). A much higher oxygen concentration (approximately 10 times greater) is needed in the muscle, however, to begin making changes that are significant in the concentration of intermediate metabolites (e.g., cytochrome c and phosphorylation potential) during exercise (Wilson, Owen, & Erecinska, 1979). The body must compensate for the increased need for oxygen in the working muscle. As oxygen consumption increases with exercise, blood flow and oxygen supply to the muscle also increase (Mizuno, Kimura, Iwakawa, Oda, Ishii et al., 2003). With increasing demands, muscles

reach a point of maximum oxygen consumption, called peak oxygen consumption (Vo_2) or maximum oxygen uptake (Vo_2max). Continued exercise past the point of maximum oxygen consumption causes ischemia (i.e., reduced oxygen). The consequences of ischemia are a decrease in oxygen supply to the mitochondria and an increase in the risk of muscle fatigue (Murthy, Hargens, Lehman, & Rempel, 2001).

3.1.3. Fiber Type Specific Metabolic Capacities Related to Muscle Fatigue

Research supports the notion that central and neuromuscular processes play a role in muscle fatigue; however, most of the evidence supports the peripheral sites within the muscle itself as the primary initiators of muscle fatigue (Fitts, 1994). This type of fatigue is of shorter duration than central fatigue and occurs primarily at the cellular level with regard to metabolic and contractile property changes. Different factors affect the onset, rate, and metabolic changes associated with peripheral fatigue. Some of these factors are exercise intensity, exercise duration, and environmental conditions (Fitts, 1994).

An increase in the onset of fatigue and decreased overall performance occurs during exercise in hot environments possibly due to dehydration or a reduction in muscle blood flow (Nadel, 1988). These results have also been noted in individual muscles. Parkin, Carey, Zhao, and Febraio (1999) conducted a study that assessed the fatigue rate of subjects in three different controlled environments (i.e., cold, normal, and hot) after cycling to the point of exhaustion. Biopsies of muscle from the vastus lateralis were taken after exercise. The samples were freeze-dried, sectioned, and analyzed enzymatically for the presence of glycogen, lactate, creatine, and creatine phosphate. Results indicated that subjects fatigued faster in the hot environment compared to the other two environments; however, analysis of the muscle samples showed no depletion of glycogen. In the normal and cold environments there was a significant decrease in

glycogen stores within the muscle samples. Due to the inconsistent results between the hot condition and the other conditions, the authors concluded that perhaps exercise in high temperatures perturbs the metabolic processes of the muscle fibers. A change in the metabolic process of the muscle could in turn contribute to an increased rate of fatigue in heat.

The intensity and duration of exercise can also affect the processes of muscle fatigue. During skeletal muscle contraction, all fiber types are not activated at the same time (Willmore & Costill, 1994). There is a particular order to the recruitment of motor units (Sherrington, 1926). A motor unit is made up of a single motor neuron and all the muscle fibers that it innervates. During isometric contraction, recruitment begins with type I fibers, proceeds to type IIA, and finally to type IIX. Although the order of recruitment is the same for all types of exercise the rate at which they are recruited is different. This process helps to prevent muscle damage and may also explain why muscular fatigue comes in stages (Willmore & Costill, 1994).

During high intensity exercise of short duration, ATP consumption quickly outpaces the aerobic capacity of type I and IIA muscle fibers (Westerblad & Allen, 2002). The muscle becomes more dependent on anaerobic metabolism relying on type IIX fibers for their rapid, glycolytic production of energy. There is, however, a trade-off for quick energy production. The ATP needed per unit force will also eventually exceed glycolytic energy production (Brooks et al., 2001). This occurs especially in type IIX fibers because of the small amount of mitochondria in these fibers. Pyruvate is not consumed by the mitochondria and begins to be converted to lactic acid. While lactic acid accumulates it begins to dissociate into lactate and hydrogen ions. Although lactate does not contribute a great deal to decreased muscle contraction and muscle fatigue (Dutka & Lamb, 2002; Posterino, Dutka, & Lamb, 2001), the increase in hydrogen ions

decreases the pH level in the muscle and causes the muscle to become more acidic. This decrease in pH level has historically been associated with muscle fatigue (Hole, 1993).

During submaximal exercise for long durations, muscle fibers rely on a more aerobic type of metabolism. For example, during endurance exercise in human skeletal muscle, the nervous system first recruits type I and type IIA fibers because of their small diameter, reduced time to peak tension, and high oxidative capacity (Westerblad & Allen, 2002). As reported in the section on oxidative phosphorylation, once a muscle reaches maximum oxygen consumption, oxidative capacity in the type I and IIA fibers decreases, and the nervous system recruits fast-contracting, glycolytic type IIX fibers. If these fibers are recruited past maximal force and fiber fatigue levels, the muscle will quickly fatigue by way of the processes described in the preceding paragraphs.

Intrinsic laryngeal muscles have been shown to contract during all aspects of laryngeal function (i.e., respiration, phonation, deglutition, airway protection) (Hillel, 2001). As noted previously, each of these muscles has been traditionally classified to function as either an adductor or abductor. Recent research, however, has indicated that some of the muscles function in both adductory and abductory tasks (e.g., interarytenoid and posterior cricoarytenoid) (Hillel, 2001). In normal functioning, intrinsic laryngeal muscles should be able to accommodate their roles in laryngeal function by following normal recruitment protocols thereby preventing overuse and subsequent muscle fatigue. During disordered function (e.g., muscle tension dysphonia), however, the muscle fibers may be called upon to overwork during phonation. Depending on the fiber type distribution and metabolic capacity of these muscles, this overwork could lead to muscle fatigue. In a muscle such as posterior cricoarytenoid which functions in both adductory and abductory tasks, this potential risk for muscle fatigue could seriously affect muscle

performance and laryngeal function. Limited research exists on the metabolic capacity of the intrinsic laryngeal muscles making it difficult to make structure to function interpretations of these muscles to disordered function.

3.1.4. Metabolic Capacity of Intrinsic Laryngeal Muscle

There is a vast amount of literature on the structure and function (i.e., metabolic capacity to muscle output) of human limb skeletal muscle. Research on these topics in the intrinsic laryngeal muscles is only in its infancy. A few studies over the years have attempted to identify metabolic capacity of intrinsic laryngeal muscles. Rosenfield and colleagues (1982) conducted studies in human intrinsic laryngeal muscles observing the presence of nicotinamide-adenine-dinucleotide tetrazolium reductase (NADH-TR) to determine glycolytic activity and succinate dehydrogenase (SDH), an enzyme present during oxidative phosphorylation (Rosenfield, Miller, Sessions, & Pattern, 1982). Results indicated that SDH showed variable staining throughout muscle sections. The authors, however, could only make general statements about the metabolic capacity of the whole section and not individual fibers because ATP-ase histochemistry was only conducted after preincubation in a pH buffer of 9.4. Thus, the only fiber type that could be definitively identified was the general type II fiber. Guida and Zorzetto (2000) performed a similar study on human vocalis muscle and determined that those muscle fibers were highly oxidative. ATP-ase histochemistry, however, was conducted after preincubation in pH buffers of 10.2 and 4.6 in this study; therefore, it was difficult to differentiate fiber types and identify the metabolic capacity of individual fibers, especially fiber types IIA and IIX and fiber type coexpressions. Another major limitation to these studies is that NADH-TR is traditionally used to determine oxidative activity in muscle fibers, not glycolytic activity (Rowlerson, 1990).

Future studies are needed with a staining protocol to determine the glycolytic capacity of the muscles.

Tellis and colleagues (2004) also performed SDH staining to measure oxidative capacity, but they used another enzyme found during glycolysis, glyceraldehyde di-phosphate (α-GPDH), to determine the glycolytic capacity of the muscle. After repeated trials, the IA muscle consistently stained darkest for the glycolytic enzyme indicating that the IA muscle may be more glycolytic and less oxidative and thus, may have the capacity to fatigue. If IA muscle fibers contract past maximal force and fatigue levels, then according to this staining, the muscle will fatigue. Results also indicated that occasionally type I fibers (traditionally considered oxidative) stained positively for the glycolytic enzyme, α-GPDH (Figure 5). More research is needed to confirm these results.

Another problem with the three studies mentioned above is that SDH staining is always paler in larger mammals (e.g., human); therefore, the results of SDH can be misinterpreted as indicating decreased oxidative capacity. Future studies should include a better marker for oxidative capacity and mitochondrial density. A combination of SDH and NADH-TR to determine oxidative capacity, as well as α-GPDH to determine glycolytic capacity may be necessary for a more complete metabolic analysis of these muscles.

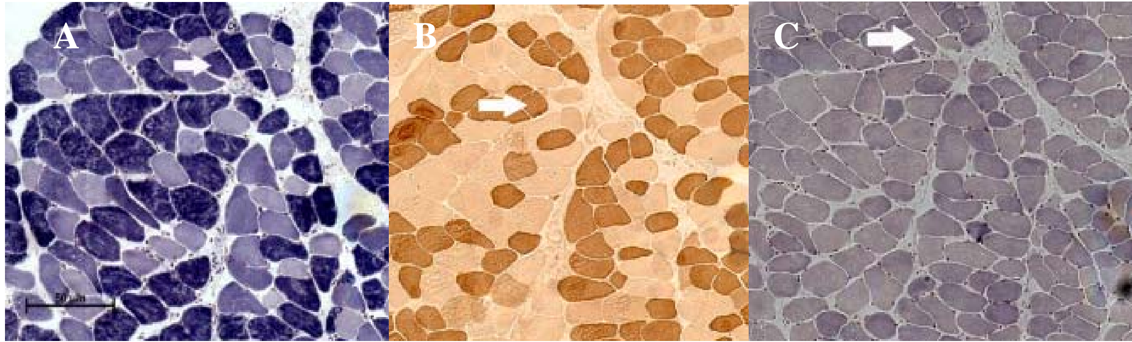

Figure 5. Glycolytic Capacity of Human Interarytenoid Muscle

Glycolytic capacity was determined by reactivity for **A)** glyceraldehyde 3-phosphate dehydrogenase (α-GPDH), enzyme in glycolytic pathway. **B)** Myofibrillar ATPase histochemical staining after preincubation in buffer of pH 4.6 allowed determination of fiber type. Oxidative capacity determined by reactivity for **C)** succinate dehydrogenase (SDH), enzyme in the citric acid cycle. Some type I fibers, however, had increased levels of α-GPDH (arrows). [Figure and figure legend adapted from Tellis et al., 2004]

4. CHAPTER IV

4.1. Oxidative Deficiency: When there is a Problem

4.1.1. Cytochrome c Oxidase Deficiency

Laryngeal muscle physiology involves the complex coordination of the intrinsic and extrinsic laryngeal muscles. The previous chapters have reviewed existing knowledge and literature regarding intrinsic laryngeal muscle histology, morphology, and metabolic capacity. Limited research, however, exists on how the biochemical and histologic properties of the intrinsic laryngeal muscles change during a disordered state or pathology. One histologic change that can occur within muscle fibers is an increase in the presence of mitochondrial abnormalities. These abnormalities can decrease the oxidative capacity of muscle fibers, reduce a muscle's ability to maintain contraction, and increase the risk for muscle fatigue. Mitochondrial abnormalities occur most often with mitochondrial disease; however, these abnormalities may also play a role in the pathogenesis of the aging muscle and a decrease in muscle function (Cao, Wanagat, McKiernan, & Aiken, 2001; Linnane, Ozawa, Marzuki, Tanaka, 1989; Pesce et al., 2001; Rifai, Welle, Kamp, Thornton, 1995).

Mitochondrial abnormalities subsequent to aging have been attributed to a variety of causes ranging from lifestyle, diet, exposure to toxins, and genotypic alterations (Pesce et al., 2001). Most biochemical and morphological changes in energy deficits of aging muscles, however, are related to reactive oxide species (ROS) (i.e., oxygen free radicals) (Beckman & Ames, 1998; Raha & Robinson, 2000; Sastre, Pallardo, Garcia de la Asuncion, & Viña, 2000).

Reactive oxide species can be generated from various sources within the cell such as the mitochondrial electron transport system (ETS), phagocytic cells, and oxidant enzymes (Cao et al., 2001). Exogenous sources of ROS include drug oxidations, cigarette smoke, ionizing radiation, and redox-cycling substances (Cross et al., 2000). A small percentage (2%-3%) of

oxygen that is taken up by the cell is chemically reduced by the addition of single electrons and thus converted into ROS, including hydrogen peroxide, superoxide anion, and hydroxyl radical (Johnson, Sinclair, & Guarente, 1999). Cigarette smoke is estimated to contain 400-500 different compounds, many of which can be a source of or contain a high concentration of ROS (e.g., micromoles per puff) (Cross et al., 2000). The presence of these free radicals in cigarette smoke has been implicated in smoking related diseases, namely cancer (Church & Pryor, 1985).

The radicals in cigarette smoke can be divided into a gas phase and a tar phase (Cross et al., 2000). The gas phase radicals consist of both organic and inorganic radicals and are oxidizing in nature. The radicals found in cigarette tar are semiquinones that can react with oxygen to produce superoxide radicals (Cross et al., 2000). These reactive oxide species are capable of producing molecular damage to all biochemical compounds including proteins, lipids, nucleic acids, carbohydrates, and free amino acids (Kalra, Chaudhary, & Prasad, 1991). By producing damage to a variety of compounds, cell activities such as metabolism and gene expression can be affected. Research indicates that the mitochondrial genome is susceptible to mutation damage from ROS (Kalra et al., 1991). The circular DNA found in the mitochondria lack histone protection and have limited repair systems, the error rate of mitochondrial (mt) DNA being 10 times greater than nuclear DNA (Brown, George, & Wilson, 1979). Unlike other cells, muscle fibers also have a constant nuclear to cytoplasmic ratio. This means that if nuclear DNA is damaged it is automatically regenerated by satellite cells that transform into nuclear DNA. Mitochondrial DNA do not have this mechanism to recover from damage (Copeland, Ponamarev, Nguyen, Kunkel, & Longley, 2003). Damage to mtDNA comes in the form of either point mutations and/or deletions. Mitochondrial DNA mutations have also been linked to abnormalities of the electron transport system. Over time the accumulation of these mutations

exceeds a critical threshold in a section of the muscle fiber which in turn will show a histochemical deficiency of the protein, cytochrome c oxidase (Chinnery, Howel, Turnbull, & Johnson, 2003).

Cytochrome c oxidase is a member of the mitochondrial electron transport system. It is an iron containing mitochondrial protein that transfers electrons during biological oxidations in eukaryotic cells. Cytochrome c oxidase protein deficiency has been implicated in age-related changes in skeletal muscle, including decreases in force production and fiber atrophy which occur through mitochondrial DNA alterations (either point mutations or deletions) or reduced mRNA levels (Cao et al., 2001; Pesce et al., 2001). Cytochrome c oxidase is coded for from mitochondrial DNA rather than from nuclear DNA. Because mitochondrial DNA has a much higher error rate than nuclear DNA, cytochrome c oxidase deficiency can occur with age and with an increase in the accumulation of free radicals. The consequences of mitochondrial mutations, especially cytochrome c oxidase deficiency, are easily detected in cells such as neurons and skeletal muscle fibers which are post-mitotic and rely heavily on oxidative metabolism for their energy supply (Papa, 1996). Abnormalities in mtDNA expressed as a poorly functioning electron transport system (ETS) would, therefore, be more easily detected in type I fibers.

Two commonly used markers to determine ETS abnormalities are the histochemical analysis for cytochrome c oxidase deficiency and the identification of ragged red fibers using the modified Gomori trichrome staining method (Edder, Lake, & Wilson, 1981). Staining for succinate dehydrogenase (SDH), an enzyme in the citric acid cycle, is often used in combination with the cytochrome c oxidase and modified Gomori trichrome staining methods to confirm cytochrome c oxidase deficient fibers (Cao et al., 2001; Pesce et al, 2001). Fibers that have

cytochrome c oxidase deficiency appear hyperactively stained with the SDH reaction. Ragged red fibers appear darker in areas where the subsarcolemmas of the mitochondria are aggregated (Di Mauro, Bonilla, Zeviani, Nagagwa, & DeVivo, 1985). The difficulty with using the modified Gomori Trichrome to confirm cytochrome c oxidase deficiency is that although all ragged red fibers are cytochrome c oxidase deficient, all cytochrome c oxidase negative fibers do not have large aggregated areas of mitochondria (Prelle, Fagiolari, Checcarelli, Moggio, Battistel et al., 1994).

4.1.2. Implications to Posterior Cricoarytenoid Muscle Function

The American Cancer Society estimates that over 10,000 new cases of laryngeal cancer will be diagnosed and over 3,000 people will die from this cancer in the United States in 2004 (American Cancer Society, 2003). The majority of these cases will occur in males with a significant smoking history. In its most virulent form, cigarette smoke can cause cancerous changes to the squamous cell epithelium of the laryngeal tissues. The effect of cigarette smoke on the histology and function of deeper layers of these tissues (namely, muscle), however, has never been investigated. Given the close approximation of the intrinsic laryngeal muscles to the mucosa of the larynx, exposure to smoking - induced free radicals is likely to be much more substantial than in any other skeletal muscles of the body

As stated above, cigarette smoke can increase the presence of reactive oxide species which can cause mutations of mtDNA in muscle fibers. Damage or mutation to mtDNA will appear as a deficiency in cytochrome c oxidase. These abnormalities can be expressed as a poorly functioning electron transport system and will therefore be observed more frequently in type I fibers. These fibers may be more easily affected because they have a larger overall number

of mitochondria and rely more on the fiber's oxidative capacity for their energy supply compared to type II fibers.

Recent research has indicated that the posterior cricoarytenoid muscle contains the highest percentage of type I fibers compared to the other intrinsic laryngeal muscles (Happak et al., 1989; Shiotani et al., 1999). If cigarette smoke and other inhaled environmental toxins can cause an increased deficiency in cytochrome c oxidase in the muscle fibers of the intrinsic laryngeal muscles, then it is hypothesized that the PCA muscle will be most affected by this deficiency. The PCA muscle, therefore, was chosen as a starting point for the present investigation. Because of the possible effects accumulated ROS can have on the histology and metabolic properties of the muscle fibers of the PCA muscle, individuals with a long history of smoking may be particularly subject to cytochrome c oxidase deficiency. Adverse physiologic consequences to the PCA muscle may therefore be possible in individuals with a significant smoking history.

4.1.3. Specific Aims of Study

Before the effects of the accumulation of ROS from cigarette smoke or other inhaled environmental toxins can be evaluated on the physiologic functioning of the human PCA muscle, it is necessary to determine if cytochrome c oxidase deficiency actually occurs in this muscle. The purpose of this dissertation, therefore, is to: (1) determine if cytochrome c oxidase deficient fibers are present in the posterior cricoarytenoid muscle and (2) determine if there is an increased presence of type I fibers with cytochrome c oxidase deficiency in the posterior cricoarytenoid muscle compared to control strap muscle harvested from the same participant. Muscle samples from individuals with a significant smoking history were used because of the increased chance of finding fibers with cytochrome c oxidase deficiency.

4.1.4. Hypotheses

Based on previous literature, it is hypothesized that cytochrome c oxidase deficient fibers will be present in the posterior cricoarytenoid muscle of individuals with a significant smoking history. The number of type I fibers with cytochrome c oxidase deficiency will be increased in the posterior cricoarytenoid muscle compared to control strap muscle because of its continuous, direct exposure to the toxins in cigarette smoke and other inhaled environmental toxins. Procedures and methodologies for this study will be described in the following chapter.

5. CHAPTER V – MATERIALS AND METHODS

5.1. Participants

Whole posterior cricoarytenoid (PCA) and strap (i.e., infrahyoid or suprahyoid) muscles were harvested from 10 male participants undergoing total laryngectomy surgery as part of their cancer treatment in the Department of Otolaryngology at the University of Pittsburgh Medical Center. One muscle biopsy, obtained from a 69 year old male during biopsy, was excluded because his smoking history was unknown[2]. Appropriate institutional review board for experiments involving human subjects was obtained – IRB #991280. Participants ranged in age from 54-78 years with a mean age of 65 years. Vocal fold function was assessed by videolaryngoscopy prior to surgical intervention such that a diagnosis of normal vocal fold function could be obtained, and only normally functioning PCA muscles were used in this study.

Immediately after removal of the larynx, the PCA muscle was exposed by resection of the postcricoid mucosa and the fibrous tissue that divides the horizontal and vertical compartments was identified. The muscular process of the arytenoid cartilage (insertion point for PCA) was marked with a suture which was then used to bisect the horizontal and vertical compartments. Each compartment was dissected from origin to insertion and oriented on gauze moistened with cooled saline such that the native in vivo orientation of the sample could be maintained. Consideration for muscle subcompartment was not made for the purposes of this study but will be investigated in future explorations. The majority (8 out of 10) of the muscle samples, however, were of the horizontal compartment. A portion of either an infrahyoid or a suprahyoid muscle was harvested along with the PCA samples and used as a control muscle for

[2] At the time of analysis it was uncertain whether the smoking history of the participant would be revealed. By the conclusion of the study, however, the participant's smoking history was still unknown. Because this muscle biopsy was the only muscle obtained during autopsy, the percentage of COX deficient type I fibers was maintained but not used during the data analysis. The percentage of COX deficient type I fibers was .035% which is comparable to other age-matched participants for this study.

the staining procedures because it does not have the exposure to inhaled environmental toxins as does the PCA muscle. For purposes of the present study, this control muscle is identified as strap muscle. Muscle samples were placed in respective, labeled containers and transported on ice to Dr. Sciote's laboratory in the School of Dental Medicine at the University of Pittsburgh. Each muscle compartment was then oriented and snap frozen separately in isopentane cooled by dry ice to -70°C with a section of the strap muscle to serve as age-matched control (Figure 6). Serial 10μm cryosections were obtained along a portion of the samples.

5.2. Staining Procedures

5.2.1. Fiber-type Identification by Immunohistochemical Staining

Antibody staining on serial sections was used to determine the type and amount of MHC isoform contained in each muscle fiber using an indirect immunoperoxidase method. Primary antibody reactivity was detected by either a peroxidase conjugated secondary antibody or a biotin-labeled secondary antibody visualized by extravidin peroxidase. The following myosin heavy chain (MHC)-specific antibodies were used: anti-Type I monoclonal (Slow skeletal MHC – Sigma Aldrich clone NOQ7), anti-fast monoclonal (Fast skeletal IIA, IIB & IIX MHC – Sigma Aldrich clone MY-32), and anti-IIA monoclonal (Fast IIA – American Type Culture Collection clone SC-71).

5.2.2. Staining Used to Identify Mitochondrial Abnormalities

Sections serial to the antibody staining were analyzed for the presence of cytochrome c oxidase (Figure 6, Panel A)). These serial sections provided a means to identify which fiber types had the most cytochrome c oxidase deficiency. This histochemical staining procedure required incubation of the cryostat sections in a medium for one hour followed by a quick rinse and fixation in formal calcium. The incubation medium contained 10mg of 3,3-diaminobenzidine

tetrahydrochloride, 18ml of .1 M of phosphate buffer, catalase solution, sucrose, and cytochrome c. Areas of enzyme activity stained brown and were distributed in a greater concentration to type I fibers in the PCA muscle. Lack of staining indicated a deficiency of complex IV of the ETS (Pesce et al., 2001).

The original succinate dehydrogenase histochemical staining protocol reported by Nachlas and colleagues (1957) was used to confirm the presence of cytochrome c oxidase deficient fibers in the PCA and strap muscles (Nachlas, Tsou, deSouza, Cheng, & Seligman, 1957) (Figure 6, Panel B). Results of this stain showed a hyperactive SDH staining in cytochrome c oxidase deficient fibers.

The histologic modified Gomori trichrome staining protocol was used to confirm questionable cytochrome c oxidase deficient fibers (Engel & Cunningham, 1963). Cryostat sections were air dried for two minutes and then stained in Harris's haematoxylin for five minutes. The haematoxylin stain was adjusted to a pH of 2.3 before use. After being rinsed, the sections were stained for ten more minutes with Gomori's modified stain adjusted to a pH of 3.4. This stain consisted of 0.3g of chromotope 2R, 0.15g of fast green FCF, 0.3g of phosphotungstic acid, 50ml distilled water, and 0.5ml of glacial acetic acid. The slide was drained and agitated for 3 seconds in a coplin jar containing acetic acid. Type I fibers were distinguished from type II fibers by their greater proportion of mitochondria and sarcoplasmic reticulum. These intermyofibrillar materials formed a more complete network around the myofibrils. Cytochrome c oxidase deficient fibers were identified as ragged red fibers by either staining darker than other fibers or having a characteristic ragged quality on the edges or throughout the fiber.

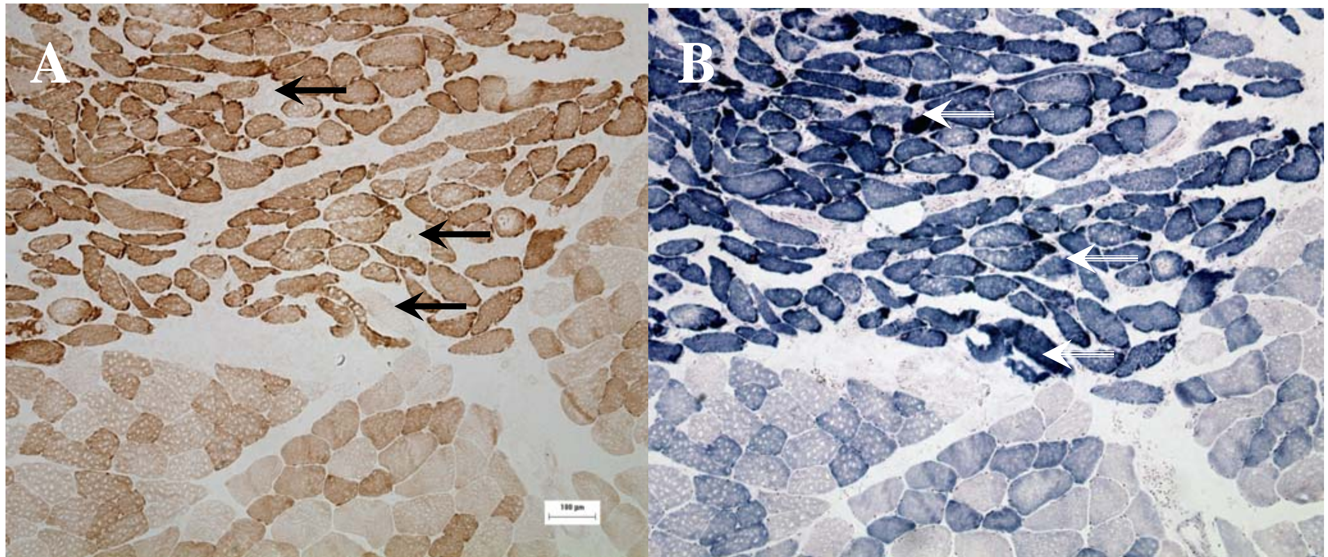

Figure 6. PCA and Strap Muscle Composite with COX and SDH Stains

Panels A and B show the PCA and strap muscles taken from the same participant frozen as a composite. The PCA muscle is located in the upper half of the panels and the strap muscle is located in the bottom half. Panel **A)** shows staining for cytochrome c oxidase and the arrows identify cytochrome c oxidase deficient fibers with their characteristic pale appearance. Panel **B)** shows staining for succinate dehydrogenase and the arrows identify hyperactive staining to confirm that the fibers are cytochrome c oxidase deficient.

5.3. Data Collection

Serial sections were viewed with a Leica DMR light microscope and projected onto a computer monitor through Image ProPlus image analysis software (version 4.5.1). All fibers in serial sections of the PCA and strap muscles were analyzed using the cytochrome c oxidase and SDH stains to determine which fibers expressed cytochrome c oxidase deficiency. Only fibers that stained pale for cytochrome c oxidase and hyperactive for SDH in serial sections were considered cytochrome c oxidase deficient. The modified Gomori trichrome was analyzed on questionable fibers to confirm cytochrome c oxidase deficiency. A total count of deficient fibers for PCA and strap muscle was recorded and placed in an Excel spreadsheet. All observed

cytochrome c oxidase deficient fibers for each muscle were compared to serial sections of the type I antibody stain to determine the fiber type of the deficient fibers. Cytochrome c oxidase deficient fibers were characterized as either type I or type II fibers. Fiber typing of deficient fibers was always done after the identification of cytochrome c oxidase deficient fibers to control for experimenter bias. A total count for each fiber type for each muscle was recorded. A point count of the total number of fibers (n ≈ 2000-4000) and the total number of type I fibers in each muscle section was documented.

The number of type I fibers containing cytochrome c oxidase deficiency was divided by the total number of type I fibers identified in a specific sample to determine the proportion of type I fibers in the muscle sample that contained mitochondrial deficiencies. This process was repeated for type II fibers to use in future comparisons.

5.4. Data Analysis

The experimental design for the present study was a paired-samples design. The independent variable included in the study was muscle sample (i.e., PCA and strap). The dependent variable in the study was the proportion of cytochrome c oxidase deficient type I fibers. Statistical analyses were computed using SPSS for Windows, version 11.5. A paired samples t-test was performed to determine if the means were equal between muscle samples. A Pearson product-moment correlation was calculated for both dependent variables with participant age to determine if there was a linear relationship between the number of cytochrome c oxidase deficient type I fibers and the age of the participant.

5.5. Reliability

Intra-rater reliability was conducted to determine the within investigator consistency in identifying cytochrome c oxidase deficient type I fibers. The primary investigator repeated the

identification and fiber typing of cytochrome c oxidase deficient fibers, as well as point counts of all fibers and all type I fibers in 50 percent (i.e., five) of the muscle pairs (i.e., PCA and strap). Inter-rater reliability was conducted to determine the consistency in identifying the cytochrome c oxidase deficient type I fibers between investigators. An independent, trained rater conducted the same procedures on 30 percent (i.e., three) of the muscle pairs. Inter-rater results were compared to initial, Time One results of the primary investigator.

Reliability coefficients were estimated using a Pearson product-moment correlation analysis and confirmed with a test for bias. The intra-rater reliability coefficient was r = 1 for the PCA muscle at time one and time two indicating a perfect correlation. Lack of rater bias over time was supported by a low mean difference (.00046, p = .069). The intra-rater reliability coefficient for strap muscle at time one and time two showed a low correlation (r = 0.354). This correlation, however, misrepresents the actual reliability because in the raw data all values for time one were zero; therefore, there was no variance calculated at time one. The mean difference for strap muscle at time one and time two was extremely low (-.00048, p = .374) and a paired t-test showed no significant difference (t = -1.0, p = .374). These results indicate that the intra-rater reliability for the strap muscle was reliable and the measures unbiased.

Inter-rater reliability coefficients for the PCA muscle at time one and time two showed a high correlation (r = 0.995). A low mean difference (-.00027, p = 347) also failed to indicate systematic differences in bias across raters. A high correlation was also found for the strap muscle at time one and time two (r = 0.945). A low mean difference (-.0004, p = 423) also indicated no measurement bias for this muscle.

6. CHAPTER VI – RESULTS

6.1. General Description of Fibers in Posterior Cricoarytenoid and Strap Muscles

6.1.1. Posterior Cricoarytenoid Muscle

This chapter will provide a description of the data obtained from the posterior cricoarytenoid muscles that were analyzed. The total number of fibers analyzed per whole muscle sample from the PCA muscle ranged from 1200 to a little over 4500. The percentage of type I fibers in three out of the eight horizontal muscle subcompartments was between 37 and 52 percent. Only three muscles had percentages slightly above 80 while the remaining two had 60 and 68 percent type I fibers. The samples from the vertical subcompartments contained 38 and 49 percent type I fibers. Fibers that coexpressed myosin, namely, type I-IIA and type I-IIX were rarely observed and were not included in the percentage count for either type I or type II fibers. During the analysis of the samples, none of the cytochrome c oxidase deficient fibers were identified as fibers that coexpressed myosin. A clear determination of either type I or type II was made for all cytochrome c oxidase deficient fibers (Figure 7).

6.1.2. Strap Muscle

The total number of fibers analyzed per control strap muscle ranged from 800 to a little over 4100 fibers. There were almost equal percentages of type I and type II fibers (~45-65%) for all but two strap muscle analyzed. One control muscle had 37% type I fibers and another muscle had 75% type I fibers. Again, fibers that coexpressed myosin were rarely observed and were not included in percentage counts for either type I or type II fibers. All cytochrome c oxidase deficient fibers were documented as either type I or type II (Figure 7).

6.2. Cytochrome c Oxidase Deficiency in Type I Fibers

A paired samples t-test was performed to determine if there was a significant difference between the percentage of cytochrome c oxidase deficient type I fibers in the posterior cricoarytenoid muscle compared to the percentage of cytochrome c oxidase deficient type I fibers in the strap muscle. Results of the t-test indicated that the paired mean difference for the two muscles was highly significant ($p = .002$, $t = 4.393$, $df = 9$) indicating a significantly larger percentage of cytochrome c oxidase deficient type I fibers in the PCA muscle. The post hoc power estimate was also high at 0.97 indicating a low chance of a making a type II error. The effect size was also very large with delta equal to 2.929. Additional results on the differences in percentage of cytochrome c oxidase deficient fibers between fiber types will be detailed in the ancillary analysis section later in the results.

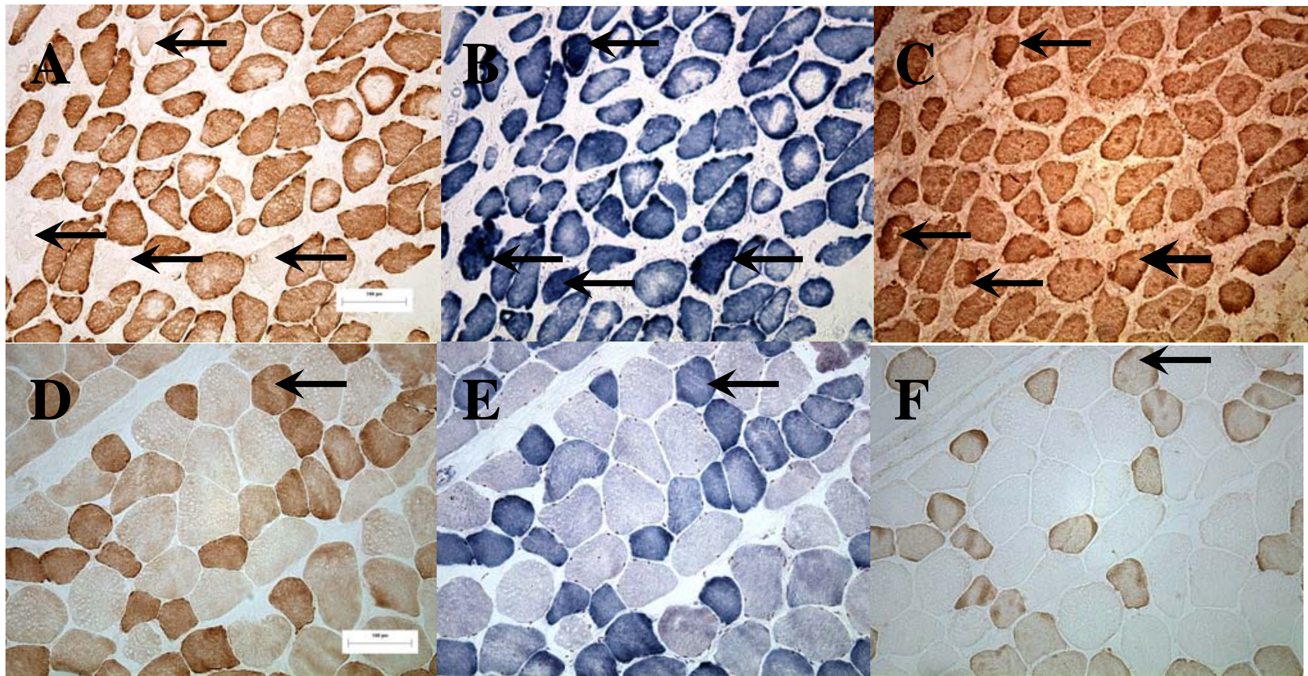

Figure 7. Panels Indicating Staining for PCA and Strap Muscle

These panels depict the difference in the proportion of type I fibers with cytochrome c oxidase (COX) deficiency in the PCA muscle compared to strap muscle. Panels **A-C** are serial cross-sections taken from the PCA muscle of a 69 year old male. Panels **D-F** are serial cross-sections taken from the strap muscle of the same participant. Type I fibers with COX deficiency were identified using the **A,D)** cytochrome c oxidase, **B,E)** succinate dehydrogenase (SDH), and **C,F)** type I antibody stains. The arrows in the top panels identify the type I fibers with COX deficiency in the PCA muscle. Four pale fibers are identified in panel A. The SDH stain in panel B confirms that the pale fibers are COX deficient by their dark appearance. In panel C, all type I fibers appear dark confirming the COX deficient fibers as type I. The bottom panels show a characteristic representation of sections taken from strap muscle. There are no COX deficient fibers observed in these panels. Arrows in the panels identify a type I fiber with positive COX activity. Fiber phenotype can be determined by the size of the fibers in panels D-F, as well as by the gradation of the stains in panels D and E. Type I fibers are characteristically smaller in diameter while type II fibers are substantially larger. Type I fibers are also darker in appearance because of their larger amount of mitochondria.

6.3. Ancillary Analyses

6.3.1. COX Deficiency between Fiber Types in the PCA and Strap Muscles

Although debatable, some research has shown that COX deficiency does not occur more often in one fiber type than another (Chinnery, Howel, Turnbull, & Johnson, 2003; Johnson,

Bindoff, & Turnbull, 1993). Ancillary analyses were completed to determine if there was a significant difference in the percentage of fibers with COX deficiency between fiber type within the PCA and strap muscles. A paired t-test indicated that there were significantly more ($p = .0035$) type I fibers with COX deficiency in the PCA muscle compared to type II fibers. There was, however, no significant difference ($p = .5450$) in the number of type I fibers with COX deficiency in the strap muscle compared to type II fibers. Total percentages of COX deficient fibers between the PCA and strap muscles were also analyzed in an ancillary fashion. A paired t-test indicated that there was a highly significant difference ($p = .0006$) between the total percentage of COX deficient fibers in the PCA muscle compared to the strap muscle, including both type I and type II fibers.

6.3.2. Correlation Analysis for Age

Previous research has indicated that COX deficiency increases with age (Harman, 1981). A Pearson product moment correlation coefficient analysis, therefore, was performed to determine if there was a relationship between age and percentage of type I fibers with COX deficiency for the posterior cricoarytenoid and the strap muscle (Figure 8). A positive correlation was significant ($r = .872$) at the 0.01 level for the PCA indicating that the percentage of type I fibers with COX deficiency increased as age increased. There was not a significant correlation for strap muscle ($r = .045$)

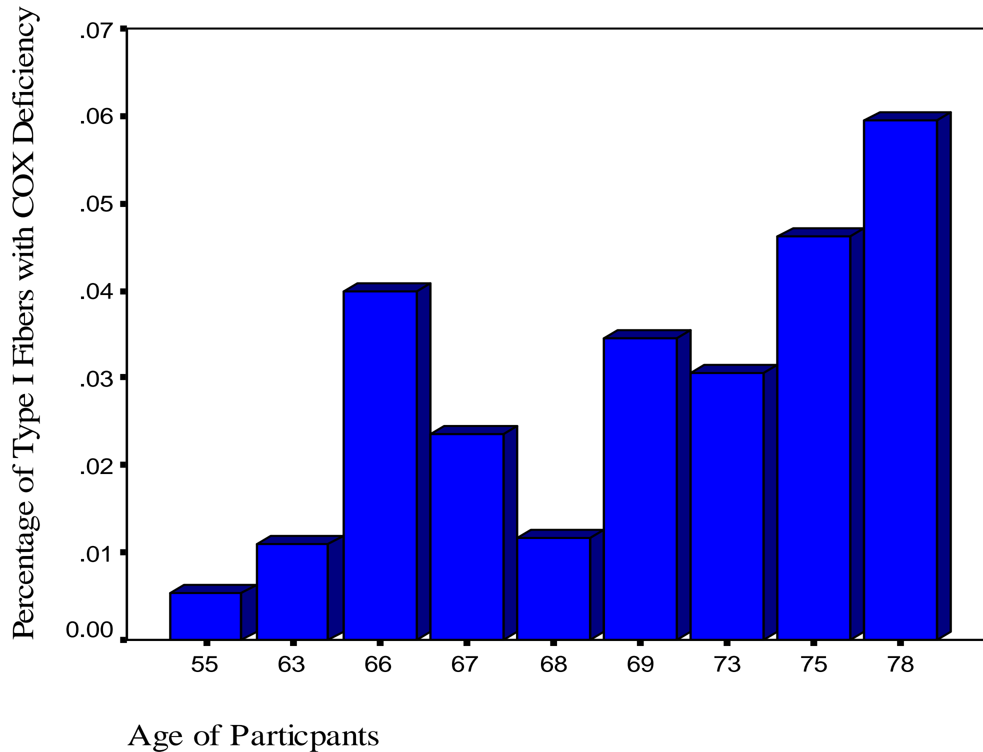

Figure 8. Percentage of COX Deficient Fibers in Relationship to Age of Participants

6.3.3. Target Fibers

During the data collection process, a large number of "interesting" fibers was observed in almost all of the PCA muscle samples (Figure 9, panel A). These fibers, however, were not observed in strap muscle. Muscle fibers that contain the empty space as do the fibers noted by the arrows in Figure 9 are either considered target or targetoid fibers (Schmitt, 1979). Traditionally it is thought that if the fiber is a target fiber then the etiology of its problem is neurophysiological (i.e., secondary to some type of denervation or denervating disease). If the fiber, however, is shown to be a targetoid fiber then the cause of the problem is unspecified. A nicodtinamide adenine dinucleotide – trans reductase (NADH-TR) stain was performed serial to the initial COX, SDH, and modified Gomori trichrome stains in the PCA muscles to determine

whether the fibers observed were mainly target or targetoid. Target fibers, with the NADH-TR stain, should appear to have a more defined circular area close to the middle of the fiber (see arrows in panel B of Figure 9). The modified Gomori trichrome stain is used to confirm target fibers. Target fibers after the Gomori stain will have a darkened circular area approximating the center of the empty space seen in the NADH-TR stain (see arrows in panel C of Figure 9). Full analysis will be conducted for future studies, however, a large portion of the fibers observed following a cursory analysis of a couple of muscle samples appeared to be target fibers.

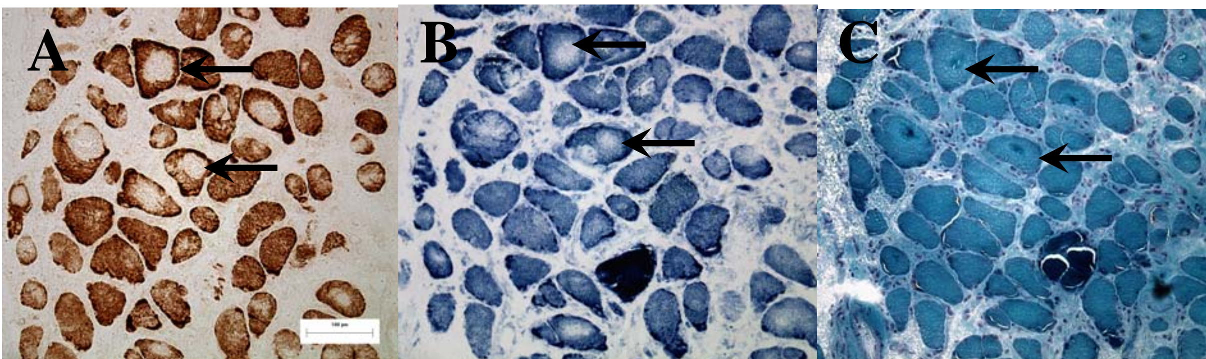

Figure 9. Target Fibers in PCA Muscle

Target fibers observed in the PCA muscle of a 69 year old male using the **A)** cytochrome c oxidase, **B)** NADH-TR, and **C)** modified Gomori trichrome stains. Target fibers are identified by their empty circular space in the middle of the fiber in the COX stain. A more defined circle will be observed in target fibers following the NADH-TR stain. The modified Gomori Trichome stain confirms that fibers are target fibers by the darker circular area in the middle of the fiber. Arrows in panels A-C indicate target fibers.

7. CHAPTER VII - DISCUSSION

7.1. Mitochondria, Cytochrome c Oxidase Deficiency, and Myopathy

7.1.1. Mitochondria and mtDNA mutations

Mitochondria are the primary energy generators in eukaryotic cells. These organelles contain their own DNA, called mitochondrial DNA (mtDNA). Mitochondrial DNA is a small circular molecule whose main responsibility is to code for 13 polypeptides, 22 transfer RNAs and 2 ribosomal RNAs all of which are elements of oxidative phosphorylation and the respiratory chain system (Anderson, Bankier, Barrell, De Bruijin, Coulson et al., 1981). Two to 10 copies of mtDNA reside in mammalian mitochondria which equal approximately 1,000-100,000 mtDNA copies per human cell (Larsson & Clayton, 1995; Lightowlers, Chinnery, Turnbull, & Howell, 1997). Wild-type mtDNA is considered normal and nonpathologic.

Unlike nuclear DNA which replicates only once during the life of the cell, mtDNA is replicated and destroyed continuously in a process called relaxed replication (Bogenhagen & Clayton, 1977). Relaxed replication occurs independently and at random helping to maintain a constant level of mtDNA within the cell. The error rate for mtDNA, however, is 10 times greater than nuclear DNA making it more likely to concede damage (Brown et al., 1979). This type of mtDNA damage comes in the form of a point mutation and/or a deletion.

Individuals with a mitochondrial myopathy contain a mixture of both wild-type and mutant mtDNA molecules (i.e., heteroplasmy) which may vary from muscle fiber to muscle fiber (Sciacco, Bonilla, Schon, Di Mauro, & Moraes, 1994). The level of heteroplasmy can change within each individual cell due to the replication rate of the individual mtDNA molecules (i.e., intracellular drift) (Chinnery & Samuels, 1999). Only when a portion of the muscle fiber contains a percentage of mutant mtDNA that exceeds a critical threshold is the muscle fiber

segment considered to have a respiratory chain defect (Schon, Bonilla, & Di Mauro, 1997). Clonal expansion of mutant mtDNA has been suggested to cause the random accumulated increase in mutant mtDNA passed threshold level in individual muscle fibers (Chinnery et al., 2003). For this reason, adjacent muscle fibers may contain different levels of mutant mtDNA, some indicating normal respiratory chain activity and others clearly showing a defect in the respiratory chain (Elson, Samuels, Johnson, Turnbull, & Chinnery, 2002). Those fibers with respiratory chain defects usually involve deficits in cytochrome c oxidase (COX) activity. Histochemical analysis of skeletal muscle cross-sections thus exhibit the mosaic pattern of COX-negative and COX-positive fibers observed in the muscle sections analyzed in the present study (see Figure 7) (Johnson et al., 1993).

7.1.2. Cytochrome c Oxidase Deficiency and Myopathy

Cytochrome c oxidase (COX) is found in the inner membrane of the mitochondria. This enzyme forms the terminal portion (complex IV) of the mitochondrial respiratory chain (Taanman, 1997; Shoubridge, 2001). COX is vital to the functioning of cellular respiration because it is primarily responsible for the transfer of the electrons from the respiratory chain to molecular oxygen. A deficiency of COX is a pathologic characteristic of mtDNA mutations and is often used to diagnosis a possible mitochondrial myopathy or encephalomyopathy (Di Mauro, Zeviano, Bonilla, Bresolin, Nakagawa et al., 1985; Di Mauro, Zeviani, Servidei, Bonilla, Miranda et al., 1987; Di Mauro, Zeviani, Rizzuto, Lombes, Nakase et al., 1988). There are many different types of mitochondrial disorders, most being maternally inherited because mtDNA comes from the mother (Schon, Bonilla, & Salvatore, 1997). When confined to skeletal muscle, mitochondrial myopathies have diverse clinical presentations including but not limited to static,

progressive, or reversible weakness; progressive external ophthalmoplegia; facioscapulohumeral syndrome; and exercise intolerance (Di Mauro, Bonilla, Zeviani, Nakagawa, & DeVivo, 1985).

In general, mitochondrial myopathy severity and the progression of weakness in the limbs are correlated with an increase in the proportion of COX-negative fibers found in cross-sectional analysis of limb skeletal muscle (Elson et al., 2002; Weber, Wilson, Taylor, Brierley, Johnson et al., 1997). For example, it has been reported that over 60% of muscle fibers can be affected in individuals with Chronic Progressive External Ophthalmoplegia (CPEO) (Müller-Höcker, 1983). Other researchers have noted that often greater than 85% of total mtDNA have to be affected before clinical consequences are reported (Boulet, Karpati, & Shoubridge, 1992; Chomyn, Martinuzzi, Yoneda, Daga, Hurko et al., 1992; Sciacco et al., 1994). Small samples sizes, no parametric statistics, and the small age ranges of participants in the referenced studies make it difficult to determine the significance of these findings. Furthermore, conflicting results exist between studies. For instance, one study which included 19 muscles from participants ranging in age from 21 - 65 noted the percentage of COX-negative fibers in patients with CPEO to be 1-23% (Collins, Byrne, & Dennett, 1995). Future research needs to be conducted in individuals with these myopathies using larger sample sizes. Considerations for future research should also include differences in ages to determine when exactly clinical symptoms or decreases in physiological functioning (i.e., oxidative capacity or power) of skeletal muscle are identifiable and, furthermore, how the percentage of COX-negative fibers correlates with the appearance of these symptoms.

7.1.3. Length of COX Deficiency

Common practice in the histochemical assessment of COX deficient fibers is to analyze thin, serial cross-sections of the muscle biopsy. This cross-sectional analysis, however,

underestimates the total number of muscle fibers that are COX-negative somewhere along the length of the fiber (Elson et al., 2002). As stated above (section on Mitochondria and mtDNA), only the portion of the muscle fiber that accumulates the percentage of mutant mtDNA passed the critical threshold will develop a respiratory chain defect. Muscle fibers, therefore, may show different COX activity along the length of the fiber. Only one study to date has analyzed COX-activity along the length of human skeletal muscle in patients with mtDNA myopathy (Elson et al., 2002). Quadriceps muscle was taken from two individuals, one 80 year-old and the other 35 years old. COX and succinate dehydrogenase (SDH) stains were conducted on 161 and 120 serial 10μm sections respectively. Results of this study found that COX-negative segments were generally shorter in the biopsy that was less severely affected. Other COX-negative fibers, however, extended throughout the 1.21mm biopsy.

There also were intermediate areas of some muscle fibers that showed COX-positive or normal enzyme activity (Elson et al., 2002). These COX-positive areas lend support to intracellular drift and continuous replication of mtDNA which causes COX deficient segments to occur at different regions of a diseased muscle fiber. Theoretically, the COX-positive areas are fusion points for the COX deficient regions that expand over time and eventually coalesce with other COX deficient areas, perhaps as the severity of the myopathy increases.

Only two animal studies to date lend support for this theory in normal, nonpathologic muscle. The first study was conducted on 14 quadriceps muscles from Rhesus monkeys (Lee, Lopez, Weidruch, & Aiken, 1998). Serial sections were analyzed using COX and SDH stains along 350-1600μm regions. Some of the COX deficient segments extended throughout the 1.6mm biopsy. Most of the COX deficient segments (70%) followed the normal COX-negative – COX-negative/SDH^{++} transition. The other 30% of the COX-negative fibers followed a SDH^{++} –

COX-negative/ SDH^{++} transition. The second study analyzed the length of COX-negative fibers in the quadriceps muscles of calorie-restricted and noncalorie restricted rats for 1000-2000μm (Bua, McKieran, & Aiken, 2004). The authors set out to determine whether rats with a restriction in calorie intake would have decreases in mitochondrial abnormalities. Results of this study indicated that the length of COX-negative fibers was at a minimum of 100μm to a mean length of 442.5±277μm (i.e., 0.44mm) in rats with no calorie restriction. The calorie restricted rats showed a reduced number of COX-negative fibers compared to the rats without calorie restriction, though information about significance was not provided.

Results of the present study indicated a highly significant difference for the proportion of type I COX deficient fibers between posterior cricoarytenoid (PCA) and control, strap muscles. Although the total proportion of type I COX deficient fibers is difficult to determine in the present study because enzyme activity was not analyzed along the length of the fiber, the significance and power ($p = .002$, power = 0.97) were so high for the particular muscle sections analyzed that the chance of making a type I or type II error is minimal. Future research on the PCA muscle should include several sections along the muscle fiber to determine the length of COX-negative fibers, as well as a more comprehensive view of the percentage of COX deficient fibers within this muscle. This research will help to identify how the length of COX-deficiency or the portion of the affected muscle fiber contributes to decreased functioning of individual muscle fibers, as well as overall muscle function.

7.2. Posterior Cricoarytenoid Muscle

7.2.1. Aging and Cytochrome c Oxidase Deficiency

The first researcher to propose a theory for mitochondrial involvement in the aging process was Harman (1972). The general concept of the theory is that accumulated exposure to

reactive oxide species throughout a lifetime can lead to an increased amount of mitochondrial deletions and mutations. Accumulated damage to the mitochondrial genome can cause decreased functioning of oxidative phosphorylation, damage to the cell, atrophy of the muscle fiber, and finally death of the cell. Müller- Höcker (1990) conducted one of the first studies on the age-related alterations in COX deficiency in normal human limb skeletal muscle. Iliopsoas and diaphragm muscle were taken during autopsy from 115 men and 109 women with no previous history of neuromuscular disease. The oldest specimen came from a 97-year-old individual. Results indicated that COX deficient fibers first appeared in the third and fourth decades of life. A significant difference in the number of COX deficient fibers was seen in both muscles throughout aging; however, there was no significant difference noted between muscles. The maximum defect in COX deficiency did not exceed 1% (.37% and .47% respectively).

In the ensuing years, many researchers have found similar results in different human, nonpathologic muscle. For example, the percentage of ragged red fibers in biopsies taken from quadriceps, biceps, and triceps was significantly higher in 13 older participants (61-77 years old) compared to 15 younger participants (21-31 years old) (Rifai et al., 1995). The percentage of COX-negative fibers also decreased with age in the vastus lateralis of eight older subjects (62-88 years old) ranging from 0.1% to 5% (Brierly, Johnson, Lightowlers, James, & Turnbull, 1998). Animal studies have also shown similar age-related results in COX deficiency. Quadriceps muscles from 14 Rhesus monkeys showed a significant age-related decrease in the percentage of COX-negative fibers with 0.05% of fibers affected during cross-sectional analysis and 0.31% when fibers were analyzed along a distance (Lee et al., 1998). The highest percentage of COX deficient fibers was noted in the oldest monkey though the proportion was still less than 1%.

The present study is the first to identify percentages of COX deficiency in the PCA and strap muscles. The results from this study are similar to the results obtained from previous studies on different human muscle. Although there was a significant difference between the two muscles, percentages in type I COX deficient fibers were less than 1% for both the PCA (.06%) and strap muscles (.007%). Percentages may have increased if fibers were followed along the length of the muscle. A significant age-related correlation was noted in the PCA muscle indicating that the percentage in COX deficiency may be a product of increased age. Some research suggests that different muscles within the same individual can contain varying amounts of deleted mtDNA (Larsson, Bodegård, Henriksson, & Öberg, 1990; Moraes, Di Mauro, Zeviani, Lombes, Shanske et al., 1989; Seibel, Degoul, Romero, Marsac, & Kadenbach, 1990; Shanske, Moraes, Lombes, Miranda, Bonilla, 1990; Zeviani, Gellera, Pannacci, Uziel, Prelle et al., 1990) which may further explain why there is a significant difference between the PCA and strap muscles. The expression of COX deficiency also varies with age in certain muscles, such as extraocular muscle (Müller- Höcker, Seibel, Schneiderbanger, & Kadenbach 1993); therefore, the difference in PCA and strap muscles could be inherent to the muscles themselves.

7.2.2. Exposure to Reactive Oxygen Species

Another possible explanation for the significant difference in the percentage of type I COX deficient fibers between the PCA and strap muscles is that the PCA muscle is constantly exposed to environmental toxins (e.g., cigarette smoke) throughout a person's life. All of the participants in the present study had significant smoking histories. For this reason, the PCA muscle may be at an increased risk for an accumulation of reactive oxide species and mtDNA abnormalities because of its position in the airway. Although there is no research on the effects

cigarette smoke has on the mtDNA of muscle, there have been a series of studies that have assessed the effect cigarette smoke has on the mtDNA of different glands and tissues.

One method to identify the effect of cigarette smoke on different glands and tissues is to analyze the presence of specific mtDNA deletions. There are several large-scale deletions that have been identified in a number of different tissues harvested from individuals with mitochondrial diseases (Chinnery et al., 1999; Lopez, Van Zeeland, Dahl, Weindruch, & Aiken, 2000). Some of these deletions have been shown to cause genes from the mitochondrial genome (e.g., multiple structural genes and transfer RNA genes) to be completely removed or truncated. Removal or truncation of these genes can cause a variety of deficits in the respiratory chain because proteins that produce certain enzymes will not be translated. Common deletion 4977 bp (base pair) is one of these large scale deletions. This 4977 bp deletion is not only seen in individuals with mitochondrial diseases but is also observed in normal aging muscle (Cooper, Mann, & Schapira, 1992; Lezza, Boffoli, Scacco, Cantatore, & Gadaleta 1994; Simonetti, Chen, Di Mauro, & Schon, 1992). This deletion occurs between two 13-bp sequence repeats that begin at nucleotides 8470 and 13447. Another deletion that occurs during muscle aging is a 7.4-kb deletion between bp 8649 and 16084 (Lezza et al., 1994). Parotid gland tissue was harvested from 23 smokers and 16 nonsmokers with a mean age of 58 and 66 respectively (Lewis, Fradley, Griffiths, Baxter, & Parry, 2002). A semi-quantitative polymerase chain reaction (PCR) technique was used to identify deletions of 4977 bp. Results indicated that there was no significant difference in the level of 4977 bp deletion in the parotid glands of smokers versus nonsmokers; however, there was a strong, significant, positive correlation between the presence of deletion 4977 bp and age. Other researchers found a significant increase in the level of 4977 bp deletion in the hair follicles of smokers versus nonsmokers (Liu, Kao, & Wei, 1997).

Two research groups conducted studies to determine if there is an association between mtDNA deletions and cigarette smoking in lung tissue (Fahn, Wang, Kao, Chang, Huang et al., 1998; Lee, Lim, Lu, Liu, Fahn et al., 1999). Fahn and colleagues (1998) harvested a small piece of lung tissue from 151 participants (mean age of 54 years old) undergoing lung resection for a variety of pulmonary diseases. The smoking index was determined in terms of pack-year and ranged from 0 to 51 years. Semi-quantitative PCR was performed to determine the presence and amount of the 4839 bp deletion. This deletion causes a fusion protein of adenosine triphosphatase (ATP6) and ND5 peptides to be generated (Anderson, Bankier, Barrel, de Bruijin, Coulson et al., 1989). Downstream from the deletion, a frameshift mutation also occurs secondary to the deletion and the fusion protein stops being translated at amino acid residue 29. Without the appropriate amount of protein, enzyme production (i.e., NADH) will decrease and muscle metabolism will be affected. Results indicated that the presence and proportion of this deletion increased significantly with an increase in pack year. There was also a significant difference in the presence and proportion of the 4839 bp deletion in the lungs of smokers versus nonsmokers. Lee and colleagues (1999) performed a similar study but analyzed the presence of the 4977 bp deletion in the lung tissue of smokers versus nonsmokers. Although the 4977 bp deletion increased significantly with age, there was no significant difference between smokers and nonsmokers.

The present study is the first to identify percentages of COX deficiency in the PCA muscles of individuals with a smoking history. Further molecular research should be conducted on the PCA muscles from nonsmokers and smokers to determine which mtDNA deletions are present that contribute to COX deficiency. This genomic research may help to identify whether

the mtDNA deletions or mutations are a product of normal aging or are enhanced by the accumulated exposure of the reactive oxide species found in cigarette smoke.

7.2.3. Implications to Function

There is scant literature on the effects mitochondrial deletions and COX deficiencies have on the physiology of individual muscle fibers as well as the whole muscle. As stated previously, there is conflicting research correlating the percentage of COX deficiency and the presence of symptoms in individuals with mitochondrial myopathies (i.e., 1%-85%) (Boulet et al., 1992; Chomyn et al., 1992; Elson et al., 2002; Müller- Höcker, 1983; Sciacco et al., 1994; Weber et al., 1997). Most researchers agree, however, that a significant proportion of the muscle fibers should be affected before a diagnosis of myopathy can be made.

Considerably less research has been conducted on the effects of mitochondrial function on normal aging muscle. As seen in the present study, the percentage of type I COX deficient fibers was less than 1% for both the PCA and strap muscles. The total number of COX deficient fibers was also less than 1% for both muscles. In the PCA muscle, the percentage of type I COX deficient fibers increased significantly with age. These percentages follow in line with research previously cited on other nonpathologic limb skeletal muscles, however, the implications to function remain elusive. Some research suggests that decreased mitochondrial oxidation throughout aging is related to a reduction in a person's physical activity (Brierly, Johnson, James, & Turnbull, 1996). This study by Brierly and colleagues, however, did not find a correlation between oxidative metabolism and age. Although decreased physical activity may partially explain age-related declines in mitochondrial oxidation in limb skeletal muscle, it does not clearly correlate to intrinsic laryngeal muscles. Muscles such as the PCA muscle continue to be activated even during old age and after other possible limb skeletal muscle compromising

situations (e.g., spinal cord injury causing the necessity of a wheelchair). The PCA muscle is activated during normal breathing and throughout phonation. Age-related declines in mitochondrial oxidative power in the PCA muscle may, therefore, be inherent to the muscle itself.

A recent study attempted to assess if aging is related to changes in the functioning of mitochondria (Tonkonogi, Ferström, Walsh, Ji, Rooyackers et al., 2003). Muscle biopsies were taken from the quadriceps muscles of seven older males (63-80 years old) and eight younger males (22-31 years old) without previous mitochondrial disease and with equal physical activity levels. Maximal oxygen uptake values for the quadriceps muscles and handgrip strength were also assessed. Individual mitochondria were isolated using a proteinase treatment with homogenization and differential centrifugation. The authors used the remaining part of the biopsy to prepare isolated skinned muscle fibers. Oxygen consumption was then assessed for both the isolated mitochondria and the skinned muscle fibers. Cytochrome c oxidase activity was also evaluated in a portion of the muscle biopsies.

Results indicated that COX activity and maximal oxidative power were significantly lower in the older group of participants compared to the younger group. Oxidative power, however, was not significantly different in isolated mitochondria. The authors' explanation for this lack of significance is that approximately 80% of the mitochondria can be lost during the isolation process; therefore, the results may be skewed because a large portion of the mitochondria are missing. The authors concluded that decreases in physical activity cannot solely explain changes in the functioning of mitochondria throughout the aging process. Perhaps these age-related changes to muscle and mitochondrial function are inherent to the biological

aspects of the aging process. The authors, however, stated that the molecular mechanisms surrounding these changes remain elusive.

Decreases in enzyme activity levels, as seen in the aging population in the studies mentioned above, are not solely responsible but can contribute to a reduction in muscle strength throughout the aging process (Vandervoort, 2002). Whole PCA muscle function, therefore, is probably not negatively affected exclusively by the small percentage (i.e., less than 1%) of COX deficient muscle fibers found in the present study. There are several other factors, however, that have been shown to contribute to decreased muscle strength and endurance in human limb skeletal muscle and evidence for these factors exist in the PCA muscle.

Along with sarcopenia (i.e., reductions in muscle mass) several researchers have indicated that the total number of muscle fibers also decreases throughout the aging process (Lexell, Taylor, & Sjöström, 1988). Researchers, however, continue to debate about whether there is a greater reduction in type II compared to type I fibers (Jakobsson, Borg, Edstrom, & Grimby, 1988; Larsson, Sjödin, & Karlsson, 1978) during aging or whether fiber type specific reductions exist at all (Grimby, Aniansson, Zetterberg, & Saltin, 1984; Lexell, Downham, & Sjöstrom, 1986). There is some evidence that indicates that type I fibers could show a disproportionate increase compared to type II fibers around the sixth and seventh decade, though fiber types are theorized to even out by the time a person is in their late 70s or early 80s (Andersen, 2003). Researchers, however, have unquestionably shown that type II fibers decrease in fiber area throughout aging though type I fibers show no reduction in fiber area (Grimby et al., 1984; Lexell et al., 1988). The significant age-related increase in type I fibers with COX deficiency compared to type II fibers in the PCA muscles in the present study shows that type I fibers are more affected as a person ages. This may be inherent to the muscle fibers in the PCA

muscle or may be secondary to a reduction in type II fibers in the PCA muscle during aging. Future research is needed to support and confirm these hypotheses.

Research has also indicated that there is a decrease in the total number of motor units in human muscle throughout aging (Brown, 1972; Campbell, McComas, & Petito, 1973), as well as an increase in the size of the innervation ratio between motor neuron and muscle fibers (McComas, 1995). An increase in the innervation ratio throughout aging indicates that as a person ages the motor neurons begin to innervate more muscle fibers than when the person was younger. Motor unit remodeling also occurs during aging. This remodeling is a natural, cyclical process whereby synaptic junctions turnover at the neuromuscular junction and involves the denervation, axonal sprouting, and reinnervation of muscle (Brooks & Faulkner, 1994). Throughout aging, however, motor unit remodeling changes such that type II fibers are denervated and reinnervated by type I motor units. The reason for this is still uncertain, though it is hypothesized that type II motor units may have a slower axonal sprouting rate compared to type I motor units, or type I motor units may have the capacity to make stronger more permanent connections in type I or type II muscle fibers. Once reinnervated by a type I motor unit the type II fiber is thought to acquire all of the physiological and biochemical properties of a type I fiber (Kugelberg, 1976). This motor unit remodeling also supports the theory that type I fibers increase throughout aging.

Histochemical evidence for motor unit remodeling in the PCA muscle exists in the form of fiber type grouping (Brandon et al., 2003a). This fiber type grouping is seen as an accumulation of tightly clustered type I and type II fibers with more type I fibers surrounding other type I fibers (Roos, Rice, & Vandervoort, 1997). The present study also shows

histochemical evidence for denervation patterns in the large number of target fibers found in the PCA muscle. Continued, ongoing research is being conducted to confirm these results.

Reductions in muscle mass, the number of muscle fibers, the fiber area of type II fibers, and the number of motor units, as well as motor unit remodeling in aging muscle (which can lead to a disproportionate increase in type I fibers) can contribute to a change in the force-generating capacity and function of human muscle (Bemben, 1998). There is some evidence that some if not all of these factors also occur in human PCA muscle throughout aging. These factors along with the enzymatic changes (i.e., COX deficiency) observed more in the mitochondria of type I fibers than type II fibers in the PCA muscle during aging could contribute to decreases in PCA muscle function over time.

Research regarding mitochondria, muscle function, oxidative power, and aging is important to understanding muscle in a disease state and during normal functioning. Studies are even being done to design a supplemental way to maintain or increase mitochondrial function and mitochondrial health. This supplement increases nitric oxide production which can increase vasodilation (i.e., oxygenation) to muscles and decrease ROS affect on COX deficiency (Gilligan, Panza, Kilcoyne, Waclawiw, Casino et al., 1994; Quyyumi, Dakak, Andrews, Gilligan, Panza et al., 1995). A new product, Oxegen AKG, is already being marketed based on the results from these studies. The present study is just the beginning of research on the functioning of the mitochondria in the intrinsic laryngeal muscles. Continued investigations related to intrinsic laryngeal muscle function throughout aging may facilitate possible pharmaceutical and exercise-induced ways to combat age-related decreases in mitochondrial functioning.

7.3. Limitations and Avenues for Future Research

A few limitations to the present study should be noted. The percentage of COX deficient type I fibers is underestimated because the PCA and strap muscles were not analyzed at different areas along the whole length of the muscle samples (Elson et al., 2003). The difference between percentages for the PCA and strap muscles was highly significant and the power was well-above acceptable levels that the risk for making a type I or type II error was low. Future research should, however, assess the whole muscle sample to determine more accurate percentages of COX deficiency and also evaluate the length of COX deficiency throughout the whole muscle sample. Results of the research from whole muscle samples will provide the groundwork for studies relating COX deficiency to muscle and mitochondrial function. Although there was a strong correlation between increases in COX deficiency and age, intrinsic laryngeal muscle biopsies should be harvested from younger populations to determine when COX deficiency develops and how it changes throughout the aging process.

Differences in subcompartment for posterior cricoarytenoid muscle were not analyzed in the present study. Preliminary results from this study do not suggest differences in subcompartments because proportions of type I and type II fibers did not always follow trends noted in other studies (Brandon et al., 2003a; Wu et al., 2000). Future research should study differences in subcompartment more rigorously to determine if COX deficient fibers differ between subcompartments and if there is a difference, assess if this difference correlates to changes in muscle function.

Although previous research has indicated that COX deficient fibers can be noted equally in type I and type II fibers (Johnson et al., 1993; Müller- Höcker et al., 1993) ancillary analysis of the percentage of type I and type II fibers noted a significantly higher proportion of type I fiber with COX deficiency compared to type II fibers in the PCA muscle. Future research may

assess changes in fiber type throughout aging to see if these changes relate to increases in one type of COX deficient fiber compared to another. For example, it is well-documented that type II fibers decrease in fiber diameter and fiber percentage over time (Tonkonogi et al., 2003); therefore, the percentage of type I fibers may increase over time leading to greater decreases in muscle fiber function, especially because of the highly significant difference noted between type I and type II fibers with COX deficiency in the PCA muscles seen in this study.

As stated previously, the accumulation of ROS can affect mitochondrial functioning throughout aging (Harman, 1972). Although the PCA is exposed to environmental toxins (regardless of the participant's smoking history) throughout the aging process, the chance of finding COX deficiency in these muscles was higher because of the participants' smoking histories. One of the most significant limitations to the present study, however, is that it was difficult to interpret whether the increase in type I COX deficient fibers seen the PCA muscle was truly age-related or due more to the accumulation of ROS subsequent to the significant smoking histories of the participants. One PCA muscle was harvested from autopsy but because of the Health Insurance Portability and Accountability Act of 1996 (HIPAA) requirements, the technicians were unable to disclose the smoking history of the participant; therefore, it was almost impossible to obtain good normal (i.e., nonlaryngectomy, nonsmoker) control muscle. Future research needs to be conducted on nonsmoking controls to determine whether the increases in COX deficiency seen in the PCA muscle are inherent to the muscle itself or secondary to the ROS found in cigarette smoke.

One of the most important avenues for future research is to study how decreases in COX activity affects the functioning of the mitochondria, muscle fibers, and whole intrinsic laryngeal muscles throughout aging. Results from these studies may provide speech-language pathologists,

laryngologists, and other voice care providers with a way to diagnose and treat voice disorders based in biological science. Unfortunately, an instrument to quantitatively measure the biological functioning of *in vivo* intrinsic laryngeal muscle function currently does not exist. Hopefully prospects will arise in the near future.

8. CHAPTER VII – CONCLUSION

The present study is the first to identify cytochrome c oxidase deficient fibers in the human posterior cricoarytenoid and control strap muscles. Results indicated a highly significant difference ($p = .002$) in the percentage of type I fibers with cytochrome c oxidase deficiency in the PCA muscle compared to the strap muscle. Mitochondrial abnormalities, such as cytochrome c oxidase, have been shown to occur during normal aging. These abnormalities, however, can increase with continued exposure and accumulation of reactive oxide species. Exogenous sources of reactive oxide species are present in cigarette smoke and other environmental toxins. Because of its position in the airway, the posterior cricoarytenoid muscle has direct exposure to these exogenous sources of reactive oxide species. To increase the chance of finding fibers with cytochrome c oxidase deficiency, the posterior cricoarytenoid and strap muscles were obtained post laryngectomy from individuals with a significant smoking history. Type I fibers were analyzed because of their increased risk of having cytochrome c oxidase deficiency and the posterior cricoarytenoid muscle was chosen as a starting point because of its position in the larynx and its large number of type I fibers compared to other intrinsic laryngeal muscles.

The exposure to exogenous sources of reactive oxide species could be a factor in the increased percentage of type I fibers with cytochrome c oxidase deficiency observed in the posterior cricoarytenoid muscle, however, there are other possible factors that could also contribute to this result. For example, previous research has indicated that some muscles inherently develop cytochrome c oxidase deficiency faster or in an increased amount compared to other muscles during aging (Müller- Höcker et al., 1993). The larger percentage of cytochrome c oxidase deficiency in the type I fibers of the posterior cricoarytenoid muscle may, therefore, be inherent to the aging process of the muscle itself. Continued research is needed to

identify the development of cytochrome c oxidase in the posterior cricoarytenoid muscle throughout aging. Normal (nonlaryngectomy, nonsmoker) control posterior cricoarytenoid muscle was extremely difficult to obtain. Research, however, is also needed to identify whether certain exogenous sources of reactive oxide species may contribute to the proportion of cytochrome c oxidase deficient fibers found in the posterior cricoarytenoid muscle using control muscle from individuals without a smoking history.

The percentage of cytochrome c oxidase deficient fibers was less than 1% for the posterior cricoarytenoid and strap muscles. This percentage is comparable to what has been previously found in other limb skeletal muscles. Cytochrome c oxidase deficient fibers, therefore, probably do not solely affect muscle fiber and whole muscle function. An alteration in muscle fiber enzyme activity is not the only way decreases in muscle strength and function occur throughout aging. Limb skeletal muscle has been shown to go through many changes throughout aging which have been identified in decreases in muscle function. Some of these changes include, reductions in muscle mass, the total number of muscle fibers, the fiber area of type II fibers, and the number of motor units, as well as motor unit remodeling. These changes are hypothesized to lead to greater increases in the proportion of type I fibers, as well as a greater number of type I motor units in aging muscle. Evidence exists to support these changes in the posterior cricoarytenoid muscle (i.e., large proportions of type I fibers, fiber type grouping, and the presence of target fibers). Ancillary analyses indicated that there were significantly more type I fibers with cytochrome c oxidase deficiency than type II fibers in the posterior cricoarytenoid muscle. Type I fibers with cytochrome c oxidase deficiency were also significantly correlated with age in the posterior cricoarytenoid muscle. Increases in type I motor units with denervation, increases in overall type I fibers with age, as well as the results that type I fibers have

significantly more cytochrome c oxidase deficiency than type II fibers in the posterior cricoarytenoid muscle indicates that there could be decreases in posterior cricoarytenoid muscle function over time.

The present study provides a starting point for continued research on mitochondrial functioning in the intrinsic laryngeal muscles. Facilitation of basic knowledge regarding mitochondria, muscle function, oxidative power, and aging is important to understanding the biochemical aspects of muscle in a disease state and during normal functioning. With continued investigations, voice therapists, laryngologists, and other voice care providers will hopefully be more equipped to base the diagnosis and behavioral treatment of certain voice disorders in biological voice science.

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