INTRODUCTION

Glaucoma is the second leading cause of irreversible blindness around the world, affecting more than 200,000 people every year. An optic neuropathy, the disease involves the death of retinal ganglion cells (RGCs) and subsequently the loss of axons in the optic nerve (ON). These nerve cells do not regenerate, causing irreversible vision loss [1,2]. Although the onset of glaucoma and loss of these RGCs is often associated with high intraocular pressure (IOP), this is not always the case and is the subject of much ongoing research [3,4].

It has been shown that primary open angle glaucoma disproportionately affects those of African Descent (AD) and Hispanic Ethnicity (HE) over those of European Descent (ED) [5,6]. Though this phenomenon may be due in part to a disparity in socioeconomic factors, it is reasonable to assume that certain biomechanical and morphological differences between the eyes of AD, HE and ED donors may play an important role. As such, it is important to study the biomechanical differences between these eyes of different races to better understand the reason for this phenomenon. This is a main aim of our laboratory.

The medical and ophthalmological records our lab receives with donor eyes are limited and not always comprehensive, so it is important to verify the difference between glaucomatous (G) and nonglaucomatous (NG) donors. Because axons of RGCs that traverse the ON become considerably degraded and are eventually lost in patients with glaucoma, the axon count of optic nerve samples is one method to confirm the normality of donor ocular tissues. Axon counting methods have been implemented on primarily non-human tissue, and the majority of research involving human ON axon counting predates 1995 [7,8].

OBJECTIVE

The goal of this work is to investigate whether there is a difference in axon count between those of AD, HE and ED race/ethnicity that may contribute to glaucoma prevalence, and to confirm via axon count that the nerves received in our laboratory without an indicated medical history of glaucoma are indeed NG. The information gathered in this work will be useful for ensuring that the biomechanical and structural endpoints being measured in separate studies in our laboratory are solely due to racial and/or ethnic differences. This data will also be useful as a control group for future experiments investigating how axon counts may be different in glaucomatous samples as a function of racial/ethnic background.

SUCCESS CRITERIA

In order for this experiment to be successful, there are a number of criteria that must be met. During processing, ONs must be stained, cross-sectioned and embedded uniformly onto slides to ensure axons are discernable. Ability to recognize and count axons is also contingent on the correct function of autofocus and other elements of image-stitching microscope software. Also, the user must be able to correctly identify what does and does not constitute an axon.

MATERIALS AND METHODS

Our laboratory received optic nerves from Midwest Eye Bank (MWEB) (n=9), San Diego Eye Bank (SDEB) (n=3), Banner Health (BH) (n=1) and Arizona Eye Bank (AEB) (n=1), containing nerves from both the left (OS) (n=8) and right (OD) (n=6) eyes, from AD (n=6), HE (n=2) and ED (n=6) donors, all over the age of 50. Four of these donors (n=2 AD, n=2 ED) were indicated glaucomatous on received medical records. Within 4 hours from death, the optic nerves were cut and fixed in Poly/LEM at their respective eye banks, at which point they were sent to our laboratory. Once received, the nerves were transferred to vials containing 2.5% glutaraldehyde and stored for 24 hours. Finally, they were transferred to PBS and stored at 4°C until they were sent out for processing. During processing, optic nerve samples were cut cross-sectionally, stained with OsO₄, and embedded onto slides that were visualized on a Nikon Eclipse 90i microscope.

Figure 1: Microscope image of full optic nerve cross section from 66 year old AD sample. Dark sections indicate bundles of axons, separated by the lighter connective tissue. A magnified section of axons is provided, characterized by their circular shape and lighter center surrounded by dark myelin sheaths.

Each cross section was individually imaged at 60x magnification using Nikon NIS-Elements image-stitching software; 15% overlap as well as autofocus capabilities were used for each pixel, allowing for successful and detailed image montaging.

Semi-automated axon counts were executed using image
processing techniques in MATLAB, and methodology adapted from that previously described in Teixeira et al [9]. Each montaged image was read one at a time through a program written to perform a semi-automated axon count across 100% of the given optic nerve cross section. A single experienced user performed all axon counts on the samples examined in this study. The user was first prompted to identify the bounds of the optic nerve cross section in order to crop the image. Contrast-limited adaptive histogram equalization was applied to the cropped image to improve user accuracy of axon identification. Ten individual small sections of axons of equal area throughout the entire image were randomly selected and presented one at a time, and the user was asked to manually click on the axons in each section. These manual counts for each section were added together and averaged corresponding to their fraction of the total cropped cross-sectional area, providing an average axon density for the section. This calculated density was then used to extrapolate across the entire cross-section in order to give an estimated axon count for the whole optic nerve. Each image was run through the program three times for repeatability, and an average and standard deviation for the axon count of each optic nerve was calculated.

RESULTS

Of the ten presumed-NG samples tested, mean semi-automated axon counts ranged from 741,679 to 1,223,581. Calculated standard deviations indicated a minimum axon count of 601,240 for 64 year old AD sample, and a maximum axon count for 66 year old AD sample at 1,351,184. For samples of African Descent (n=4), mean axon count was 931,603 ± 92,863. Samples of European Descent (n=4), mean axon count was 979,421 ± 93,560, and mean axon count for samples of Hispanic Ethnicity (n=2) was 873,250 ± 28,700. A one-way ANOVA was performed across the three races, and no significant difference in axon count between races was found (P>.05). The mean axon count for all (n=10) samples with no indication of glaucoma in their medical history was 939,547 ± 81,514. When individually compared to the axon count of glaucomatous donors (248,483 ± 35,351), all presumed-NG donor ON axon counts were significantly higher.

Figure 2. Graph showing the difference in mean axon count between the glaucomatous and confirmed nonglaucomatous optic nerves examined in this study.

DISCUSSION

A one-way ANOVA between races indicated that there was no difference in axon count for those of different racial/ethnic backgrounds. This indicates that axon count is not a contributing factor to the difference in prevalence of glaucoma between races. However, considering our limited sample size, more axon counts between races are needed to fully confirm this observation.

All n=10 samples tested were within the normal range for human eye axon counts as reported by Mikelberg et al, indicating healthy non-glaucomatous eyes [7]. Our axon count range was slightly lower than the range reported in Jonas et al. This disagreement is likely due to the difference in age of subjects in the two studies, which is significant (P<.05). It has been shown in multiple studies that there are generally fewer axons in the eyes of older individuals, as a number of RGCs are lost every year and do not regenerate [8]. When axon counts were compared individually to donors with a medical history of glaucoma, the presumed-NG donors’ axon counts were found to be significantly higher (P<.05). From this data, we are able to confirm that the eyes in this study are indeed nonglaucomatous.

Identifying these baseline axon counts for normal and glaucomatous eyes is useful in order to better understand the adverse effect glaucoma has on RGC axons, since the reason for the loss of these axons is still unclear. Further research involving axon counting will help to better characterize of the mechanism of RGC axon death, and hopefully shed light on possible solutions to the irreversible blindness that results.

Our lab will continue to perform axon counts on eyes of different races as a means of verifying normality, so that other parts of these undiseased eyes may be studied and compared between different racial backgrounds. We also intend to develop a fully automated code for axon counting, in order to improve count accuracy.

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REFERENCES
