A Study of the Change in Mitochondrial DNA from Various Tissues of Mice of Varying Ages

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Abstract

The nucleus is not the only organelle containing DNA, the mitochondria also contains its own DNA. This experiment was conducted to determine how much the mitochondrial DNA (mtDNA) decreases with age in mice using real-time PCR. Samples previously extracted from nine varying tissues of three different mice of four different ages were used. The DNA was extracted from those samples and the concentration of DNA per cell was measured using a real-time PCR machine. The mtDNA concentration in the first three ages had already been calculated prior to the experiment. It was found in the majority of samples that during the twelfth month the mtDNA reached its peak, and during the 18th month it decreased. The amount it decreased depends on the tissue. There were two samples that did not decrease the 18th month, both of which would require further research to determine the cause. This experiment provides further research into the quantitative changes in mtDNA throughout age.

Contents

Abbreviations

AL	Contains guanine hydrochloride (for more information contact QIAGEN)
ATL	Animal Tissue Lysis
AW2	Contains 70% EtOH (for more information contact QIAGEN)
CT	Threshold cycle
mtDNA	Mitochondrial DNA
mt-Nd1	Mitochondrial NADH-ubiquinone oxidoreductase chain 1
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
Rpph1	Ribonuclease P RNA Component H1
TAE	Tris base, acetic acid, EDTA
UV	Ultraviolet

1 Introduction

A common misconception is that the nucleus holds all of the DNA in the cell. However the mitochondria, organelles in the cell, also contain their own DNA. Mitochondrial DNA (mtDNA) is crucial to the function of the mitochondrion. The mitochondrion is best known for being the main energy source in the cell, due to the aerboic ceullular respiration that takes place in the mitochondrion. It was first discovered in 1963 by Margit and Sylvan Nass [1]. Since then, many other researchers have discovered more about the mitochondrion, its DNA, and diseases caused by defects in mtDNA.

Not only are the mitochondria the energy sources of the cell, but they also contribute to: apoptosis i.e. programmed cell death, and the production of cholesterol and heme groups - two important components of hemoglobin [2]. Therefore when the mtDNA begins to degrade the mitochondrion begins to lose these vital functions, causing the cell to become senescent and decease. Other causes of mitochondrion deaths include a number of diseases caused by mtDNA depletion. One of these diseases is mitochondrial depletion syndrome (MDS), an inherited disorder that can result in 98% depletion of mtDNA [3]. If the mtDNA is degrading, then the mitochondria are not able to create energy for the cells, therefore hindering the growth and development of the organism. A study conducted at Karolinska Institute revealed the effects on mice if they continuously lose mtDNA; their lifespan shortens to two weeks as opposed to the expected lifespan of one and a half to two years, in addition to being twice as small as the mice with normal mtDNA [4]. This is important, because it reveals that an organism can not live long with very little mtDNA.

Previous work has already been conducted on the mitochondrial copy number in mice of different ages. Masuyama et al. (2005) was one of the first studies discussing the change in mtDNA copy number with age. It was found that, with age, the mitochondrial copy number changes in various tissues depending on the amount of oxygen that the tissue uses [5]. For example, at two weeks old the liver, brain, and heart had the highest concentrations of mitochondria while the skeletal muscle had a moderate amount. At

15 months old, however, the skeletal muscle, the brain, and the heart had the highest concentrations while the liver remained mostly unchanged. The skeletal muscle, the brain, and the heart are large consumers of oxygen and need a lot of energy, hence they have more mitochondria [6].

There are many reasons explaining why mtDNA degrades, a significant factor being age [7]. This study measures the mtDNA concentration in nine different tissues in mice of four different ages; 1, 6, 12, and 18 months old. Discovering the mtDNA changes in mice will give insight into how human mtDNA changes with age as mouse mtDNA is similar to human mtDNA. Determining how much the mtDNA degrades with age is important, as it allows further studies to be conducted in order to determine the effects of mtDNA degradation on the human body. The purpose of this study is to investigate how the quantity of mtDNA changes with time, using real-time PCR.

2 Method

All products used in this study, except for the tissues and the machines, are manufactured by QIAGEN.

Samples were extracted from nine varying tissues (brain, tongue, heart, liver, kidney, spleen, skeletal muscle, lung, and intestine) from twelve different healthy mice of varying ages (1 month old, 6 months old, 12 months old, and 18 months old). The data from three mice during the first three time periods (1 month old, 6 months old, and 12 months old) had already been collected. This project measured the mtDNA in nine varying tissues from three different 18 month old mice. The samples had already been collected from three different mice of 18 months old. The samples were placed in tubes filled with 180 µL of Buffer ATL to help maintain the pH and create the enzyme, proteinase, which aided the experiment by initiating lysis of the cell proteins, exposing the DNA. 20 µL of proteinase K was then added to the tissue solution to degrade the proteins in order to extract the DNA. Once the solution was complete, it was vortexed for five seconds. The solutions

were then put into a thermomixer at 56°C for 16 hours in order to break down all the proteins to extract the DNA.

The solutions were vortexed 16 hours later for 15 seconds, 400 µL of a mixture, half Buffer AL, the other half ethanol, was pipetted into the tubes with the samples and then vortexed once more. The mixture was then pipetted into DNeasy Mini Spin column tubes and centrifuged for one minute at 8000 rpm. The flow-through and collection tube from centrifuging were discarded and the remaining tube was placed into a new DNeasy Mini Spin column. From there, 500 µL of Buffer AW1 was pipetted into each tube and the tubes were centrifuged for 1 minute at 8000 rpm. The flow-through and collection tube from centrifuging were discarded and the remaining tube was placed into a new DNeasy Mini Spin column. From there, 500 µL of Buffer AW2 was pipetted into each tube and the tubes were centrifuged for 3 minutes at 1300 rpm. The flow-through and collection tube from centrifuging were discarded and the remaining part of the tube was placed into a micro-centrifuge tube. 100 µL of distilled water was pipetted directly onto the DNeasy membrane where it then stayed for 1 minute at room temperature and was centrifuged one last time for 1 minute at 8000 rpm.

Once all the DNA was collected the concentration of mtDNA was calculated by first calculating the concentration of DNA. The DNA was diluted with a 1:10 ratio of distilled water: 10 µL of the sample and 90 µL of water were pipetted into a micro-centrifuge tube. The remaining DNA was then placed in the fridge for later use. The concentration of the DNA in the tissues was measured using the diluted samples and a spectrophotometer. A spectrophotometer makes use of UV radiation at 260 nm to measure the concentration of the diluted solution.

Gel electrophoresis was also used to determine the quality of the DNA. The gel was prepared by dissolving 1.2 g of Agarose in 150 mL of TAE buffer and then adding 4 µL of ethidium bromide. The mixture was then poured into a mold and a comb was placed into the mixture to create wells for inserting the DNA into the gel. While the gel was hardening from liquid to gel form, the DNA that was in the fridge was withdrawn. 5 µL of

the samples and 1 μL of loading dye was pipetted into a micro-centrifuge tube and then mixed. 6 μL of the mixture was then placed into the wells of the hardened gel and the system was connected to 90 V, causing the samples to migrate from one end to the other. After 45 minutes the gel block was transported to a UV Transilluminator, capturing an image of the DNA migratory path.

The samples were then diluted to 10 ng/μL with the formula in Equation 1, (where C_i represents the concentration that was measured, V_i represents the volume that is being calculated, C_f is equal to 10 ng/μL, and where V_f is equal to 100 μL) the necessary concentration for real time PCR.

$$C_i V_i = C_f V_f \tag{1}$$

1 μL of the diluted samples respectively was submerged in a mixture consisting of: 5 μL of Universal PCR Master Mix, 0.5 μL of the primer/probe solution, and 3.5 μL of distilled water.

This mixture was then placed into different wells. For accuracy, the samples were repeated three times, meaning that there were three wells that contained the same sample. The container was then placed in a centrifuge machine for 2 minutes on 1500 rpm. After centrifuging, the container was placed into the real-time PCR machine. The results were then graphed and analyzed.

In order to obtain the mtDNA concentration within each cell, a standard curve was used. The slope of the standard curve, as seen in Equations 2 and 3, which can also be found in Appendix A, provides an equation to find the number of nuclear copies in the sample that was measured (Equation 2) and another equation to find the number of mtDNA copies in the sample (Equation 3).

$$y = -1.445 \ln(x) + 40.431 \tag{2}$$

$$y = -1.471 \ln(x) + 41.154 \tag{3}$$

Once the x variable was found for both the Rpph1 and mt-Nd1, of each tissue, the mt-Nd1 copy number was divided by the Rpph1 copy number in order to find the number of mtDNA copies in each cell. Rpph1 is a gene found in the DNA of the nucleus. Mt-Nd1 is a gene found in the DNA of the mitochondrion. These genes are used to determine the mtDNA content in each cell, due to the fact that the DNA only has one copy of this gene and the real-time PCR machine detects that copy with a fluroescent light and determines how much of the gene is in the sample. The threshold cycle (CT) is the cycle when a significant increase in ΔRn is first detected. ΔRn is the indicator of the amount of fluorescent signal generated by the real-time PCR. The steps in this method were repeated three times for each mouse.

3 Results

Table 1: The mtDNA concentration in 9 various tissues from 3 different mice of varying ages averaged together.

Type of Tissue	1 month	6 months	12 months	18 months
Brain	2,074	3,870	8,258	1,982
Tongue	520	871	767	4,325
Heart	14,695	19,537	22,544	6,308
Liver	2,268	3,979	4,934	4,119
Kidney	623	1,092	2,213	1,473
Spleen	435	548	497	596
Skeletal Muscle	4,317	7,563	10,982	7,760
Lung	587	831	845	531
Intestine	490	647	785	323

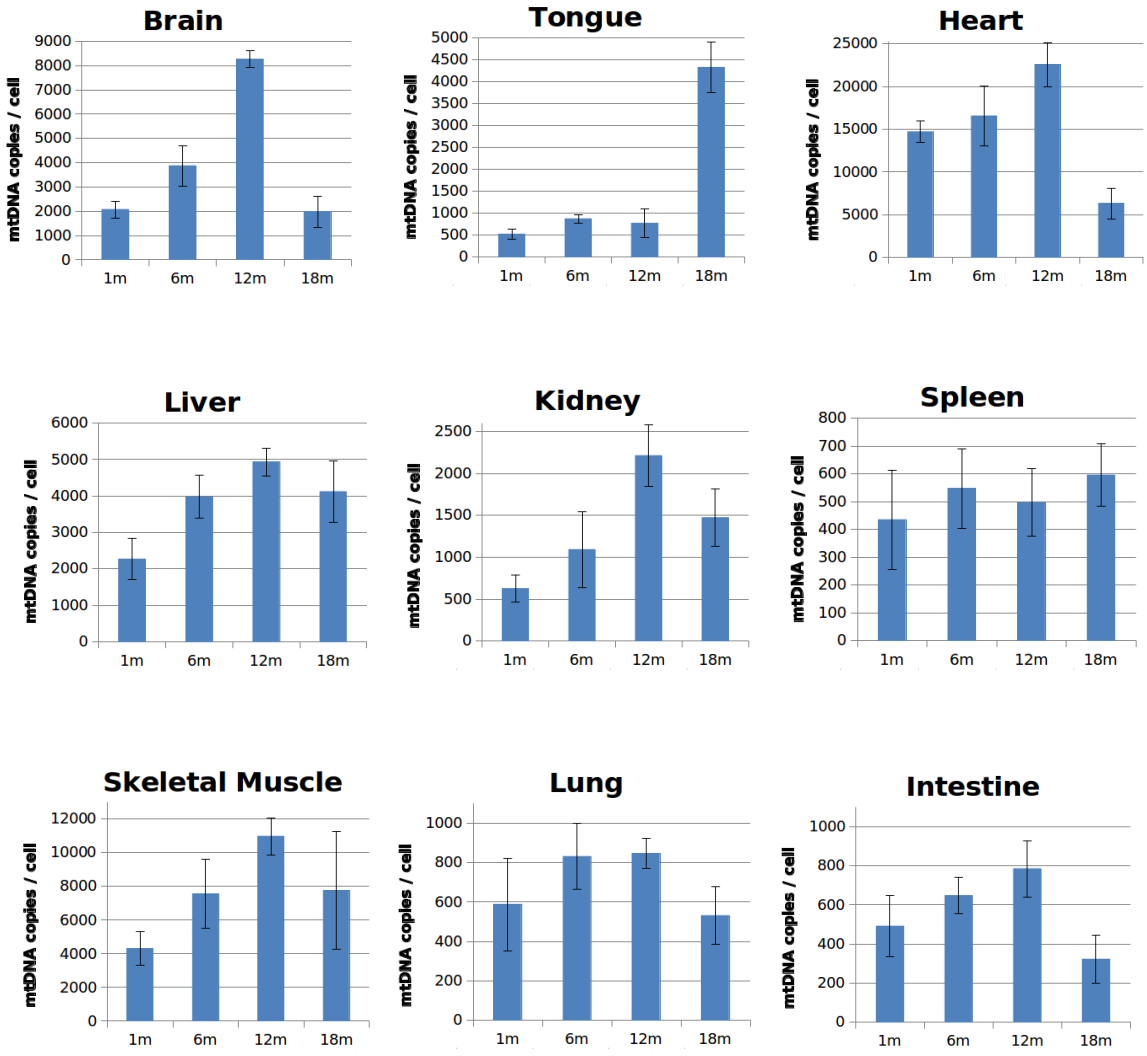

Figure 1: Quantitative Changes in mtDNA over 18 months.

As seen in Figure 1 the results are presented in seperate bar graphs representing the quantitative change of mtDNA during the four different time periods of each tissue. A bar graph with all the data combined into one graph can be found in Appendix B. The error bars represent the standard deviation in the samples.

At one month old, the mouse brain consisted of a lower mtDNA concentration compared to the concentration at 12 months. During 18 months the brain mtDNA began to degrade and dropped by a factor of four, close to the initial concentration at one month.

The mtDNA values of the tongue were low between the one month mark and 12 month

mark, staying between 520 ng/μL and 871 ng/μL. At 18 months the mtDNA values from the tongue experienced a sharp increase, almost quadrupling in concentration.

The mtDNA concentration of the heart steadily increased throughout the first three time periods. During the 18th month the value decreased, approximately, by a factor of four.

The mtDNA values of liver increased throughout the first, sixth, and twelfth months. The value experienced a slight drop during the 18th month. Here, the concentration of mtDNA decreased by one fifth.

The values of the kidney rapidly increased from one month to six months to twelve months, almost doubling in size each time. However, during the eighteenth month the value decreased by approximately a third.

The values of the spleen fluctuated up and down consistently over the four time periods with the highest values at 18 months old.

The mtDNA concentration of the skeletal muscle began low and experienced a sharp increase during the time between the first month and the twelfth month. The value decreased by a third from the twelfth month to the eighteenth month.

The values of the lung increased by a third from the first time period to the second time period. During the second and the third time period the values remained relatively consistent, until the values decreased by a third from the twelfth month to the 18th month.

The mtDNA concentration of the intestine experienced a consistent increase from the first month to the twelfth month. However, the values decreased by approximately a third from the twelfth month to the 18th month.

4 Discussion

As observed previously in the results, the common trend line is a peak at 12 months and then a decline at 18 months. There were only two tissues that did not follow this trend, the

tongue and the spleen. The brain, heart, liver, kidney, skeletal muscle, lung, and intestine all peaked at 12 months and declined at 18 months. These results are the expected results for all tissues, because with age comes degradation. The decrease in mtDNA concentration per cell in these tissues proves that the mice are becoming more senescent throughout aging and that it has an impact on their tissues. These results aid the project, because they now give indication into the mtDNA concentration of varying tissues at 18 months, a study that has rarely been conducted before. Some of the samples experienced a sudden decline from their peak, for example the brain and the heart. The sudden decline clearly shows that both of those organs are slowing down and becoming decrepit. With mtDNA degradation comes problems, meaning that if the mtDNA concentration suddenly declines the mouse will experience some problems in those tissues. The heart, the brain, and the skeletal muscle all had very high mtDNA concentrations to begin with, meaning that they used the most oxygen and are vital organs for life.

The other samples that peaked at twelve months and then declined were the liver, kidney, skeletal muscle, lung, and intestine. These samples had lower concentrations overall compared to the heart, brain, and skeletal muscle. This is because they do not use as much oxygen as the other three tissues. These samples still decreased, supporting the expected results, meaning that they do become senescent.

There were two samples that did not coincide with the measurements from the other tissues, the tongue and the spleen. The spleen intermittently increases and decreases, until it has peaked on the 18th month. Since the difference is not large compared to the other samples, the values are not detrimental to the experiment. However the mtDNA values from the tongue spiked by 464% between the third and fourth time period. All of the tongue samples taken from the mice contained similar values, below 1,000 ng/μL during the first three time periods; whereas after a total of 18 months the value increased to over 4,000 ng/μL. This is not an ordinary occurrence, but one explanation could be a type of cancer in the mouth. Cancer would result in a sudden generation of cells, hence a spike in the mtDNA concentration during the 18th month.

This project revealed that in the majority of tissues mtDNA concentration decreases during their final months of life. There were two samples that did not decrease and one that spiked immensely. Another conclusion that can be drawn from this experiment is that the tissues that consume a large amount of oxygen have a larger amount of mtDNA compared to the tissues that do not consume a large amount of oxygen.

Even though the results came out as expected, there were still errors when performing the lab. First of all there were only three mice used per time period, which will not provide enough data to come to an accurate conclusion. In order to improve the accuracy of the experiment at least eight mice should be used per time period. Secondly, when transferring the tissues they were exposed to light and other bacteria in the air. This could have resulted in particles contaminating the samples and providing inaccurate results. Transferring the samples in a sterile environment would avoid inaccuracy.

As mentioned previously, the tongue had a surprising spike during the last time period. A hypothesis for this occurrence would be that the mice have developed some type of mouth cancer. If more time were provided for this experiment, the causes of this spike would be researched. This experiment could also be furthered by determining the diseases the mice contracted from their mtDNA degradation, if there were any. Further research would provide more insight into mtDNA degradation and its effects on the human body.

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A CT Values and Standard Curve Graph

This Appendix consists of Table 1, Table 2, and Figure 2. Table 1 is a data table consisting of all of the measurements of CT. Table 2 is the average of all of the measurements. Figure 2 is the standard curve graph that is made from Table 2.

Table 2: A table of all of the CT values found in the different number of copies of DNA.

Target Name	CT	CT Mean	CT SD	Quantity
Rpph1	20.43	20.36	0.0626	1000000
Rpph1	20.35	20.36	0.0626	1000000
Rpph1	20.30	20.36	0.0626	1000000
Rpph1	23.73	23.77	0.0359	100000
Rpph1	23.80	23.77	0.0359	100000
Rpph1	23.76	23.77	0.0359	100000
Rpph1	27.41	27.31	0.0864	10000
Rpph1	27.28	27.31	0.0864	10000
Rpph1	27.25	27.31	0.0864	10000
Rpph1	30.74	30.63	0.2056	1000
Rpph1	30.39	30.63	0.2056	1000
Rpph1	30.76	30.63	0.2056	1000
Rpph1	33.58	33.56	0.0281	100
Rpph1	33.56	33.56	0.0281	100
Rpph1	33.56	33.56	0.0281	100
mt-Nd1	20.24	20.42	0.3403	1000000
mt-Nd1	20.21	20.42	0.3403	1000000
mt-Nd1	20.81	20.42	0.3403	1000000
mt-Nd1	25.10	24.64	0.4455	100000
mt-Nd1	24.62	24.64	0.4455	100000
mt-Nd1	24.21	24.64	0.4455	100000
mt-Nd1	27.88	27.84	0.0502	10000
mt-Nd1	27.84	27.84	0.0502	10000
mt-Nd1	27.78	27.84	0.0502	10000
mt-Nd1	30.99	30.87	0.2341	1000
mt-Nd1	31.01	30.87	0.2341	1000
mt-Nd1	30.60	30.87	0.2341	1000
mt-Nd1	34.28	34.25	0.0415	100
mt-Nd1	34.25	34.25	0.0415	100
mt-Nd1	34.22	34.25	0.0415	100

Table 3: CT value averages for standard curve.

Quantities	Rpph1	mt-Nd1
1000000	20.36	20.42
100000	23.77	24.64
10000	27.31	27.84
1000	30.63	30.87
100	33.56	34.25

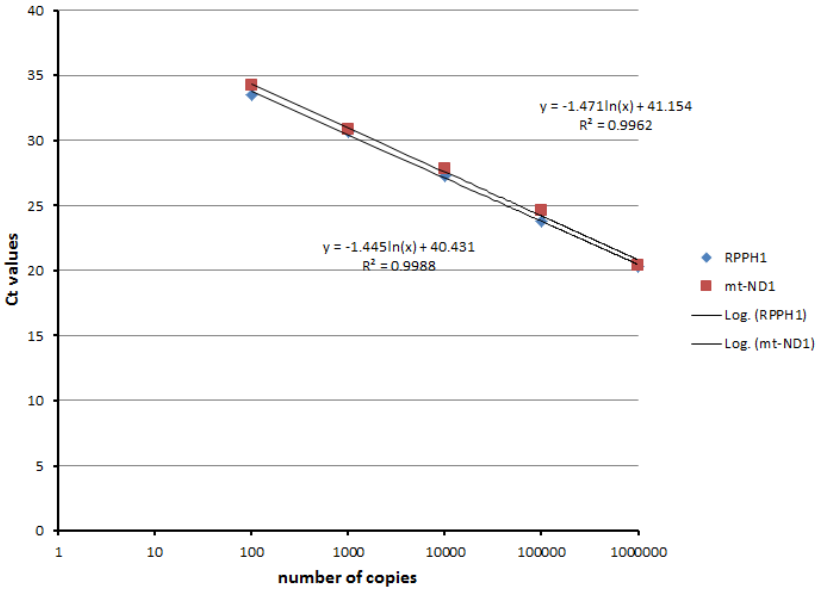

Figure 2: Standard curve with slope formulas to find the mtDNA concentration in each cell.

B Data of mtDNA Concentrations

This Appendix consists of Figure 3. Figure 3 is a bar graph that represents the quantitative changes in the varying tissues over age. It uses the data from Table 1 in the results. The error bars represent the standard deviation between each mouse.

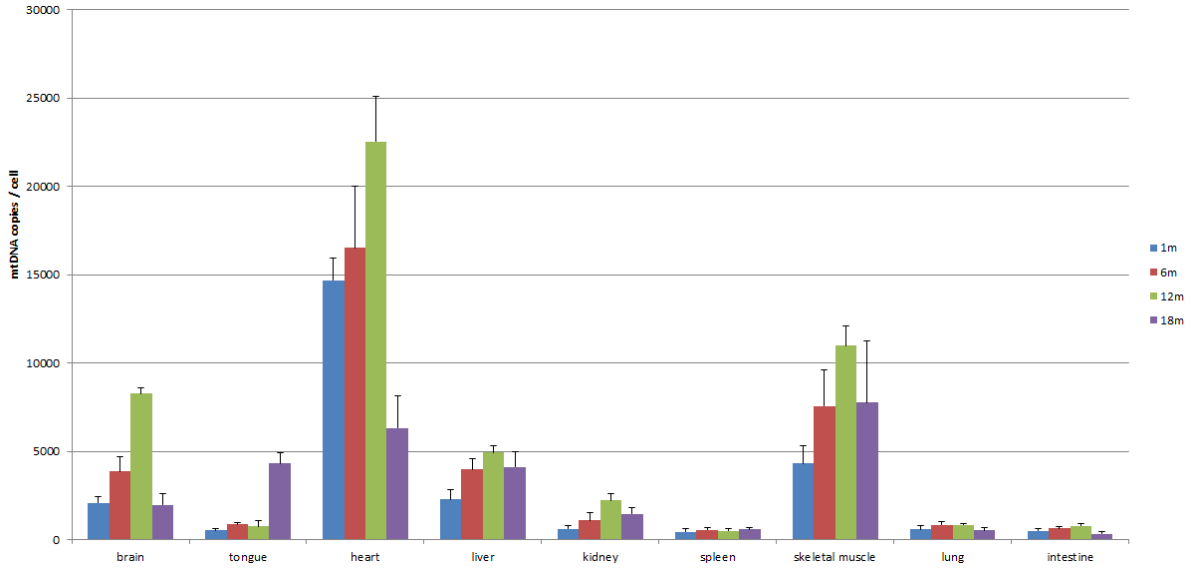

Figure 3: Quantitative changes in mice over 18 months from 9 varying tissues.