تأثير حرمان الفئران من فيتامين ج على حدوث الطفرات الجنينية

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ملخص البحث :

يعتبر أنزيم Gulonolactone مهم في المرحلة الاخيرة من الانتاج الحيوي لحمض أسكوربك. يحول أنزيم جلولونولاكتون أوكسيديز بواسطة جولو جينGulo gene معظم الحيوانات مثل الفئران لديها الجين جولو Gulo Gene، والذي تنتج من خلاله الاسكوربيك من الجلوكوز ، بينما يحمل الانسان وخنازير غينيا والحيوانات من رتبة الرئيسيات جين Gulo gene غير وظيفي ولهدا يعتبر الاسكوربيك اسيد عنصر ضروري للعمليات الحيوية في داخل الجسم و يقلل من مضادات الاكسدة. يلعب حمض الاسكوربيك دورا حيويا في حماية الحمض النووي (DNA) من الجدور الحرة free radicals التي تنتج من الاجهاد التأكسدي oxidative stress الذي قد يسبب العديد من الامراض مثل السرطان وأمراض القلب. تركز هذه الدراسة على بحث دور حمض الاسكوربيك في إزالة التغير الجيني الناشئ عن الاجهاد التأكسدي. للتحقق من كيفية استخدام فيتامين ج (vitamin C) لتقليل مستوى معدل التغير الاحيائي للحمض النووي (DNA) وحماية الحمض النووي من الجدور الحرة free radicals . تم استخدام نوعان من الفئران (الفئران الزرقاء العملاقة Big Blue mice) و (Knockout Gulo mice) كنموذج لتحديد قدرة فيتامين ج على التقليل من الاجهاد التأكسدي . تحمل فئران الزرقاء العملاقة على *cll* reporter gene وهو جين يمكن تحديد معدل التغير الأحيائي للحمض النووي (DNA) في أجزاء الجسم . بناء عليه ، قمنا بتكوين فئران معدلة وراثيا ينقصها الجين جولو (Knockout Gulo mice) و تحمل *cll* reporter gene ، وتم الحصول على فئران متجانسة الزيجوتية (positive Gulo-+Homozygote Gulo) cll -/ من خلال تهجين نوع ايجابي غير متجانس من الجين جولو وفئران متجانسة الجين جولو. خمسة فئران أعطيت تغذية ينقصها فيتامين ج وتم اعطاء خمسة فئران تغذية يوجد بها فيتامين ج . تم تحليل معدل تحول الحمض النووي في المجموعتين . فكان هناك اختلافات كبيرة بين المجموعة المتجانسة التي ينقصها فيتامين ج (vitamin C) وبين الفئران التي أعطيت غذاء غنى بفيتامين ج (vitamin C). فكانت درجة التحول الاحيائي لجين جولو في الفئران التي لم تعطى فيتامين ج أعلى بشكل كبير من المجموعة الاخرى (104) التي أعطيت فيتامين ج (vitamin C) . هده النتائج تدعم دور فيتامين ج (vitamin C) كعامل مضاد للأكسدة وتسلط الضوء على أهمية العمل المستقبلي لتحديد أنواع التحور الأحيائي الناشئ عن الاجهاد التأكسدي في عدم وجود فيتامين ج (vitamin C) .

Effect of vitamin C deprivation diet to increase mutation by reporter gene knockout Gulo$^{-/-}$cII^{+} mice Abstract

Gulonolactone oxidase enzyme is important in the final stage of ascorbic acid biosynthesis. Gulonolactone oxidase is encoded by the *Gulo* gene. Most animals, such as mice have the *Gulo* gene, through which they produce ascorbic acid from glucose, while humans, guinea pigs and primates carry a nonfunctional *Gulo* gene. Ascorbic acid plays an important role in many biological processes. However, it is primarily an essential as an antioxidant. Ascorbic acid protects genomic DNA from free radicals which result from oxidative stress that may cause a variety of diseases such as cancer or heart disease. This study focuses on investigating the role of ascorbic acid in the elimination of oxidative stress-induced mutagenesis.

To investigate how vitamin C decreases level of the DNA mutation rate and protects DNA from free radicals, knockout *Gulo* and Big Blue mice were used as models to determine the ability of vitamin C to minimize oxidative stress. The Big Blue mice carry the *cII* gene which is a reporter gene through which DNA mutation rate can be detected in any part of body. Therefore, we generated double transgenic mice which are Gulo deficient or a Big Blue background. Homozygote *Gulo cII* positive (*Gulo*$^{-/-}$ *cII*$^{+}$) were obtained by crossing heterozygote *Gulo cII* positive and homozygote *Gulo* mice. Five *Gulo*$^{-/-}$*cII*$^{+}$ mice were vitamin C deficient diet from water and another five were supplemented with vitamin C. DNA mutation frequency were analyzed in the two groups. There were significant differences mutation frequencies between homozygote *Gulo*$^{-/-}$ *cII*$^{+}$ mice on vitamin C deficient diet and homozygote *Gulo*$^{-/-}$ *cII*$^{+}$ mice fed vitamin C rich diet. In fact mutation frequencies of *Gulo*$^{-/-}$ *cII*$^{+}$ mice on vitamin C deficient diet were significantly (10^{-4}) higher than on vitamin C group . These founding support the role of vitamin C as a potent antioxidant reagent highlight the importance future work that may identify the mutation types generates by oxidative stress in absence of vitamin C.

Introduction

Vitamin C is an important nutrient for humans and primates (30). This nutrient plays many roles in the human body. It is necessary for humans to obtain vitamin C

through their diet, as humans do not synthesize vitamin C due to the absence of the gene, *Gulo* (26;30) . This gene was present in early ancestors but was lost through selection, since more distantly related mammals contain the gene, such as mice (24). Vitamin C works as an antioxidant to protect DNA from damage that is caused by reactive oxidative species (ROS) (13). Ascorbate also induces immunity to minimize lung pathology during influenza infection (23). In this study, is use to determine whether vitamin C can be synthesized in these transgenic animals and the effect vitamin C was on preventing DNA damage. A cross of knockout *Gulo*$^{-/-}$ mice with Big Blue mice provides a reporter gene that detects mutations resulting from a vitamin C deficient diet (10). This research is significant as it will determine whether this vector can be administered as a therapy to people who are extremely deficient in vitamin C (10). It will also investigate the effectiveness of vitamin C in minimizing the oxidation of DNA, a condition which leads to complicated diseases like cancer (20). Furthermore, this work will contribute to the use of adenoviral vector for gene therapy in general (13). Vitamin C, also known as ascorbic acid, is a water-soluble vitamin, an essential in nutrient for cells, especially connective tissue cells (04). Ascorbic acid has a therapeutic effect in many biological molecules, such as DNA, lipid and protein (20). The daily recommended intake of vitamin C is 60 mg/day for adults (20). Ascorbic acid is not stored in the body but it is eliminated by the kidney when it accumulates (20). Vitamin C acts as an antioxidant, which maintains both intracellular components and important processes that are sensitive to free radicals, is a co-factor in many processes in the body, and plays a role in collagen synthesis, which is the main component of connective tissue in humans and animals (20;27). Decrease in collagen synthesis leads to impairment and weakness in blood vessels. This can cause bleeding as a result of a rupture in blood vessel walls, thus appearing purple and causing pain, especially in the legs, hands, and skin (15).

Importance of vitamin C

As an antiscorbutic agent, ascorbic acid helps the formation of connective tissues (39). Ascorbic acid plays an important role in collagen synthesis, and it is an essential nutrient in humans, guinea pigs and primate animals that lack the enzyme for

ascorbic acid production (39). Low levels of ascorbic acid in cells cause the collagen-deficient disease, scurvy. L-ascorbic acid is required for collagen biosynthesis in the connective tissues of cells (33;36). L-ascorbic acid acts as a co-factor for the enzyme prolyl hydroxylase and catalyzes the hydroxylation of peptidyl proline in post-translational processes (16;33). Ascorbic acid reacts with the oxidized iron bound to prolyl hydroxylase, reduces the iron and helps to produce prolyl hydroxylase enzyme, which is important for the synthesis of hydroxyproline (36). Hydroxyproline is an amino acid which is a component of type II collagen, the main structural protein in connective tissues, such as tendons, bone, blood vessels, ligaments, teeth and skin (27;36). The protein that constitutes collagen has three polypeptide chains, forming triple helices. An inadequate amount of ascorbic acid in the cell leads to a breakdown of the triple helices of collagen, which results in scurvy (36).

Water-soluble vitamins, such as vitamin C, play an essential role as an antioxidant and a co-factor in many hormonal processes (38). Ascorbic acid is primarily a component used in minimizing oxidative stress, which normally is a by-product of normal biochemical processes in the presence of excess glutathione (32). (ROS) are formed by normal processes in the body, such as cell proliferation and cell signaling. Free radicals are produced, which leads to a disturbance of the bimolecular components of the cell, especially DNA, proteins and lipids. In particular, ROS leads to DNA damage, which may cause a variety of diseases such as cancer and neurodegenerative diseases (03;08;09). To minimize this disturbance in cell molecules, some enzymes work defensively and protect against DNA damage (03;08;09). Ascorbic acid acts as a therapy to protect the cell membrane and other intracellular components and processes that are sensitive to oxidative species (30). Many cell studies have shown beneficial antioxidant effects of ascorbic acid when using ascorbic acid as a supplement in cell biology and vascular disease (22). Vitamin C is a micronutrient in food which has the beneficial effect of enhancing the immune system in the human body (07). Intracellular vitamin C provides protection for neutrophils from apoptosis during any inflammation (42). In addition, there is a lot of evidence that vitamin C has the ability to inhibit NO production that is necessary for macrophage proliferation and activity during phagocytosis (41). Therefore, adequate

amounts of this antioxidant improves the function and restoration of leukocytes (01).

Experimental models of vitamin C

Knockout mice and Big Blue mice

Small laboratory animal models have been used to study some human diseases (26). Many endogenous and exogenous factors have been shown to cause genetic diseases, through the use of mutant mice (26). Targeted modification of the mouse *Gulo* gene was done by using the neomycin resistance gene as a target vector inserted into the Gulo gene and introduced in embryonic stem cells, which can be verified by Southern blot analysis (26). This modification inactivated the *Gulo* gene, and therefore, halts the synthesis of vitamin C (26). Embryonic stem cells are used to generate knockout mice. These knockout mice like humans, require 330 mg/L of vitamin C as a supplement in water to survive (26).

Big Blue transgenic mutation models (mouse or rat), carrying *cII* or *lacI* as reporter genes, can used to analyze mutations in DNA (18;28). Lambda *lacI* or *cII* is inserted in the chromosome of Big Blue mice and can be packaged into a lambda vector. It then is infected into *Escherichia coli* to analyze the quantity of mutations (28) . The *lacI* phage particles, when infected in *E.coli* appear as blue plaques on plates in the presence of a chromogenic substrate X-gal (5-bromo-4chloro-3-indoyl-β-D-galactopyranoside) (19). Mutation frequency can be measured and the DNA sequence can be analyzed. For the *cII* gene, the Big Blue model DNA is collected, packaged into the lambda *cII* vector and infect into *E. coli* (G1250 *hfl*-) (19). The phage infected *E.coli* (carrying *cII*) is grown in two different conditions: (1) incubation at 30^{o}C for enumerates the number of phage and (2) incubation at 24 ^{o}C to enumerate the packaged lambda *cII*. Mutation frequency will be measured (19). This transgenic mouse mutation assay is able to identify spontaneous and somatic mutations, as well as determine the mutation frequency. DNA sequence can be analyzed determine to the type of mutation (02;18). The significance of this recent Big Blue mouse strain is the ability to determine the mutation frequency in any part in the body (28;29). Since, mutations represent a main cause of most types of cancer (28).

Research question:

- Does vitamin C reduce DNA mutation during stress?
- How can vitamin C protect the human body from diseases, such as cancer and cardiovascular disease?

Research Hypotheses:

Vitamin C reduces DNA mutation frequency in vivo

Experimental design

1- Create Gulo-/- cII+ mice:

Big Blue mice:

Is formed from two λ phage-based Transgenic mice

- In this system, both *lacZ* and *cII* genes are integrated into the mouse chromosome by λ shuttle vectors.
- *lacZ* and *cII* are reporter genes for mutation detection (18;28).

Knockout *Gulo* mice:

- Have a deletion of *Gulo* gene exons (15).

2- First cross knockout Gulo-/- with Big Blue Gulo+/+ cII+ mice
3- Second cross Gulo-/- with Gulo+/- cII+ mice.
4- Confirm Gulo expression using Western Blot.
5- Gulo-/- cII+ mice on vitamin C deprivation diet or on vitamin C.
6- Weight measurement.
7- Mutation assay.

Definition of terms:

Free radicals: are produced, which leads to a disturbance of the bimolecular components of the cell, especially DNA, proteins and lipids. In particular, ROS leads to DNA damage, which may cause a variety of diseases such as cancer and neurodegenerative diseases(03;08;09)

Oxidative stress:

Oxidative stress is also referred to as oxidation or free radical damage

Mutation : Mutation is a change in the base pair sequence of genetic material (DNA or RNA)

Reporter genes: can used to analyze mutations in DNA such as carrying *cII* or *lacI* as(18;29)

RESULTS: Crossing

The plan for crossing the mice was to start with homozygous *Gulo* mice and Big Blue mice. The first generation produced the expected results. There were four heterozygous *Gulo* and two *cII* positive mice, each confirmed by *Gulo* and *cII* genotyping. The body weight of these pups (heterozygote *Gulo cII* positive) was less

than wild type Gulo after weaning, but pups (heterozygote *Gulo cII* positive) were maintained from the first crossing on vitamin C supplementation. Then the heterozygous *Gulo cII* positive mice were crossed with homozygous mice. After that, results of this cross were confirmed by genotyping in both cases. Four homozygote *Gulo cII* positive mice were obtained, two males and two females. In addition, some were heterozygous *Gulo cII* positive. For our experiment at least twelve mice homozygous for *Gulo* and *cII* positive were needed. Therefore, homozygous *Gulo cII* positive males were crossed with homozygous *Gulo cII* positive females, producing twenty homozygous *Gulo cII* positive mice. All homozygous *Gulo cII* positive mice were on vitamin C supplementation in drinking water (330 mg per liter) for protection. Seven heterozygous *Gulo cII* positive were also obtained. The homozygous *Gulo cII* positive were kept and crossed with other homozygous *Gulo cII* positive and confirmed by genotype. Thirty homozygous *Gulo cII* positive mice were used for the experiment. Five groups of homozygous *Gulo cII* positive were placed on vitamin C deficient diet while continuing another group of age, genotype and gender matched mice on ascorbic acid supplementation. Five groups were started on treatment at different times and also harvested at different times.

Genotype

Knockout *Gulo* mice were used to study the effect of ascorbic acid on preventing DNA mutation as a result of oxidative stress. Big Blue mice (carrying the *cII* gene) were used to analyze mutation frequencies. PCR was used to detect alleles of the *Gulo* gene as well as the *cII* gene. Wild type *Gulo cII* negative, wild type *Gulo cII* positive, heterozygote *Gulo cII* negative, heterozygote *Gulo cII* positive, homozygote *Gulo cII* negative and homozygote *Gulo cII* positive mice were obtained during the experiment (Figure 1). These results were confirmed by PCR results, which are 330 bp for wild type *Gulo* and 230 bp for a *Gulo* deletion, and 381bp if the mouse is *cII* positive but no band if *cII* negative (Figure 1: lane 1 and 2). In heterozygous *Gulo* mice there are two different bands: one for the deletion (230 bp) and one for the wild type allele (330 bp) (Figure 1: lane 3 and 4). Also, *cII* primers were used to detect the

presence or absence of the *cII* gene in homozygote or heterozygote *Gulo* mice. The PCR amplificon for *cII* positive mice was 230 bp in size (Figure 2: lane 5 and 6).

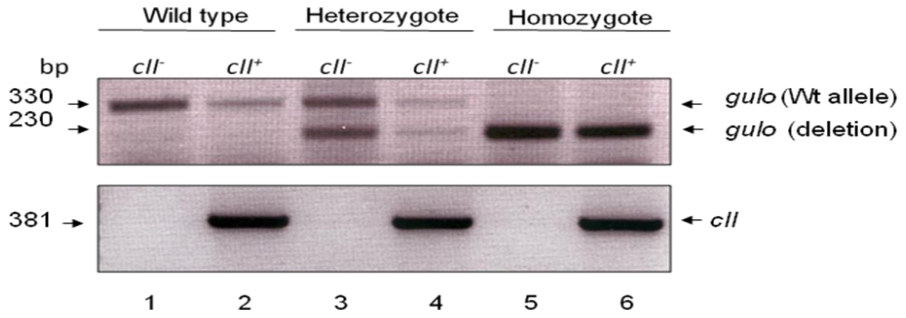

Figure 1: Gel photo showing PCR products from amplification of genomic DNA isolating from mice using *Gulo* and *cII* primers. As well, the different genotypes resulting from crossing Gulo and Big blue mice are shown, such as Wild type *Gulo cII*$^{-}$ (lane: 1), Wild type *Gulo cII*$^{+}$ (lane: 2), Heterozygote *Gulo cII*$^{-}$(lane: 3) , Heterozygote *Gulo cII*$^{+}$ (lane: 4), Homozygote *Gulo cII*$^{-}$ (lane: 5), Homozygote *Gulo cII*$^{+}$(lane: 6).

First generation results

Homozygous *Gulo* (*Gulo*$^{-/-}$) mice were crossed with Big Blue mice (*cII*$^{+}$) while on vitamin C supplemented water, and four mice were obtained: two males and two females, referred to as the first generation. Once these mice were weaned, tissue samples were taken from these mice for genotyping. Genotyping was done by PCR using *Gulo* primers and *cII* primers (Figures 2). All first generation mice were heterozygous for *Gulo* while two mice were positive *cII*. (Figure 6 for Gulo and *cII* genotyping). For *Gulo*, Each mouse tissue sample shows two bands: one is 230 bp and the other is 330 bp. The same DNA from these mice was then taken and used with *cII* primers to detect the *cII* gene from Big Blue mice. Two mice were carrying the *cII* gene, with one 381 bp band (Figure 2). The conclusion of results was two mice were heterozygote *Gulo cII* positive (Referred as first generation Gulo$^{+/-}$ *cII*$^{+}$) (Figure 2: lane 1 and 3).

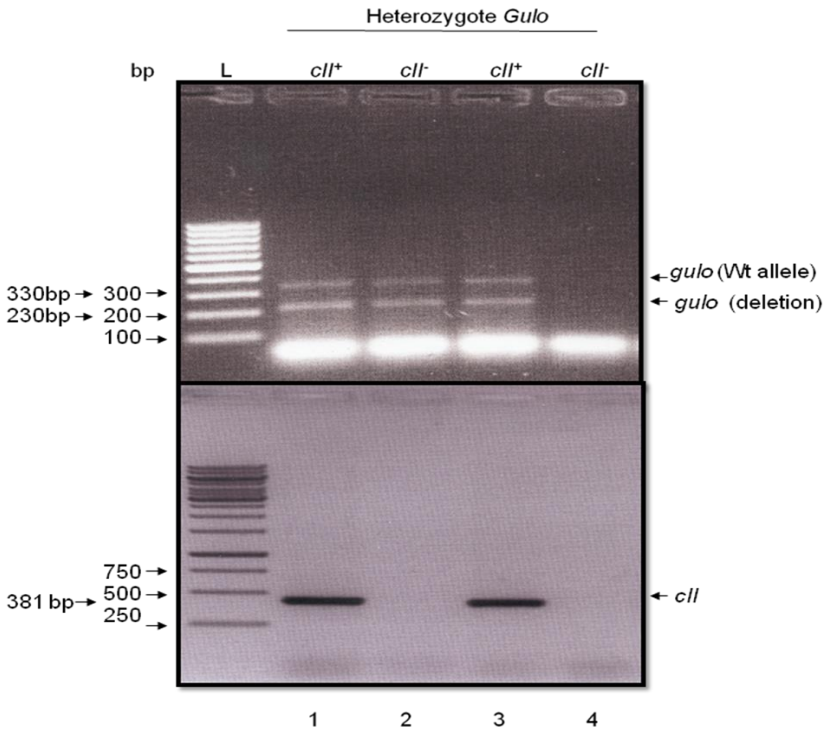

Figure 2: First generation results of PCR genotyping from amplification of genomic DNA isolated from first generation mice (crossed *Gulo*$^{-/-}$ with *Gulo*$^{+/+}$ *cII*$^{+}$). The results were two heterozygote *Gulo* and two heterozygote *Gulo cII*$^{+}$ mice (lane: 1 and 3).

Second generation result

The first generation Gulo$^{+/-}$ *cII*$^{+}$ was re-crossed with homozygous *Gulo* mice, producing six mice. Tissue samples were collected from these mice for DNA extraction. DNA samples were used for PCR reactions (using *Gulo* primers and *cII* primers), and the result was two homozygote *Gulo cII* positive mice and one heterozygote *Gulo cII* positive mouse (Figure 3). The homozygote *Gulo* was re-crossed to obtained more homozygote *cII* positive mice. After DNA extraction and PCR, as seen in the result, the mice were

homozygous *Gulo cII* positive (Figure 3).

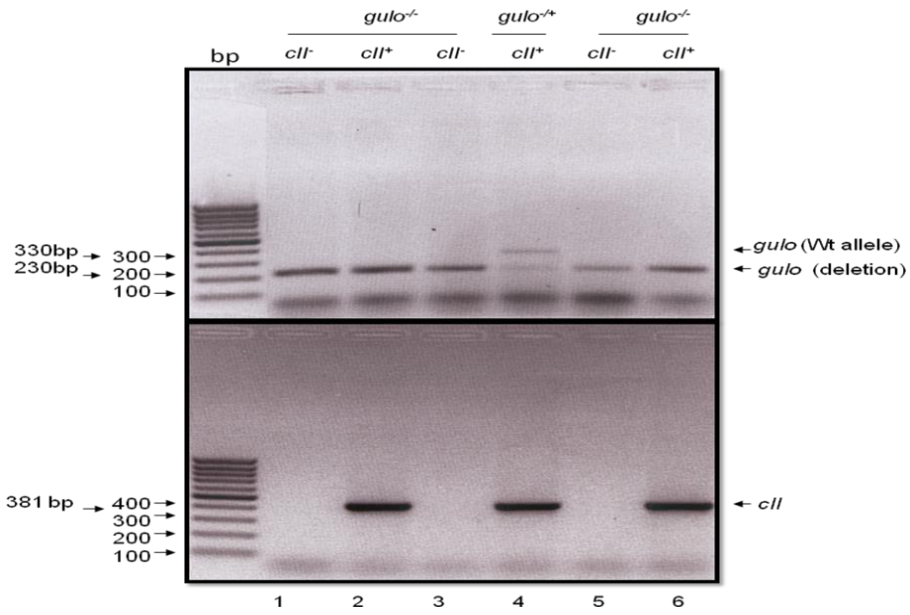

Figure 3: Genotype results of second generation mice (F2). Genomic DNA was extracted from F2 (crossed *Gulo*$^{+/-}$ *cII*$^{+}$ with *Gulo*$^{-/-}$), and the results were three mice homozygote *Gulo* (lane: 1, 3 and 5), two homozygote *Gulo*$^{-/-}$ *cII*$^{+}$ (lane: 2 and 6) and one heterozygote *Gulo*$^{+/-}$ *cII*$^{+}$ mouse (lane: 4).

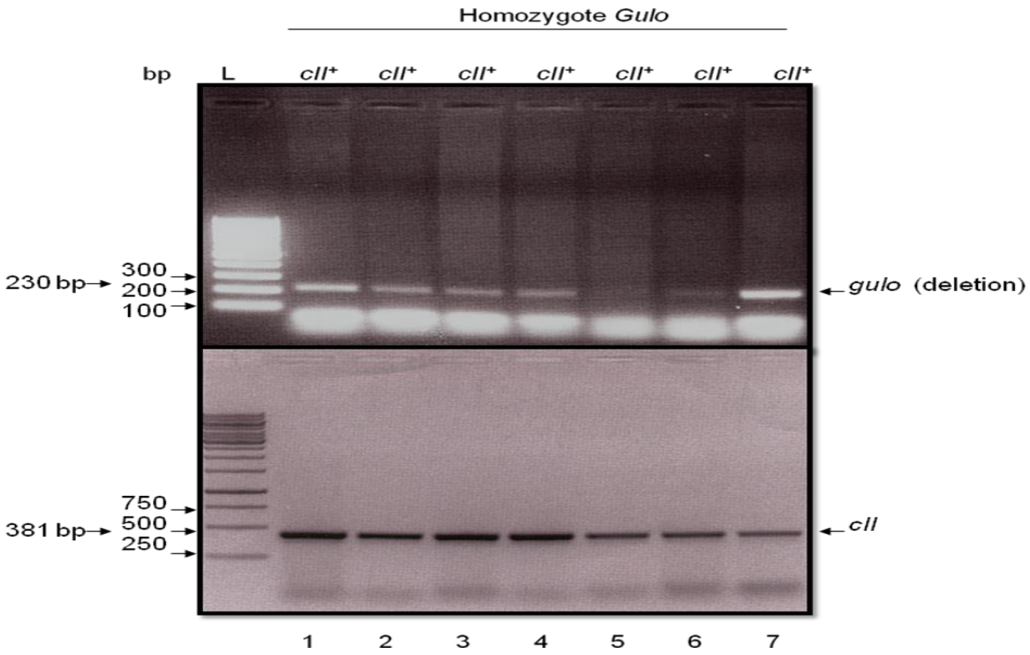

Figure 4: Image of PCR amplification of genomic DNA from mice with *Gulo*$^{-/-}$ *cII*$^{+}$ parents. All mice shown were homozygote *Gulo*$^{-/-}$ *cII*$^{+}$.

Effect of vitamin C deficiency on weight of homozygote Gulo cII positive mice

The initial sign of scurvy is weight loss, as shown in guinea pigs (17;25). They start to lose weight after 2 weeks on a vitamin C deficient diet. To determine whether this weight loss is noticeable in the homozygous *Gulo cII* positive mice and investigate how long it take to start to be scurvy after both them on vitamin C deficient diet, six groups (A, B, C, D, E and F) of mice were used for treatment. The first group was three males on vitamin C deficient diet and three males on vitamin C, as well, in a second and third group. These (six groups) homozygote *Gulo*$^{-/-}$ mice were under treatment at age 35 days and weighed once a week and results are discussed below.

Effect of vitamin C deficient diet on homozygote *Gulo cII* positive mice (Group A and B)

Group A (*Gulo*$^{-/-}$ *cII*$^{+}$) and group B (*Gulo*$^{-/-}$ *cII*$^{+}$) were 86 days old when first weighed. They were under treatment for four months. Figure 12 shows weight of mice in cage A and B. Mice A1 (*Gulo*$^{-/-}$ *cII*$^{+}$), A2 (*Gulo*$^{-/-}$ *cII*$^{+}$) and A3 (*Gulo*$^{-/-}$ *cII*$^{+}$) are under treatment of water without vitamin C. Mice B1 (*Gulo*$^{-/-}$ *cII*$^{+}$), B2 (*Gulo*$^{-/-}$ *cII*$^{+}$) and B3 (*Gulo*$^{-/-}$ *cII*$^{+}$) are under treatment of water with vitamin C, which is a control of group A. There is a difference between the weights of mice under water with and those without vitamin C. The mice B1 (*Gulo*$^{-/-}$ *cII*$^{+}$), B2 (*Gulo*$^{-/-}$ *cII*$^{+}$) and B3 (*Gulo*$^{-/-}$ *cII*$^{+}$) are heavier than mice A1 (*Gulo*$^{-/-}$ *cII*$^{+}$), A2 (*Gulo*$^{-/-}$ *cII*$^{+}$) and A3 (*Gulo*$^{-/-}$ *cII*$^{+}$). However mouse B1 in (control) has a high weight compared to the others. It has a high variation as shown in figure 12. Overall, the mice under water with vitamin C (B1, B2 and B3) gain weight more than mice without vitamin C treatment (A1, A2 and A3). Mice A2 and A3 had a rise and fall in their weight, followed by a big drop in weight after being given water without vitamin C. However, Mouse A1 started to gain weight (Figure 5).

Effect of vitamin C deficient diet on homozygote *Gulo cII* positive mice (Group C and D)

Six male mice were 66 days old when first weighed. Figure 13 shows the weight of mice in groups C and D. Mice C1, C2 and C3 are under treatment of water without vitamin C, and mice D1, D2 and D3 are supplied with vitamin C as a control of group C. There is no significant weight loss observed until two months after taking away vitamin C. Then, after three months of absence of vitamin C, they started losing weight. The mice on C1, C2 and C3 are between 28 g and 31 g. Also, during the treatment mice on a vitamin C deficient diet were in stable weight as in lane C3. But mice in the control group have variation as shown by the weight of mouse D2, and mouse D3 ($Gulo^{-/-}$ cII^{+}) had a low weight compared with other mice on vitamin C (Figure 6).

Effect of vitamin C deficient diet on homozygote *Gulo cII* positive mice (Group E and F)

As mention before, the difference between homozygote $Gulo^{-/-}$ cII^{+} mice on vitamin C deficient diet and on vitamin C, group E ($Gulo^{-/-}$ cII^{+} on vitamin C deficient diet) mice and F ($Gulo^{-/-}$ cII^{+} on vitamin C), was significant in weight loss. Group E mice started to lose in weight one month after vitamin C withdrawal. After nineteen days of losing weight, they lost 22% of their weight and the experiment was discontinued. However, mice in Group F gained weight and were still healthy until the end of the experiment (Figure 7).

Estimated cII mutant frequencies in the liver of homozygote Gulo cII positive mice on vitamin C deficient diet

Spontaneous production of hydrogen peroxide has been recognized as a mutagenic factor (14). Ascorbic acid is an antioxidant that can reduce the harmful effect of oxidative stress (14). Knockout $Gulo^{-/-}$ mice have been used to investigate the role of ascorbic acid on free radicals and 7,8-dihydro-8-oxoguanine (8-oxoG)(12). Big Blue mice have been used to detect spontaneous mutations (31). To determine whether ascorbic acid can suppress production of free radicals following oxidative stress, we

placed one group of *Gulo*$^{-/-}$ *cII*$^{+}$ mice on water without ascorbic acid for forty eight days while maintaining another group of same age and gender mice on ascorbic acid supplementation. After 48 days the ascorbic acid deficient mice had lost 22% of their weight. The mutation frequency was calculated (Table 1). The total plaques in an ascorbic acid deficient (E3) mouse were an insufficient plaque which means no significant number to calculate the mutation frequency (Table 8 and 9)**.** But, the total plaques in another ascorbic acid deficient mouse (E2) were 108,750 pfu and a total of 13 mutant plaques. The mutation frequency in this mouse (E2) was 1.25×10^{-4}. Also, in mouse E4 the total number of plaques screened was 108,750 pfu and 7 were mutant plaques, making the mutation frequency in this mouse (E4) 6.5×10^{-5}. The total number of plaques measured in the ascorbic acid supplemented (F1, F2 and F3) group included one mouse with no significant result (F3). However, the F1 mouse had a mutation frequency of 1.96×10^{-5} and mouse F2 had 6.9×10^{-5} (Table 2)**.** The level of mutation frequencies of Gulo$^{-/-}$ cII^{+} (untreated) was 1.25×10^{-4} mutation frequency (MF) and of (treated) was 6.9×10^{-5} mutation frequency (MF). Therefore the number of spontaneous mutations was greater in an ascorbic acid deficient diet than in the group on ascorbic acid supplementation. To this point, the level of mutations in other groups must be confirmed as well as in the rest of mice (E2 and E3) because a low number of plaques were obtained in tittering. Furthermore, the Package DNA sample will be amplified and then sequenced to identify mutation type.

Table 1: Titration of the packaged DNA samples of *Gulo*$^{-/-}$ *cII*$^{+}$ treated and untreated.

Group E &F	Genotype	Treatment Ascorbic Acid (AA)	Mean number of plaques Titer 20	Total Plaques Screened Titer 20
E2	*Gulo*$^{-/-}$ *cII*$^{+}$	No AA	69	103,500
E3	*Gulo*$^{-/-}$ *cII*$^{+}$	No AA	0	0
E4	*Gulo*$^{-/-}$ *cII*$^{+}$	No AA	108.75	108,750
F1	*Gulo*$^{-/-}$ *cII*$^{+}$	On AA	34	51000
F2	*Gulo*$^{-/-}$ *cII*$^{+}$	On AA	106.25	159,375
F3	*Gulo*$^{-/-}$ *cII*$^{+}$	On AA	0	0

Table 2: Measurement of mutation frequency on *Gulo*$^{-/-}$ *cII*$^{+}$ treated and untreated.

Group E &F	Genotype	Treatment Ascorbic Acid (AA)	Total Mutant (screened)	Mutant Frequencies
E2	*Gulo*$^{-/-}$ *cII*$^{+}$	No AA	13 plaques	1.25 X 10^{-4}
E3	*Gulo*$^{-/-}$ *cII*$^{+}$	No AA	0 plaques	0
E4	*Gulo*$^{-/-}$ *cII*$^{+}$	No AA	7 plaques	6.4 x 10^{-5}
F1	*Gulo*$^{-/-}$ *cII*$^{+}$	On AA	1 plaques	1.96 x10^{-5}
F2	*Gulo*$^{-/-}$ *cII*$^{+}$	On AA	11 plaques	6.9 X10^{-5}
F3	*Gulo*$^{-/-}$ *cII*$^{+}$	On AA	0 plaques	0

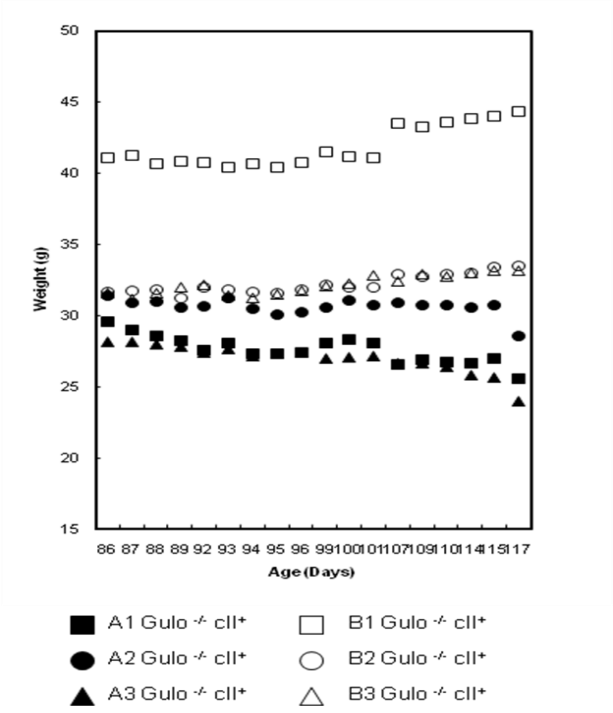

Figure 5: Weight data of mice in groups A and B. A1, A2 and A3 are *Gulo*$^{-/-}$ *cII*$^{+}$ and under water without vitamin C. B1, B2 and B3 are *Gulo*$^{-/-}$ *cII*$^{+}$ and under water with vitamin C (control). There is a different between mice on water without vitamin C and on vitamin C.

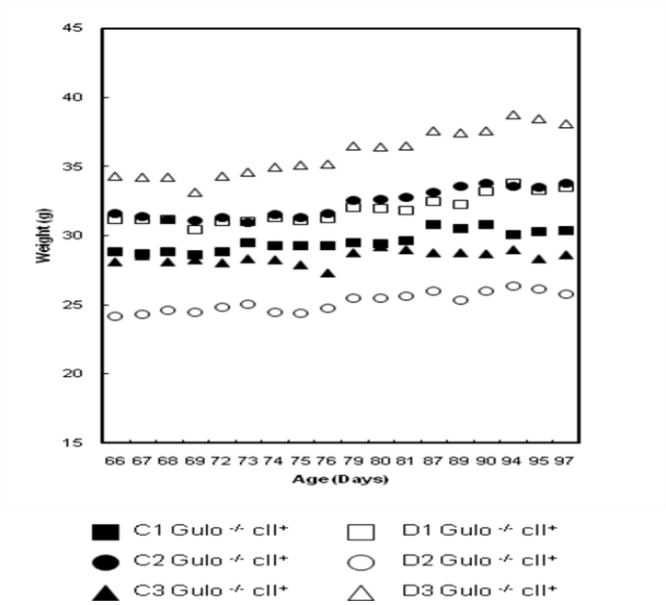

Figure 6: Weight data of mice in groups C and D. C1, C2 and C3 are *Gulo*$^{-/-}$ *cII*$^{+}$ and under water without vitamin C. D1, D2 and D3 are *Gulo*$^{-/-}$ *cII*$^{+}$ and under water with vitamin C (control). There is a different between mice on water without vitamin C and on vitamin C.

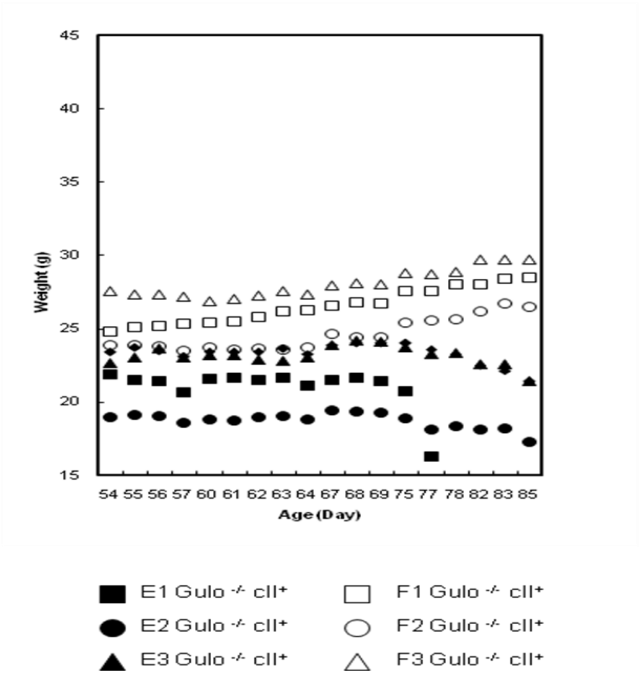

Figure 7: Weight data of mice in groups E and F. E1, E2 and E3 are *Gulo*$^{-/-}$ *cII*$^{+}$ and under water without vitamin C. F1, F2 and F3 are *Gulo*$^{-/-}$ *cII*$^{+}$ and under water with vitamin C (control). Figure shown; there is a difference between mice on water without vitamin C and on vitamin C.

DISCUSSION

Vitamin C is required for several important biological processes in the body. It minimizes mutations by reducing the effect of free radicals during oxidative stress (32;37). Ascorbic acid has been implicated to be important in processes such as iron absorption, spermatogenesis, wound healing, blood formation and in boosting the immune system (06; 07; 11; 36). It acts as a co-factor to help in hydroxylation of proline to protect against cardiac vascular diseases, cancer and common colds (07). In this study, we investigated 1) whether vitamin C acts as an antioxidant and 2) the ability of vitamin C to eliminate the harmful effects of free radicals, and therefore the development of certain diseases. Reactive oxidative species (ROS) are a byproduct of

normal processes that occur in the body, and these free radicals lead to DNA damage (03;08;09). There is an increase in the level of 8-oxoG during any oxidative stress (40). In addition, one study reported that vitamin C acts as antioxidant, and described the damage that can happen during vitamin C deficiency (32;37). Animal models are used to investigate the role of ascorbic acid in oxidative stress in vivo (12). Knockout *Gulo*$^{-/-}$ mice, guinea pigs, and Osteogenic Disorder Sionogi rats are common animal models used to study vitamin C (03;23). These model organisms cannot form L-gulonolactone oxidase, which is the main enzyme for the production of vitamin C (35;40). In this study, we utilized knockout *Gulo*$^{-/-}$ mice to investigate the function of ascorbic acid in decreasing the mutation rate and Big Blue mice to quantify and identify the type of mutations caused during vitamin C deficiency. Big Blue mice can detect DNA mutations in any part of the body because they have prokaryotic shuttle vectors carrying a reporter gene, such as the *cII* and the *lacI* genes (19;21). These reporter genes are integrated into chromosome 4 in Big Blue mice (21). Here, we crossed Big Blue mice with homozygote *Gulo*$^{-/-}$ mice. The first generation, which was heterozygote *Gulo*$^{+/-}$ *cII* positive, was then re-crossed with homozygote *Gulo*$^{-/-}$ mice to produce homozygote *Gulo*$^{-/-}$ *cII* positive mice. We had several problems with the first cross. It took a long time to breed, pups often died soon after birth, litter sizes were small, and pups were skinny. Pups from the first generation were weak, and we had to keep them more than four weeks with their parent. The second generation mice were healthy. Their weights, once weaned as normal mice, were between 18 g and 19 g. The main symptom of knockout *Gulo* mice on a vitamin C deficient diet is weight loss. This symptom is apparent in guinea pigs when also on a vitamin C deficient diet. Vitamin C deficiency in the guinea pig may affect the digestion of food (17;25), which may also affect their weight. In homozygous *Gulo*$^{-/-}$ mice, animals were fed without vitamin C, and control animals were fed with vitamin C in water. Our results show that vitamin C deficiency affects the weight of homozygous *Gulo*$^{-/-}$ mice. The knockout *Gulo*$^{-/-}$ *cII* positive mice were observed for more than five months for weight loss while on a vitamin C deficient diet. Some of these mice, after two months, lost more than 22% from their original weight. Post-mortem inspection showed some hemorrhaging in the muscles and an emaciated carcass. The control mice matches with vitamin C water

were healthy, and showed no sign of weight loss, and in fact gained weight. Another group of mice on a vitamin C deficient diet had fluctuated in their weight. These mice remained healthy, and did not show signs of scurvy. Their overall weight, however, was less than the control mice on vitamin C. Our results show two general trends in mice without vitamin C: mice losing weight after two months of treatment and mice a fluctuation in weight. Our investigation was about the difference in weight of knockout *Gulo*$^{-/-}$ *cII* positive mice, but how these mice can survive for a long time and no scurvy symptom shows? Also, why did one group had early signs of scurvy and lost 22 % of their weight in a short period? To answer to questions, the level of vitamin C will be measured in their blood by using HPLC and the food and shelter will be reanalyzed in the future. We then investigated the level of mutation in knockout *Gulo*$^{-/-}$ cII positive mice in a vitamin deficient diet. After a period of weight observation, group E and group F were killed and different tissue samples were collected. After DNA extraction, the DNA was packaged into phage and infected into an *hfl*$^{-}$ strain (*E.coli* G1250). Cells were grown at two different temperatures (24^{o}C and 37^{o}C). Only mutant phage could grow at 24^{o}C and make plaques at low temperature. On the other hand, all infected bacteria could make plaques at 37^{o}C. The mutant frequency that resulted from vitamin C deficiency can be determined by the ratio of lambda *cIl* plaques to the total number of plaques screened. Mutant plaques can be sequenced, and the type of mutation determined. The results show a high level (10^{-4}) of mutation in the vitamin C deficient group - more than those on vitamin C. Similarly, Big Blue mice were used to detect spontaneous mutations during vitamin E deficiency (10^{-5}) of mutation frequency (31). However, our result was a higher level of mutation which means that there is something stressful that may increase the mutation frequency. It could be that the absence of vitamin C results in a greater number of DNA mutation. To confirm this result we need to obtain data from all mice and all groups. In addition, analysis of the mutation frequency for all the groups at that time can be compared to make conclusions of the final result and to see any differences. Then we can analyze and confirm the result by DNA sequencing and identify the types of mutation (spontaneous or transition). In addition, we are going to detect the level of 8-oxo-7, 8-dihydro-2-deoxtguanosine (oxo^{8}dG) that result from oxidative stress and cause

spontaneous mutation (05;31). Moreover, the results show that vitamin C is important as a nutrient, in both spontaneous mutation and body weight experiments. This study demonstrated that body weight in mice decreased during a vitamin C deficient diet, compared with a control group which received vitamin C. But the variation between these groups was large, which means that there could be other variables. These mice may have been in a stress condition or been infected which could affect body weight. Also the level of mutations was high, making it surprising that mice can survive with such a high level of mutations.

FUTURE WORK

a) PCR amplification and sequence analysis of lambda *cII*⁻ mutants:

The *cII*⁻ mutant will be amplified and sequenced by PCR by using *cII* primers. Primer sequences are listed in table 1. As well, we can analyze the sequence of the PCR product and compare to the wild type lambda *cII* gene and surrounding regions in Big Blue shuttle vector.

b) Group A, C, G, and I will be tested by using lambda select-*cII* mutation detection system and compared with results of group E.

c) Measurement of vitamin C concentration in blood by using HPLC.

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