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大專學生研究計畫研究成果報告

計　畫
名　稱：　Klotho小鼠作為一個新興的先天性乾眼症模型

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Klotho mice as a novel congenital dry eye disease model

Abstract

The mutation of the *klotho* (*Kl*) gene expression has been well-established as having phenotypes like accelerated aging. Although many studies on *Kl* defect mice have been conducted, the role of *Kl* defect on ocular secretory system remains not explored. Therefore, this study investigates the effects of *Kl* gene deficiency on the ocular secretory system by observing the morphological changes in lacrimal gland and meibomian gland as well as the function of extraorbital lacrimal gland (ELG) in postnatal mice. Klotho seemed to act with FGFR1(IIIc), with the later directly converted by Klotho into FGF23 receptor. Hence FGF23 expression was also examined in this study. Mice were divided into groups according to different genotypes, namely: (1) wild-type C57BL/6 mice (+/+); (2) heterozygous C57BL/6 *klotho* mice (+/-) and (3) homozygous *klotho* mice (-/-). Changes of eye's tear secretory system were studied through Schirmer's test for quality assessment. Furthermore, ocular surface indexes, including smoothness, topography, and opacity were examined. The structural alternations were investigated by histology, immunohistochemistry, and electron microscopy. Although gradual increase of tear volume from week 4, 6, to 8 was found, irrespective of genotypes, the (-/-) secreted less tear than their littermates. Besides, extraorbital lacrimal glands from (-/-) mice were smaller and meibomian glands were degenerated at week 8 in the (-/-) mice, in contrast to the normal status of (+/+) and (+/-) littermates. Furthermore, the lacrimal glands of (+/+) mice were formed of saturated, well arranged acinar with uniform size, in contrast to the acinar ploymegethism and pleomorphism in the (-/-) mice. By electron microscopy, the wild type mice showed an euchromatic nucleus surrounded by a regular smooth nuclear membrane, whereas the (+/-) mice showed margination of heterochromatin with loss of normal structure. The ductal epithelial cells in (-/-) mice showed the loss of polarity compared to the wild type mice and (+/-) mice. Moreover, the wild type mice showed numerous electron dense and moderately electron dense granules, while light electron dense granules were shown in heterozygous and homogenous *klotho* mice besides the dense and moderate dense granules. The cristae of the mitochondria in homozygous *klotho* mice were disrupted and some of them were fused together. The rough endoplasmic reticulum (RER) in the wild type mice were arranged in parallel stacks and regular in shape. In contrast, *klotho*(-/-) showed a fingerprint-like arrangement of RER that were arranged in non-parallel orientation. Immnohistochemistry showed downregulation of FGF23 receptor expression in the (-/-) mice. These results supported that Kl defect mice were congenitally dry-eyed with accelerated age-related atrophies in the lacrimal gland and meibomian gland, with underlying decreased FGF23 receptor expression as a causing factor.

Key words: Klotho, aging, secretory system of the eye, lacrimal gland, mouse model

Introduction

Klotho (*Kl*) gene was discovered by Kuro *et al.* as related to aging. The *Kl* defect mice showed growth retardation after 3-4 weeks old, hypokinesis and gait disturbance, atrophy of genitial organs and thymus, arteriosclerosis, ectopic calcification, osteoporosis, skin atrophy, impaired mutation of gonadal cells, emphysema, as well as abnormalities in the pituitary gland (Kuro-o *et al.*, 1997). This mutant was named as "*klotho*", which is one of the three goddesses of fate in Greek mythology that spins the thread of life (Kuro-o *et al.*, 1997; Dalton, G. D., 2017; Kuro-o, 2012, 2018). *Kl* gene-deficient (-/-) mice accelerate a variety of degeneration features and shorten their life span and mice with overexpression of *Kl* gene were alleviated of degenerative symptoms and prolonged of their life span (Kuro- o *et al*., 1997; Kurosu *et al.,* 2005; Wang, Y., 2009; Kuro-o, 2011).

In the previous studies, it was shown that the *Kl* gene encodes a single-pass transmembrane protein that binds to a variety of fibroblast growth factor receptors (FGFRs) and acts as a co-receptor for Fibroblast Growth Factor 23 (FGF23). FGF23 is a bone-derived hormone that inhibits phosphate reuptake in the kidney and biosynthesis of vitamin D. In addition, the extracellular domain of Klotho protein is shed and secreted, possibly causing the action of humoral factors. The secreted Klotho protein regulates several growth factor pathways, including insulin/IGF-1 and Wnt, as well as the activity of multiple ion channels. Klotho proteins also protect cells and tissues from oxidative stress (Yamamoto *et al*., 2005; Kuro-o, 2008; Kuro-o, 2011).

FGF is a large family of growth factor proteins consisted of 22 proteins of the FGF family in vertebrates (Ornitz *et al*., 2001). The FGF family is mainly composed of two major classes of proteins: (1) secreted signaling proteins (secretory FGFs) which transmit the signal to tyrosine kinase through heparin and activation of heparin sulfate protein; (2) intracellular non-signaling proteins (intracellular FGF, iFGFs) is a cofactor for voltage-gated sodium channels and other molecules (Ornitz *et al*., 2015). The secreted FGFs bear an important role in the tissue maintenance, repair, regeneration, and metabolism which function as autocrine or paracrine factors (canonical FGFs; also called paracrine FGFs); however, three of them were specifically labelled as endocrine FGFs, which were found to reduce the affinity of heparin to heparin sulfate and required αKlotho, βKlotho as a cofactor (Ornitz *et al*., 2015).

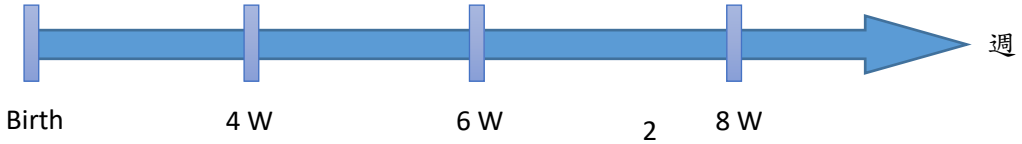
Dry eye disease (DED) is one of the common diseases in ophthalmology clinics. Tear hyperosmolarity has been referred as the core mechanism of DED and is the hallmark of the disease. The main causes of tear hyperosmolarity can be divided into two categories: (1) decreased amount of tear secretion which is recognised as ADDE (Aqueous-deficient dry eye); (2) excessive evaporation of tears or uneven distribution of the tear film, resulting in tears that cannot properly retain the wetness on eye surface which then turns into EDE (Evaporative dry eye). (Bron, A. J., *et al*., 2017).

A healthy tear film is made up of three layers included a lipid layer, a watery layer and a mucin layer (Gayton, 2009; Conrady *et al*., 2016). In general, assessments of dry eye syndrome include Schirmer's test for tear quantity and tear quality by Tear film Break-up Time (TBUT), and must be tested four weeks after birth to reflect the insufficient function of tear secretion for a congenital dry eye syndrome.

Methods

Animal experiment. A group of Klotho mice have been imported from the United States. After extensive breeding, genotyping was conducted to differentiate their genotypes (+/+; +/-; -/-), so subsequent studies will have sufficient supply of mice. The following experiments were performed:

Postnatal：



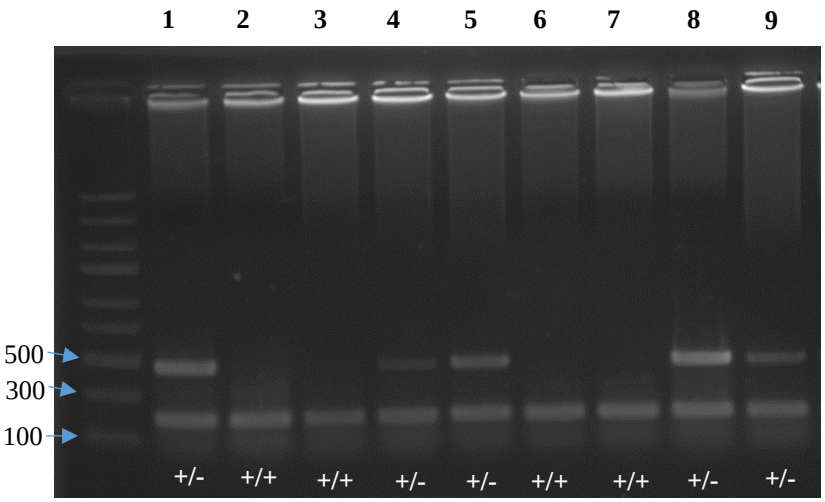
W: weeks (postnatal)

Methods	After birth	4 W	6 W	8 W
Genotyping	✓	-	-	-
Schirmer's test	-	✓	✓	✓
Tear film Break-up Time, TBUT	-	-	-	✓
Photography of ocular surface pathology	-	-	-	✓
Gross morphology analysis	-	-	-	✓
Hematoxylene Eosin stain, H.E	-	-	-	✓
Immunohistochemistry stain, IHC	-	-	-	✓
Electron microscopy analysis	-	-	-	✓

The tear volume of the mice were measured by Schirmer's test at 4th, 6th, and 8th weeks. The mice were then sacrificed, the right eyeball and the left extraorbital lacrimal gland (ELG) were taken to observe the gross appearance and photographed, and were embedded in paraffin and sliced for the histological observations by light microscope, whereas the right ELGs were used for electron microscope to identify the ultrastructure of ELG.

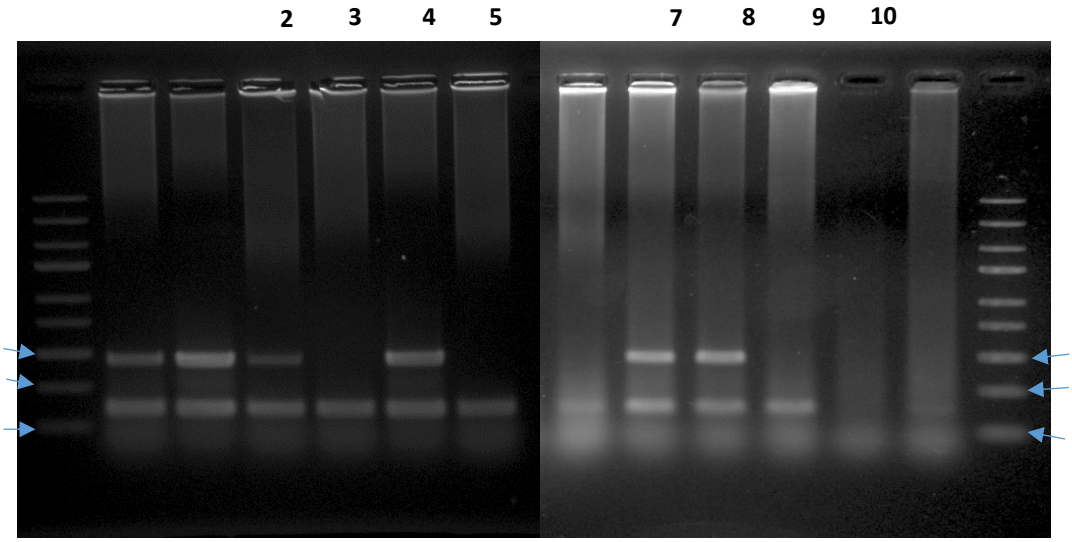
Genotyping. The mice's toes were cut and DNA was extracted, the DNA was amplified by polymerase chain reaction (PCR). The results were determined by the gel electrophoresis that shown below. (wild type: 186 bp; mutant: 455 bp).

male ♂



+/+ = 4
+/- = 5

female ♀



+/+ = 4
+/- = 6

Breeding. When the mice reached sexual maturity (3-4 weeks), male *klotho*(+/-) mice were mated with female C57BL/6 mice to obtain more heterozygous (+/-) mice, stabilizing their genetic background. In order to obtain more homozygous (-/-) mice, we mated male *klotho*(+/-) mice with female *klotho*(+/-).

Schirmer's test. After the mice were anesthetized, a 1 mm-wide tear test strip paper was inserted into the lower fornix of eyelid, and after 20 seconds, the wet length was measured and recorded.

Tear film Break-up Time (TBUT). The mice were anesthetized, and the eyeballs of the mice were photographed by a microscope for 25-30 seconds in a dark room, and the timing for tear film rupture was observed and recorded.

Light and electron microscopy examination. Left ELG was fixed in 10% of the formalin, and then dehydrated with a series of alcohol (40%, 75%, 85%, 95%, and 100%) and then embedded in the paraffin. H.E and IHC staining were performed after sectioning. 1 mm^3 of right ELG was cut for electron microscopy examination.

Results

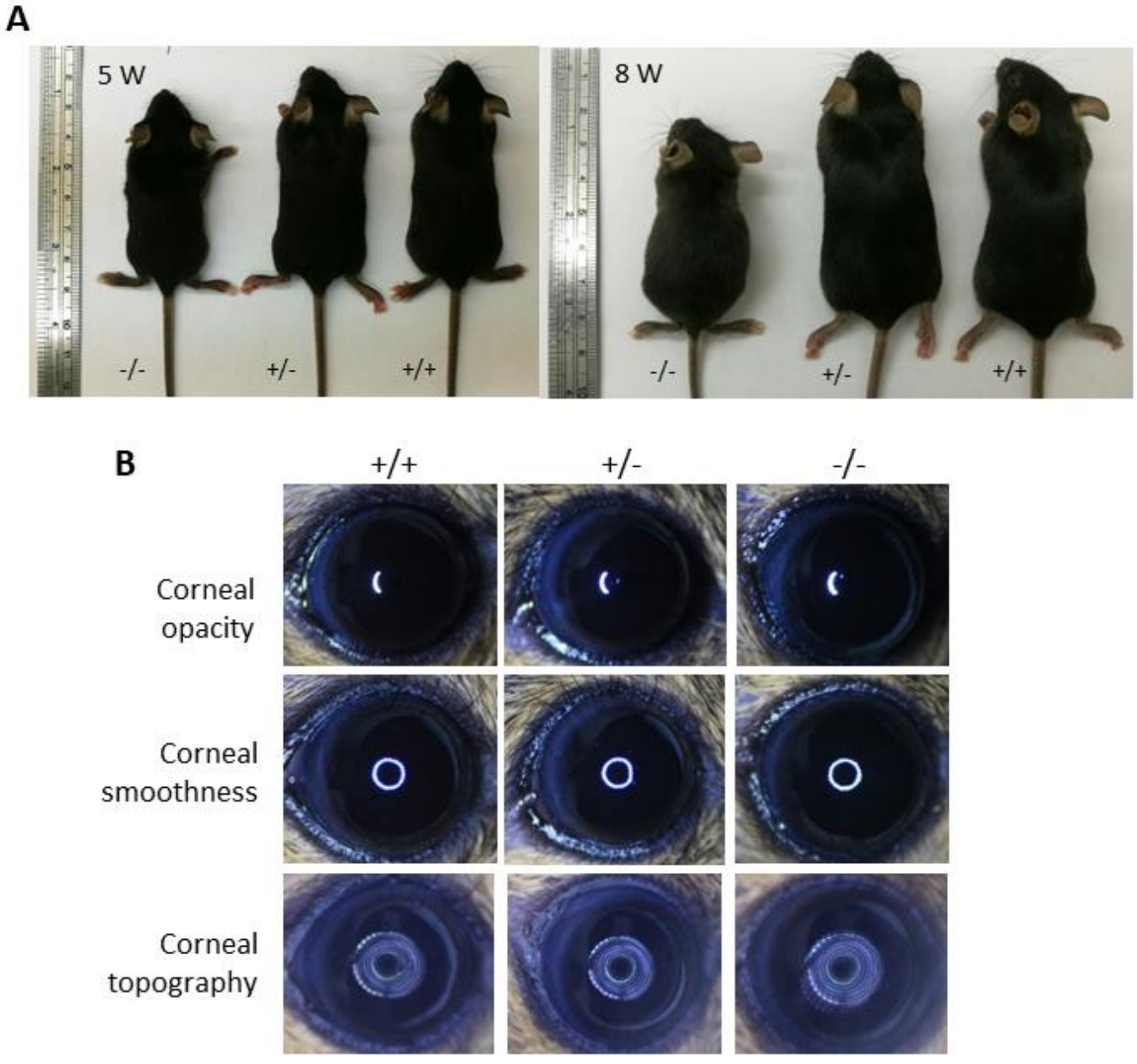
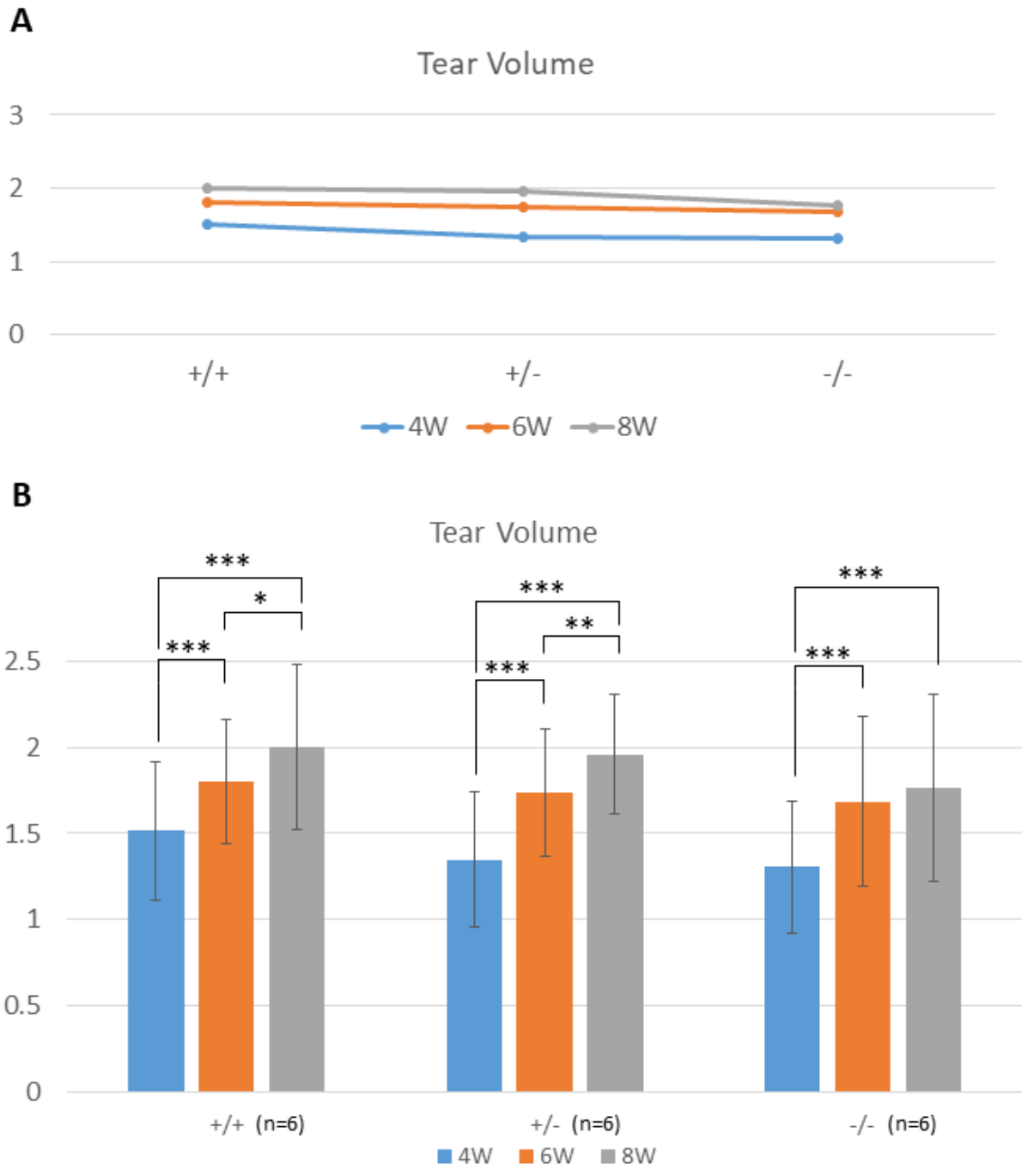


Figure 1.(A) Macroscopic findings of *klotho* mice while 5 weeks old and 8 weeks old. (B) Ocular surface conditions of three genotypes mice in 8 weeks old.

1. **Growth retardation seen through external appearance of the mice**

As the previous study shown, *klotho*(-/-) mice grew up normally up to the age of 3-4 weeks. However, growth retardation accelerated after the age and many organs shrank (Figure 3), showing similar degradation and died at the 8th to 9th week of age. *Klotho*(+/-) mice showed the same lifespan and growth with the wild type (+/+) mice (Kuro-o *et al.*, 1997). We can see the same argument in our study. As the results shown in Figure 1A, the *klotho*(-/-) mice was relatively smaller than the wild type even the *klotho*(+/-) in 5 weeks old. As they grew older, the disparity was getting more obvious. However, *klotho*(-/-) mice didn't show the variation in the ocular surface during 8 weeks old (Figure 1B).



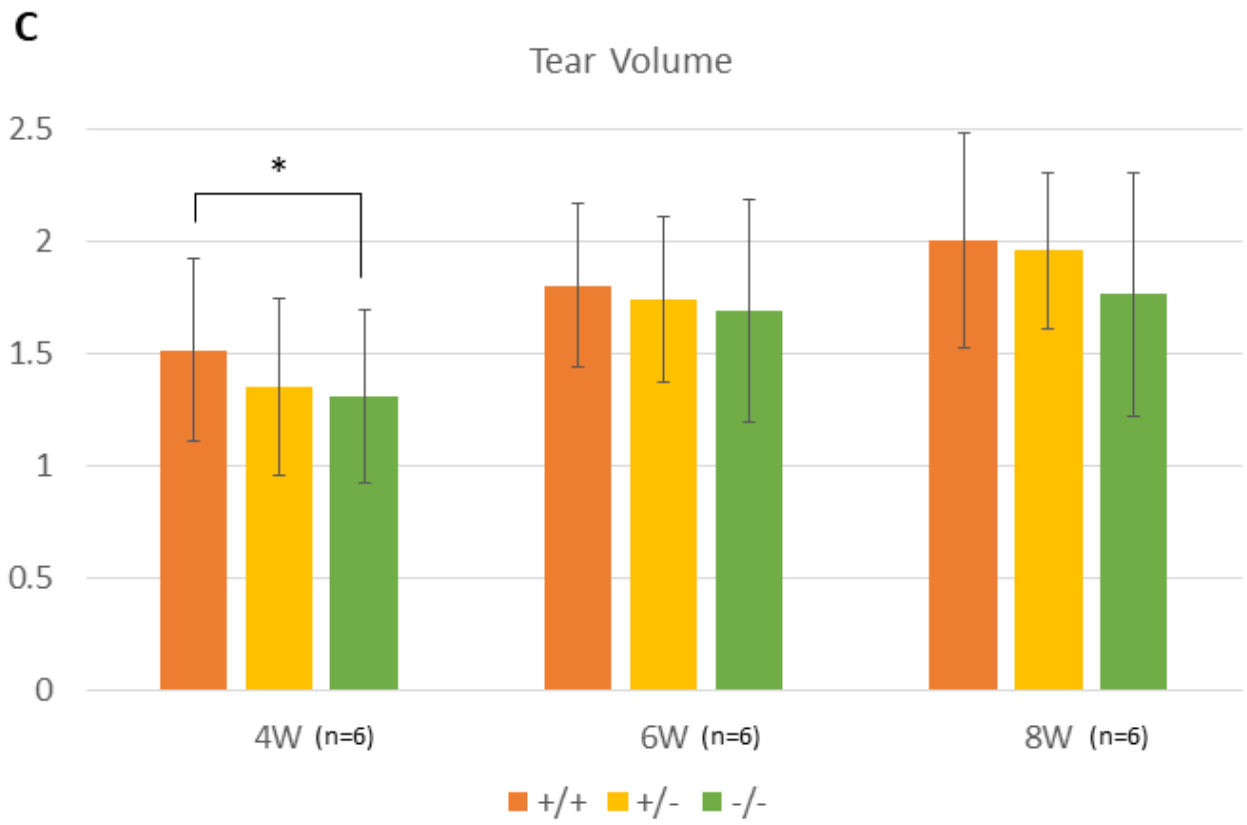


Figure 2. Tear volume of different genotypes of mice during 4th weeks, 6th weeks and 8th weeks. (A) Line chart shows the volume of tear production in various weeks. (B) Column chart shows the changes of tear production of each genotypes of mice in different weeks. (C) Column chart shows different genotypes of mice's tear production during 4th weeks, 6th weeks and 8th weeks. Data are means ± SD, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by analysis of T-test.

2. **Effect of aging in lacrimal gland's function.**
   The lacrimal gland's function can be observed through the volume of tear production (Figure 2A, B). As the mice grew older from 4 weeks old to 6 weeks old, the tear production increased significantly either in the wild type mice or *klotho*(+/-) or *klotho*(-/-) mice. But when they getting more older, there was only slightly increment, especially in *klotho*(-/-) mice. When the mice were 4 weeks old, there are significant difference ($p = 0.028$) between wild type and *klotho*(-/-) mice, yet no significant difference during 6 weeks old and 8 weeks old.

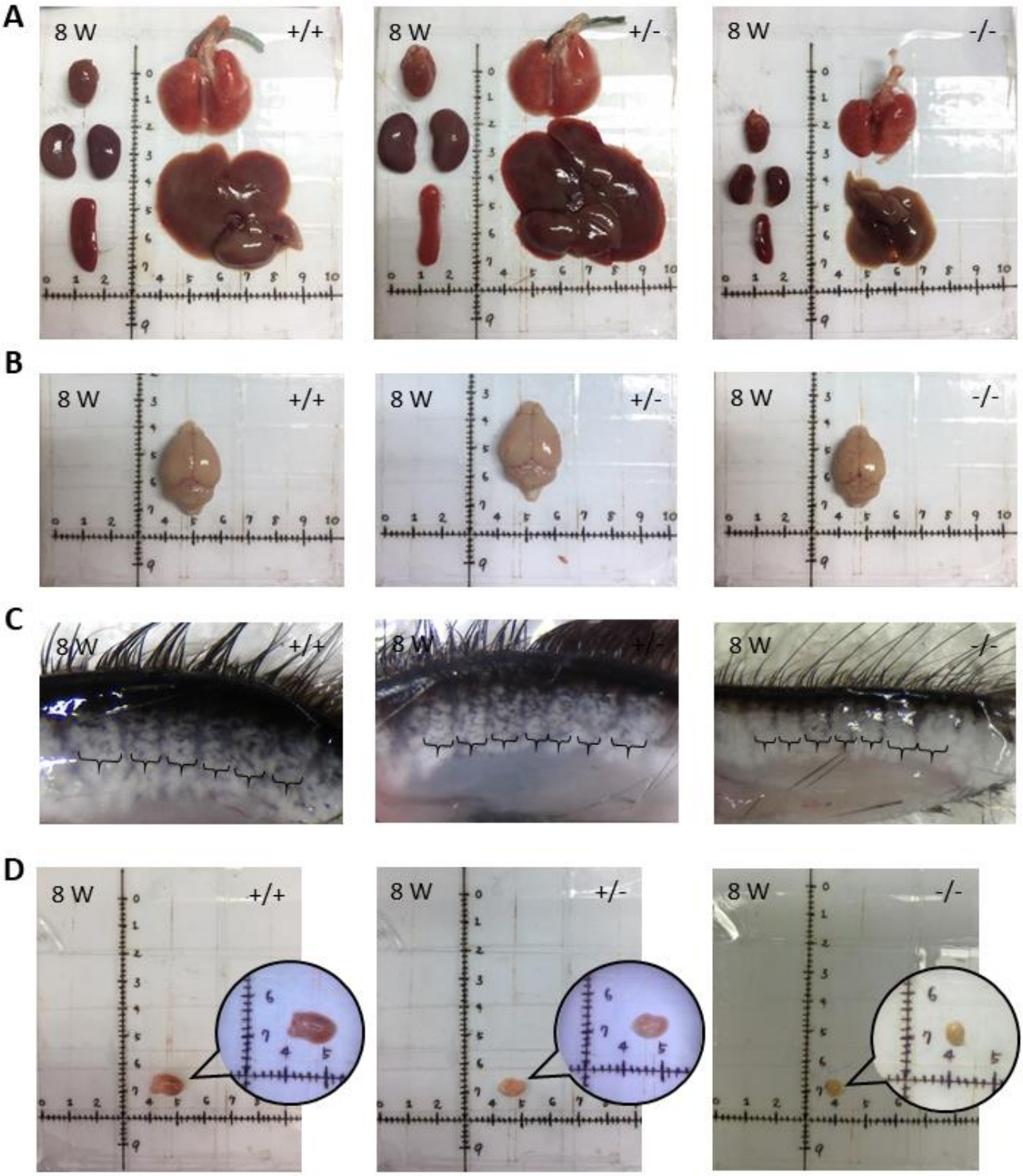


Figure 3. Macroscopic findings of mice's organs in 8 weeks old. (A) Heart, liver, spleen, lung and kidneys. (B) Brains. (C) Meibomian glands. (D) Lacrimal glands. Left panel is wild type (+/+); middle panel refers to *klotho*(+/-) mice while right panel is *klotho*(-/-) mice.

3. Difference of the organs' external appearance

Apart from the body size getting smaller (Figure 1A) in *klotho*(-/-) mice, their organs, including heart, liver, spleen, brain, meibomian glands and lacrimal gland, also showed evident smaller size than other genotypes (Figure 3). The meibomian glands are less saturated and shrink in the *klotho*(-/-) mice during 8 weeks of age.

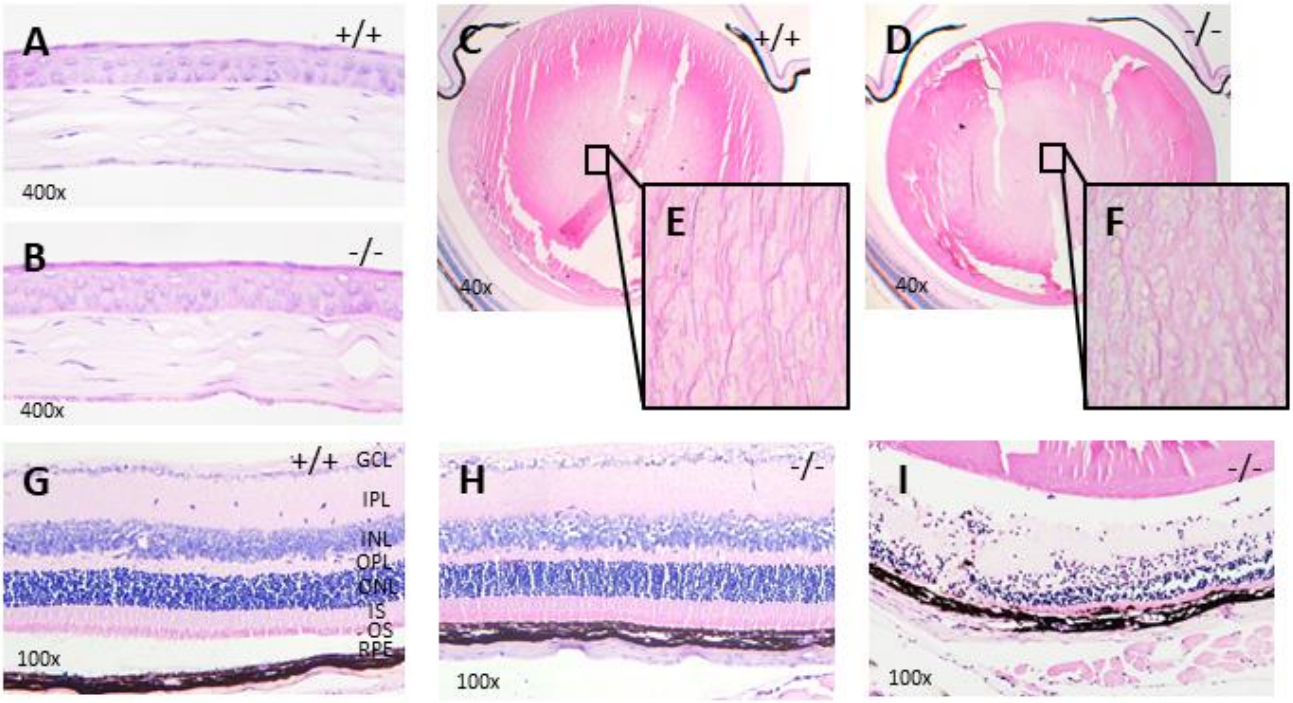


Figure 4. Photomicrograph of the eye structures histology in 8 weeks old in H.E. stain. (A)-(B) Cornea. (C)-(D) Lens. (G)-(I) Retina. (RPE, retinal pigment epithelium; OS, outer segment; IN, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer)

4. Pathological changes of the eye structures

Histological analysis showed no significant difference in cornea and lens between wild type and *klotho*(-/-) mice (Figure 4A-F). However there were several changes in retina histology (Figure 4G-H). The ratio of the inner segment and outer segment became smaller. Many of the cells in inner nuclear layers of the *klotho*(-/-) mice were undergoing atrophy (Figure 4H). Besides, we also found that one of the *klotho*(-/-) mice's retina lose its structure (Figure 4I).

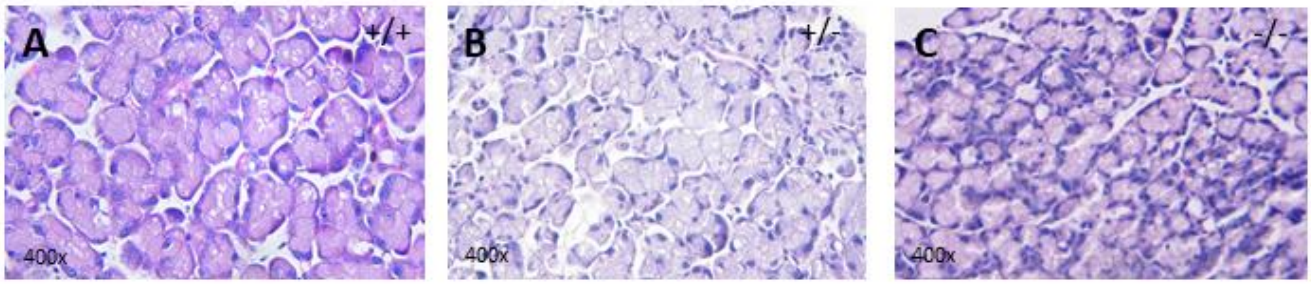


Figure 5. Microscopic findings of lacrimal gland in 8 weeks old. (A) wild type (+/+) (B) *klotho*(+/-) mice (C) *klotho*(-/-) mice.

5. Assessment of pathological changes in lacrimal gland

The lacrimal gland of wild type mice was formed of saturated, well arranged acinar with uniform size. Wild type mice had a larger acinar area but smaller acinar density than *klotho*(-/-) mice (Figure 5A). *Klotho*(-/-) mice showed the acinar that loss of the normal architecture and the immature nucleus increased (Figure 5C). While some of the *klotho*(+/-) mice showed the same phenotype with wild type mice (Figure 5B) whereas some same with *klotho*(-/-) littermates.

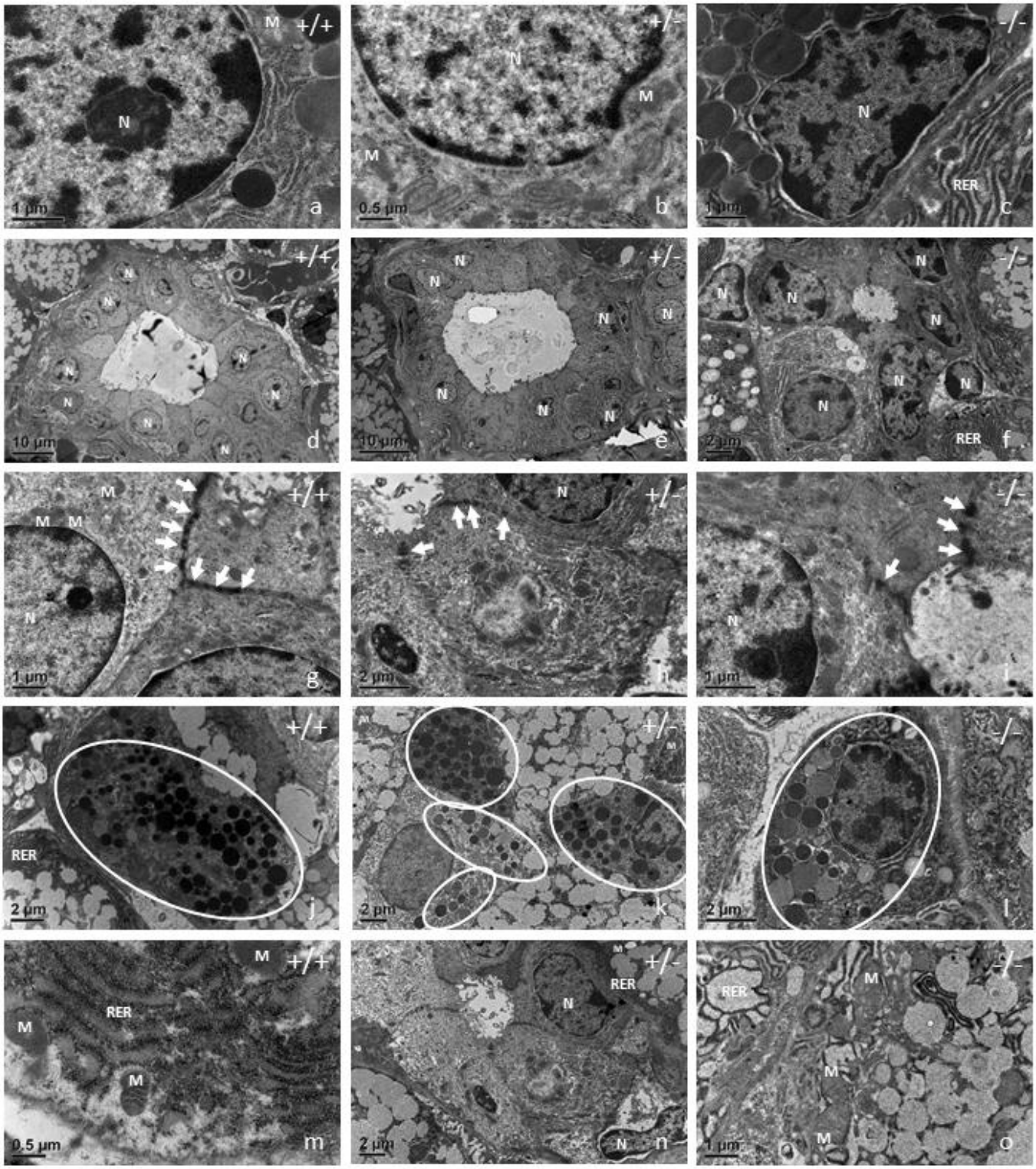


Figure 6. Electron photomicrograph of lacrimal gland in 8 weeks old. Left panel is wild type (+/+); middle panel refers to *klotho*(+/-) mice while right panel is *klotho* mice (-/-).
(N: nucleus; M: mitochondria; RER: rough endoplasmic reticulum; white arrow: desmosomes)

6. Assessment of ultrastructural alterations in the lacrimal gland

Wild type mice showed an euchromatic nucleus surrounded by a regular smooth nuclear membrane (Figure 6a). *Klotho*(+/-) mice showed a similar nucleus phenotype with wild type mice (Figure 6b) while margination of heterochromatin can be seen in *klotho*(-/-) mice with nucleus which was loss of structure (Figure 6c). The swollen nucleus were also been shown near the lumen (Figure 6f). The ductal epithelial cells in *klotho*(-/-) mice showed the loss of polarity compared to the wild type mice and *klotho*(+/-) mice (Figure 6 d-e). Moreover, wild type mice showed a clear lateral cellular boarder near the lumen (Figure 6d) with numerous of desmosomes that arranged regularly (Figure 6g). The lateral cellular boarder near the lumen still clear to be seen in *klotho*(+/-)

mice while it is indistinguishable in *klotho*(-/-) mice (Figure 6e-f). There were only a few separated desmosomes showed in the *klotho*(+/-) and *klotho*(-/-) mice (Figure 6h-i). Wild type mice showed numerous electron dense and moderately electron dense granules (Figure 6j). Light electron dense granules shown in heterozygous and homogenous Klotho mice besides of dense and moderate electron dense granules (Figure 6k-l). There are several mitochondria around the nucleus that have uniform clear cristae in both wild type mice and *klotho*(+/-) mice (Figure 6k, m, n). The cristae of the mitochondria in homozygous Klotho mice were disrupted and some of them were going to fused together (Figure 6o). The rough endoplasmic reticulum (RER) in the wild type mice were arranged in parallel stacks and regular in shape even no signs of deformation(Figure 6m). In contrast, *klotho*(-/-) showed a fingerprint-like arrangement of RER that were arranged in non parallel orientation (Figure 6o).

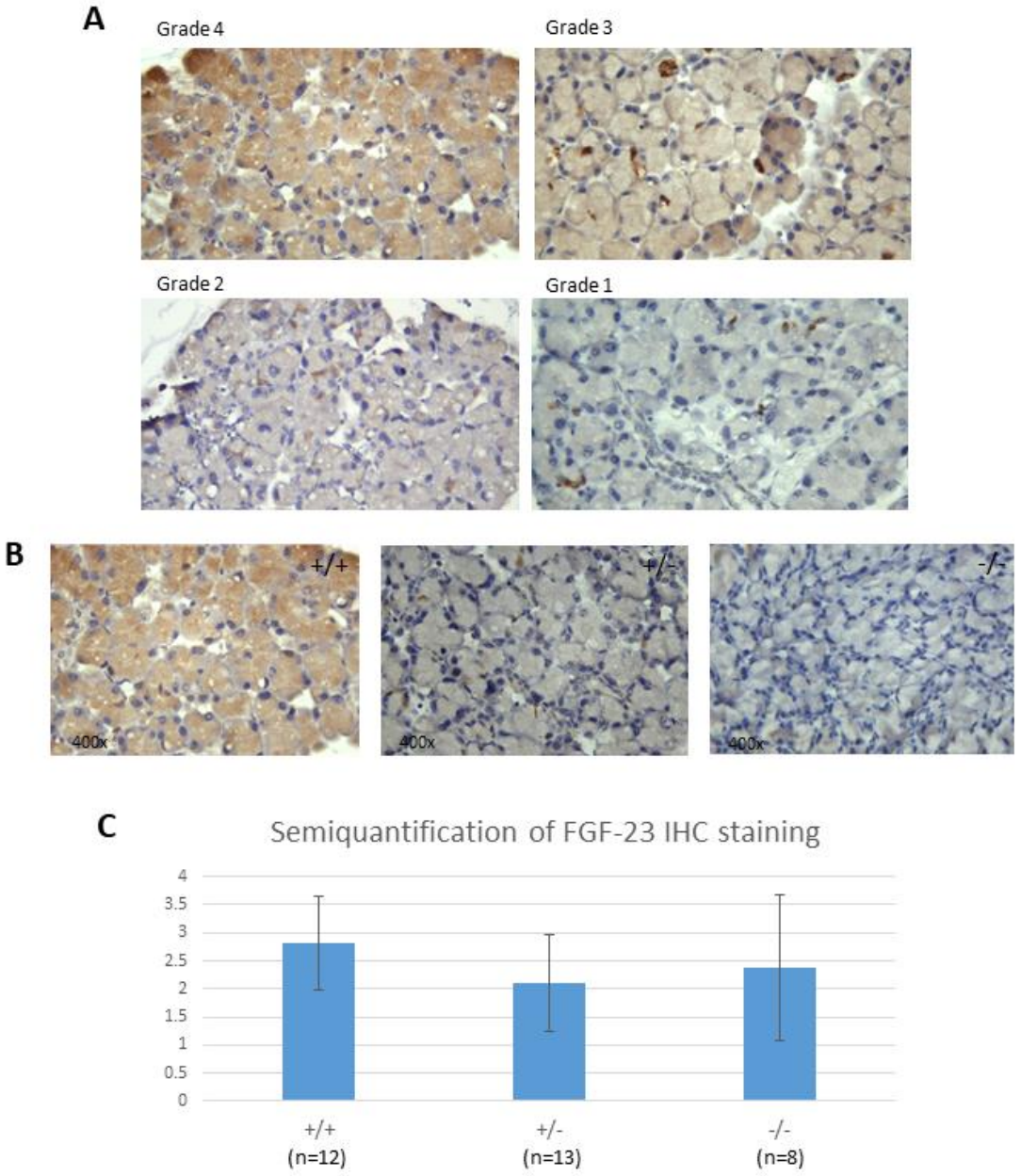


Figure 7. FGF-23 Immunohistochemistry staining (IHC) of lacrimal gland in *klotho* mice. (A) Grade of staining. Grade 4 is the most dense staining while Grade 1 is the lightest. (B) Staining results of lacrimal gland of mice in 8 weeks old. From left to right are wild type (+/+), *klotho*(+/-) mice and *klotho*(-/-) mice respectively . (C) Semiquantification of FGF-23 IHC staining of lacrimal gland according to grade shown in (A).

7. FGF-23 expression in the Klotho mice

Wild type mice showed the highest expression of fibroblast growth factor-23 (FGF-23) while heterozygous and homozygous Klotho mice were lack of FGF-23 (Figure 7B). After digitization, the grading of the FGF-23 IHC staining in homogenous Klotho mice was lower than the wild type mice.

Discussion

We have consolidated the argument of a defect in *klotho* gene accelerates degradation-related symptoms in several tissues, including those relating to tear production.

One of the key points to understand dry eye diseases (DED) is to identify two distinct components of the disease - tear evaporation and insufficient tear production - and their roles individually in the DED or simultaneously (Bron, A. J., *et al.*, 2017). The identification of tear film instability is also an enhancement for the statement of DED (Gayton, J. L., 2009; Perry *et al.*, 2004) which can be determined through the measurements of fluorescein tear film break-up time and Schirmer's test (Isreb *et al*., 2003). In the present study, because of the fluorescein tear film break-up time (TBUT) was unobservable, we did not completed the test. Nevertheless, the volume of tear production was measured by the Schirmer's test with anaesthesia. As the data shown, the tear volume increased with age which exhibited the same results in some researches. (Marko, C. K., *et al.*, 2013; McClellan, A. J., *et al*., 2014). The tear secretion is regulated by a complex system; it is not surprising that the evidence for the effect of age on the tear film and ocular surface is inconsistent. One important reason of the incompatible condition is that compensation mechanism. Normally the decreased reflex tear secretion capacity in the older eye may be compensated by a reduced lacrimal drainage (Van Haeringen, N. J.,1997). Alternatively, inaccuracy or variability of the methods used to measure tear function might also cause the inconsistency. The last but not least, either increase (McClellan, A. J., *et al*., 2014) or decrease (Kojima, T., *et al.,* 2012) in tear volume associated with advancing age are depending on the genetic background of the mice(Marko, C. K., *et al.*, 2013).

Besides of tear secretion, aging also induces the alternations in the lacrimal gland structure and function (Rocha, E. M., *et al*., 2008). El-Fadaly *et al*. described the acinar atrophy, variation in acinar area and acinar density which were explained in more detail by the electron microscopic appearance of the acinar cells. In the study, they found that the nature of the secretion changed in the aged lacrimal gland with the secretory granules associated with the changes in rough endoplasmic reticulum. We described a same observation. In spite of the alternation of the lacrimal gland, there was no obvious gross defect in cornea or eyelids.

The meibomian glands secrete the outermost lipid layer of the tear film who aims to prevent the evaporation of tears and maintain the ocular surface tension (Isreb *et al*., 2003). In our study, we observed the atrophy and low density of the meibomian glands in the *klotho*(-/-) mice. This may affects both the quality and quantity of meibum, resulting in changes of tear film composition (Chhadva, P., *et al*., 2017) though decrease of lipid layer thickness. Therefore, further investigation of meibomian glands should be done. The Klotho protein has been confirmed for its role in adipocyte maturation and intracellular lipid accumulation (Razzaque, M. S., 2012; Kobayashi, K., 2015). That's the reason why lipid droplets or lipid accumulation cannot be found in the lacrimal gland in the homozygous (-/-) mice. Klotho seemed to be incapable of intracellular signalling alone and FGFR1(IIIc) was founded directly converted by Klotho into the FGF23 receptor (Urakawa, I., et al., 2006). Hence the FGF23 expression is relevant to Klotho expression which had also been proved in our experiment.

As a conclusion, the *klotho* defect mice may not be regarded as a perfect model of congenital dry eye syndrome as tear volume data had not totally fulfil significant criteria of dry eye syndrome. However, since dry eye is complicated in many aspects, not just reduction of tear volume, but also lacrimal gland and meibomian gland degeneration, the *klotho* defect mice may remain a good model for the study of glands atrophy.

References

Bron, A. J., de Paiva, C. S., Chauhan, S. K., Bonini, S., Gabison, E. E., Jain, S., ... & Uchino, Y. (2017). Tfos dews ii pathophysiology report. *The ocular surface*, *15*(3), 438-510.

Bron, A. J., de Paiva, C. S., Chauhan, S. K., Bonini, S., Gabison, E. E., Jain, S., ... & Uchino, Y. (2017). Tfos dews ii pathophysiology report. *The ocular surface*, *15*(3), 438-510.

Chhadva, P., Goldhardt, R., & Galor, A. (2017). Meibomian gland disease: the role of gland dysfunction in dry eye disease. *Ophthalmology*, *124*(11), S20-S26.

Conrady, C. D., Joos, Z. P., & Patel, B. C. (2016). The lacrimal gland and its role in dry eye. *Journal of ophthalmology*, *2016*.

Dalton, G. D., Xie, J., An, S. W., & Huang, C. L. (2017). New insights into the mechanism of action of soluble klotho. *Frontiers in endocrinology*, *8*, 323.

El-Fadaly, A. B., El-Shaarawy, E. A., Rizk, A. A., Nasralla, M. M., & Shuaib, D. M. (2014). Age-related alterations in the lacrimal gland of adult albino rat: a light and electron microscopic study. *Annals of Anatomy-Anatomischer Anzeiger*, *196*(5), 336-351.

Gayton, J. L. (2009). Etiology, prevalence, and treatment of dry eye disease. *Clinical ophthalmology (Auckland, NZ)*, *3*, 405.

Isreb, M. A., Greiner, J. V., Korb, D. R., Glonek, T., Mody, S. S., Finnemore, V. M., & Reddy, C. V. (2003). Correlation of lipid layer thickness measurements with fluorescein tear film break-up time and Schirmer's test. *Eye*, *17*(1), 79.

Kobayashi, K., Tanaka, T., Okada, S., Morimoto, Y., Matsumura, S., Manio, M. C. C., ... & Fushiki, T. (2015). Hepatocyte β-Klotho regulates lipid homeostasis but not body weight in mice. *The FASEB Journal*, *30*(2), 849-862.

Kojima, T., Wakamatsu, T. H., Dogru, M., Ogawa, Y., Igarashi, A., Ibrahim, O. M., ... & Nakamura, S. (2012). Age-related dysfunction of the lacrimal gland and oxidative stress: evidence from the Cu, Zn-superoxide dismutase-1 (Sod1) knockout mice. *The American Journal of Pathology*, *180*(5), 1879-1896.

Kuro-o, M. (2011). Klotho and the aging process. *The Korean journal of internal medicine*, *26*(2), 113.

Kuro-o, M. (2012). Klotho in health and disease. *Current opinion in nephrology and hypertension*, *21*(4), 362-368.

Kuro-o, M. (2018). *The Klotho proteins in health and disease. Nature Reviews Nephrology.*doi:10.1038/s41581-018-0078-3

Kurosu, H., Yamamoto, M., Clark, J. D., Pastor, J. V., Nandi, A., Gurnani, P., ... & Shimomura, I. (2005). Suppression of aging in mice by the hormone Klotho. *Science*, *309*(5742), 1829-1833.

Marko, C. K., Menon, B. B., Chen, G., Whitsett, J. A., Clevers, H., & Gipson, I. K. (2013). Spdef null mice lack conjunctival goblet cells and provide a model of dry eye. *The American journal of pathology*, *183*(1), 35-48.

McClellan, A. J., Volpe, E. A., Zhang, X., Darlington, G. J., Li, D. Q., Pflugfelder, S. C., & de Paiva, C. S. (2014). Ocular surface disease and dacryoadenitis in aging C57BL/6 mice. *The American journal of pathology*, *184*(3), 631-643.

Modulo, C. M., Filho, E. B. M., Malki, L. T., Dias, A. C., de Souza, J. C., Oliveira, H. C., ... & Rocha, E. M. (2012). The role of dyslipidemia on ocular surface, lacrimal and meibomian gland structure and function. *Current eye research*, *37*(4), 300-308.

Ornitz, D. M., & Itoh, N. (2001). Fibroblast growth factors. *Genome biology*, *2*(3), reviews3005-1.

Ornitz, D. M., & Itoh, N. (2015). The fibroblast growth factor signaling pathway. *Wiley Interdisciplinary Reviews: Developmental Biology*, *4*(3), 215-266.

Perry, H. D., & Donnenfeld, E. D. (2004). Dry eye diagnosis and management in 2004. *Current opinion in ophthalmology*, *15*(4), 299-304.

Razzaque, M. S. (2012). The role of Klotho in energy metabolism. *Nature Reviews Endocrinology*, *8*(10), 579.

Rocha, E. M., Alves, M., Rios, J. D., & Dartt, D. A. (2008). The aging lacrimal gland: changes in structure and function. *The ocular surface*, *6*(4), 162-174.

Tsubota, K. (2007). Oxidative stress and inflammation: hypothesis for the mechanism of aging. *Nippon Ganka Gakkai Zasshi*, *111*(3), 193-205.

Urakawa, I., Yamazaki, Y., Shimada, T., Iijima, K., Hasegawa, H., Okawa, K., ... & Yamashita, T. (2006). Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature*, *444*(7120), 770.

Van Haeringen, N. J. (1997). Aging and the lacrimal system. *British journal of ophthalmology*, *81*(10), 824-826.

Wang, Y., & Sun, Z. (2009). Current understanding of klotho. *Ageing research reviews*, *8*(1), 43-51.

Yamamoto, M., Clark, J. D., Pastor, J. V., Gurnani, P., Nandi, A., Kurosu, H., ... & Kuro-o, M. (2005). Regulation of oxidative stress by the anti-aging hormone klotho. *Journal of Biological Chemistry*, *280*(45), 38029-38034.