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The effect of the mtDNA4834 deletion on hearing

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Abstract

Mutations in mitochondrial DNA (mtDNA) are associated with diverse pathological states in humans, notably sensorineural deafness. In humans, mtDNA4977 deletion, known as common deletion, is thought to play a critical role in presbyacusis. A similar mtDNA deletion occurs in the naturally aging rats is mtDNA4834 deletion. Today, it is still obscure about the effect of common mtDNA deletion on the presbyacusis and hearing loss. We establish a model of rat associated with mtDNA4834 deletion in inner ear by D-galactose. It was found that the malondialdehyde (MDA) increased with superoxide dismutase (SOD) decreasing in the inner ear of the rat treated with D-galactose than of the control. However, there was no significant difference in elevation of ABR threshold between the rat with mtDNA4834 deletion induced by D-galactose and control. After aminoglycoside antibiotic injected, the hearing threshold of the rats carrying mtDNA4834 deletion increased significantly compared with the rats without mtDNA4834 deletion. The results show that resembled accelerated aging in the inner ear of the rat could be induced by injecting D-galactose. Moreover, those suggest that mtDNA4834 deletion can not directly induce the hearing loss, but acting as a predisposing factor which can greatly enhance the sensitivity of the inner ear to the aminoglycoside antibiotic.

Keywords: D-Galactose; Aminoglycoside antibiotic; Mitochondrial DNA; Common deletion; Presbyacusis; Stress

Mutations in mitochondrial DNA (mtDNA) have been found to be associated with both syndromic and nonsyndromic forms of sensorineural deafness [1–3]. Several studies in human being have reported an association of mitochondrial DNA mutations and presbyacusis [4,5]. Since mtDNA mutations and the resulting loss of oxidative phosphorylation activity seem to play an important role in the aging process, it is not unlikely that mitochondrial mutations in the auditory system can also lead to presbyacusis [6]. In humans, a 13 bp direct repeat (at 8470–8482 and 13,447–13,459) in the mtDNA leads to the frequent occurrence of a 4977 bp 'common deletion' (CD) by recombination between the repeats, which heteroplasmic inheritance of the mtDNA4977 deletion causes Pearson syndrome or Kearns–Sayre syndrome [7,8]. However, it has been reported that the mtDNA4977 deletion also occurs frequently during aging [9]. In the current concept, the mtDNA4977 deletion is considered to play an important role in presbyacusis. Bai et al. [10] found that 14 out of 17 patients with presbyacusis had detectable levels of the 4977 bp deletion in archival temporal bone and this deletion was present in only eight of the 17 human specimens with normal audiograms. Ueda et al. [11] also reported that older patients with presbyacusis had a higher frequency of the common mtDNA deletion (4977 bp) compared with similar aged patients without presbyacusis. On

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the other hand, we found that mtDNA4977 deletion occurred also in young people, even in child with hearing loss between 3 and 5 years old [12]. Thayer et al. [13] identified the presence of the CD in leukocytes from all 71 individuals including an infant aged 8 months. So far, there are some controversial results in the previous study: (1) Not all patients with presbyacusis carry the common deletion. (2) The common deletion occurred in the individual with normal hearing. (3) The common deletion was found not only in aged but also in young people. Therefore, the effect of the mtDNA common deletion on presbyacusis and hearing loss is still obscure.

The study of presbyacusis associated with mtDNA mutation in human being is a challenge due to the confounding nongenetic factors during the individual life span and the unavailability of the suitable materials for research. Labyrinth tissue is embedded in the bony capsule of the inner ear. It is impossible to obtain labyrinth tissue of inner ear by biopsy. Because of many practical benefits, including the varied degrees of hearing loss and short life span of the rat, the rat may be a suitable model associated with common deletion. It is also an ideal subject for molecular studies because of the availability of the rattus genome and the previous characterization of mutations that affect hearing. A similar common mtDNA deletion occurs in the rat due to a 16 bp repeat at 8103–8118 and 12,937–12,952; recombination between these repeats leads to a 4834 bp deletion [14]. The mtDNA4834 deletion also increases in aged rats [15]. The D-galactose treated mice and rats model has been widely utilized for brain aging or anti-aging pharmacology research. The behavioural impairment and the change of oxidative biomarkers in these models are similar with those in naturally aged animals [16–18]. But there was no paper available about the mtDNA common deletion in these mimetic aging induced by D-galactose. With the aim of understanding the mechanisms involved in hearing loss associated with mtDNA mutation, we established a rat model associated with mtDNA4834 deletion in inner ear by injecting D-galactose.

The incidence of hearing loss increased remarkably due to more and more environment stress which people suffered from. Stress, such as noise and ototoxic drug, was thought to play an important pathogenic role in aging and deafness. Nedic et al. [19] proposed noise as a stress factor for the onset of hearing disorders in workers. Oxidative stress has been implicated in cochlear injury following severe noise exposure, and administration of aminoglycoside antibiotics and the antineoplastic agent, cisplatin [20]. Oxidative stress is also thought to play a role in potentiation of noise-induced hearing loss [21]. So far, the mechanism involved in the hearing loss exposure to stress has not been completely clarified.

In this study, we choose the aminoglycoside antibiotic as a representative of stress to elucidate this relationship by means of mtDNA deletion model. Our results suggested that mtDNA4834 deletion did not cause the hearing loss directly, but the rat with mtDNA4834 deletion was much more sensitive to the stress than control rat.

Materials and methods

Animals and treatment. Wistar rats aged 1 month ($n = 50$) were obtained from experimental animal center of Tongji Medical College, Huazhong Science and Technology University. The rats were divided into four groups randomly: group A (D-galactose group, $n = 14$), group B (D-galactose and kanamycin group, $n = 14$), group C (kanamycin group, $n = 12$), and group D (control group, $n = 10$). The procedure of treatment for all animals was separated into two stages. Rats of group A were treated with hypodermic 5% D-galactose (150 mg/kg/d, AMERESCO) for 8 weeks in stage 1 and then with intraperitoneal saline for 10 days in stage 2. Rats of group B were given the same dose of D-galactose in stage 1 but kanamycin (500 mg/kg/d) instead of saline in stage 2. Rats of group C were given the same dose of kanamycin in stage 2 but saline instead of D-galactose in stage 1. Saline was administered to rats of group D all the protocol. The animals were housed in the standard animal facility under normal rat rearing conditions. All experimental procedures involving the rat were approved by the Animal Care and Use Committee.

Evaluation of auditory function. Auditory thresholds were tested by evoked auditory brain stem responses (ABR) thrice (SpiritTM,Nicolet), first before the drug administered, then after 8 weeks the stage 1 drug used, third after all drug treated. The rats were anesthetized with ketamine (30 mg/kg, i.m.) and chloropromazine (15 mg/kg, i.m.). A differential active needle electrode was placed subcutaneously below the test ear and a reference electrode at the vertex. A ground electrode was positioned below the contralateral ear. Click stimulations were given from a speaker (10 ms duration, 1 ms rise/fall time). The average responses from 1024 stimuli were obtained by reducing the sound intensity in 10-dB steps and finally at 5-dB intervals near threshold. Threshold was defined as the lowest stimulus level at which a positive waveform in the evoked response was evident. Thresholds at each frequency were verified at least twice. Threshold shifts were calculated for individual animals by comparison to their prestudy thresholds. The ABR score of each animal was given and interpreted by an observer without knowledge of the group and administration.

SOD and MDA assay. After detection of hearing threshold, the rats were killed (aged about 3.5 months). The bilateral membrane labyrinth tissues were harvested and homogenated with 150 μl liquid (0.01 M Tris–Cl, 0.1 mM EDTA, and 0.01 M sucrose). One hundred microliters of homogenate was used to DNA isolation, the rest of it was used for biochemical detection. Malondialdehyde (MDA) evaluated by measuring the content of thiobarbituric acid reactive substances and superoxide dismutase (SOD) activity was determined by measuring the dismutation of the toxic superoxide radical as described by RANSOD. The method was referred to previous report [16].

DNA isolation and PCR-based deletion analysis. The total DNA was extracted from membranous labyrinth of each inner ear by standard extraction protocol using Proteinase K and sodium dodecyl sulphate (SDS). Primers for the rat D-loop and the rat mitochondrial deletion from the rat mitochondrial genome (GenBank Accession No. X14848) were designed using primer 5.0 (primer sequences and amplification conditions available on request).

Nest-PCR verified specific amplification of the mtDNA 4834 deletions was detected using the primer pair S2/A2(nt7682–13129):S3/A3 (nt7825–13117) for amplification of a 459-bp PCR product, and using the primer pair A1/S1 (nt4395–5164) resulted in the fragment of the D-loop region in the amplified product size to 770–bp. The mitochondrial DNA was amplified using 150 ng DNA, 50 pmol of each primer, 10 mM Tris–HCl, (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates (MBI), and 0.5 U *Taq* DNA polymerase (MBI) in a volume of 20 μl. Polymerase chain reaction was done according to standard protocols in GeneAmp PCR system 9700 (Perkin-Elmer, Norwalk, CT) using annealing temperatures calculated by OLIGO 6.0.

The PCR products were electrophoresed through 1.0% agarose and visualized with ethidium bromide. Each product obtained with primers 7825–13117 was gel-purified and direct sequencing was done using sequencing (ABI PRISMTM377SXL DNA sequencer).

Statistical analysis. Data values are presented as means ± standard deviation (SD). SPSS 12.0 was used.

Results

SOD activity and MDA levels

The SOD activity of the membranous labyrinth was lower in rats (group A) than in controls (group D) (Student's t test, 41.13 ± 3.59 vs. 80.67 ± 2.75 U/mg, $p < 0.01$), while the levels of MDA increased (Student's t test, 5.01 ± 0.82 vs. 1.43 ± 0.19 μmol/g, $p < 0.01$) (Table 1).

Table 1
Total superoxide dismutase activity and MDA levels

Group	T-SOD (U/mg HB)	MDA (μmol/g protein)
A D-galactose group	41.13 ± 3.59[a]	5.01 ± 0.82[a]
B D-galactose and kanamycin group	31.24 ± 7.15[a]	9.04 ± 2.59[a]
C kanamycin group	73.84 ± 6.91	3.55 ± 0.83
D control group	80.67 ± 2.75	1.43 ± 0.19

[a] Denotes significant results compare with group D ($\alpha = 0.01$). Data values are presented as means ± the standard deviation (SD).

Mitochondrial DNA analysis and sequencing

The 770-bp PCR product can be detected in all samples of each group (Fig. 1A), which indicated the effective extraction of mtDNA. Almost all rats treated with D-galactose in group A (100%, 28/28 ears) and group B (92.86%, 26/28 ears) were detected the presence of the mtDNA4834 deletion (Fig. 1B). The occurrence of the mtDNA4834 deletion in the ear from these groups was bilateral. We noted that only one rat treated with D-galactose (group B) was detected of no mtDNA4834 deletion, meanwhile no mtDNA4834 deletion was found in rat only treated with kanamycin and control rat treated with saline. We concluded that mtDNA common deletion could be induced effectively by D-galactose. The 459-bp products were sequenced to prove the presence of 4834-bp deletion (Fig. 2).

The data of ABR hearing threshold

To determine whether mtDNA4834 deletion has effect on the hearing, hearing thresholds were tested by evoked auditory brain stem responses (ABR) (Table 2). ABR hearing thresholds were measured thrice before and after stage 1 administration, and after stage 2 administration, respectively (Fig. 3). Only 5–10 dB variation in ABR threshold was observed in each group before and post stage 1 administration. No difference was found between the rats treated with D-galactose (groups A and B) and control rats treated with

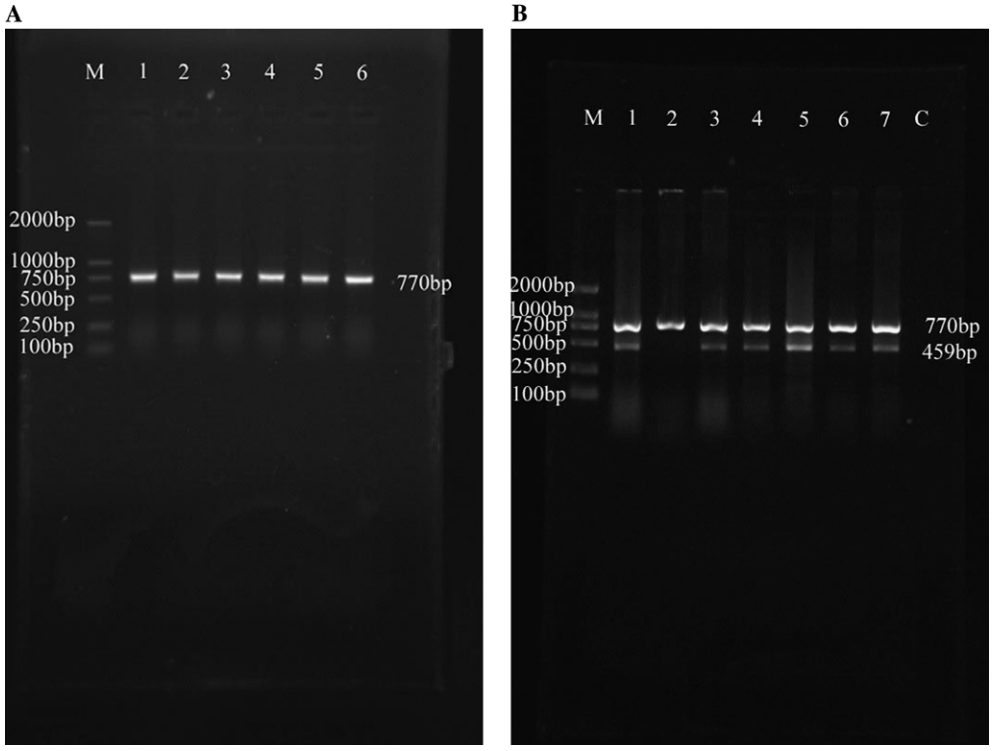

Fig. 1. (A) 770-bp PCR product. Lane 1, A1; lane 2, A10; lane 3, B5; lane 4, B7; lane 5, C4; lane 6, D3. (B) Some samples of it have 459-bp product (lane 1, A1; lane 2, B5; lane 3, B9; lane 4, A4; lane 5, A7; lane 6, B11; lane 7, A10; C, negative control; M, Marker, DL-2000).

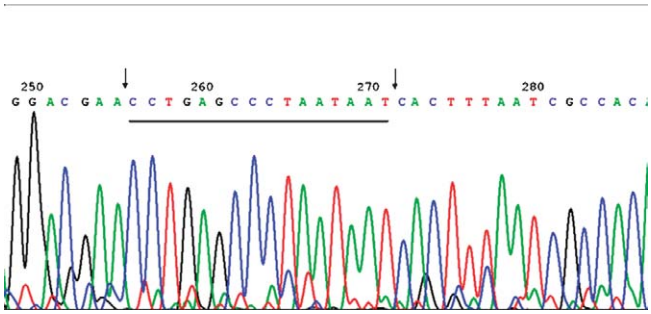

Fig. 2. Left arrow indicates the breakpoint in which a fragment of 4834-bp defect. The sequence between the two arrows is repeats.

saline (groups C and D) (ANOVA, $p > 0.05$). Previous report showed that the aging individual with hearing impairment was often accompanied with mtDNA4977 deletion, but it could not be excluded that other factors participate in this pathophysiological processes in addition to mutations of mitochondrial DNA. Thus, we applied the aminoglycoside antibiotic, one of the stress agents, to elucidate the effect of the mtDNA common deletion on the hearing. In group B, the rat treated with kanamycin followed D-galactose presented significantly elevated ABR thresholds. Kanamycin (group C) only increased the mean threshold by 34.17 ± 4.69 dB while D-galactose together with kanamycin (group B) increased the mean threshold by 61.79 ± 11.20 dB. The difference in shift of ABR threshold between group C and group B is significant (ANOVA, $p < 0.001$). In same group, the shift of ABR was found in similar degree. Moreover, it is very interesting to find that the only rat, which was treated with D-galactose and kanamycin together but detected no mtDNA4834 deletion increased the hearing threshold only by 35 dB. It is noteworthy that this ABR threshold shift is similar to the average shift of ABR threshold of group C, which were injected only with kanamycin and detected no mtDNA4834 deletion. These results have provided the direct evidence that mtDNA4834 deletion cannot cause the hearing loss directly, but can greatly enhance the sensitivity of the inner ear to the stress being a predisposing factor.

Discussion

In this study, mtDNA4834 deletion was found in almost all the inner ear of rats treated with D-galactose compared with control in which no mtDNA4834 deletion was

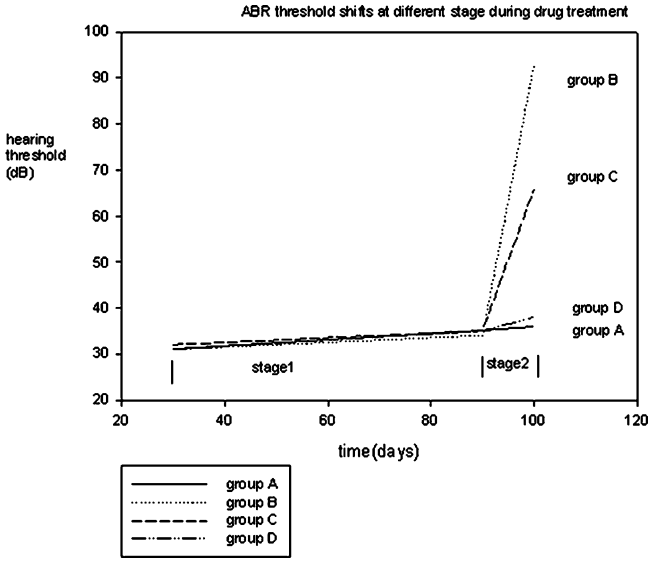

Fig. 3. ABR threshold shifts at different stages during drug treatment.

observed. These findings indicated the successful establishment of the animal model associated with mtDNA common deletion of inner ear. Prithivirajsingh et al. [22] reported tumor cell lines showed only a modest induction of the mtDNA common deletion by ionizing radiation. It has been reported that 12 of 17 young and 11 of 11 old mice were induced mtDNA common deletion in cerebrums by incompletely ligated to reconstruct cerebral ischemia [23]. Compared with these methods, our induction of mtDNA common deletion of inner ear is stable, safe, and high efficiency, which would provide a way to study the relationship between the common deletion and hearing. During the past few years, extraordinary progress has been made in elucidating the mechanisms involved in galactose-induced aging in cell. Several aged animal models, such as cataract, neural degeneration, were established by using different doses of galactose [16–18]. In this study, we proved that D-galactose could induce mtDNA4834 deletion in inner ear. Meanwhile, we found that the activity of SOD decreased and MDA increased after D-galactose was injected. The changes of these two biochemical markers for aging are similar to those happened in naturally aging process in human being and other animals. It is indicated that we could establish the animal model of mimetic aging in the inner ear by D-galactose. Although the mechanism of

Table 2
ABR threshold shifts

Group (dB peSPL)	Before the drug administration	After the stage 1	Hearing threshold elevation	After the stage 2	Hearing threshold elevation
A	31.07 ± 3.50	34.64 ± 4.58	3.57 ± 3.63	36.43 ± 3.63	5.36 ± 3.08
B	31.43 ± 3.06	34.29 ± 3.85	2.86 ± 2.57	93.21 ± 9.73	61.79 ± 11.20[a]
				(95.00 ± 7.36)	(63.85 ± 8.45)[a]
C	32.08 ± 3.96	35.42 ± 3.34	3.33 ± 3.26	66.25 ± 6.08	34.17 ± 4.69[a]
D	31.00 ± 3.16	35.00 ± 3.33	4.50 ± 2.84	37.50 ± 3.54	6.50 ± 3.37

[a] Denotes significant results compare to group D. ($\alpha = 0.01$). The data in parentheses in the group B have excluded the ABR threshold of the rat without mtDNA4834 deletion. Data values are presented as means ± the standard deviation (SD).

mtDNA deletion in this animal model was not clear, it was the general opinion that galactitol is formed by reduction of galactose after metabolism of the cell. Galactitol could not be decomposed, and accumulated in the cell to affect the osmotic pressure, which caused the metabolism of sugar and reactive oxygen species (ROS) in disorder [24]. More specifically, these ROS damage mitochondrial DNA, resulting in the production of specific mtDNA deletions. These specific mtDNA deletions are also known as the common aging deletion. Our results provided a direct evidence that mtDNA common deletion could be induced by special potentially noxious agents, which could explain why aging people have higher incidence of mtDNA4977 deletion than young individuals.

The mtDNA common deletion has recently been proposed as important contributors to aging and presbyacusis. However, it is still uncertain whether the mtDNA common deletion cause presbyacusis directly and what role this deletion played on the hearing loss. In this study, we found that no significant difference of ABR threshold shift was detected between the rats associated with mtDNA4834 deletion treated with D-galactose (group A) and control rats treated with saline (group D). It seemed controversial with the result that Bai et al. [25] found 1 of 10 young rats has mtDNA4834 deletion and this rat had worse auditory sensitivity. We speculated that it cannot be excluded that other factors participate in this pathophysiological processes in addition to mutations of mitochondrial DNA. Stress was thought to play a role in hearing loss associated with the mtDNA mutation. The novel features of mtDNA deafness is that the same mutation produces a clinical phenotype that varies considerably [26–28]. Genetic background and environmental stress may be involved in the process of deafness in the presence of this mutation. In order to understand the effect of mtDNA4834 mutation on hearing, we used the kanamycin as a representative of stress agents. The mean threshold elevation induced by kanamycin was much higher in the rats with mtDNA4834 deletion (group B) than the rats without deletion (group C). It is noteworthy that there is one rat without induction of mtDNA4834 deletion by D-galactose from group B, which has 35 dB elevation of ABR auditory threshold after the treatment of kanamycin. It is different from other rats in the same group which were induced mtDNA4834 deletion have a much severe hearing loss, but it has the same value as the average shift of ABR threshold of the rats which were only treated with kanamycin from group C. We proposed that the mtDNA4834 deletion alone is not sufficient to produce the clinical phenotype directly but can enhance the sensitivity of inner ear to the stress such as aminoglycoside antibiotic. The region of the 4834 bp deletion encodes several proteins which are important to OXPHOS, such as ATPase subunit 6, ND3, ND4, ND4L, ND5, and cytochrome oxidase III. So this deletion might result in OXPHOS relating enzyme defect. The mtDNA4834 deletion accumulated together with the decrease in mitochondrially encoded proteins in inner ear cause the cumulative OXPHOS disordered. In addition, it may result in dysfunction of the hair cell and imbalance of ion in cochlear. This lesion might be slight to cause the detectable hearing loss directly, but it could induce inner ear in a "sensitive status" to the damage of stress. Being in this condition, exposure to harmful agents which could do potential damage to hearing might trigger a series of biochemical and pathological changes in inner ear and lead to deafness phenotype. The mtDNA4834 deletion and environment stress interacted in a vicious cycle are central to a potion of the sensorineural deafness pathogenesis.

In summary, we established a rat model associated with mtDNA4834 deletion by D-galactose administration, which provides a way to study the mechanisms underlying the role this mutation played in the deafness. It also shows that this mutation can develop the sensitivity to the environmental stress and aggravate the hearing impairment. Though much work remains to be done to further explore the mechanisms underlying hearing loss, this finding could help in the prevention of hearing loss related with mtDNA mutation.

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