Evaluation of the Effects of Chronic Injection of D-Galactose on Auditory Brainstem Responses as a Model for Studying Age-Related Hearing Loss

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Received: 28 Jan. 2018; Accepted: 28 Feb. 2019

Abstract- The D-galactose induced mimetic aging rat model has been widely used in studies of age-associated diseases recently. Evidence indicates that D-GAL could also play a key role in age-related hearing loss. However, there is conflicting data about the relationship between the D-GAL injection and tone-burst auditory brainstem responses (ABRs). The present study aimed to compare ABRs in D-GAL injected rats compared with young and naturally aged rats. Tone-burst ABR was recorded and analyzed at the frequencies of 4,6,8,12 and 16 kHz in male young (3-month-old, n=10), naturally aging (18-month-old, n=10) and D-GAL injected (3-month-old, 500 mg/kg D-GAL injection for 8 weeks, n=10) Wistar rats. When the ABRs thresholds obtained in the D-GAL group and the natural aging group were compared with the thresholds in the young group, we observed a significant increase in thresholds, which affected all of the frequencies (P<0.05). A statistically significant decrease in amplitude of wave PI at 4 and 8 kHz, PII at 4,8 kHz, PIV at 4,6,8,12 and 16 kHz was also observed in naturally aging group compared with young group. However, in D-GAL group, a significant difference was exclusively detected in amplitude of PIII at 4 kHz. Latency did not reveal any significant difference between the groups (P>0.05). The present study confirmed that experimental injection of 500 mg/kg/day D-GAL for 8 weeks to Wistar rats could lead to ABRs threshold shifts but not latency. Because there are several types of presbycusis, further studies are needed to determine what type of presbycusis is induced by D-GAL and where is the first region affected by it to provide the best treatment and prevention methods.

Acta Med Iran 2019;57(5):281-288.

Keywords: D-galactose; Age-related hearing loss; Auditory brain stem responses

Introduction

Age-related hearing loss (ARHL), also referred to as presbycusis, is the most common sensory disorder in the aged population (1-5).

The pathogenesis of ARHL is not yet well understood. However, there is a consensus that it is a multifactorial disorder and numerous contributing risk factors including extrinsic (noise, exposures to environmental ototoxic agents, trauma, vascular insults, metabolic changes, hormones, diet, immune system), superimposed upon an intrinsic, genetically driven, physiologic aging process (6-10). Among several underlying mechanisms, mitochondrial malfunction has been supported by several studies, and it has been suggested that aging is strongly related to accumulated mitochondrial damage over time that caused mainly by reactive oxygen/nitrogen species (11-20). The malfunction of mitochondria is often associated with large-scale mtDNA deletion (14-20).

Because of the gradual nature of age-related hearing loss in humans, animal models provide a better understanding of the underlying mechanism of aging and anti-aging treatments. Although several animal models have been designed for aging so far among them, D-galactose-induced model of aging is the best one because provide closer results to clinical studies (21). An oversupply of D-GAL leads to its conversion to aldose, hydrogen peroxide, and galactose oxidase and speed up the generation of superoxide anion and oxygen-derived free radicals. These products can accumulate in cells, which in turn cause osmotic and oxidative stress that may account for the acceleration of aging (22,23). Hence rodents treated with D-GAL (100-500 mg/kg) for consecutive 6-8 weeks can be also used as an animal model for oxidative stress as well as age-related disease (22,23). This model exhibits accelerated aging in several

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tissues such as brain and liver (22). In addition, mimetic rat models of aging in auditory system using D-GAL have been established previously (15-17,20,24). It was suggested that D-GAL could be a suitable agent to produce an age-related hearing loss model (15,16,25) because it can increase the mtDNA deletion (14-16,20,26-28), a biomarker for aging, through a process similar to naturally aging process. In particular, it was demonstrated that D-GAL led to mtDNA 4977-bp deletion/mutations (also known as the ‘‘common deletion’’, CD) in humans, and the corresponding mtDNA 4834-bp deletion in rats, that have been postulated to play a key role in age-related hearing loss (15-18,26,27,29,30).

While histopathological changes in the cochlea and central auditory system following the D-GAL injection are addressed in some studies, limited studies have investigated the relationship between the D-GAL injection and auditory tests. This is important because in both humans and animal models, age-related changes are also routinely seen in auditory tests, such as auditory brain stem responses (ABRs) which is an objective auditory test, evaluating the integrity of the hearing system from the level of the cochlea up through the lower brainstem in response to auditory (31,32). ABR is used for threshold estimation in neonates and neurological disorders.

Therefore, for determining a suitable model for age-related hearing loss, it is necessary to have an accurate examination of auditory brain stem responses, particularly following the D-GAL injection.

Although the relationship between auditory brainstem responses and D-GAL injection has been evaluated in previous studies but mostly click stimuli have been used (11,15,17,24,27,28,33). Tone-bursts have been used in a few studies with conflicting results (15,24). Tone-burst ABRs enable us to evaluate the auditory system using specific frequencies, and the test has greater frequency-specific sensitivity than click ABR (34). Given the gradual nature of age-related hearing loss which primarily involves the high-frequencies and then gradually extends to lower frequencies, it seems reasonable to use tone-burst stimuli for studying age-related hearing loss. So, the present study utilized tone-bursts to accurately compare auditory brainstem responses between young, D-GAL treated and naturally aging rats at several frequencies.

Materials and Methods

Male Wistar rats at the age of 3-month-old (young group, n=20) and 18-month-old (naturally aging group, n=10) were prepared. Rats in the young group were randomly divided into D-GAL injection group (n=10) and young control group (n=10). D-GAL group received 500 mg/kg/day D-GAL for consecutive 8 weeks. The rats were housed standard polypropylene cages with wired-net top in a controlled room (temperature 23±1° C, humidity 55±10%, an altering 12 hr light-dark cycle) and were allowed free access to standard laboratory pellet diet and water during the experiments. All ethical issues on the use of animals were carefully considered. For acclimation to the new living condition, animals kept in a quiet room for 7 days before the starting experiment.

For baseline ABR measurements, rats were anesthetized with a mixture of xylazine (10 mg/kg) and ketamine (40 mg/kg) given intraperitoneally. ABRs with tone burst stimuli at the frequencies of 4, 6, 8, 12, 16 kHz were performed for all animals using Biologic Navigator pro system (Natus, USA). Tone-burst stimuli were delivered through a speaker who was positioned directly above the animal’s right ear at the height of 10 cm. An inverting needle electrode was placed subcutaneously below the test ear and a non-inverting electrode at the vertex. A ground electrode was positioned below the contralateral ear. The electrical responses from the recording electrodes were amplified (×100000), filtered (100-3000 Hz). The sound intensity was varied in 10dB steps down, and then 5 dB steps up near threshold. One thousand and twenty-four tone presentations delivered at the rate of 23.1 were averaged to obtain a waveform at each level. Hearing threshold was defined as the lowest level at which clear ABR peaks can be detected. Replications were obtained at stimulus levels around threshold. The threshold was defined as the lowest intensity to elicit a reliably scored ABR component. Threshold measurements were performed with PII wave. Because of high rate of otitis media in rats housed in conventional cages (35), rats were sacrificed after last ABR testing, and middle ear were accurately observed. Rats with middle effusion were removed from the analysis.

Statistical analysis

SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Comparisons between three groups were performed using one-way analysis variance (ANOVA). If necessary Scheffe post-hoc-analysis were performed. $P<0.05$ indicated a statistically significant difference between the groups. The data of two rats in each group were excluded because of otitis media. A sensitivity analysis showed their measurements could not change the results.

Results

Effect of D-GAL on ABR threshold:

ABRs in Wistar rats include 5 distinct waves (PI, PII, PIII, PIV, PV). Consistent with previous studies, in the present study, wave II was the largest of all waves. The sample of ABR waves at 4 kHz in the study groups was illustrated in figure 1.

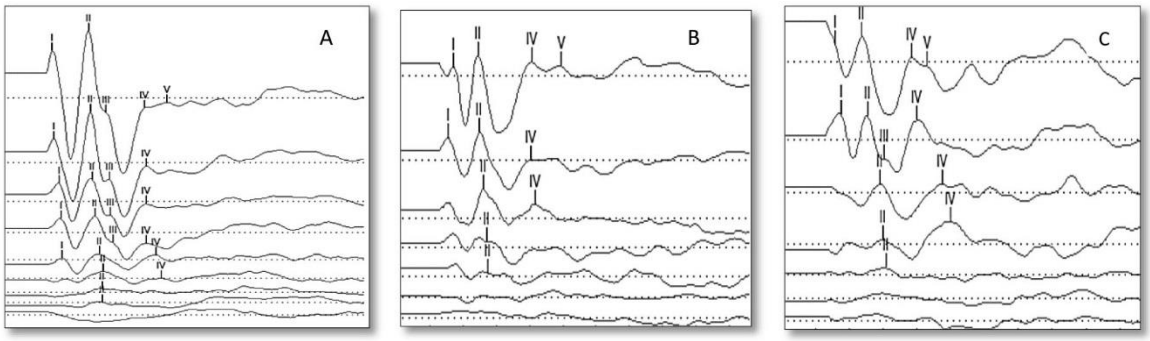

Figure 1. ABR waves at 4 kHz in the study groups (A: young group, B: naturally aging group, C: D-GAL group before the injection)

The auditory threshold was defined as the stimulus intensity that evoked waveforms with a peak-to-peak voltage greater than 2 standard deviations (SD) of the background activity. The mean value of ABR thresholds of young rats was similar to the D-GAL treated rats at the baseline measurement ranging from 26.57±3.59 to 28.14±2.73 dB SPL in the young group and 24.38±4.17 to 26.25±2.31 dB SPL in D-GAL treated group across frequencies. There was no significant difference between them (P>0.05). The average ABR thresholds in D-GAL treated rats increased significantly after 8 consecutive weeks ranged from 32.50±5.24 to 35±3.16 dB SPL across all of the frequencies.

When the auditory thresholds obtained in the D-GAL and naturally aging groups were compared with the thresholds in the young group, we observed a significant decrease in the audition, which affected all of the frequencies. ANOVAs analysis revealed a significant difference in ABR thresholds for the natural aging as well as D-GAL group compared with control young group. The average threshold shifts for the naturally aging compared with young rats (at frequencies of 4, 6, 8, 12,16 kHz) were 5.43 (P<0.01), 5.57 (P<0.05), 6.21 (P<0.000), 8.36 (P<0.000) and 9.36 (P<0.000) dB SPL and for D-GAL treated rats were 7.60 (P<0.001), 6.24 (P<0.001), 5.21(P<0.05), 6.86 (P<0.000) and 6.86 (P<0.000) dB SPL. It appears that the threshold shift was more at higher frequencies.

In naturally aging rats, the average of ABR thresholds was higher than those in the D-GAL group at all the frequencies evaluated except for 4 and 6 kHz. However, there was no significant difference between D-GAL and naturally aging rats at any of frequencies (P>0.05). Figure 2 shows the average of ABR thresholds for three groups.

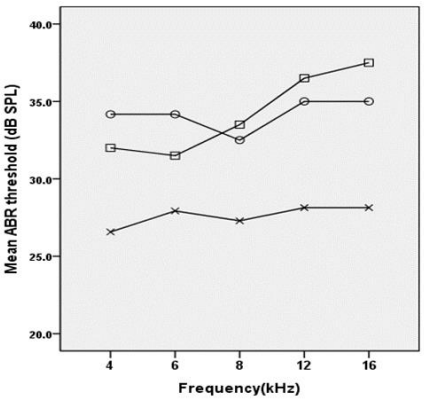

Figure 2. The mean ABR threshold as a function of frequency in the study groups

D-GAL group ○ Naturally aging group □ Young group ×

Effect of D-GAL on ABR amplitude

The wave amplitude was defined as the peak-to-peak amplitude from the positive peak to the subsequent negative trough of each wave. As shown in figure 3, the amplitude of all ABR waves in young group was greater than other groups. ANOVAs analysis revealed a statistically significant difference in amplitude of wave PI at 4 (P<0.01) and 8 kHz (P<0.05), wave PII at 4,8kHz (P<0.05), wave PIV at 4, 6 (P<0.05), 8, 12 (P<0.01) and 16 kHz (P<0.05) between naturally aging and young rats. It should be noted that amplitude of waves PI at 6 kHz (P=0.08), PIII at 8 kHz (P=0.08), PI and PII at 12 kHz (P=0.07, P=0.06) were smaller in the natural aging group compared to young control group such that the

differences were near the significance level.

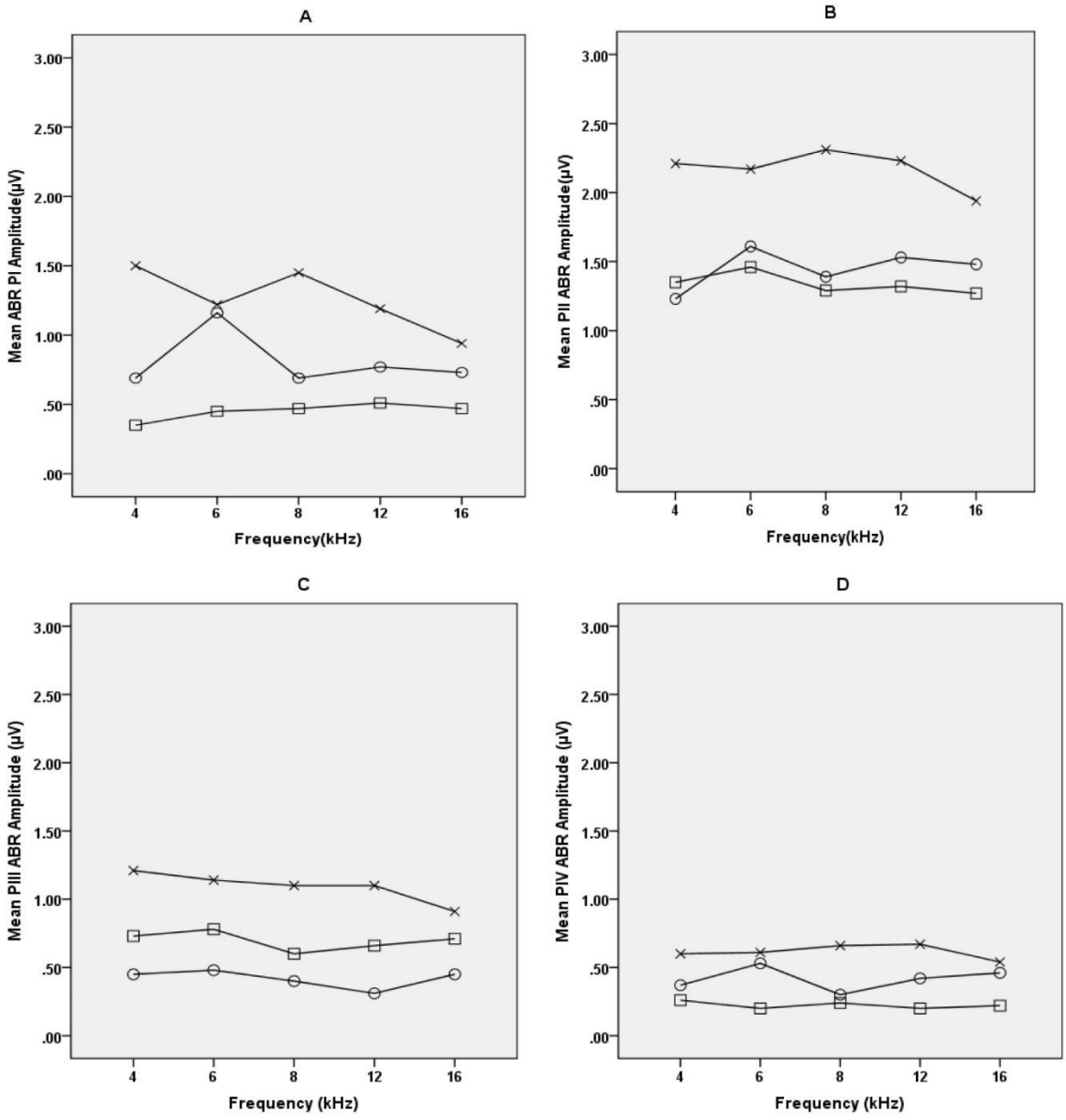

Figure 3. The mean ABR waves amplitude in the study groups; PI(A), PII(B), PIII(C), PIV(D)
D-GAL group ○ Naturally aging group □ Control young group ×

In the D-GAL treated rats, a significant difference was exclusively detected in the amplitude of wave PIII at 4 kHz (P<0.05). Wave PIII at 8 kHz was also showed a marginal effect near the significance level (P=0.08). Wave PIII had a smaller amplitude in the D-GAL group compared with young group. Additionally, amplitude of wave PIII at 4 kHz showed a significant difference between D-GAL and naturally aging rats being the amplitude of wave PIII in the D-GAL aging groups were smaller than those in the natural aging group.

Effect of D-GAL on ABR absolute and inter-peak latency

Two latencies were measured for each ABR wave: (1) absolute latency: the latency comprising the time between the stimulus onset and the positive peak, and (2) the inter-peak latencies between I-II, II-IV, and I-IV waves .There was no significant difference in latencies of ABR waves

between young rats compared to natural aging and D-GAL aging rats (P>0.05).There was just a significant difference in the latency of wave PIII at 6 and 12 kHz (P<0.05) between D-GAL and naturally aging rats in which latency in D-GAL rats was longer than naturally aging rats.

Discussion

The evaluation of tone-burst ABRs in the Wistar rats demonstrated that there is a significant difference in the auditory thresholds of all frequencies and in the wave amplitudes at some frequencies in naturally aging 18-month-old and D-GAL treated rats compared with young 3-month-old rats. These findings were consistent with previous studies that demonstrated increased threshold and decreased amplitude in aged Wistar rats compared with young rats. However, latency did not show any significant changes neither in naturally aging, nor D-GAL treated rats. There are some inconsistencies in the human and also animals in literature on aging and the ABR. The increase in latency as function of age has been seen in study of Alvarado *et al.*, (2015), on Wistar naturally aging rats (18-20-month-old) (36). In another study on C57BL/6J mice (16-month-old) which undergo severe progressive age-related sensorineural hearing loss during aging, ABR thresholds increased and amplitudes of early waves, but not late waves decreased greatly in aging C57 mice. There was also a non-significant trend toward increased latencies, but only when threshold elevations were substantial. However, study on CBA/J mice (to 19-months), which show only mild loss late in life, showed no significant changes in ABR parameters (37). So, it seems that changes in latency as a function of age is dependent on threshold shift.

The average of the ABR threshold shifts in the present study was lower than those on Alvarado's study (2015), in which tone-burst ABR was compared between the age range of 6-8, 12-14 and 16-18 months in Wistar rats. The 18-20-month-old rats showed a range of 30-46 dB threshold shift across frequencies compared to 6-8-month-old rats that are considerably higher than the present study. In the present study, the maximum threshold in the natural aging and D-GAL treated rats were 37.50±4.24 dB SPL and 35±3.16 dB SPL respectively compared with 28.14±3.22 dB SPL in younger rats.

The findings of the present study were closer to the study of Chen and colleagues (2010). In the study of Chen *et al*., (2010), the average of threshold in the naturally aging (22-month-old) and D-GAL treated rats was 53.04±8.23, 42.57±3.44 dB SPL respectively compared to control group that had thresholds about 41.33±4.17dB SPL (28). However, there is still an apparent difference between the ABR thresholds of the naturally aging rats between the studies. One of likely reasons might be the high rate of otitis media in the animals housed within conventional cages that were not considered in the mentioned studies. Otitis media can interfere with the ABR test results (35). In the present study, the findings of some rats were removed from analysis because of otitis media.

The present study also demonstrated that there was no significant difference between D-GAL and naturally aging groups in the ABR threshold. There was just a significant difference between latency and amplitude of PIII between D-GAL and naturally aging groups. Hence, the findings of the current study indicate D-GAL can somewhat mimic ARHL by shifting of the ABR thresholds across all the frequencies evaluated. These findings were in consistence with Du *et al.*, (2012, 2015) who reported that D-GAL could change ABR threshold at several frequencies (11,15). However, in study of Zhong *et al*., (2011) (16), Kong *et al.*, (2006) (17,27) ,Wu *et al.*, (2012) (24) and Peng *et al*., (2010) (33), ABR threshold remained unchanged. In addition, Chen *et al*. (2011) found a small but significant latency increase in D-GAL rats, that did not confirm in the present study. The latency of ABR waves was not evaluated in other studies.

As mentioned, the role of D-galactose in inducing oxidative stress in the auditory system has been well documented in several studies (15-17,20,24). Accordingly, daily injection of 500 mg/kg D-galactose for 8 weeks could be also one of the aging models to imitate presbycusis (15,16,25) because it can lead to several histopathological changes in the peripheral and central portions of auditory system; that was similar to the process of changes in natural presbycusis. In particular, D-GAL causes the elimination of mitochondrial DNA that is similar to the process of changes in natural age-related hearing loss. Chen and colleagues (2010) found increased mtDNA deletion, lipid peroxidation level, apoptosis and neurodegenerative changes in cochlear nucleus, inferior colliculus and auditory cortex that was similar to changes in naturally aging rats after 150 mg/kg D-GAL injection (28). Du *et al*., (2012) also evaluated oxidative stress, mitochondrial dysfunction, and inner ear apoptosis after D-GAL-induced aging rats. In addition, increased in the expression of NOX, uncoupled proteins, cleaved caspase-3 has been found after D-GAL injection. There was also a link between high-fat diet and age-related hearing loss as such the changes were more

prominent in rats received D-GAL plus high-fat diet (15).

In another study, Du *et al*., (2015) showed D-GAL-induced oxidative damage to DNA in the auditory cortex and ventral cochlear nucleus (11). They found that D-GAL at doses of 150, 300, 500 mg/kg can increase oxidative stress in auditory cortex by increasing NOX2 and mtDNA deletion compared with the control group. They also showed that D-GAL at the dose of 500 mg/kg led to more changes. Zhong and colleagues (2011) also reported common deletion of mtDNA4834, which is a molecular marker of aging and plays an important role in presbycusis using three doses of 150, 300 and 500 mg/kg D-galactose for 8 weeks (16). Similar to the previous studies, 500 mg/kg led to more changes compared with lower doses. Reduction of cytochrome c oxidase (COX) activity, increasing of the mtDNA deletion and ultrastructural changes in the inner ear were also observed in D-GAL treated rats (25).

Several studies also used D-GAL in animal models to investigate the effectiveness of therapeutic interventions in age-related hearing loss. Peng *et al*., (2010) evaluated the protective effect of Alpha-lipoic acid on ARHL in animals receiving 150 mg/kg D-GAL injection. The SOD and MDA levels were significantly higher in D-GAL group than other groups butABR thresholds were not significantly different between the groups(33). However, the relationship between auditory brain stem response and D-galactose injection has been evaluated in some studies with conflicting results (11,15, 17,24,27,28,33). It seems that the difference in D-GAL dose, starting age of injection, otitis media, and ABR stimuli are factors which can lead to conflicting results. It seems they are important factors that should be considered when using D-GAL for studying the age-related hearing loss. According to studies, it seems D-GAL injection at dose of 500 mg/kg was more effective than 150 mg/kg. However, in study of Chen *et al*., (28), Kong *et al*., (17,27), Peng (33) that D-gal could not cause threshold shift, 150 mg/kg of D-GAL were used. One of another factor is ABR acquisition parameter particularly auditory stimuli which are used for eliciting ABR waves. Except the study of Wu *et al*., (2012) (24) and Du *et al*., (2012) (15), all of the studies had been used click stimuli to record ABR while deterioration of hearing in aging process is more accurately measured at acute frequencies using tone-bursts. The findings of the present study also showed that higher frequencies exhibit more threshold shifts. The starting age of D-GAL injection is a probable another factor. The findings of previous studies indicated that for the establishment of D-GAL-induced aging model in rats, the optimal starting age of the D-GAL injection was mature rats (3-month-old or higher) (23). However, in all of the above-mentioned studies, animals were younger than 3 months. Therefore, the mentioned factors should be considered when we study the age-related hearing loss in animal models. In addition, except the auditory threshold, other ABR parameters such as latency remained unchanged in D-GAL as well as natural aging rats. Several types of presbycusis have been explained including sensory, neural, metabolic (strial), mechanical and mixed (7). We must determine what type of presbycusis can be induced by D-GAL and where is the first region affected by it. The effect of D-GAL on threshold but not latency reveal that sensory or metabolic components or a combination of them are likely to be induced by D-GAL more than neural component. Although, the effects of D-gal on central levels of auditory system has been shown in previous studies (11,28), however, it probably makes a slight effect compared with peripheral effects. So, it seems experimental administration of D-GAL in rats can be one of animal models for studying sensory or metabolic age-related hearing loss but not neural. However, it is not clear that which component has more effect in the age-related hearing loss in humans. Further studies are needed to discover the contribution of each component. Currently, there is no animal model that cover all aspects of age-related hearing loss in humans. Advancement of other genetic and pharmacological tools, promise approaches for a better understanding of the presbycusis mechanisms taking place in the peripheral and central auditory system and for best animal models that cover all aspects of age-related hearing loss in humans.

The present study confirmed that experimental administration of D-GAL could change ABR thresholds and so can be used as one of the animal models for studying the age-related hearing loss mechanism and therapeutic interventions, but it is not the best model. D-GAL injection and natural aging process share somewhat same mechanisms. However, the exact mechanism of D-GAL on the auditory system is remained to be elucidated. Because there are several types of age-related hearing loss involving different portions of the auditory system, further studies are needed to determine the what type of presbycusis is likely to be induced by D-GAL and where is the first region affected by it to provide the best treatment and prevention methods.

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