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ORIGINAL RESEARCH

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Beneficial effects of time-restricted feeding on gentamicin cytotoxicity in mouse cochlea and vestibular organs

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Abstract

Objective: Time restricted feeding (TRF), which is an intermittent fasting protocol, has been reported to decrease the toxicity and mortality rate associated with systemic gentamicin (GM) administration. The aim of this study is to evaluate the effect of TRF on GM-induced vestibular and auditory function in mice.

Methods: Japan Central Laboratory for Experimental Animals:Institute of Cancer Research (Jcl:ICR) mice were housed in a light–dark (LD) cycle (12:12) and were divided into three groups: (1) GM treatment at a dose of 220 mg/kg with TRF (feeding time: 8 h [9:00–17:00] during the light phase [7:00–19:00]) (GM + TRF group), (2) GM treatment at a dose of 220 mg/kg without TRF (GM group), and (3) saline injection with TRF (NS + TRF group). GM or saline was injected subcutaneously for 18 days (three courses of 5 days' injection + 2 days' rest, and an additional 3 days' injection). The auditory brainstem response (ABR) and vestibular evoked potential (VsEP) were tested after the treatments. The number of sensory hair cells in the cochlear organs and the vestibular organs were quantified using microscopic images.

Results: All animals survived until the end of the experiment. One day after the last injection, GM + TRF mice showed significantly lower ABR thresholds at 4 kHz compared to GM mice, and there was no significant difference between the GM + TRF and NS + TRF groups. There was a significant difference of VsEP between GM and GM + TRF mice only in symmetric parabolic waves with linear acceleration and ramps waveform stimulation. GM + TRF mice showed significantly less outer and inner hair cell loss compared to GM mice. GM + TRF mice showed significantly less type II hair cell loss in the utricle and the ampulla compared to GM mice.

Conclusion: TRF with daytime feeding reduced GM cytotoxicity in the cochlea and vestibular organs of ICR mice.

Level of Evidence: NA

Ying Gao and Teru Kamogashira were equally contributed to the study.

KEYWORDS
cochlea, gentamicin, time restricted feeding (TRF), vestibular evoked potential (VsEP), vestibule

1 | INTRODUCTION

Time restricted feeding (TRF) is an intermittent fasting regimen, in which mice are fed for certain hours every day. In mice, TRF prevents obesity and improves clinical outcomes, such as hyperlipidemia and insulin sensitivity, in a circadian clock gene deficiency model.[1] TRF in mice on a high-fat diet has been found to improve motor coordination and protect against obesity, high blood insulin, liver fat, and inflammation.[2] In rats, TRF increases the expression of Sirtuin 1 and brain-derived neurotrophic factor (BDNF), decreases the expression of serum IGF-1, and has an antitumor effect.[3] In humans, TRF improves insulin-sensitivity, blood pressure and oxidative stress in pre-diabetic men,[4] and improves glucose tolerance in men at risk of type 2 diabetes.[5] TRF is also reported to prevent metabolic disorders such as obesity and diabetes, and can improve cardiometabolic disorders.[6] These effects of TRF may occur when the timing of food intake triggers synchronization with the circadian clock gene, thereby regulating circadian rhythms.[7,8] In the cochlea, the expression of BDNF has been reported to have a circadian rhythm and reduces its susceptibility to noise exposure.[9,10] Thus, a positive effect of TRF in protecting the auditory system through entrainment of circadian clock genes and their downstream cascades is expected.

Systemic administration of gentamicin (GM), a bactericidal antibiotic belonging to the aminoglycoside family, is well known to cause vestibular and auditory ototoxicity as the main side effect in humans and rodents, and it also has high mortality rates in mice due to acute kidney injury.[11] Therefore, it is crucial to clinically protect the inner ear from potential side effects during GM treatment. In the current study, we focused on the fact that GM exhibits dose-and-time-dependent kinetics in both rodents and humans and that the circadian changes in drug disposition can contribute to the drug effects and toxicity.[12] We hypothesized that TRF with daytime feeding may protect inner ears from GM cytotoxicity. Certain types of TRF have been reported to reduce the mortality rate after GM administration in rats and mice,[12,13] but the direct effect of TRF on the inner ear is not well known. Therefore, we examined the beneficial effects of TRF on GM-induced cytotoxicity in the cochlea and vestibular organs in mice.

2 | MATERIALS AND METHODS

2.1 | Experimental animals and design

Eight-week-old male Jcl:ICR mice (weighing 28–30 g) were housed in a light–dark (LD) cycle (12:12) with free access to water and access to food for 8 h daily (09:00–17:00, TRF) during the light phase (07:00–19:00) (Figure 1A,B).[13] All procedures regarding the use and care of animals were approved by the Institute for Animal Care and Use Committee of Medical Science, University of Tokyo (I-P14-132, I-P19-083). We selected male ICR mice because the referenced article which analyzed GM subchronic toxicity with TRF used male ICR mice.[13] In addition, the experiments in hearing research are predominantly performed on male animals because of the possibility of variances in hearing levels due to the reproductive cycle.[14] The hearing thresholds in ICR mice match well with those in other mouse stains[15] and do not deteriorate at least until the age of 12 weeks.[16] ICR mice are less likely to exhibit congenital hearing loss and are considered to be suitable for studying hearing function.[17]

Mice were divided into three groups and were assigned to different feeding regimens randomly, as described in Figure 1A–C. The same amount of food was provided to all groups of animals, almost all of which was consumed. In the GM injection with TRF (GM + TRF) group ($n = 8$) and GM injection without TRF (GM) group ($n = 10$), a

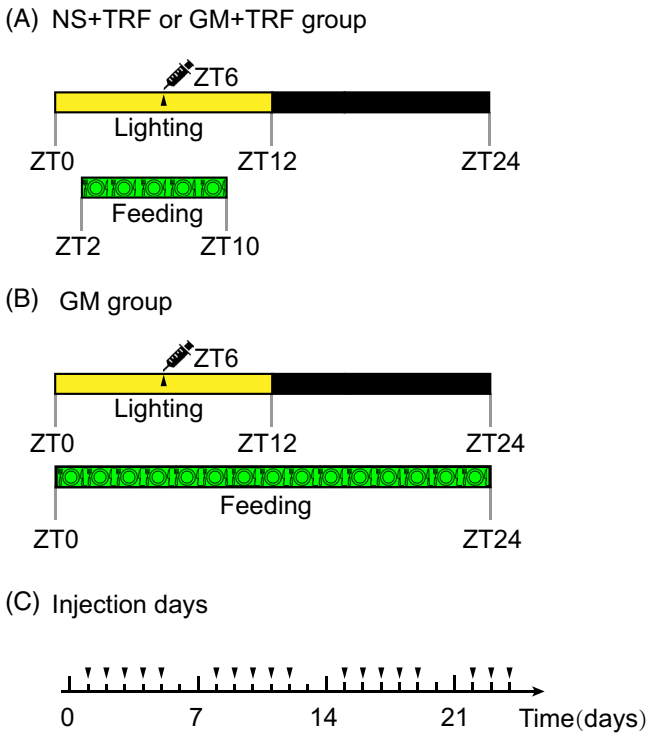

FIGURE 1 Experimental design. (A) Schematic representations of the feeding method and the injection time in the NS + TRF group and GM + TRF group (ZT: Zeitgeber time). (B) Representations of feeding method and the injection time in GM group. (C) Representation of every injection day during the 24-day course. GM was administered on 18 days of the 24-day course: from Monday to Friday for 3 weeks and from Monday to Wednesday of the 4th week. GM, gentamicin; TRF, time restricted feeding

dose of 220 mg/kg GM was given at 13:00 by subcutaneous (SC) injection. GM (Nacalai Tesque) was prepared at 20 mg/ml in saline using 1 M NaOH 20 μl/ml to adjust to pH 7. In the saline with TRF (NS + TRF) group ($n = 3$), saline was subcutaneously injected at 13:00. GM or saline was injected subcutaneously for 18 days (weeks 1–3: injections 5 days a week [Monday to Friday] with 2 days rest at

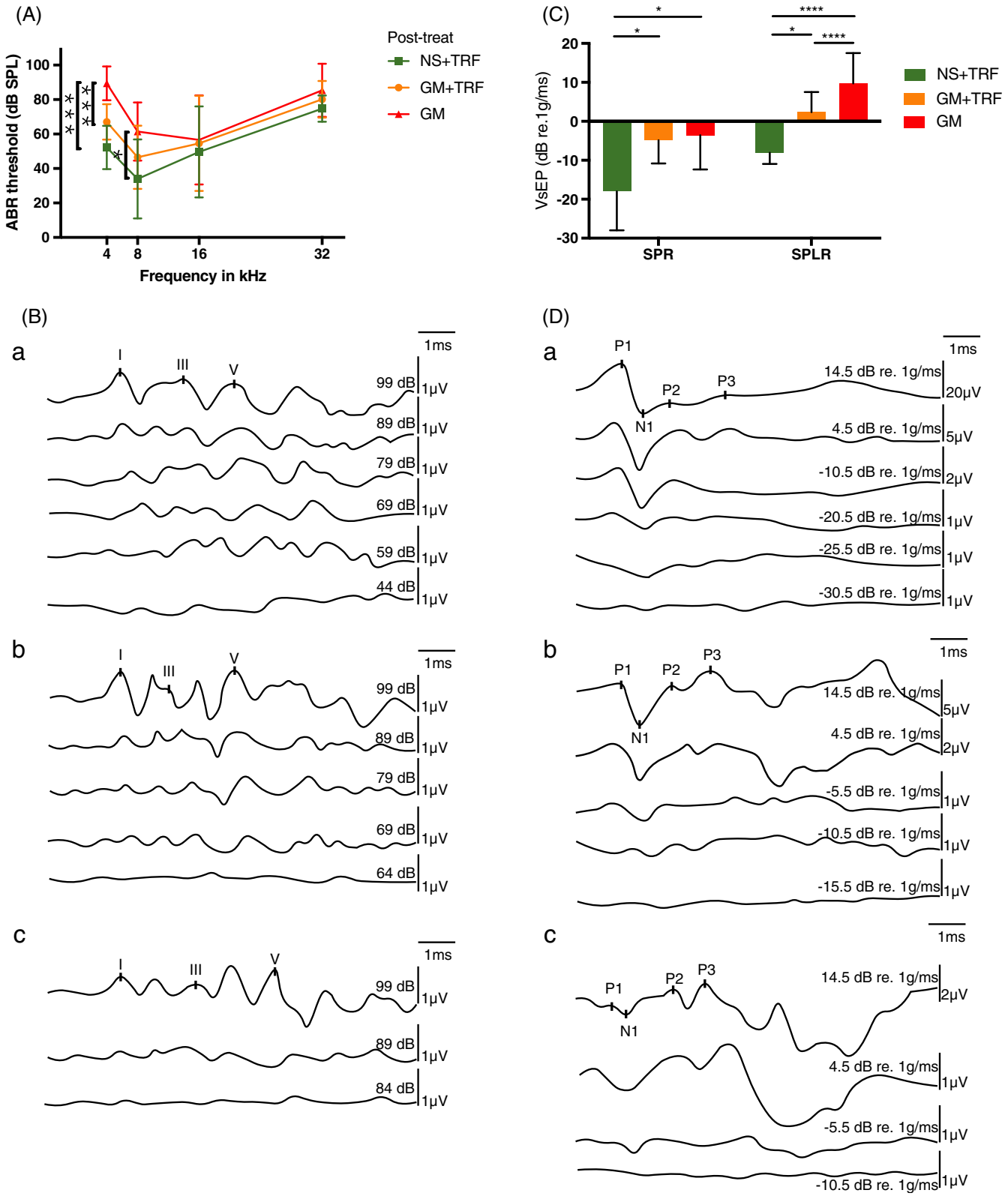

FIGURE 2 Legend on next page.

the weekend due to facility circumstances; week 4: injections Monday to Wednesday only), taking a total of 24 days to complete. One day after the last GM or saline injection, the animals were examined with auditory evoked brainstem responses (ABR) and then vestibular evoked potentials (VsEP), after which they were euthanized for histological analysis.

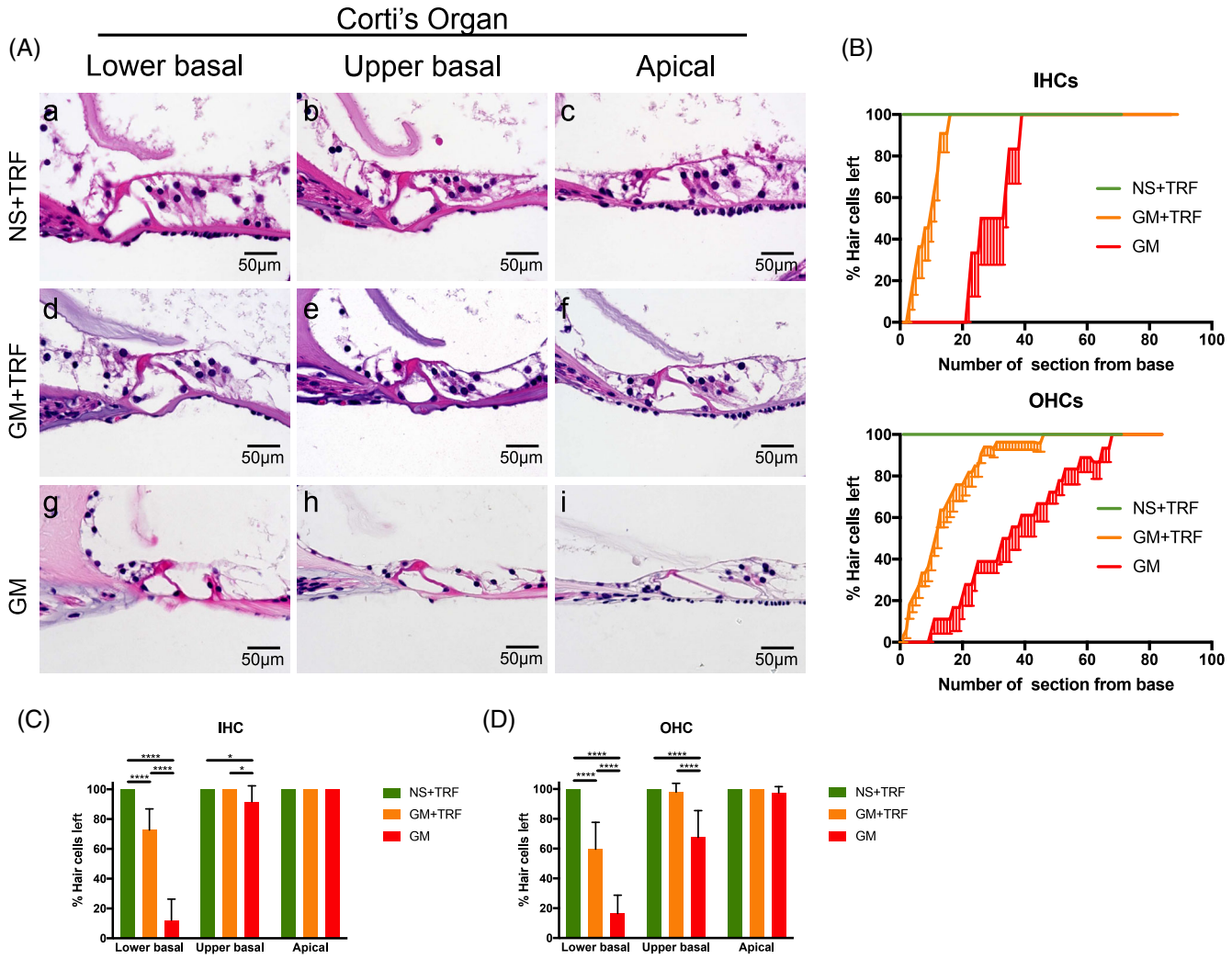

FIGURE 3 Histopathology and numerical evaluation of Corti's organ. (A) HE staining of Corti's organ. No significant shape changes were detected in the IHCs or OHCs between the GM + TRF and NS + TRF groups, while significant decreases in the number of HCs were present in the GM group. (B) Average cytocochleograms of the NS + TRF group, GM + TRF group, and GM group. Graphs show the percentage of remaining IHCs and OHCs. (C) Percentage of the remaining IHCs in the three regions of the cochlea in the three groups. (D) Percentage of the remaining OHCs in the three regions of the cochlea in the three groups. $^{*}p < .05$, $^{****}p < .0001$. (B–D: $n = 8$ in GM + TRF group, $n = 3$ in NS + TRF group, $n = 10$ in GM group. Error bars represent the standard error of the mean.) GM, gentamicin; HCs, hair cells; HE, Hematoxylin and Eosin; IHCs, inner hair cells; OHCs, outer hair cells; TRF, time restricted feeding

FIGURE 2 The ABR and VsEP results. The administration of GM under TRF did not alter the ABR but increased thresholds in VsEP. (A) ABR thresholds after treatment are shown for NS + TRF group, GM + TRF group and GM group. (B) Typical waveforms of ABR under 4 kHz for the NS + TRF group (a), GM + TRF group (b), and GM group (c). (C) Average VsEP thresholds for the NS + TRF group, GM + TRF group, and GM group. Two types of vibration acceleration, SPR and SPLR (Figure SS1), were used in the evaluation of VsEP. Thresholds of VsEP were −4.9 ± 2.1 dB (SPR), −2.4 ± 1.8 dB (SPLR) in GM + TRF group, −3.6 ± 2.9 dB (SPR), 9.7 ± 2.6 dB (SPLR) in GM group, −18.0 ± 5.7 dB (SPR), and −8.1 ± 1.7 dB (SPLR) in NS + TRF group, respectively. (D) Typical waveforms of VsEP using SPLR waveform stimulation in the NS + TRF group (a), GM + TRF group (b), and GM group (c). From top to bottom within each plot, waveform in response to: (a) 14.5, 4.5, −10.5, −20.5, −25.5 or −30.5 dB re. 1 g/ms stimulation. (b) 14.5, 4.5, −5.5, −10.5 or − 15.5 dB re. 1 g/ms stimulation. (c) 14.5, 4.5, −5.5 or −10.5 dB re. 1 g/ms stimulation. $^{*}p < .05$, $^{***}p < .001$, $^{****}p < .0001$. $n = 8$ in GM + TRF group, $n = 3$ in NS + TRF group, $n = 10$ in GM group. The error bars represent the standard error of the mean. ABR, auditory brainstem response; GM, gentamicin; SPLR, symmetric parabolic waves with linear acceleration and ramps; SPR, symmetric parabolic waves with ramps; VsEP, vestibular evoked potential

2.2 | Auditory brainstem response measurement

The measurement method of ABR was reported previously.[18] ABR were measured in all mice 1 day before and 1 day after the 18-day course of GM or saline administration. Mice were anesthetized with a mixture of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (40 mg/kg). Needle electrodes were inserted subcutaneously at the vertex (active electrode), beneath the pinna of the measured

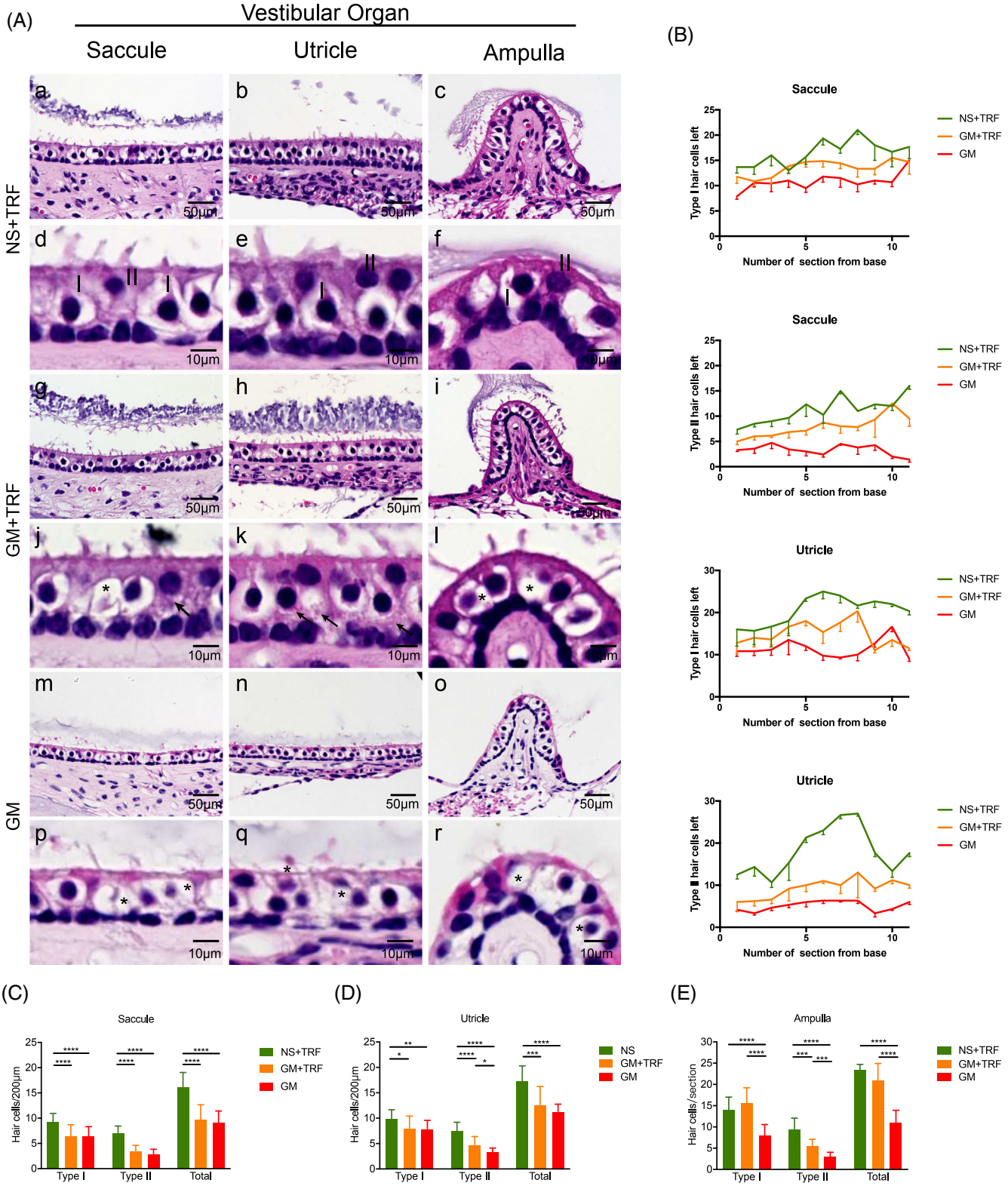

FIGURE 4 Legend on next page.

ear (reference electrode), and beneath the opposite ear (ground electrode). The sound frequencies presented were 4, 8, 16, and 32 kHz. The repetition rate was 20 Hz, with a duration of 5 ms, which consisted of a 3 ms plateau and a 1 ms rise/fall time. The 500 responses were bandpass filtered (4–32 kHz) and averaged with the Neuropack MEB-2208 measuring system (Nihon Koden). The threshold was defined as the lowest intensity level at which a clear reproducible waveform was visible in the trace.

2.3 | VsEP measurement

VsEP were measured immediately after the ABR measurements after the 18-day course of treatments. The VsEP measurement system consisted of an acceleration stimulation system and the evoked potential measuring system. Under general anesthesia with a mixture of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (40 mg/kg), the head of the mouse was fixed on a stimulation frame system using a SG-4 N mice head holder (Narishige). Two types of vibration acceleration, symmetric parabolic waves with ramps (SPR) and symmetric parabolic waves with linear acceleration and ramps (SPLR) (Supporting Information S1), were used to stimulate the head holder. The acceleration stimulation was generated with a WaveMaker Mobile S-0105 vibration exciter (Asahiseisakusyo) by an in-house designed direct current power amplifier. The acceleration was measured with a 352C65 accelerometer and 482A21 signal conditioner (PCB Piezotronics) which was used to calibrate the peak jerk (expressed in 0 dB re. 1 g/ms) of the VsEP stimulation. The evoked potential measurement system was identical to the ABR measurement system, and the typical waveforms, which start in the range of 1 to 2 ms (peak jerk shape), were regarded as effective responses.[19]

2.4 | Histological analysis

We evaluated entire inner ear tissues using thin sectioning method, not surface preparation, in order to avoid the risk of losing tissue specimens during the manipulation of surface preparation. One of the aims of the current study was to compare the degree of damage and the effect of TRF against it between the cochlea and vestibular tissues, and therefore if we damaged one sensory epithelium during surface preparation of the cochlea and vestibular endorgans, we could not analyze data obtained from this mouse. Analyzing all sensory endorgans from a single mouse simultaneously is also considered to reduce the variability of the results due to individual differences of susceptibility to GM compared to analyzing samples separately from different animals.

After ABR and VsEP measurements, all mice were euthanized and decapitated, and the inner ears were removed and fixed by immersion in 4% formaldehyde overnight at room temperature. The inner ears were decalcified in 10% EDTA for 1 week, then dehydrated and embedded in paraffin for cutting with the following steps: 100% ethanol, seven changes, 1.5 h each, 100% chloroform, three changes, 45 min each, and paraffin wax (56°C–58°C), two changes, 1.5 and 2 h. Paraffin-embedded specimens were cut with semi-thin (4 μm) transverse sections. Tissue slices were collected on SuperFrost Plus slides (Thermo Fisher Scientific), dried on a heating plate at 37°C overnight and stained with Hematoxylin and Eosin (HE) staining. The observer-blinded counting of the inner hair cells (IHCs) and the outer hair cells (OHCs) at each of the lower basal, upper basal, and apical cochlear locations was performed on mid-modiolar sections obtained from seven non-overlapping radial sections in each cochlea, as reported previously.[18] The percentage of remaining hair cells (HCs) from the basal end to the full length of the basilar membrane was assessed and was plotted as a cytocochleogram. For the quantitative assessment of the vestibular endorgans, the numbers of type I or type II hair cells of the saccule and utricle were calculated on each slide from the first slide nearest to the cochlea to the most distant slide where the HCs were visible. For the ampulla analysis, the middle part of the posterior semicircular canal ampullary cristae was selected and the type I or type II HCs were counted at five or more transverse sections.[20] For the utricle and the saccule analysis, the number of remaining HCs was counted in their middle part and expressed as the average number per 200 μm.[21] The HCs were regarded as present when their nucleus was clearly visible. In further morphometric evaluations, type I HCs were identified based on their prominent nerve calyx. For the regional analysis, the total length of the saccule or utricle in sections was divided into three equal parts and the number of cells was evaluated separately as a part of lateral extrastriola, striola, or medial extrastriola.

2.5 | Statistical analysis

Microsoft Excel 2006 was used for processing data. ABR thresholds, VsEP thresholds, and HC counting results were analyzed with one-way analysis of variance followed by Bonferroni's multiple comparison testing using Prism (v.7.0 for Apple Macintosh,

FIGURE 4 Histopathology and numerical evaluation of vestibular endorgan. (A) HE staining of the saccule, utricle and ampulla in NS + TRF (a–f), GM + TRF (g–l), and GM (m–r) groups. Type I and type II hair cells are shown in the magnified areas of the upper images (d–f, j–l, p–r). In the GM + TRF and GM groups, both type I and type II hair cells in the peripheral vestibular organ showed degenerative changes, such as shrinkage (marked with a *) or vacuole formation (marked with an arrow). (B) Systematic quantification of hair cells in vestibular endorgans. Graphs show the average number of the remaining type I and type II hair cells per slide in the saccule and utricle. (C–E) Averages of the number of type I and type II hair cells per 200 μm in the saccule (C) and utricle (D) and those per section in the ampulla of the posterior semicircular canal (E). *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$. (B–E: $n = 8$ in GM + TRF group, $n = 3$ in NS + TRF group, $n = 10$ in GM group. Error bars represent the standard error of the mean.) GM, gentamicin; HE, Hematoxylin and Eosin; TRF, time restricted feeding

GraphPad Software). Data were presented as mean ± s.d. A confidence level of 95% was considered statistically significant. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$ were used to depict the significance level in bar graphs.

3 | RESULTS

3.1 | TRF protects auditory and vestibular function from GM ototoxicity and vestibulotoxicity in ICR mice

All animals survived until the end of the experiment with a change of body weight in all three groups being less than 15%. At the end of the experiment (day 24), none of the mice in the GM or GM + TRF groups were able to stand to access food and water bottles, and they moved only in a certain zone of the cage, suggesting that vestibular function was impaired in both the GM group and GM + TRF group. In contrast, mice in the NS + TRF group showed no difficulty in getting food and water and moved in a large area of cage.

Baseline ABR thresholds before the experiment did not differ among groups. The ABR results after the 18 days of injections showed that GM + TRF mice showed significantly lower ABR thresholds at 4 kHz compared to GM mice ($p < .001$). The average ABR thresholds were not significantly different between the GM + TRF and NS + TRF groups ($p > .05$) (Figure 2A). Typical ABR waveforms at 4 kHz in all three groups are shown in Figure 2B. These results suggest that auditory function was more significantly impaired in the GM group compared with the GM + TRF group.

As for VsEP responses (Figure 2C,D), significant differences were detected between the GM group and NS + TRF group and between the GM + TRF group and NS + TRF group in both SPR and SPLR waveform stimulation. There was a significant difference between the GM group and GM + TRF group only in SPLR waveform stimulation. Typical waveforms of VsEP in SPR stimulation in all three groups are shown in Figure 2D. These results suggest that both GM and GM + TRF groups developed vestibular dysfunction, while TRF protected vestibular function to some extent from GM.

3.2 | TRF protects cochlear and vestibular hair cells from GM cytotoxicity

The loss of IHCs and OHCs was significantly lower in the GM + TRF group compared to the GM group (Figure 3). Remarkably, the IHCs and OHCs in the apical turn of the cochlea remained nearly intact in the GM + TRF group (Figure 3A). Average cochleograms showed that TRF attenuated GM-induced damage of both IHCs and OHCs especially in the basal turn of the cochlea (Figure 3B). The numbers of IHCs and OHCs were markedly decreased in the GM group in the summarized analysis; there was a significant decrease of IHCs and OHCs in the lower basal turn and the upper basal turn of the cochlea in the GM group compared to the NS + TRF group. The IHCs and OHCs in the lower basal turn and the upper basal turn of the cochlea in the GM + TRF group were significantly protected compared to the GM group. There was no significant difference of the numbers of IHCs and OHCs between the GM + TRF and NS + TRF groups in the upper basal turn and the apical turn of the cochlea (Figure 3C,D), suggesting that TRF can protect IHCs and OHCs in the apical turn of the cochlea from GM-induced damage.

Many type I HCs in the vestibular endorgans showed shrinkage or loss in the saccular macula, utricular macula, and the ampulla of the semicircular canals in both the GM and GM + TRF groups (Figure 4A). In addition, numerous hydropic or vacuolar degenerations were detected in type I HCs in the GM + TRF group (Figure 4A). TRF protected both type I and type II HCs from GM-induced damage evenly throughout the saccule and utricle (Figure 4B; Figures S1 and S2). The regional difference in GM-induced damage was observed, and the striolar/central zone of the saccule or utricle was damaged more significantly compared to the peripheral zone of the saccule (Figure 4B; Figures S1 and S2). In addition, type I HCs in the striolar/central zone of the saccule or utricle was significantly protected with TRF compared to the peripheral zone of the saccule or utricle (Figures S1 and S2). The summarized analysis showed that the loss of type II HC in the utricle and the ampulla was significantly lower in the GM + TRF group compared to the GM group (Figure 4C–E), suggesting that TRF can protect vestibular HCs from GM-induced damage to some extent.

4 | DISCUSSION

We evaluated the protective effect of TRF with daytime feeding against GM-induced cochlear and vestibular damage and demonstrated that TRF with daytime feeding alleviated cochlear damage and hearing loss due to GM administration in 8-week-old male Jcl:ICR mice, whereas the protective effect of TRF on GM-induced vestibular damage was limited.

Based on a previous study,[13] we selected TRF with daytime feeding instead of nighttime feeding, because the mortality rate with daytime feeding has been reported to be lower. Nephrotoxicity was alleviated when GM was administered at 13:00 during TRF with daytime feeding (restoring circadian rhythms) (08:00–16:00), because TRF induced a shift in the peak and the trough of gentamicin nephrotoxicity in rats.[22] Based on this previous study, we hypothesized that TRF could affect the toxicity of GM to the cochlea and vestibular endorgans because renal tubular cells have clinical similarities with inner ear cells.[23,24] To our knowledge, there is no report on the beneficial effects of TRF on GM ototoxicity and vestibulotoxicity.

TRF is one of the hot topics in anti-aging research because intermittent fasting regimes have been reported to delay aging and increase lifespan by improving cardiometabolic health in rodents[3] and prevent obesity by improving clinical outcomes such as body weight, blood pressure, and insulin sensitivity in both rodents and humans.[1,25] These positive effects of TRF are based on two important mechanisms. First, TRF is thought to regulate circadian clock gene because the timing of food intake can work as an entraining signal to the

circadian clock, which synchronizes rhythmic clock gene expression and restores rhythmicity in mice.[7,8,26] Daytime or nighttime TRF can synchronize the phases of the circadian clock, altering the expression of oxidative metabolism and DNA repair genes in the skin tissue of mice exposed to ultraviolet radiation to induce DNA damage.[27] In the current study, we confirmed that TRF with daytime feeding and GM injection at the midpoint protected cochlear HCs and ampulla type I HCs from GM ototoxicity and vestibulotoxicity, thereby preserving hearing levels and partially protecting vestibular function. These results suggest that using TRF to synchronize circadian clock genes and restore rhythmicity may lead to the mitigation of cytotoxicity of GM in the cochlea and vestibule by altering the expression of oxidative metabolism and DNA repair genes in the inner ear of mice. Recent research has demonstrated that several genes in the cochlea are regulated by the circadian clock and that there are diurnal variations in susceptibility to noise exposure.[9,10] These reports also support the idea that TRF with daytime feeding and GM injection at the midpoint protects cochlea and vestibular organs from GM cytotoxicity.

The second important mechanism is that TRF leads to calorie restriction. The circadian metabolic homeostasis changes resulting from TRF with daytime feeding or nighttime feeding can vary depending on the tissues or genes. Daytime feeding lowers fasting glucose and insulin in the morning, increases fasting insulin in the evening, and decreases 24-h glycemic excursions.[26] Lipids and hormones are also affected by meal timing. Early time-restricted feeding increases LDL and HDL cholesterol in the morning, which may be attributed to the prolonged fasting period and greater reliance on fat oxidation in the early time-restricted feeding arm.[28] The evaluation of internal organs, locomotive activities, oxygen consumption rate, hormone levels, serum glucose levels and serum amino acid levels is helpful to understand the systemic effect of TRF with daytime feeding or with nighttime feeding.

As shown in the current study, GM administration significantly affects VsEP thresholds and vestibular HC numbers. VsEP waveforms in response to a brief head acceleration stimulus have been reported to be a compound action potential originating from the otolith organs.[29] A decline in vestibular function after GM administration has been reported in another study,[11] in which a shorter injection period and a low dose of GM also achieved selective injuries in peripheral vestibular organs. This selective vestibulotoxicity may depend on the dose concentration and the type of aminoglycoside drug, although the detailed mechanism is unknown.[30,31] One of the possible mechanisms is the difference in pharmacokinetics including drug accumulation in the inner ear fluid. The drug concentration in the inner ear is reported not to rise above the levels found in serum[32] and an excessive imbalance in free radical generation in vestibular organs may also alter the toxicity regardless of the drug concentration.[33] The relatively selective vestibulotoxicity of GM due to TRF found in the current study might have a similar explanation. Another factor which can be affected by TRF is the regeneration process in the cochlea and vestibular organs.[34] Although it is possible that TRF affected the regeneration of the HCs in the vestibule, many previous studies have reported that it takes weeks to months for the HCs to regenerate after vestibular injury.[35,36] The spontaneous HC regeneration might influence the counting of type II hair cells because regenerated immature HCs resemble morphology of type II HCs.[36] However, the experimental duration in the current study was 24 days, which was not long enough for the HCs to induce robust regeneration. Further, the type I HCs in the ampulla were significantly protected with TRF. Therefore, the influence of the HC regeneration on the effect of TRF is considered extremely low.

It has been reported in the histological studies analyzing GM vestibulotoxicity using guinea pigs or chincillas[37–39] that the ampulla is more susceptible to GM than the maculae, the utricle is more susceptible than the saccule, the type I HCs are more sensitive to GM than type II HCs, and the central part of the crista ampulla or the striolar/central part of the maculae are more damaged than the peripheral part of the ampulla or the maculae. In the current study, there was no clear difference of the susceptibility to GM between type I and type II HCs nor the ratio of HC loss between the ampulla and the maculae. Possible causes include the use of different GM dosage, the use of a different route such as inspiratory administration, and the use of different mouse strains such as C57BL/6. The regional difference between the central and peripheral parts was not observed in the crista ampulla, but the striolar/central part of the saccule and utricle tended to be more significantly damaged than the peripheral part of the saccule and utricle by GM (Figure 4B; Figures S1 and S2), which was consistent with the findings of previous studies.[37,40] The regional difference of the effect of TRF was also observed and type I HCs in the striolar/central zone of the saccule or utricle was significantly protected with TRF compared to the peripheral zone of the saccule or utricle (Figures S1 and S2).

One of the limitations of the present study is the use of male ICR mice based on a previous study examining the effect of TRF on GM subchronic otoxicity.[13] The number of previous studies in GM experiments using ICR strains is limited and therefore, further studies using other strains are necessary to confirm the beneficial effect of TRF. Also, we counted only the hair cells in both the cochlea and vestibular organs. Although the HCs in the basal turn were histologically normal in the NS + TRF group, the ABR thresholds were higher than other reports.[16] The ICR mice used in our study may have had some abnormalities in cochlear tissues other than the HCs. A detailed histological analysis of other tissues, such as nerve fibers, spiral ganglion cells, and the stria vascularis, may also be necessary.

Another limitation of this study is the utilization of thin section in the quantitative analysis of cochlear and vestibular tissues. Although the quantitative analysis using thin sections is widely utilized in human temporal bone studies and animal studies,[38,41–45] the tissue embedding process can shrink some parts of the tissue, making the analysis slightly difficult. The analysis of spatial changes of the vestibular tissues also requires surface preparation of the tissues. Further regional quantification of the tissues can provide deeper aspect of the effect of TRF.

5 | CONCLUSION

We observed the protective effect of TRF with daytime feeding, known to restore circadian rhythms, on GM-induced inner ear

damage. The protective effect was significant in the cochlea and present to some extent in the vestibular endorgans. TRF can be a pivotal treatment for protecting cochlea hair cells and, to a lesser extent, vestibular hair cells, from GM-induced damage.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest in this study.

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SUPPORTING INFORMATION

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