ORIGINAL ARTICLE

Correlation of signal to noise ratio (SNR) value on distortion product otoacoustic emission (DPOAE) and expression of nuclear factor erythroid 2-related factor 2 (NRF2) in cochlear organ of Corti in rat exposed to noise

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ABSTRACT

Aim To investigate the changes in the value of the signal to noise ratio (SNR) and to assess changes in the expression of nuclear factor erythroid 2-related factor 2 (NRF2) in the organ of Corti of rat exposed to noise.

Methods This study used a randomized post test only control group laboratory experimental design with 27 male Wistar strain *Rattus norvegicus*. The study group was divided into 3 groups (n = 9): group I (control), group 2 (2 hours of 100 dB noise exposure) and group 3 (2 hours of 110 dB noise exposure).

Results There was no significant difference in the SNR in the group 1 on day 0, 2 and 4 ($p>0.05$). However, there was a significant difference in the SNR in the group 2 and the group 3 on day 0, 2 and 4 ($p<0.05$). There was a significant difference in the mean levels of NRF2 expression in the cochlear organ of *Rattus norvegicus* in all groups ($p<0.05$). There was no correlation between the SNR and the NRF2 expression in group 2 ($p> 0.05$), but there was a correlation between the SNR and the NRF2 expression in the group 3 ($p<0.05$).

Conclusion There was found a correlation between the SNR value on distortion product otoacoustic emission (DPOAE) examination and NRF2 expression in the cochlear organ of Corti of *Rattus norvegicus* exposed to 110 dB noise.

Key words: animal, experimental, immunohistochemistry

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INTRODUCTION

It is estimated that there are 1.3 billion people with hearing loss in the world, and according to WHO, around 10% who are exposed to noise have the potential to get noise-induced hearing loss (NIHL) (1). Based on the multi-centre study, there were several countries that experienced a high prevalence of hearing loss due to noise including Nepal (16.6%), Thailand (13.3%), Sri Lanka (9%), Bangladesh (9%), Myanmar (8%), Maldives (6%), India (6%), and followed by Indonesia (4.6%) (2).

Hearing loss can be caused by strong metabolic activity which is caused by noise stimulation that can cause the hair cells to experience oxidative stress and will cause cell death until NIHL occurs. This will interfere with blood flow to the cochlea which is an important factor for hearing function, vasoconstriction caused by noise can also cause NIHL (3). Excessive noise exposure is associated with damage to sensory cells in the inner ear, especially the outer hair cells. Noise with an intensity of 85 dB or more can cause damage to the hearing receptors, especially the frequency of 3000-6000 Hz and the heaviest of 4000 Hz (4).

Noise-induced hearing loss is one of the most common sensorineural hearing disorders (5).The study shows that the pathogenesis of NIHL is closely related to cochlear ischemia-reperfusion injury, which is caused by decreased blood flow and free radical production due to excessive noise. Nuclear factor erythroid 2-related factor 2 (NRF2) is a transcription activator that plays an important role in defence mechanisms against oxidative stress and also maintaining the reduction of intracellular homeostasis (6). Biological function of NRF2 is to activate transcription factors from cytoprotective gene sequences that are able to counteract the harmful effects of oxidative stress (7).

The NRF2 is a regulatory factor that arises from cellular resistance to oxidants. It also controls basal expression and induction of a ARE-dependent genes series to regulate physiological and pathological outcomes from oxidant exposure (8). The NRF2 is a central mediator of the foremost cellular defence system, the transcription factor of NRF2 protects against oxidative tissue damage through ARE-mediated transcription activation of several phase 2 detoxification enzymes and antioxidant enzymes (9).

Hearing loss due to noise that causes damage to the outer hair cells of the cochlea is irreversible and cannot be operated or treated (10). Since the discovery of otoacoustic emissions (OAE) by David Kemp, there have been many investigations regarding the relationship between NIHL and the damage to hair cells outside the cochlea, where OAE is one of the technologies in the field of audiology used to detect damage to hair cells outside the cochlea due to noise exposure (1,11). Distortion product otoacoustic emission (DPOAE) is a hearing test that is more sensitive than conventional audiometry to detect a NIHL (12).

This study is different from the previous study by Honkura et al. (6). We used normal rat, while Honkura et al. (6) used 2 types of mice (wild mice and $Nrf^{-/-}$ type mice); in our study we were checking the length of time to rest after giving noise after 2 days, while Honkura et al. (6) after 7 days; the difference in an amount of giving noise in the Honkura study was 96 dB, while we used 100 db and 110 dB; while Honkura et al. used auditory brainstem response (ABR), we used DPOAE.

The aim of this study was to investigate a correlation between the signal to noise ratio (SNR) values in the DPOAE examination of NRF2 expression in the cochlear organ of Corti in the rats exposed to noise.

MATERIAL AND METHODS

Material and study design

In this study a randomized post-test only control group laboratory experimental design was used with 27 healthy, adult, male (2-3 months), *Rattus norvegicus* Wistar rats, weighing 150-250 grams. The animals were divided into 3 groups (nine animals in each): group 1 (control), group 2 (2 hours 100 dB noise exposure) and group 3 (2 hours 110 dB noise exposure).

The maintenance of experimental animals was carried out at the Animal House Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, the manufacture of tissue paraffin blocks was carried out at the Anatomical Pathology Laboratory of Medical Faculty, Universitas Sumatera Utara, and cutting of tissue blocks, hematoxylin eosin staining, immunohistochemical examination techniques carried out in the Biochemistry and Molecular

Biology, Faculty of Medicine, Brawijaya University, Indonesia.

The study has received ethical approval from the Ethics Commission of the Faculty of Medicine, Universitas Sumatera Utara.

Methods

Noise exposure was given to *Rattus norvegicus* rats in a voice box measuring 64.5 x 45 x 40 cm and made of foam-coated cork. The speaker was placed on the roof of the box cover, then a hole was made at the bottom of the box to measure the intensity of the noise where a tool for measuring noise intensity was called sound level meter; this measurement was carried out at eight points where the noise difference did not exceed 1 dB. The sound source was provided by a Compact Disc (CD) which contained a sound recording, CD player, and amplifier that produces noise with a frequency of 1-10 kHz; intensities of 100 dB and 110 dB given for 2 hours and 2 days. DPOAE (Elios Elito Otodia brand, Echodia Ltd, London, UK) examination was carried out on all experimental animals with anaesthetic with ketamine 50 mg/kg body weight (13). The probe was adjusted in size and placed in the ear canal. DPOAE was assessed 3 times: the initial assessment before the treatment, 2 days after noise exposure, and then 2 days after the noise rest was over.

The examination of NFR2 expression was carried out by exposing the rats first with ether inhalation, then performing necropsy of the rat's temporal bone tissue. Tissue samples were taken, fixed with 10% formalin buffer solution and decalcified with EDTA for 4 weeks. Furthermore, laboratory examinations were carried out through tissue fixation by making tissue paraffin blocks and sliced into 4 μm thick sections, then placed in a slide to be stained with haematoxylin-eosin and immunohistochemical staining, namely NRF2 with primary antibody NRF2 H-300: sc-13032 (Santa Cruz, United States). To assess the NRF2 expression, an Olympus XC 10 microscope was used using 40x magnification.

The expression assessment was carried out by assessing the distribution and intensity of the immunohistochemical streaks. The distribution assessment was carried out by counting the percentage of cells stained with brown in the entire microscope field of view using 40x magnification. Where to assess the NRF2 expression, the IRS (Immunoreactive Score) formula was used by multiplying the percentage of cells stained brown with the intensity of the streaks. The distribution of cells dyed brown was divided into 4 categories: 0 = no cells dyed brown, 1 = <10%, 2 = 11-50%, 3 = 51-80 % and ≥4 = 81% of cells stained brown; then the intensity assessment was carried out by category; 0 - no colour, 1 - weak intensity, 2 - moderate intensity, and 3 - strong intensity. The calculation results will show a minimum score of 0 and a maximum score of 12 (14). After all the results had been calculated, a statistical analysis was carried out.

Statistical analysis

Data were analysed using the One Way Anova test, the Post-Hoc Bonferoni test and Pearson correlation test was used to analyse the differences/correlation in each group,. The statistical test was considered significant if $p<0.05$.

RESULTS

The difference in NRF2 expression was found: decreased in the group 3 compared both to the groups 1 and 2 (Figure 1).

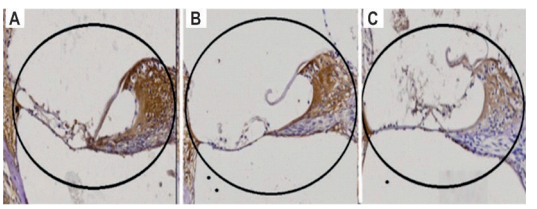

Figure 1. An overview of NRF2 expression (at 40x magnification). The circle shows the depiction of NRF2 expression in the cochlear organ of corti of *Rattus norvegicus*, which is marked in brown. A) group 1 (control); B) group 2 (2 hours of 100 dB noise exposure); C) group 3 (2 hours of 110 dB noise exposure)

There was no significant difference in the SNR value of the DPOAE examination on day 0, 2 and 4 ($p>0.05$). However, in the group 2 and 3 there was a significant difference in the SNR value from the DPOAE examination on days 0, 2 and 4 ($p<0.05$). There was a significant difference in the mean of NRF2 expression in the cochlear organ of corti of *Rattus norvegicus* in all groups ($p<0.05$) (Table 1).

In the group 2 (110 dB) there was no correlation between the SNR value on the DPOAE examination and the NRF2 expression ($p>0.05$), with a positive correlation direction ($r=0.040$). In the group 3 (110 dB) there was a correlation between the SNR value on the DPOAE examination and the NRF2 expression ($p<0.05$), with a strong positive correlation direction ($r=0.792$) (Table 2).

Table 1. Differences in the mean of signal to noise ratio (SNR) value on distortion product otoacoustic emission (DPOAE) examination on the day 0, 2 and 4, and differences in nuclear factor erythroid 2-related factor 2 (NRF2) expression in *Rattus norvegicus* cochlear organ of corti

SNR	Mean±SD on the day			p
	Day 0	Day 2	Day 4	
Group 1	11.36±2.50	10.62±2.43	9.44±2.14	0.245
Group 2	10.63±2.05	4.23±0.96	4.26±1.38	<0.001
Group 3	9.73±3.09	4.11±1.15	4.33±1.21	<0.001
NRF		**Mean±SD**	**Min-Max**	
Group 1		8.33±3.08	3.00-12.00	
Group 2		3.67±1.12	2.00-6.00	<0.001
Group 3		4.00±2.00	1.00-8.00	

Table 2. Correlation of signal to noise ratio (SNR) value on day 4 on distortion product otoacoustic emission (DPOAE) examination and nuclear factor erythroid 2-related factor 2 (NRF2) expression at 100 dB & 110 dB noise

Group	Variable	Mean±SD	r	p
2 (100 dB)			0.040	0.918
	SNR	3.66±1.11		
	NRF2	4.27±1.38		
3 (110 dB)			0.792	0.011
	SNR	4.00±2.00		
	NRF2	4.27±1.38		

r, Pearson correlation;

DISCUSSION

The susceptibility of developing NIHL varies greatly, there are some individuals who can tolerate high noise levels for a long time, but there are some individuals in the same environment with rapid hearing loss, which is related to the duration and intensity of exposure and genetic susceptibility to noise trauma (15). Grondlin et al. also found that individual susceptibility to NIHL depends on two factors, namely environmental factors that can increase physiological stress, inflammation and oxidative stress as well as genetic factors (16).

Honkura et al. compared wild-type and NRF2-/- mice were exposed to 96dB noise for 2 hours continuously, and then ABR were observed 1 day before, 4 hours and 7 days. On the second day after noise exposure, the ABR threshold before noise exposure was almost comparable between the two groups of mice; this indicates that NRF2 does not play a role in the development of cochlear function (6). The ABR in 4 hours after noise did not show any difference between the two groups of mice, but after 7 days of rest from noise exposure, there was a permanent threshold change which was significantly greater in NRF2-/-β; type ratio was NRF2-/- in wild types which were more susceptible to noise, and will impair the recovery ability of threshold shift (TTS) in the absence of NRF2 (6). Our research showed no significant difference in the SNR before rats exposed to noise, in group 1 on day 0, 2 and 4, suggesting that NRF2 does not play a role in the development of cochlear function, similarly with Honkura's study (6). After giving 100 dB and 110 dB noise for 2 hours and 2 days our results showed that the SNR value decreased (TTS occurred) and after 2 days of rest there was an improvement in the SNR value but did not return to the initial value before the treatment, which indicated that PTS had occurred.

We found that the correlation between SNR values on DPOAE examination and NRF2 expression in the cochlear organ of corti of *Rattus norvegicus* exposed to 110 dB noise on day 4 could be due to an increase in the reactive oxygen species (ROS). Kim et al. showed that increased noise exposure can be harmful, which triggers the formation of molecules, such as ROS, and induction of inflammatory genes in the ear, which results in damage or death of hair cells. These cells are very delicate and sensitive and do not regenerate once they are damaged or lost. In addition, there are no known therapeutic treatments to restore damaged hearing (3). In our study there was a decrease in SNR value due to noisy administration, which can also damage the hair cells in the cochlea.

Under normal circumstances NRF2 binds to Kelch-like ECH-associated protein-1 (Keap1) in the cytoplasm where this prevents NRF2 from translocating to the nucleus and binding to the antioxidant response element (ARE). If there is noise exposure that exceeds the threshold, the bond between NRF2 and Keap1 will be released and NRF2 translocate to the nucleus and binds to the ARE located in the gene promoter region that encodes cytoprotective proteins, which are antioxidant enzymes (6,17). The phosphorylation process is an important factor in the detachment of the NRF2 bond with Keap-1 and translocating to the nucleus (6,17).

We conducted this study because there was no previous study on the correlation of SNR values in DPOAE examination with NRF2 expression in noise-exposed rat's cochlea. Urono and Motohashi in 2011 stated that NRF2 is very important for protection from oxidative stress and xenobiotic detoxification (18), so that cell damage on Cortiary organs caused by oxidative stress due to noise exposure can be repaired and protected through NRF2 by triggering the formation of endogenous antioxidant enzymes to prevent cell damage which led to NIHL.

In conclusion, our study showed a correlation between the SNR value on DPOAE examination and NRF2 expression in the cochlear organ of *Rattus norvegicus* exposed to 110 dB noise. The research we conducted was based on the lack of research on the correlation of SNR values on DPOAE examination with NRF2 expression in noise-exposed rat cochlea, so it is hoped that this study can provide new knowledge and future benefits for NIHL patients where with endogenous antioxidants that are still unable to help repair, it is expected that exogenous antioxidants are given.

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TRANSPARENCY DECLARATION

Conflicts of interest: None to declare.

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