ORIGINAL ARTICLE

Effect of Spirulina on Cochlea Histopathological Changes in Wistar Rats Induced by Kanamicin

Prima Erlangga Harinto, Dian Ayu Ruspita, Dwi Marliyawati, Pujo Widodo, Zulfikar Naftali

Ear Nose Throat Head and Neck Surgery Unit, Department of ENT-HNS, Faculty of Medicine, Diponegoro University, 50275, Semarang, Central Java, Indonesia

ABSTRACT

Introduction: Streptomycin and kanamycin are aminoglycosides that are toxic to the cochlea vestibular system, can causing hearing loss. This antibiotic is used for the treatment of tuberculosis and its ototoxicity occurs in 20% of tuberculosis patients. Spirulina is a cyanobacterial species that is used as a dietary supplement and contains phycocyanin compounds that function as antioxidants and anti-inflammatory. The aim of this study was to determine the effect of spirulina on histopathological changes in the cochlea in Wistar rats after kanamycin induction. **Methods:** this study is a form of posttest-only controlled group design research with a sample of 24 wistar rats divided into 4 groups, namely negative control group, positive control group, treatment group 1 and treatment group 2. Observations of the study took place in November-December 2021. Histopathological measurements in hair cells, macrophages and cochlear vasculature. The analysis used non-parametric Kruskal-Wallis and post-hoc Mann-Whitney tests. **Results:** There were more hair cell damage, macrophage cell count, and significant vascular dilatation in the kanamycin group than in the without kanamycin group with the value p=0.001. There was significantly less number of hair cell damage in the kanamycin group with spirulina at a dose of 1000 mg than in the kanamycin group with spirulina at a dose of 400 mg p=0.045. **Conclusion:** There was a significant effect on the administration of spirulina on histopathological changes in the cochlea of rats.
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Corresponding Author:
Prima Erlangga Harinto, MD
Email: primaerlangga.thtkl@gmail.com
Tel: +6281322666440

INTRODUCTION

Hearing loss requiring rehabilitation affects more than 5% of the world's population, of about 430 million adults and 34 million children. By 2050 it is estimated that more than 700 million people or 1 in every 10 people, will experience hearing loss (1). Streptomycin and kanamycin are aminoglycosides that are toxic to the cochlea and vestibule (2). The mechanism of ototoxic drugs is known to induce cell apoptosis and inflammation in the cochlea either directly or through the formation of reactive oxygen species (ROS) (3). Disruption of mitochondrial protein synthesis, formation of free oxygen radicals, activation of c-Jun N-terminal kinase (JNK), and activation of caspases and nucleases (4).

Spirulina (SP) is a fibrous cyanobacteria species that has been used as a dietary supplement and has antioxidant effects derived from phycocyanins, carotenoids, flavonoids, polyphenols, -lipoic acid, glutathione and others (5), also functions to activate cellular antioxidant enzymes, inhibit lipid peroxidation, DNA damage, free radicals, increase the activity of superoxide dismutase and catalase (6,7). Rats are widely used for research because they have similar anatomical characteristics to humans, good adaptability to the environment and the treatment given during the study. Besides that, it is easy to handle and maintain because the body is small, healthy and clean (8).

The mechanisms of inflammation in the cochlea can be caused by 3 main mechanism, which are noise exposure, ischemia and drugs. For example, some ototoxic drugs can induce cell apoptosis and inflammation in the cochlea either directly or through the formation of reactive oxygen species (ROS). Trauma caused by noise also induces an inflammatory reaction in the inner ear (9).

Aminoglycosides are a class of conventional antimicrobials and are fermentation products or semisynthetics derived from Streptomyces or Micromonospora species, for example, such as kanamycin, gentamycin, tobramycin, and streptomycin.

They work by inhibiting protein synthesis by binding to the 30S ribosomal subunit (2). Some aminoglycoside antibiotics are reported to be more cochleotoxic, while others are more vestibulotoxic. Gentamicin, for example, at any level in serum can cause vestibulotoxicity. In a prospective study of patients receiving variable doses of aminoglycosides, vestibular dysfunction was reported more often than hearing dysfunction (10). Research on rats at a dose of 800 mg/kg bw subcutaneously (SC) given twice a day for 15 days gave the effect of histopathological changes on the cochlea (11,12). Administration by injection will cause the process of inhibition of auditory neuron cells in a period of 1-2 hours; this is thought to be caused by ototoxic drugs, which work by blocking cholinergic activity at efferent synapses in the cochlea that are submerged in perilymph fluid. Experiments show that aminoglycosides are rapidly absorbed through the mechano-electrical transduction channel into the cochlea. Aminoglycosides circulating in the strial capillaries (C) are preferentially transported across the strial blood-labyrinth barrier consisting of endothelial cells attached by tight junctions, into the intra-strial space. From there, the aminoglycosides pass through the marginal cells into the endolymph, and cross the apical surface via endocytosis and non-selective cation channel permeation mechanisms. This strong electrophoretic force most likely drives cations, including aminoglycosides. Aminoglycosides are absorbed by most other cochlear cells, including lateral wall fibrocytes, spiral ganglion neurons, and supporting cells in the organ of Corti. Aminoglycosides can persist in hair cells in the cochlea for a fairly long time span of about 6 months (1).

Spirulina is a bluish-green autotrophic microorganism with columnar cells forming spiral-like twisted filaments (helix), so they are called filamentous blue-green algae (cyanobacterium) (13). The nutritional composition of SP has a very high value which contains, among others, protein, essential amino acids, minerals, essential fatty acids, vitamins, and fat-soluble antioxidants (vitamin E and carotenoids) (13,14). The main advantage is the antioxidant effect of SP is that of phycocyanin. SP contains phycocyanin about 20% of its dry weight, Phycocyanin is a protein complex that is able to increase immunity, has anticancer, anti-inflammatory and antioxidant properties (12,14–16). and also block free radicals (5). The reported side effects in humans are insomnia and gastric problems, the cause of which is still uncertain (14) in people who consume 1g of Spirulina per day side effects include facial flushing, headache, stomach pain, sweating, and muscle aches (17), whereas in mice there were no negative side effects other than weight gain (18) and several studies are still assessing the effects of spirulina on the liver, kidneys and testes of rats (17). Antioxidants are compounds that donate electrons or electron donors that are able to inactivate the development of an oxidation reaction, by preventing the formation of ROS (5). Currently, antioxidants that are widely used in foodstuffs are generally synthetic antioxidants such as Propyl Galat (PG), Tertiary Butylhydroquinone (TBHQ), Butylated Hydroxy Toluene (BHT), and Butylated Hydroxy Anisole (BHA) (13). In an in vivo study, antioxidant levels were seen to increase in plasma and liver of experimental animals receiving a daily dose of 5 mg SP for 2-7 weeks (19). The effects of decreased blood pressure, triglycerides and increased antioxidant levels occurred after administration of 500 mg/kg body weight (bw) for 21 days in Wistar rats (20).

Giving SP is expected to have a role as an autoprotector that will inhibit the occurrence of irreversible damage to the cochlea due to the inflammatory process. There are no studies that observe the histopathological changes of the cochlea after administration of SP with graded ones, so the researchers will observe the effect of SP with graded doses on the histopathological improvement of the cochlea of wistar rats induced by kanamycin.

MATERIALS AND METHODS

Materials

The dose of spirulina used at a dose of 400 mg and the kanamycin group with spirulina at a dose of 1000 mg and the dose of kanamycin used in this study was kanamycin with a dose of 800 mg/kg bw, following previous journals that have been shown to have an ototoxic effect in experimental animals (20,21). The technique of administering kanamycin in this study was by subcutaneous injection in mice.

Animals and treatment

The study was conducted on 24 wistar rats aged 8 weeks with a weight of 150-200 grams. Before the treatment started, they were acclimatized for 7 days, given food and drink. Then randomized and divided into 4 groups, numbers of rats per group NC, PC, T1 (n=6) and T2 has n=6, found 2 dead rats in this group; the Negative Control group (NC), namely the group of rats that were not given anything; the Positive Control group (PC), namely the group of rats that were given kanamycin 800 mg/ kg body weight. Kg body weight for 2 weeks on days 15-30, Treatment Group 1 (T1) is a group of rats that were given SP at a dose of 400 mg/kg body weight for 30 days followed by the addition of kanamycin 800 mg/kg body weight for 2 weeks on days 15-30, the last is Treatment group 2 (T2) was a group of rats that were given SP at a dose of 1000 mg / kg for 30 days followed by the addition of kanamycin 800 mg / kg for 2 weeks on days 15-30. This research was conducted for 30 days. This animal research has received ethical approval no 98/EC/H/FK-UNDIP/VIII/2021.

Histopathology

On the 31st day, termination was carried out so that the cochlear organ could be taken for making slides and reading the results of the preparations. Each group consisted of 6 wistar rats, and from each wistar rat 5

preparations were made. The preparations were viewed under a light microscope at 100x magnification to determine the location of the cochlea and continued to describe histopathological changes with 400x magnification on each cochlear tissue preparation. The data observed in terms of the suitability of the 3 parameters of the histopathological changes of the cochlea include: the number of hair cells, the number of macrophages and vascular dilatation.

Statistical analysis

Histopathological score data were analyzed using comparative hypothesis testing. The data was checked for normality tests. If the data is normally distributed, the data is homogenized and followed by the One-Way Anova parametric test with post-hoc LSD. However, if the data are not normally distributed, hypothesis testing is carried out using the Kruskal-Wallis nonparametric test with post-hoc Mann-Whitney. P value <0.05 is considered significant.

RESULTS

At the end of the study, 22 wistar rats were terminated on the 31st day; using chloroform, then the cochlear organ was taken after termination, all samples were examined to make preparations to see the histopathological changes of the cochlea. Histopathological cell changes in this study were assessed as hair cells, macrophage cells, and the incidence of vascular dilatation as a result of the inflammatory process of cells (Figure 1).

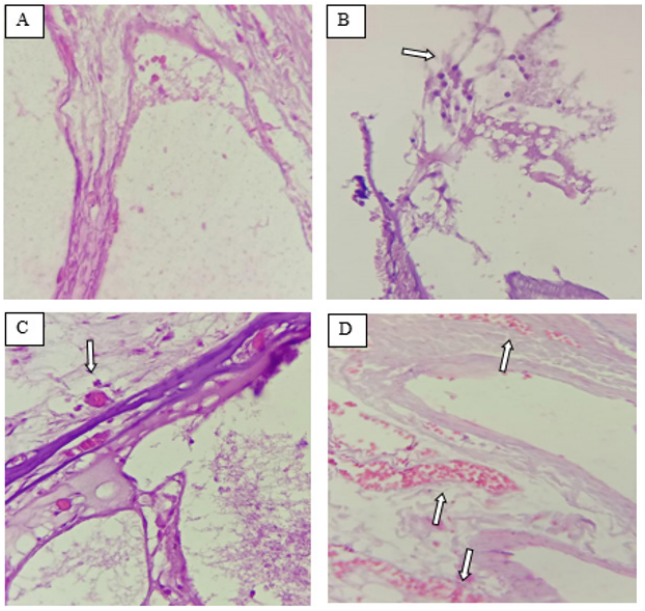

Figure 1: A normal histopathological picture 100x magnification B. hair cell damage (white arrow) 400x magnification, C. Macrophage cells (white arrow) 400x magnification, D. vascular dilatation (white arrow) 400x magnification.

Histopathological analysis of hair cells

Table I shows the characteristics of cochlear hair cell damage. The most damage is found in PC Group and in T1 Group with less number of cell damage.

In table II there is a significant difference in the amount of hair cell damage between the PC and NC groups (p=0.001). There is a significant difference in the amount of hair cell damage between the NC and T1 groups (p=0.025). There was a significant difference in the amount of hair cell damage in PC and T2 groups (p=0.001). There was a significant difference in the amount of hair cell damage between Group T1 and T2 (p=0.045).

Table I: Histopathological characteristics of the number of damaged hair cells

Group	N	Mean	Standard deviation
NC	6	0.00	0.00
PC	6	8.28	0.20
T1	6	3.60	0.41
T2	4	0.00	0.00

Table II: Histopathological analysis of the amount of hair cell damage

Sampel	p Value			
	NC	PC	T1	T2
NC	-	-	0.010*	0.362
PC	0.001*	-	0.103	0.004*
T1	-	-	-	0.162
T2	-	-	-	-

Mann Whitney; *Significant if p< 0,05; CI 95%

Histopathological analysis of macrophage cells

Table III shows the characteristics of the number of macrophage cells. Most found in Group PC and in Groups T1 and T2 with reduce of macrophage cells.

In table IV there is a significant difference in the number of macrophage cells between the NC and PC groups (p = 0.001). There was a significant difference in the number of macrophages between Group NC and T1 (p=0.010). There was a significant difference in macrophage cells between PC and T2 groups (p=0.004).

Histopathological analysis of vascular dilatation

Table V shows the characteristics of the amount of vascular dilatation. The highest number was found in Group PC and in Groups T1 and T2 with less number of vascular dilatation.

In table VI there is a significant difference in the number of vascular dilatations between the NC and PC groups (p = 0.001), there is a significant difference in vascular dilatation between the NC and T1 groups (p = 0.007) and there is a significant difference in vascular dilatation between the PC and T2 groups (p = 0.008).

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Table III: Histopathological characteristics of macrophage cell count

Group	N	Mean	Standard deviation
KN	6	0.00	0.00
PC	6	4.03	0.15
T1	6	1.67	0.20
T2	4	0.12	0.09

Table IV: Histopathological analysis of macrophage cell count

Sampel	p Value			
	NC	PC	T1	T2
NC	-	-	0.025*	0.999
PC	0.001*	-	0.093	0.001*
T1	-	-	-	0.045*
T2	-	-	-	-

Mann Whitney; *Significant if p< 0,05; CI 95%

Table V: Histopathological characteristics total vascular dilatation

Group	N	Mean	Standard deviation
NC	6	0.00	0.00
PC	6	3.70	0.21
T1	6	1.37	0.08
T2	4	0.25	0.10

Table VI: Histopathological analysis of the amount of vascular dilatation

Sampel	p Value			
	NC	PC	T1	T2
NC	-	-	0.007*	0.225
PC	0.001*	-	0.103	0.008*
T1	-	-	-	0.225
T2	-	-	-	-

Mann Whitney; *Significant if p< 0,05; CI 95%

DISCUSSION

Effect of Kanamycin on Cochlear Histopathological Changes

Aminoglycosides are one of the most effective antibiotics used to treat infections caused by Pseudomonas, Salmonella and Enterobacteria species (12). The aminoglycoside group has a side effect that is ototoxic, through damage to cochlear hair cells. Antibiotics in this group include kanamycin, gentamicin, tobramycin, and streptomycin (10,20).

Kanamycin works in the body, causing the emergence of ROS. The effects of ROS directly contribute to vascular dysfunction and remodeling through oxidative damage by reducing bioavailability, interfering with endothelial-dependent vasodilation and endothelial cell growth, causing cell apoptosis or anoikis, stimulating cell migration. endothelium, and activate adhesion molecules and inflammatory reactions (22). Dilation of blood vessels will cause more inflammatory cell migration and activate cell apoptotic pathways (23).

In the positive control group, there were significant differences in hair cell damage, macrophage cell count, and vascular dilatation compared to the negative control group. Kanamycin exposure within a month in this study has made changes in the histopathological assessment of the cochlea. It is proven that kanamycin has caused damage at the tissue and cellular level. Hair cell damage was found to be the most common. This is in accordance with the theory that kanamycin causes damage to the mitochondrial level so that it interferes with the metabolism of hair cells and causes loss of basal hair cells (24).

In a previous study carried out histopathological examination of hair cells in adult CBA, C57BL and BALB mice and Sprague-Dawley mice receiving kanamycin and the results showed that the outer hair cells were completely lost and the damage to the supporting cells extended to the space in the basal region, whereas in the apex morphological damage was not very assessable after administration of kanamycin at a dose of 700 mg/kg/day given twice a day for 15 days (25).

Effect of Spirulina on histopathological assessment of the rat cochlea

Hair Cell

The most damage to hair cells was found in the group given kanamycin. In the group T1, there were fewer number of damaged hair cells than the group with kanamycin, but not statistically significant. In the group T2, there were significantly less number of damaged hair cells than the group with kanamycin.

Hair cell damage between the treatment groups T1 and T2 was found to be significantly different. Assessment of the histopathological condition of hair cells in the SP group T2 was the same as in the negative group, and it was statistically proven that there was no significant difference. This shows that dose SP 1000 mg/kg bw is more effective as protection against the ototoxic effects of kanamycin.

Macrophages

The highest number of macrophage cells was found in the kanamycin-induced group of mice. In the treatment group T1, there were fewer macrophage cells than the kanamycin group, but not statistically significant. In the treatment group T2 there were significantly fewer macrophage cells than the kanamycin group. There was no significant difference in the number of macrophages between the treatment groups at T1 and T2. The histopathological assessment of macrophage cells in the SP group at T2 was the same as in the untreated group, and it was statistically proven that there was no significant difference. This shows that SP plays a role in

the work of macrophage cells.

Macrophages found in the cochlea play a role in the inflammatory process due to the oxidation reaction of kanamycin (26). Macrophage cells work to react to various cytokine or chemokine signals to eliminate pathogens and apoptotic cells (27). The mechanism of action of SP on macrophages in previous studies was found increase the activity of macrophages through phagocytic activity and nitrite production (28). Macrophages work for phagocyt of damage cochlear hair cells so the more damage hair cells caused by kanamycin, the more macrophages in the cochlea will also increase.

Vascular Dilation

The highest amount of vascular dilatation was found in the kanamycin-induced rat group. In the treatment group T1 there was less vascular dilatation than the group with kanamycin, but not statistically significant. In the SP group, at a T2, there was significantly less vascular dilatation.

There was no significant difference in the amount of vascular dilatation between the treatment groups at T1 and T2. The histopathological assessment of vascular dilatation in the SP group at a T2 was the same as in the untreated group, and it was statistically proven that there was no significant difference. This shows that the SP dose of 1000 mg plays a role in less inflammatory processes with changes in vascular dilatation.

ROS such as superoxide (O^{2-}) and NO are produced due to an acute inflammatory process. ROS induces cell membrane damage. NO itself has an important role in regulating vasodilation and protecting neuronal networks. When there is physical stress in the form of acute inflammation, NO is produced in excess due to the activity of the enzyme NO synthase (iNOS). Superoxide is produced by reperfusion after temporary arterial occlusion. Superoxide reacts with NO to form nitrite (NO^{2-}) and peroxynitrite (NO^{3-}) (28,29).

In this study, the use of SP showed a protective function in the cochlea induced by kanamycin, which had been tested on experimental animal wistar rats. Significant changes were seen between the positive control group, the SP treatment group at T1 and T2; namely hair cell damage, changes in the number of macrophages and vascular dilatation at different levels. These results can be concluded if SP has the effect of inhibiting the occurrence of hair cell damage, more macrophage cell numbers, and vascular dilatation due to the induction of kanamycin which causes ototoxicity.

The dose in the 1000 mg treatment group gave better results than the 400 mg treatment group. Thus, giving SP at a dose of 1000 mg/kg bw/day was proven to give significantly better results in terms of changes and damage to hair cells than SP at a dose of 400 mg/kg bw/day. Although the changes in macrophage cells and vascular dilatation did not show statistically different results, there was a change in the number compared to SP at a dose of 400 mg/kg bw/day. The antioxidant effect of SP can inhibit the formation of free radicals from toxic substances produced by kanamycin (5,12,14,15,30).

In this study, observations were made on the toxic effects of SP in the form of diarrhea and loose stools. During the observation, there was no change in the consistency of the rat droppings and the vomiting reaction during feeding. During the study, it was found that 2 rats died in the treatment group with a dose of 1000 mg SP. This is in accordance with the literature which says that the mortality rate of rats treated with kanamycin is 20%-25% (24).

The limitation of this study is that no tests such as immunohistochemical tests were carried out, to prove the presence of an inflammatory reaction and in this study, the rats were not weighed at the end of the study to assess whether the effect of rat death was caused by an overdose of kanamycin, and there was no examination of the antioxidant content of spirulina.

CONCLUSION

Spirulina has been shown to be effective in reducing inflammation in the cochlea given kanamycin as an ototoxic substance in wistar rats. Further research is needed using immunohistochemical tests, to prove the presence of an inflammatory reaction and checking the levels of antioxidants contained in spirulina. Further research is needed on human subjects to determine and assess the clinical response of the protective effect of spirulina against ototoxicity due to drug preparations.

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