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RESEARCH ARTICLE

Mechanism of Apoptosis Retinal Ganglion Cells *Rattus norvegicus* Caused by Ethambutol

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ABSTRACT:

Background: The cause of cell death is thought to be due to the pathological apoptotic process in Retinal Ganglion Cells (RGCs), but how the exact mechanism of what is most influential is still not explained. **Objective:** This study aimed to explain the mechanism of RGCs apoptosis *Rattus Norvegicus* which is thought to underlie the occurrence of ethambutol toxic optic neuropathy. **Methods:** A total of 42 male, adult *Rattus norvegicus Sprague-Dawley* strains were divided into 6 groups with 3 control groups and 3 treatment groups in a randomized design with time series test. The treatment groups were given ethambutol 15 mg/kg/day for each group within 5, 10 and 15 days orally using a gauge.Expressions of SOD2, MDA, PKC δ, p53, Cyt c, Caspase 3 and apoptosis were examined by immunohistochemical methods. **Results:** Ethambutol affected significant decreased expression of SOD2 with p=0.002 in 5 days, p=0.013 in 10 days and p=0.018 in 15 days; significant increased MDA in 5 days with p=0.05, 10 days with p=0.017, 15 days with p=0.002; significant increased p53 in 5 days with p=0.012, 10 days with p=0.002, 15 days with p=0.001; significant increased Cyt c in 5 days with p=0.004, 10 days with p=0.001, 15 days with p=0.001; significant increased Caspase 3 in 5 days with p=0.001, 10 days with p=0.003, 15 days with p=0.001 and apoptosis in 5 days with p=0.001, 10 days with p=0.001, 15 days with p=0.001. **Conclusion:** The mechanism of apoptosis of *RGCs* caused by ethambutol was showed via decreased expression SOD2, increased expression of MDA, p 53, Cyt c, Caspase 3 andapoptosis. These biomarkers are essential to detect apoptosis as one of mechanism in cell death.

KEYWORDS: Apoptosis, ethambutol, toxic, *Rattus*, mechanism, SOD2.

1. INTRODUCTION:

Ethambutol is routinely used as an anti-microbacterial drug, especially in the treatment of tuberculosis. Ethambutol can cause even irreversible loss of vision, known as Ethambutol-induced optic neuropathy (EON), in small but significant amounts[1]. Depending on the dose and duration of Ethambutol administration, the incidence of EON has been reported to be in the range of 1-5%. The incidence of tuberculosis globally in 2018,

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there were around 140 cases per 100,000 population. The five countries standing out as having the largest number of incident cases in 2018 are (in descending order) India, Indonesia, China, the Philippines, and Pakistan which together account for 56% of the total global TB sufferers worldwide, and Ethambutol is still a line drug first thing that still needs to be used [2].

A study at the Tambakrejo Hospital in Surabaya, Indonesia on 40 Tuberculosis's patients found that there was visual field defect after one month of administration and increased in number after two months of ethambutol administration Likewise,other study at Surabaya pulmonary hospital also found a visual evoked potential (VEP) disorder and an impairment picture of OCT as a marker of toxic optical neuropathy due to the use of ethambutol[3].

Another study shows a sharp decrease in vision, contrast sensitivity, and Relative Afferent Pupillary Defect(RAPD) as a marker of toxic optic neuropathy due to ethambutol in drug use under two months by 0.9% and 100% in use over two months in poly DOTS Wahidin Sudirohusodo Hospital, Indonesia and Makassar Center for Lung Health, Indonesia[4]. A study conducted in 30 tuberculosis patients using ethambutol at the Sanglah General Hospital, Denpasar,Indonesia showed sharp visual disturbances and red-green dyschromatopsia in 7 people and optic nerve lesions in 5 people. Visual impairment, color discromatopsia, and VEP disorders were found in the use of ethambutol therapy for fivemonths[5].

Ethambutol often causes quite severe side effects such as liver disorders, toxic optic neuropathy, visual field disorders and has been used to treat TB widely[6]. In toxic optic neuropathy, visual disturbances can be restored after discontinuation of ethambutol use. Some patients still experience severe or even permanent visual loss, even though using standard doses of ethambutol[7,8].

Toxic ethambutol in retinal *ganglion cells (RGCs) work* via an excitotoxicity pathway. This study shows the presence of glutamate neurotoxicity characterized by N-methyl-D-aspartate (NMDA) overstimulation which causes excessive intracellular Ca levels. Increased mitochondrial Ca and decreased cytosolic Ca which is a marker of the flow of Ca from the cytosol to the mitochondria-mediated by a disturbance in the permeability of the mitochondrial membrane result in an increase in mitochondrial membrane potential. Retinal ganglion cells become more sensitive to normal extracellular glutamate levels, which can result in decreased ATPase activity and mitochondrial energy homeostasis [9].

Previous studies have shown that ethambutol induces cytosolic vacuole formation and reduces phagocytic activity in human-derived cells and rat retinal cells[10]. Autophagy is one of the key mechanisms of homeostasis and stress adaptation in cells in which this process facilitates lysosomes to degrade unnecessary proteins or organelles by forming autophagosome-lysosome fusion and activating the hydrolytic enzyme lysosome. Ethambutol induces neutralization of lysosomes, causing lysosomal swelling and Zn buildup in lysosomes thereby inhibiting fusion between auto phagosomes and lysosomes [6].

Ethambutol has chelating agent properties, the ethambutol metabolite, which is ethylenediimino-dibutyric acid, is binding Cuprum (Cu) and Zinc (Zn). Cu and Zn are needed as cytochrome c oxidase cofactors that play a role in the oxidative phosphorylation process in the mitochondria to form energy in the form of ATP for cellular activities. Disruption of ATP production will damage the axonal transportation system[11], [12]. The cytotoxicity caused by reduced EMB and phagocytosis in retinal cells is thought to be mediated through the protein kinase C (PKC) signaling pathway [10]. PKC is a serine/threonine kinase family involved in a variety of neuronal development, such as proliferation, differentiation, survival, and apoptosis [13].PKC was first indicated as a substrate of Caspase-3, and the proteolytic activity of PKC has been directly linked to apoptosis[14].

Damage to the retinal nerve fibers occurs, especially in RGCs in the use of ethambutol. Damage to even the occurrence of death in retinal ganglion cells is believed to exist. The cause of cell death is thought to be due to the pathological apoptotic process in RGCs, but how the exact mechanism of what is most influential is still not explained. This research was conducted to find the model of retinal ganglion cell death through apoptosis, which is the most influential cause of RGCs death based on the standard dose and duration of administration of ethambutol.

2. MATERIALS AND METHODS:

This study was a true experiment in animal model with a randomized control group design with time-series test. A total of 42 male, adult *Rattus norvegicus Sprague-Dawley* strains were divided into 6 groups with 3 control groups and 3 treatment groups. The treatment groups were given ethambutol at a dose of 15 mg/kg/day for each group within 5, 10 and 15 days orally using gauge. The rats in the control and treatment groups were then sacrificed using cervical dislocation method under rat cocktail anesthesia, and then the eyes were enucleated for the sake of tissue examination. Expressions of SOD2, MDA, PKC δ, p53, Cyt c, Caspase 3, and apoptosis were examined by immunohistochemical

methods and counted using H- score method. This was observed at five field views with 400x magnification confirmed previously at 1000x magnification. In term of reliability on the evaluation of the same observation object, researchers used the same researchers or laboratories and to maintain the validity of the assessment of variables selected tools and test materials with high sensitivity and specificity. This is consistent and can be accounted for.

The collected data were analyzed statistically with the R program.Bivariate analysis was tested with Kruskal Wallis test and then with Mann-Whitney test or t2-free sample test. Multivariate analysis was tested with gradual correlation using Spearman test.

3. RESULTS:

The results of this study were obtained based on histopathological examination with immunohistochemical methods, obtaining in a description of the expression of each variable representing each treatment group, i.e., protein expression in RGCs, including SOD2, MDA, p53, PKCδ, Cyt c, Caspase 3 and apoptosis. The expression of apoptosis using tunel test is shown in Figure 1.RGCs protein expression in the treatment group was compared to the control group, and then a statistical analysis was performed to determine the differences in each group.

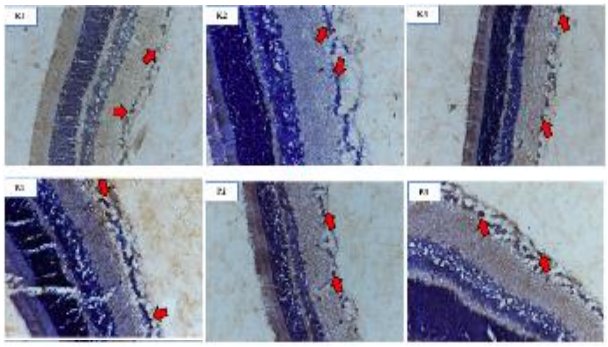

Figure 1.The retinal eyes of various groups (K.1 = control 5 days, K.2 = control 10 days, K.3 = control 15 days, P.1 = treatment 5 days, P.2 = treatment 10 days and P.3 = treatment 15 days). The red arrows show the expression of apoptosis in retinal ganglion cells which are marked by the presence of brown chromogen (arrow). IHC. 400x

Normality test was performed on all variable data with the Shaphiro-Wilk test. The results showed that all data were not normally distributed, with p <0.05. The next test examined differences between treatment groups, and further tests were performed to determine the most different groups, carried out with the Kruskal-Wallis test, which was then continued with the Mann-Whitney test for normally distributed data or t2 free sample test for abnormally distributed data, shown in Table1. There were significant differences between control and treatment group in all variables, except PKCδ at 5 days with p=0.881,10 days with p=0.160, and 15 days with p=0.100.

Table 1. Bivariate analysis table for each variable (* t2 free sample test, ** Mann Whitney test)

	Kruskal–Wallis Test - p	5 days p	10 days p	15 days p
SOD_2	0.002	0.011**	0.017**	0.003*
MDA	0.001	0.037*	0.020*	0.001**
PKCδ	0.06	0.881*	0.160*	0.100*
p53	0.001	0.012*	0.002**	0.001**
Cyt c	0.001	0.004*	0.001**	0.001**
Caspase 3	0.01	0.001*	0.003**	0.001**
Apoptosis	0.001	0.001**	0.001*	0.001**

Ethambutol affected significant decreased expression of SOD2 with p=0.002 in 5 days, p=0.013 in 10 days and p=0.018 in 15 days; significant increased MDA in 5 days with p =0.05, 10 days with p=0.017, 15 days with p=0.002 ; p53 in 5 days with p=0.012, 10 days with p=0.002, 15 days with p=0.001; Cyt c in 5 days with p=0.004, 10 days with p=0.001, 15 days with p=0.001; Caspase 3 in 5 days with p=0.001, 10 days with p=0.003, 15 days with p=0.001 and apoptosis in 5 days with p=0.001, 10 days with p=0.001, 15 days with p=0.001.

This study analyzed the effect of ethambutol induction on the decrease in the amount of RGCs, which is characterized by the occurrence of apoptosis through a series of SOD2, MDA, p53, PKCδ, Cyt c, Caspase 3, and apoptosis proteins. The results of the stepwise correlation analysis of these variables were:Ethambutol on SOD2 showed that there was a significant effect with p=0.001. The magnitude of rs=-63% was negative, indicating that ethambutol would stimulate a decrease in SOD2.SOD2 on MDA showed that there was a weak influence with p=0.003. The magnitude of rs=-0.45 was negative, indicating that a decreasing SOD value would cause an increase in MDA or, vice versa, a high SOD level was found to decrease MDA.SOD2 on PKCδ showed that there was no significant effect with p=0.34, indicating that the decreased SOD value was not quite significant to cause an increase or activate the expression of PKCδ. SOD on Cyt c showed that there was a significant effect of p=0.001.

The magnitude of rs=-57% was negative, indicating that a decreasing SOD value would cause an increase in Cyt c. MDA on CCP showedthat there wasno significant effect with p=0.64, indicating that the increased MDA value was not quite significant to cause an increase in CCP expression.MDA on Cyt c showed that there was a significant effect with p=0.001 The magnitude of rs=60% of positive, indicating that MDA which experienced an increase in change would cause an increase in Cyt c.PKCδ and p53 showed that there was

no substantial effect with p=0.64, indicating that the increased value of PKCδ was not quite significant to cause an increase in p53.P53 against Cyt c showed that there was a significant effect with p=0.001. The magnitude of rs=71% was positive, indicating that p53 which experienced an increase in change would cause an increase in Cyt expression c.Cyt c against Caspase 3 showed that there was a significant effect with p=0.001. The magnitude of rs=66% had a positive value, indicating that the expression of Cyt c which experienced an increase in change was quite significant to cause an increase in Caspase 3. Caspase 3 on apoptosis showed that there was a significant effect with p=0.001. The magnitude of rs=0, 79% waspositive, indicating that Caspase 3 which experienced a significant increase in change caused an increase in apoptosis.

4. DISCUSSION:

Death of RGCs in the eyes of ethambutol toxic optic neuropathy and experimental animal models occurs through the process of apoptosis. Apoptosis is program cell death, and there is no visible inflammation. Apoptosis occurs during the process of development and aging as a mechanism of homeostasis to maintain cell populations and the surrounding microenvironment. Initially, apoptosis is found in a physiological state, but later this condition can be triggered quickly by agents in stressed cells known as pathological apoptosis[15].

Giving ethambutol causing toxicity through the occurrence of apoptosis has been carried out on several researchers, including Yoon et al. (2000) where ethambutol induces vacuole formation in the cytoplasm and loss of neurons through the process of apoptosis; Karacurt et al., 2018 who observed the effect of Lutein on optic nerve injury in the administration of apoptosis and ethambutol and isoniazid; Clinici et al., 2015 investigating gene expression and evaluating the histopathology of RGCs undergoing apoptosis after administration of ethambutol at a dose of 30 mg/kgBB for 90 days orally after adding thiamine pyrophosphate; Huang et al., 2015 conducting a study on adult rats that received ethambutol at a dose of 40% above the recommended dose examining, how administering ethambutol induces autophagic fluk disorders and induces apoptosis in RGCs; and Ahmed et al., 2016 administering ethambutol at a dose of 100 mg/kgBW/day for 28 days in Sprague Dawley mice undergoing apoptosis through the effect of memantin as NMDA receptor inhibitors on RGCs.

Apoptotic expression had p-value of 0.001, with the Mann-Whitney test showing the difference between the two control groups and treatment group at 5 days of observation, at 10 days of observation at p 0.001 of 15 days of observation. This shows that ethambutol significantly causes apoptosis in RGCs *Rattus norvegicus* rats based on comparative bivariate between different groups of control and treatment groups.

This study aimed to uncover various aspects that may occur regarding the mechanism of RGCs death through apoptosis intrinsic pathway, which begins with impaired permeability of the outer membrane of the mitochondria due to ethambutol induction, triggering oxidative stress marked by an increase in superoxide radicals, hydrogen peroxidase, and hydroxyl groups where the body attempted to fight with the formation of scavenger enzymes such as SOD, CAT, GPX and PRX, and the observed markers were SOD2 expression. There was a significant decrease between the control and treatment groups on observation after administration of ethambutol for 5, 10 and 15 days.

The negative effects of reactive oxygen compounds can damage three types of compounds important for cell integrity, i.e.,(1) fatty acids, especially unsaturated fatty acids, which are important components of phospholopids making up cell membranes. Fat peroxidation occurs which in turn will form increased MDA expression measured in the treatment group that has been initiated with treatment 5 days, 10 days and 15 days;(2) DNA which is a genetic device of cells; and (3) proteins, which play important roles, such as enzymes, receptors, antibodies and cytoskeleton. H2O2 will expose oxidized GSH to GSSH with the help of GSH peroxidase. If oxidative stress accumulates, the GSH/GSSH ratio cannot be maintained at physiological values, so an increase in GSSG levels and glutathione reductase occur.

This will affect the oxidative status of the protein, and the protein that signals the oxidative stress reaction is PKC δ due to oxidation of the thiol group by ROS to induce apoptosis through PKC-dependent signaling pathway. In this study, the pathway through the CCP δ was not quite strong to induce p53 at treatment for up to 15 days. This might be influenced by the dose and time dependent nature of ethambutol, which may be induced by using larger doses and even with longer treatment times. It can also occur due to strong ROS activity causing damage to proteikinase C. The induction of p53 protein was quite significant to directly induce intrinsic apoptotic pathways through the induction of proapotosis proteins, such as BAX/Bak and inhibit anti-apoptotic proteins of the BCL2 family to then induce Cyt c secretion into the cytosol by opening the pore in the mitochondrial membrane. Cyt c in sitosol release triggers the activation of Caspase 9 and apaf 1 to form apoptosomes which then induces Caspase 3 for apoptosis [16],[17].

Path analysis using stepwise correlation shows that there are two pathways that can explain the mechanism in this study that led to the expression of apoptosis:

a) Ethambutol induces a decrease in SOD2, an increase in Cyt c, an increase in Caspase 3 and an increase in apoptosis.
b) Ethambutol induces a decrease in SOD2, an increase in MDA, an increase in Cyt c, an increase in Caspase 3, and an increase in apoptosis.

Both of these pathways can be analyzed through the magnitude of the path coefficients resulting from the Spearman test on each variable path traversed. It can be concluded that the effect of apoptosis due to ethambutol administration at a dose of 15 mg/kgBW/day for 5, 10 and 15 days in *Rattus norvegicus* rats can be estimated to cause retinal damage of 28.5%.

The limitation of this study is that the duration of treatment was only limited to 5,10 and 15 days. Further research needs to be done with shorter treatment duration to find out earlier when the side effects of ethambutol begin to emerge and even longer to find out the extent to which ethambutol exerts the effects of apoptosis, and whether other mechanisms of cell damage are generated, such as inflammation, and the possibility of necrosis in RGCs *Rattus norvegicus*. This study used animal models that can only be done for one treatment period but could not be for continuous observation because the animal must be killed to find out the effect on its RGCs.

5. CONCLUSION:

The mechanism of apoptosis of RGCs caused by ethambutol was showed by the decreased of expression SOD2, increased expression of MDA, p 53, Cyt c, Caspase 3, and apoptosis. These biomarkers are essential to detect apoptosis as one of mechanism in cell death.

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