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Ethionamide Modulates of Thyroid Stimulating Hormone, Thyroxine, and Triiodothyronine Levels in White Rats

(ETHIONAMIDE MEMODULASI KADAR THYROID STIMULATING HORMONE, THYROXINE, DAN TRIIODOTHYRONINE TIKUS PUTIH)

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ABSTRACT

Multidrug-Resistant Tuberculosis (MDR-TB) infection is not solely caused serious impact for patient and cause long recovery process. Multidrug-Resistant Tuberculosis needs specific treatment approach using second-line TB drugs. Ethionamide as one of medication used in MDR-TB are known to cause greater side effects compared to the first-line drugs. One of side effect like hypothyroidism is remain unclear. There is limited study about role of ethionamide cause hypothyroidism. In this present study, 24 male, wistar rats were divided into three groups: ethionamide group; positive control positive (propylthiouracil) and negative control group (received distilled water only) and treated accordingly for 12 week. Under ketamine and xylazine anesthesia, blood sample were collected from retroorbital vein at 8 and 12 week, put in EDTA tube and stored -20ËºC until use. After last sampling, rat were sacrificed using CO2 chamber. Our data showed that thyroid stimulating hormone (TSH) levels in ethionamide group is increased from 8 weeks to 12 weeks significantly, but there is no significant changes in control groups. Thyroxine (T4) levels is trend decreased from 8 weeks to 12 weeks compared to control groups. There is no change of Triiodothyronine (T3) levels. Effect of ethionamide on thyroid hormone (T3 and T4) levels was not prominent and there were no significant changes observed. Interestingly, we observed an increase of TSH level after ethionamide treatment. Taken together, 12 week treatment of ethionamide tablet might potentially modulate thyroid hormone (T3 and T4) levels that is reflected from an increase of TSH levels in the plasmas feed back negative mechanism.

Keywords**:** **e**thionamide; thyroid stimulating hormone (TSH); thyroxine (T4); Triiodothyronine (T3); hypothyroidism

ABSTRAK

Infeksi *Multidrug-Resistant Tuberculosis* (MDR-TB) tidak hanya berdampak serius bagi pasien, tapi juga menyebabkan proses pemulihan yang lama. Penyakit MDR-TB memerlukan pendekatan pengobatan khusus dengan menggunakan obat TB lini kedua. Ethionamide sebagai salah satu obat yang digunakan pada MDR-TB diketahui menyebabkan efek samping yang lebih besar dibandingkan dengan obat lini pertama. Salah satu efek samping seperti hipotiroidisme masih belum dipahami dengan baik. Ada sejumlah kecil penelitian tentang peran etionamida yang menyebabkan hipotiroidisme. Pada penelitian kali ini, 24 ekor tikus wistar jantan dibagi menjadi tiga kelompok: kelompok etionamid; kontrol positif positif (*propylthiouracil*) dan kelompok kontrol negatif (hanya menerima air suling) dan diberi perlakuan

selama 12 minggu. Di bawah pengaruh anestesi ketamin dan xylazine, sampel darah tikus diambil dari vena retroorbital pada minggu ke-8 dan 12. Sampel darah yang diperoleh dimasukkan ke dalam tabung EDTA dan disimpan -20°C sampai digunakan. Setelah pengambilan sampel terakhir, tikus dikorbankan nyawanya dengan memasukkan tikus kedalam ruang yng diberi gas karbon dioksida (CO_2). Data penelitian ini menunjukkan bahwa kadar *thyroid stimulating hormone* (TSH) pada kelompok etionamid meningkat secara signifikan dari minggu ke-8 hingga minggu ke-12, tetapi tidak ada perubahan yang signifikan pada kelompok kontrol. Kadar tiroksin (T4) cenderung menurun dari minggu ke-8 sampai minggu ke-12 dibandingkan dengan kelompok kontrol. Tidak ada perubahan kadar triiodothyronine (T3) pada penelitian ini. Pengaruh etionamid terhadap kadar hormon tiroid (T3 dan T4) tidak menonjol dan perubahannya teramati tidak signifikan. Menariknya, dalam penelitian ini teramati peningkatan kadar TSH setelah pengobatan etionamid. Secara keseluruhan, pengobatan tablet etionamid selama 12 minggu berpotensi memodulasi kadar hormon tiroid (T3 dan T4) yang tercermin dari peningkatan kadar TSH dalam mekanisme umpan balik plasma negatif.

Kata-kata kunci: etionamida; hormon perangsang tiroid (TSH), tiroksin (T4), triiodotironin (T3), hipotiroidisme.

INTRODUCTION

The World Health Organization estimated that in 2017, more than 10 million people developed TB (as newly infected TB patient or relapsed TB patient), and about 5.58% of those patients were infected with rifampicin resistant (RR) bacterial strain and 82% of these rifampicin resistances were multidrug-resistant (MDR) TB (MDR; resistance against isoniazid and rifampicin with or without resistance to other first-line TB drugs (WHO 2018). The number of MDR-TB cases around the globe is increasing alarmingly as compared to earlier in 2013 that was only 3.5% of the estimated nine million new TB cases having MDR/RR-TB (WHO 2014). Therefore, MDR-TB incidence rate has gone up, and as the consequences, its therapy has become critical to be understood very well.

The major concerns regarding the second line drugs (SLDs) for MDR/RR-TB are expensive, have relatively low efficacy, need longer treatment period, and exert more side effects or toxicities as compared to first-line antituberculosis drugs. Hypothyroidism is one of the potential side effects that may emerge and often observed among patients that undergo the MDR-TB treatment. Hypothyroid of MDR-TB patient is various between 3.9%-78% (Cheung *et al.,* 2018), and also often underdiagnosed due to vague symptoms that are masked by other conditions (e.g., arthralgia, depression, ectodermal dysplasia, psychosis, and xeroderma). Thus, observing and diagnosing a hypothyroid case based on clinical symptoms commonly leads to underdiagnosed, moreover if the changes or thyroid hormone level is subtle. Therefore, there is a protocol treatment which recommend a TSH routine serological test in patients undergoing MDR-TB treatment (Munivekatappa *et al.,* 2016). It had been reported that ethionamide/prothionamide and para-aminosalicylate sodium (PAS) used in MDR-TB treatment might cause hypothyroidism (Drucker *et al*., 1984; Munivekatappa *et al.,* 2016; Cheung *et al*., 2018; Tola *et al*., 2019). The combination of ethionamide/prothionamide and PAS often causes a high incidence of gastrointestinal side-effects and hypothyroidism (WHO 2014).

Ethionamide itself is a derivative of isonicotinic acid and is classified into carbothionamides group. It acts as weak bactericidal by blocking mycolic acid synthesis of the *Mycobacterium tuberculosis* (WHO 2014). Ethionamide has a good clinical efficacy against MTB but has poor tolerability because of considerable gastrointestinal adverse effects, such as nausea, vomiting, anorexia, a metallic taste, and abdominal pain, prohibiting this drug from being used as a first-line therapy (Hallbauer and Schaaf, 2017; Donald 2009). Ethionamide has thioamide side chain correlated with methimazole (an antihyperthyroid drug) and believed as inhibitor for formulating of thyroid hormone like propylthiouracil and methimazole mechanism of action (Hallbauer and Schaaf, 2017). Thus, one of well-recognized adverse effects of ethionamide is hypothyroidism which is observed frequently in adults and children. Other adverse effects are hepatotoxicity, nervous system abnormalities similar caused by isoniazid, and in long-term therapy of MDR TB, causing gastrointestinal disturbances (Akshata *et al*., 2015; Thee *et al*., 2015). There is limited informations about correlation of ethionamide treatment induced hypothyroidism, availability

of evidence about the mechanism and modulation level of thyroid hormones in MDR-TB patients consuming ethionamide drug. The available evidence is limited to clinical-based finding in patient and hospital setting. Therefore, the proper protocol for managing the side effect from hypothyroid in MDR-TB patient is also still unjustified. We believe that it is very important to know the significance of the thyroid hormones modulation effect caused by ethionamide treatment in MDR-TB patient for optimizing management approach. Taken together, understanding of ethionamide modulates thyroid hormone levels will be very beneficial for considering optimal management for MDR TB patient in the future.

RESEARCH METHODS

Animal Model

This study was approved by the Research Ethics Committee of the Faculty of Medicine of UniversitasPadjadjaran (KEPK) with Number 1465/UN6.KEP/EC/2019 and followed international standard care of experimental animal (Research NRC 2004). This study was an experimental analytic research using 24 healthy male wistar strain rats (*Rattus norvegicus*), 10 weeks old, and body weight 200-250 g. Rats were divided into three groups (Ethionamide, Propylthiouracil (PTU), and Control (Placebo)). The rats were purchased from PT. Biofarma, Bandung and acclimatized for one week before treatment. Rat received PTU, Ethionamide or dd H_2O treatment for 12 weeks using small oral gavage.

Ethionamide and PTU Preparation

The ethionamide and propylthiouracil tablets were purchased from OTC Pharmacy store. Ethionamide tablet 250 mg was diluted in water with the ratio 5:1. Propylthiouracil tablet 100 mg was diluted in water with ratio of 2:1. Ethionamide and PTU tablet were administreted orally. Ethionamide dose is 20 mg/day derived and calculated from human dose conversion to animal equivalent dose/AED (Munivekatappa *et al.,* 2016).

AED (mg / kg) = Human dose (mg / kg) x K_m ratio

*K_m ratio: factor that is estimated by dividing the average body weight (kg) of species to its body surface area (m^2)

The control positive group was given propylthiouracil oral supplementation with the dose of 6 mg/day also based on animal equivalent dose calculation. The control negative group was given placebo treatment containing distilled water once a day with same administration route and amount similar to those of the ethionamide and propylthiouracil treatments. This was done to give the same amount of stress resulted from the treatment administration.

Blood Sampling

The blood sampling was taken at two different times, at eighth week and twelfth week of the treatment. The technique used in this process was retro orbital puncture. Before the puncture procedure, the anesthetic procedure using ketamine and xylazine anaesthesia was performed to each rat by injecting them intramuscularly. Under anesthetic, retro-orbital puncture was performed using micro-hematocrit non heparine capillary tube, tube was inserted carefully via the gap near the tear duct (medial canthus) to puncture the retrobulbar venous sinus. After the capillary tube reach and puncture the blood vessel, the blood will flow out through the capillary tube and then the blood was directly collected into the EDTA-Eppendorf tube (1,5 mL). To minimize the bleeding, a gentle press with sterile gauze pad was given on the rat's eyeball with closed eyelids to help stop the bleeding. Serum were separated using centrifugation with a speed of 6000 rpm for 15 minutes at 4ºC and then stored in -20ºC until use.

Serum Analysis Using ELISA

Serum samples were analyzed for Thyroid Stimulating Hormone (TSH), Thyroxine (T_4), and Triiodothyronine (T_3) using sandwich ELISA method. ELISA were based on the principle of biotin double antibody sandwich technology with the sensitivity for TSH at 0.1 mU/L and the range of the standard curve at 0.5-12 mU/L. For the T4 and T3 ELISA kits, the sensitivity was 0,1 ng/mL and the standard curve ranged from 5 ng/mL to 120 ng/mL. The antigen of the corresponding hormone in the rat's serum was analyzed in the Greiner 96 Flat Bottom Transparent Polystyrene ELISA plate. As much as 10 µL sample and 40 µL diluent were pipetted into labeled testing sample well, and then, 50 µL standard solutions to the standard well. The HRP-conjugate reagent was then added to each well (standard and testing sample). Plate was shaken to mix up the solution for 60 minutes at 37ºC incubation. After wash properly, 50 µL

chromogen solution A was added to each well followed by chromogen solution B and followed by incubation the solution for 15 minutes at 37°C with cover. Finally, stop solution was added and read the O.D value within 15 minutes. The quantitative measurement of the hormones concentration was measured using a standard curve that was generated by plotting the average optical density (O.D) from the six standard concentrations on the vertical Y axis versus the corresponding concentration on the horizontal X axis. First, we calculated the mean O.D value both for each standard and sample as mentioned above, and then we generated the standard curve. All the O.D values were subtracted by the mean value of the zero standard before result interpretation.

Statistical Analysis

The results were statistically analyzed using Statistical Product and Service Solutions (SPSS) software version 20. The data were tested for the normality using Sahpiro-Wilk test to determine the normal distribution of data. Then, the test of homogeneity of variance (Levene's Test) was conducted. We compared the means of data using One way analysis of variance for each TSH, T_3, and T_4 analysis from each three group of treatment with 95% Confidence Interval. Non-parametric Kruskal-Wallis test was used if the data were not normally distributed. The difference between weeks was analyzed using paired sample T-test with alternative Wilcoxon test if the data were not normally distributed.

RESULT AND DISCUSSION

Serum TSH Modulation

The mean concentration of TSH in the eighth week of intervention showed the differences among groups which were not statistically significant ($p>0.05$). After twelve weeks of treatment, the differences between ethionamide group and control group were still not statistically significant. The fluctuation of the TSH concentration was observed by comparing the mean concentration of TSH from the 8th week to the 12th week (Figure 1; Table 1). The results showed statistically significant differences in ethionamide group.

Serum Thyroxine (T_4) Modulation

In the 8th week of treatment, the statistical analysis showed that the differences of serum T_4 concentration among treatment groups were insignificant ($p>0.05$). in the 12th week of treatment, the statistical analysis showed that the differences were still insignificant. The fluctuation of T_4 serum concentration was observed by comparing the T_4 concentration in the 8th week and the 12th week in all treatment groups. The result showed that there was no significant difference between 8th week and 12th week (Figure 2; Table 2).

Serum Triiodothyronine (T_3) Modulation

The Triiodothyronine serum mean concentration in the 8th week showed that the differences among groups were not statistically significant ($p>0.05$). After 12 weeks of treatment, we observed that there was differences among groups. However they were still not significant (Figure 3; Table 3). The fluctuation of hormone concentrations was analyzed by comparing the results of the 8th week and the 12th week in the treatments in each group and the result showed that there was no significant difference of the concentration between weeks.

The role of ethionamide-induced hypothyroidism is reported in MDR-TB patient in Africa and Asia (Hallbaueer and Schaaf, 2017). MDR-TB patient usually also has other confounding risk factors which may contribute to the alteration of thyroid hormone function, such as Human Immunodeficiency Virus (HIV) (especially in resource poor countries where TB and HIV are endemic), antiretroviral drug use, and nutritional and iodine deficiencies that may also contribute to thyroid status (Cheung *et al.,* 2018; Munivekatappa *et al.,* 2016; Madeddu *et al.,*2006). The effect of ethionamide in regulation of thyroid hormone in MDR-TB patients is still unclear. This present study aimed to assess role of ethionamide modulate of thyroid hormone levels in rat model.

In the present study, we used the rats were treated using same OTC PTU and Ethionamide, similar dose as in human (15 mg/kg/day) and given orally to mimic drug metabolism in human. Thyroid stimulating hormone (TSH) showed an increase in ethionamide after 12 weeks treatment by 4% (Figure 1). In hypothyroidism, TSH is important as sensitive marker to identify the alteration of thyroid hormone. TSH levels should be increased as a result from feedback stimulation to Hipothalamus Pituitary and Target organ axis (HPT axis) driven by the decreased of thyroid hormone (T_3 and T_4) to the anterior pituitary and hypothalamus. Our data

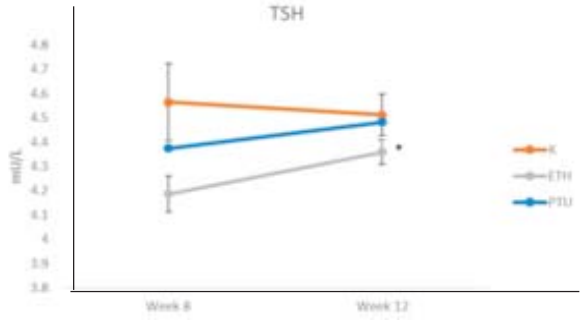

Figure 1. Comparison of mean TSH concentration among three groups of treatment in the 8th and the 12th week of intervention. Data representation in mean ± SEM of experiment. It showed significant difference in ethionamide group between week 8 and week 12 (* $p<0.05$ compared to week 8).

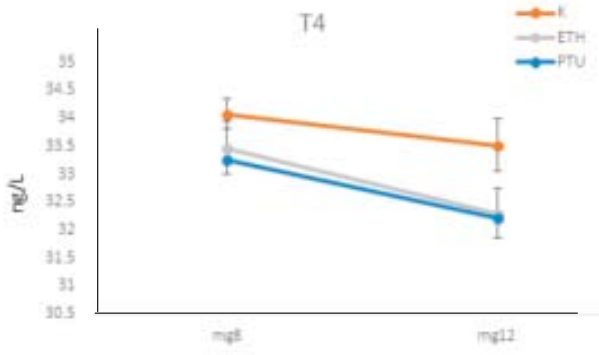

Figure 2. Comparison of mean T_4 concentration among three groups of treatment in the 8th week and the 12th week of intervention. Data representation in mean ± SEM of experiment.

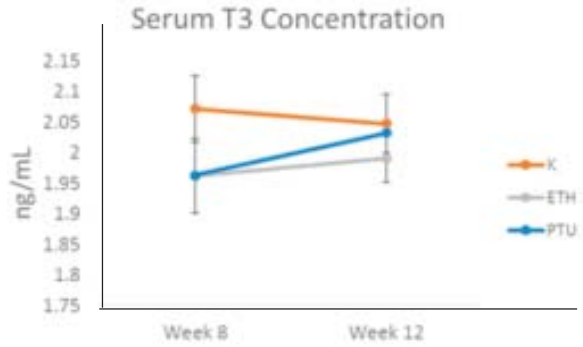

Figure 3. Comparison of mean T_3 concentration among three groups of treatments in the 8th week and the 12th week. Data representation ratio in mean ± SEM of experiment.

Table 1. Results of thyroid stimulating hormone concentration after eight weeks and 12 weeks of ethionamide (ETH), propylthiouracil (PTU) and placebo (K) treatments.

	Mean of Average ± SEM (mU/L)	
	Week 8	Week 12
Placebo (K)	4.56±0.16	4.51±0.08
Ethionamide (ETH)	4.19±0.09	4.36±0.05
Propylthiouracil (PTU)	4.37±0.08	4.48±0.08

Table 2. Results of serum thyroxine concentration after eight weeks and 12 weeks of ethionamide, propylthiouracil and placebo treatments.

	Average±SEM (ng/L)	
	Week 8	Week 12
Placebo (K)	34.08±0.48	33.52±0.53
Ethionamide (ETH)	33.46±0.44	32.30±0.66
Propylthiouracil (PTU)	33.24±0.48	32.21±0.46

Table 3. Results of serum triiodothyronine concentration after 8 weeks and 12 weeks of ethionamide, prophytiouracil and placebo treatments.

	Average ±SEM (ng/L)	
	Week 8	Week 12
Control	2.07±0.05	2.05±0.06
Ethionamide	1.96±0.05	1.99±0.04
PTU	1.96±0.05	2.03±0.04

showed that there small but significant increasement of TSH levels in Ethionamide group at the 12 week, and observed that there is no changes in control group. However, TSH level is very sensitive to some stress factors in the body and this might altered and masked the it

level changes in plasm. Thus, there are some limitations of our the study, like induction of anesthesia and stress factor during blood sampling, dose of ethionamide, and duration of treatment which could alter the result of TSH in control, PTU and Ethionamide groups.

Interestingly, in line with TSH results, total Thyroxine (T_4) levels was lowered by the use of PTU and ethionamide (Figure 2). Similar to PTU, ethionamide could inhibit the incorporation of iodine into trichloroacetic acid-precipitable protein and also the uptake of iodine but it does not necessarily remove thyroid hormones which are already in the thyroid or in the bloodstream (Drucker *et al.*, 1984; Jastrzebsksa 2015). T_4 itself is the main product of the thyroid gland production and most T_4 produced is stored in the bloodstream and body attached to thyroxine binding globulin (TBG), albumin, transthyretin (prealbumin), and other lipoproteins. While attached to these molecules, the T_4 is not biochemically active and provides a reservoir of stored T_4, therefore maintaining thyroid function for days even if thyroid function were decreased (Dubbs and Spangler, 2014).

The Triiodothyronine (T_3), as the active form of the thyroid hormone, is mostly made in the extrathyroidal tissues by deiodination of T4 (about 80% of T_3 is made in extrathyroidal tissue (Dubbs and Spangler, 2014). The deiodination process is done by the Deiodinase (D) enzyme. There are three types of D (D1, D2, and D3) with different properties in terms of activity in various tissues and roles in states of hypothyroidism and hyperthyroidism. In states of iodine deficiency and hypothyroidism, D2 activity is markedly up-regulated to convert T_4 to active form T_3. D3 activity is decreased to reduce conversion of T_3 to the inactive r T_3. D1 activity is also increased by stimulation of TSH on thyroid gland resulting in increased conversion of T4 to T_3. These mechanisms are in place to maintain the T_3 within the normal range (Tarym 2011; Brent 2012). Our data showed that T_3 level was almost constant in all groups that might be caused by this mechanism (Figure 3). However, even though this mechanism took place, the alteration of ethionamide and PTU was still insignificant for the twelve week treatment. We assume that the treatment time was not long enough as in a previous study in which the effect of hypothyroidism in MDR-TB patient ranged between 132-365 days (19 to 52 weeks) with median time of 260 days (Medongo and Zetola, 2012). Also, in our studywe had utilized tablet of PTU and Ethionamide for patient, so that we did not use pure powder of PTU and ethionamide from Sigma company as commonly used. Therefore, it might reduce the effectiveness of PTU and Ethionamide. In addition, for PTU, its dose approach for rat was not given in divided dose as in human, therefore it might alter the effectiveness since pharmacodynamic and metabolic effect of PTU only lasted for 12-24 hours (Jastrzebsksa 2015).

CONCLUSION

Ethionamide treatment for 12 weeks stimulates a little but significant of TSH levels as feedback mechanism caused thyroid hormone alteration. Some physiological compensation might take place and masked the hypothyroidism condition in our model. Future detailed experiment should consider duration, purity of ethionamide and route of treatment. In addition, study about genetic modulation of thyroid receptor expression will be very interesting to be explored.

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