ORIGINAL ARTICLE

Immunoreactive Scoring of Metallothionein-3 (MT-3) Expression as an Indicator of Metallic Taste Rats which are Induced by Doxorubicin and Antioxidant

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ABSTRACT

Introduction: Cancer is one of the leading causes of deathworldwide. Chemotherapy like as doxorubicin is the most common treatment procedure given to cancer patients. Doxorubicin is a cytotoxic drug that triggers the production of Reactive Oxygen Species (ROS) affect to body cells including taste bud cells to induced the expression of Metallothionein 3 (MT-3), eventually cause cell damage that leads to a metallic taste. Antioxidant therapy can be an alternative to overcome metallic taste as it counters ROS effect and lowers the expression of MT-3. The aim of this study is to evaluate MT-3 expression in the taste bud cells of male Wistar Rats after induction of doxorubicin combined with vitamin E and mung bean sprouts (*Phaseolusradiatus L.*) juice. **Methods:** 27 male Wistar rats weighing 250-300 g aged 3-4 months were divided into 3 groups randomly; control group, treatment group 1 (receiving doxorubicin and vitamin E), and treatment group 2 (receiving doxorubicin and mung bean sprouts juice). After 5 days, the rats were sacrificed, and the tongue was taken for immunohistochemistry analysis. Data were then analyzed by One Sample Kolmogorov-Smirnov, Levene Test, and Oneway-ANOVA statistical test ($p<0.05$). **Results:** The MT-3 expression increases in the following order; control group (4.93), treatment group 2 (7.08), and treatment group 1 (9.95). Treatment group 1 and 2 both show remarkable increases of MT-3 expression compare to control. **Conclusion:** The induction of doxorubicin and antioxidant can increase the level of MT-3 expression.

Keywords: Antioxidant, Doxorubicin, Metallothionein-3, Mung bean sprouts

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INTRODUCTION

Cancer is one of the leading causes of death in the world. In 2018, around 9.6 million people death were caused by cancer. Lung, liver, stomach, colorectal, and breast cancer are the biggest causes of cancer every year (1). Nationally, the prevalence of cancer in all-ages population in Indonesia in 2013 was 1.4% or an estimated 347,792 people. Based on a research, East Java Province is the province with the most cancer patients, which is around 61,230 people (2).

Chemotherapy is one of the most common treatment procedures for cancer patients. This therapy relies on the ability of special drugs to destroy cancer cells. These drugs work by slowing down or stopping the proliferation of cancer cells (3). One of the broad-spectrum chemotherapy drugs is doxorubicin. Doxorubicin is an anthracycline class antibiotic that is widely used for the treatment of various cancers such as acute leukemia, breast cancer, bone and ovarian cancer. This compound was isolated from Streptomyces peucetius in the 1960s and used widely (4,5).

Doxorubicin is a cytotoxic drug that triggers the formation of radical Reactive Oxygen Species (ROS) (6). The formation of radical ROS due to doxorubicin can damage body cells including taste budcells. Taste buds damage due to ROS occurs in several ways, namely a decrease in the number of normal taste receptors, an increase in the threshold for umami taste, and neurotoxicity in cranial nerve VII, XI, X which functions as a sensory nerve on the tongue. Damage of taste buds can cause metallic taste in cancer patients associated with doxorubicin chemotherapy (7,8). Taste buds cells that are exposed to oxidants due to chemotherapy can induce the expression of Metallothionein 3 (MT-3) as an immune's defense protein against oxidant exposure (9,10).

Increased sensation of metallic taste in cancer patients

can cause a decrease in appetite, food intake and quality of life. This metallic taste was felt by 56.3% of the majority of cancer patients with chemotherapy. Decreased appetite will result in malnutrition in cancer patients and reduce the survival rate (11).

The current therapy for reducing metallic taste is the use of the zinc gluconate supplement. Zinc gluconate supplement of 140 mg per day is equivalent to 20 mg per day of zinc elements. Zinc gluconate supplements must be taken on an empty stomach alongside with a lot of water. The time period for consuming zinc supplements is also longer, which is around 1-3 months to get the maximum removal of metallic taste. Long-term consumption of high doses of zinc can cause side effects such as gastrointestinal disorders, hematological disorders, and zinc poisoning (12).

Antioxidant therapy can be an alternative to overcome metallic taste effectively and faster. One of the popular food ingredients in Indonesia as a source of antioxidants is mung bean sprouts. Mung bean sprouts contain high levels of bioactive compounds, antioxidants and zinc minerals. The content of vitamin E (α-tocopherol) from mung bean sprouts per 100 g is 90 mg which is equivalent to 138 Intra Units (IU), meanwhile the content of bioactive compounds (phenols and flavonoids) with antioxidant activity reaches 91%, so that it has the potential as a source of natural antioxidants (6). Vitamin E also plays a role as a source of natural antioxidants. Vitamin E is a phytonutrient which naturally has 8 isomers, which are grouped into 4 tocopherols (α, β, γ, δ) and 4 tocotrienols (α, β, γ, δ). Most vitamin E supplements in nature are in the form of α-tocopherol. This compound has been known as an anti-oxidant capable of maintaining cell membrane integrity. This compound is also reported to work as an oxygen free radical scavenger, fat peroxidation and singlet oxygen (13).

Giving antioxidants in the form of mung bean sprouts and supplementation of vitamin E as a source of antioxidants is expected to induce MT-3 expression to eliminate metallic taste in cancer patients with chemotherapy, so that improvement in appetite and quality of life can be improved. The aim of the study was to evaluate the MT-3 expression in taste bud cells of male Wistar Rats after induction of doxorubicin combined with vitamin E with the group of receiving doxorubicin intraperitoneal injection and mung bean sprouts juice (*Phaselusradiatus L.*) juice.

MATERIALS AND METHODS

Samples

The type of research used was experimental analytics using wistar (Rattus novergicus) rats as experimental animals. The research subjects were male wistar rats, weighing 250-300 g, aged 3-4 months obtained from the Faculty of Veterinary Medicine, Airlangga University, Surabaya. The number of research subjects was 27 and then divided into 3 groups so that the number of each group was 9 rats. The research was conducted at the Faculty of Veterinary Medicine, Airlangga University, Surabaya. This research has been approved by the research commitee ethics of Faculty of Dental Medicine Airlangga University with number of 131/HRECC. FODM/VII/2018

Preparation of mung bean sprouts juice

The research material, fresh green bean sprouts, is obtained from the germination process for 48 hours, given in the form of juice. Mung bean sprout juice is made by the retail method, namely green bean sprout mass (g) added with a certain volume of water (ml) at a dose of 10 mg/gBW. Vitamin E used in this study was commercial vitamin E (Sea-quill, Watson, Indonesia).

Inducing chemotherapy in animal model

Mice were adapted first for 7 days and given standard pellet diet (expanded pellets; Stepfield, UK) and distilled water. After the adaptation period was completed, the rats were divided into 3 groups (C, T1, T2) with each group consists of 9 rats. Group C is the control group which was given standard pellet, distilled water and injection of doxorubicin (Doxil©, ALZA Corp., Mountain View, CA) 1.17 ml/kg body weight. Group T1 is treatment group (1) which was given standard pellet, distilled water, injection of doxorubicin (Doxil©, ALZA Corp., Mountain View, CA) 1.17 ml/Kg body weight and supplement Vitamin E 138 IU. T2 group is treatment group (2) which was given standard pellet, distilled water, injection of doxorubicin 1.17 ml/Kg body weight and mung bean sprouts juice 10 mg/g body weight.

Immunohistochemical staining and scoring methods

On the 5th day, the rats were sacrificed, and the tongue was taken. The tongue was cut at the base of the tongue with a foliate papilla structure. The tongue pieces containing foliate papillae were fixed with 10% formalin buffer pH 7.3 for 8 hours. The tongue tissue preparation then was made paraffin blocks. After paraffin block, the next step were deparaffinization and staining process of IHC (Immunohistochemical) with anti-MT-3 monoclonal antibodies.

MT-3 expression in each sample was assessed semi-quantitatively according to the modified Kaemmerer method (14). Immunoreactive Score (IRS) is scored by multiplication percentage of positive cells score with the intensity of staining score produced in that cell (table I). The percentage of positive cells score based on: 0 = no positive cells, 1= positive cells less than 30 %, 2= positive cells 30 %- 60%, 3= positive cells more than 60%. The intensity of staining score produced in that cell indicator based on: 0 = no color reaction, 1 = light intensity, 2= medium intensity, and 3 = strong intensity. Positive cells with anti MT-3 indicated brown color.

Table I: The IRS semiquantitative scale is a product of the percentage of the positive cells score (A) with the intensity of staining score (B), becoming an immunoreactive score (IRS) = (A x B)

A	B	A x B
0 : there are no positive cells	0: no color reaction	0 – 1 = negatif
1 : Positive cells less than 10%	1 : Low color intensity	2 – 3 = low
2 : Positive cells between 11% - 50%	2: Medium color intensity	4 – 8 = medium
3 : Positive cells between 51% - 80%	3 : Strong color intensity	9 – 12 = strong
4 : Positive cells between more than 80%		

Statistical Analysis

Data obtained from MT-3 scores are displayed in averages and standard deviations. Data were analyzed using Statistical Package for the Social Sciences (SPSS 17) program. The correlations between groups analyzed using Spearman.

RESULTS

This study was conducted to examine the effect of doxorubicin and antioxidants on MT-3 expression scores in 27 wistar rats. Based on statistical tests, the mean expression of MT-3 in the control group was 2.31, in the treatment group 1 there was an increase in MT-3 expression with a mean of 3.29 (Fugre 1). In the treatment group 2 also increased the expression score of MT-3 with a mean of 3.07. Treatment group 1 had a higher mean expression of MT-3 than treatment group 2 and control group (Figure 2).

The next statistical test is the non parametric correlation test with Spearman. Correlation test results obtained by the control group correlation with treatment 1 is -0.49 (negative), which means strongly correlated. While the correlation of the control group with treatment 2 is 0.244 (positive), which means weak correlation.

DISCUSSION

Cancer is one of the leading causes of death in the world.

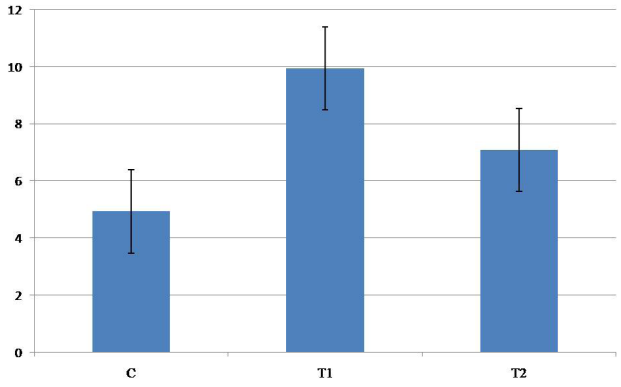

Figure 1: Differences of MT-3 expression scores between groups by Immunoreactive Score (IRS). Immunohistochemical staining of rat foliate papillae on MT-3 expression at 400x magnification were percentaged of positive cells score with the intensity of staining using light microscope. C. Doxorubicin, T1. Doxorubicin and vitamin E, T2. Doxorubicin and green bean sprouts. The X Axis is the divided rats on this research (three groups, consist of: Control (C), Treatment 1, and Treatment 2. The Y axis mentions the different levels of Immunoreactive Score (IRS) of each group.

In 2015, around 8.2 million people death were caused by cancer. Chemotherapy is one of the most common treatment procedures for cancer patients. This therapy relies on the ability of special drugs to destroy cancer cells.

This study was conducted to compare MT-3 expression in the taste bud cells of Wistar Rats after induction of doxorubicin, in combination with antioxidant therapy using vitamin E or mung bean sprouts (*Phaseolusradiatus L.*) juice. Doxorubicin is a chemotherapy drug that is often used. Doxorubicin is classified as a cytotoxic agent that triggers the formation of radical radical (ROS). The formation of radical ROS due to doxorubicin can cause negative effects in the form of metallic taste. ROS radicals can damage body cells including cell taste buds. Damage to taste buds can result in a change in taste. Changes in taste due to radical ROS occur due to a decrease in the number of normal taste receptors, an increase in the threshold for umami taste and neurotoxicity in VII nerves, XI, X which functions as a

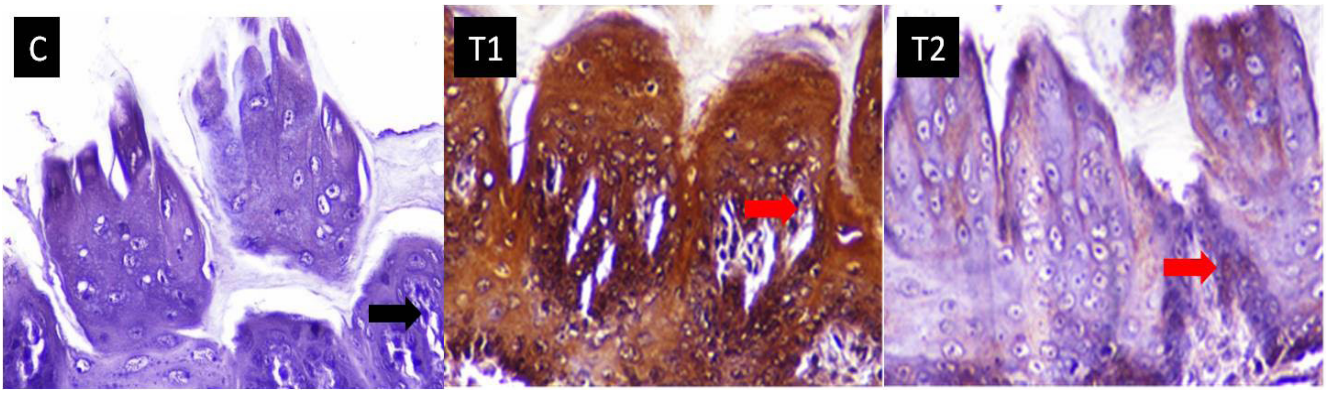

Figure 2: 2: IHC staining of rat foliate papillae at 400x magnification light microscope. C. doxorubicin, T1. Doxorubicin and vitamin E, T2. Doxorubicin and green bean sprouts. Black arrow not show MT-3 expression, Red arrow show MT-3 expression

sensory nerve on the tongue. Damage to taste buds can cause a sensation of metallic taste in cancer patients after chemotherapy (7).

MT-3 expression in each sample was assessed semi-quantitatively according to the modified Remmele method. Immunoreactive Score (IRS) is the product of a percentage of positive cells score with the intensity of staining score produced in that cell (14). According to a previous study, giving antioxidants 2 days before intraperitoneal injection of doxorubicin 1.17 ml/kg body weight in mice functioned as a preventive measure to prevent fat peroxidation in the membranes of the liver and mouse brain which could be aggravated by administration of doxorubicin on the next day. High fat peroxidation can result in a decrease in the body's resistance to mice and the possibility of death before treatment time is complete because doxorubicin is a cytotoxic drug (15).

In the positive control group (C), The group given standard food and induction of doxorubicin was 1.17ml/kg body weight, the mean MT-3 expression score was 4.93. The positive control group is the lowest score of MT-3 expression cause the promotor MT-3 in only depend on doxorubicin.

In the first treatment group (T1), the group given doxorubicin injection was 1.17 ml/kg body weight + vitamin E 138 IU, the mean MT-3 expression score was 9.95. The treatment group 1 had a higher MT-3 expression score compared to the control group and treatment 2, because in this group besides giving exposure to doxorubicin, antioxidant was also given in the form of vitamin E of 138 IU. The expression of MT-3 on increased taste buds was caused by exposure to doxorubicin which is a pro-apoptotic agent by inducing radical formation of Reactive Oxygen Species (ROS). The increased number of MT-3 expressions in group 1 indicates an attempt to taste buds of foliate papillae that can express MT-3 to carry out protective mechanisms against the pro-apoptotic effects of cytotoxic drugs such as doxorubicin (16).

Increased expression of MT-3 in treatment group 1 (T1) also occurred because in this group rats were given vitamin E which was quite potent at a dose of 138 IU. MT-3 expression can be induced by various pro-inflammatory and anti-inflammatory mediators such as cortisol, ROS, antioxidants, endotoxin and acute inflammatory phase cytokines. Vitamin E is one source of antioxidants that works to break the chain of free radicals, so the administration of vitamin E in the body of mice can induce the expression of MT-3 as its role to protect the body from exposure to cytotoxic drugs such as doxorubicin (16,17).

In treatment group 2 (T2), the group with administration of doxorubicin and mung bean sprouts with a dose of 10 mg/g body weight was obtained as an increase in MT-3 expression score compared to the control group which was equal to 7.08. MT-3 expression on taste buds increased in treatment group 2 caused by exposure to doxorubicin which induced the formation of Reactive Oxygen Species (ROS) and was triggered also by zinc cofactors contained in mung bean sprouts. ROS can damage body cells including cell taste buds. Damage to taste buds can result in a change in taste. Changes in taste can occur because ROS causes a decrease in the number of normal taste receptors, an increase in the threshold for umami and neurotoxicity in VII, XI, X nerves as sensory nerves in the taste bud. Changes in taste can lead to metallic taste in cancer patients due to chemotherapy (7).

The results of this study indicate that the highest average MT-3 expression score was in the treatment group 1. The role of MT-3 to neutralize the presence of metallic taste can be explained that MT-3 is a special protein that functions to reduce ROS levels in the body and regulate zinc homeostasis which plays an important role in taste. Decreased taste buds ROS levels will minimize taste receptors damage, stabilize the umami taste threshold and avoid sensory taste buds damage. Zinc homeostasis by MT-3 also helps improve taste because zinc plays an important role in the synthesis of gustin protein and alkaline phosphatase for taste transduction (18,19).

Increased expression of MT-3 can neutralize the sensation of metallic taste in cancer patients after chemotherapy. Increasing the MT-3 expression score with a combination of doxorubicin and vitamin E showed a higher increase in the MT-3 expression score compared to the combination of giving doxorubicin and mung bean sprouts. So, it can be concluded that the administration of antioxidant vitamin E is more effective in inducing MT-3 expression related to metallic taste after chemotherapy (20).

The weaknesses of this research are the rats used as objects of research are easily dead, the treatment is difficult and requires patience in taking care of mice (feeding hours, etc.). In addition, there are other cancer markers besides ROS, which have not been done in-depth research and only discuss the metallic taste study of ROS. Future studies can use a metallic taste approach that is associated with a review of apoptosis, BAX genes, BCL-2, and links to AGE-RAGE.

CONCLUSION

This summary of this research is the increase in MT-3 expression scores in mice induced by doxorubicin and antioxidants from vitamin E or mung bean sprouts can be used as a neutralizing agent of metallic taste in cancer patients due to chemotherapy.

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