

Effectiveness of Vitamin C and Vitamin E Antioxidant Combination on Caspase-3 Expression in Wistar White Rat Pulmonum Contusion

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Abstract

Introduction: Pulmonary contusions can cause a progressive inflammatory response. Activation of TNF- α cytokines and reactive oxygen species (ROS) can cause pulmonary cell death. Antioxidants can have the potential to neutralize ROS. The purpose of this study is to determine the effectiveness of antioxidant administration in maintaining pulmonary cell function in wistar rats that have been induced to experience pulmonary contusions through caspase-3 levels.

Methods: This study was an in vivo experimental study conducted on thirty male wistar rats and divided into five groups (n = 6): control, pulmonary contusion + asthaxanthine 5 mg/kgBW, pulmonary contusion + vitamin C and E 50 mg/kgBW, pulmonary contusion + vitamin C and E 100 mg/kgBW, pulmonary contusion + vitamin C and E 200 mg/kgBW. The value of Caspase-3 is evaluated by the IHC. All data analyzes used SPSS 18.

Results: Low doses of antioxidants have the potential to reduce pulmonary cell death in wistar rats induced by pulmonary contusions.

Conclusion: Vitamin C and E effective to reduce pulmonary cell death in pulmonary contusion.

Keywords: antioxidants, vitamin C, vitamin E, pulmonary contusions animal model, apoptosis, caspase-3

1. Introduction

The lungs are the second most frequent organ involved in trauma cases, which often have a peri-hilar distribution and have a high risk of mortality. Pulmonary contusions are the most common intra-thoracic injuries associated with thoracic blunt trauma, about 17-25% of thoracic blunt trauma patients suffer from pulmonary contusions.^{1,2}

The exact mechanism of pulmonary contusions due to blunt trauma is still not understood, possibly due to various pathophysiological changes, including inflammatory processes, increased alveoli-capillary permeability, pulmonary edema, imbalance between perfusion-ventilation, increased intrapulmonary shunting, and loss of lung strength (lung compliance).¹ Clinically, patients with pulmonary contusions will show symptoms of hypoxemia, hypercemia, and increased respiratory work of varying severity.³ However, in some cases there is often a mismatched correlation between the volume of the lung that is controversial and the clinical severity of patients experiencing respiratory failure. This shows that there are inflammatory processes and injuries at the cellular and sub-cellular levels.

Pulmonary contusions are associated with a progressive inflammatory response mediated by the presence of immunological changes both locally and systemically. Lung damage directly triggers an inflammatory reaction which includes activation of blood leukocytes, activation of macrophages in lung tissue, and production of several mediators such as cytokines and chemokines.⁴ Neutrophils will significantly increase the severity of inflammation in pulmonary contusion injuries. The inflammatory response to the pulmonary contusion will induce cell damage and increase apoptosis / necrosis of pulmonary epithelial cells, as well as damage the integrity of the alveoli-capillary membrane. In general, lung injury will cause the formation of *reactive oxygen species* (ROS) by lung endothelial cells. In order to avoid undesired oxidative damage caused by ROS, every organism has an antioxidant mechanism.⁵

Antioxidant is a molecule that can delay or inhibit the oxidation process of other molecules. Oxidation is a chemical reaction that can produce free radicals, thus triggering a chain reaction that can damage cells. Antioxidants such as thiols or ascorbic acid (vitamin C) end this chain reaction. To maintain a balanced level of oxidation, plants and animals have a complex system, such as glutathione and enzymes, for example: catalase (CAT) and

Superoxide dismutase (SOD) produced in the body itself or can be obtained from intake of vitamin C and Vitamin E.

Astaxanthin is an antioxidant which is a group of natural pigments from carotenoids. Astaxanthin can prevent apoptotic cell death in mice due to oxidative stress and increased ROS through the MEK pathway. Astaxanthin can also deactivate caspase 3 so that cell death does not occur.

At Dr. Mohammad Hoesin Hospital himself patients with pulmonary contusions so far have received standard therapy. Therefore, researchers want to see the efficiency of providing a combination of antioxidants such as vitamin C and vitamin E as an additional therapy for pulmonary contusions by looking at decreasing caspase 3 expression. Researchers want to assess the effect of the addition of both types of antioxidants together on the incidence of pulmonary contusions, whereas in previous studies only one of them was given.

2. Methods

This research is an *in vivo* experimental study using pre-test and post-test control group. This research was conducted in the pre-clinical testing laboratory and bio-molecular laboratory of the Faculty of Medicine, Sriwijaya University. The study was approved for 1 month. The sample of this study was white Wistar male rats (*Rattus norvegicus*), which were 2-3 months old, with mice weighing between 150-200 grams.

Retrieval of 36 research subjects was done by random sampling. At the time of grouping the sample randomization, which is called the random allocation of mutations. All mice are numbered 1 to 36. This number is drawn, and each mouse has the same opportunity to be included in each group. The first collection was included in group 1, the second collection was included in group 2. The six collection was included in group 1, and so on, until all the rats were evenly divided into 6 groups.

The procedure of this research was started by preparing the mice. Experimental animals were obtained from the Pre-Clinical Test Laboratory Faculty of Medicine, Universitas Sriwijaya, housed in stainless steel cages, with the required cage volume of at least 500 cm² for two mice and a minimum height of 20 cm cage, fed with pellets and given adequate drinking water.

After that, pulmonary contusion was induced. Before induction of pulmonary contusions, experimental animals were anesthetized with ketamine 50 mg/kgBW. Experimental animals were made to experience pulmonary contusions using techniques described by Raghavendran etc. A cylindrical load (400 grams) is dropped from a certain height (50 cm) through a vertical stainless steel tube positioned on the Lexon platform. This device is supported by Teflon on all four sides to minimize friction and allow energy transfer. Then the animal will be placed under the platform and given a precordial shield to avoid contusions in the heart myocardial, so that the resulting trauma will only cause bilateral pulmonary contusions. Furthermore, rats will be grouped into six groups consisting of control groups and given antioxidants with various doses. In addition, a perfusion technique with perfusion was performed to fix the white wistar rat lung organs before organ evacuation was carried out to prevent organ damage.

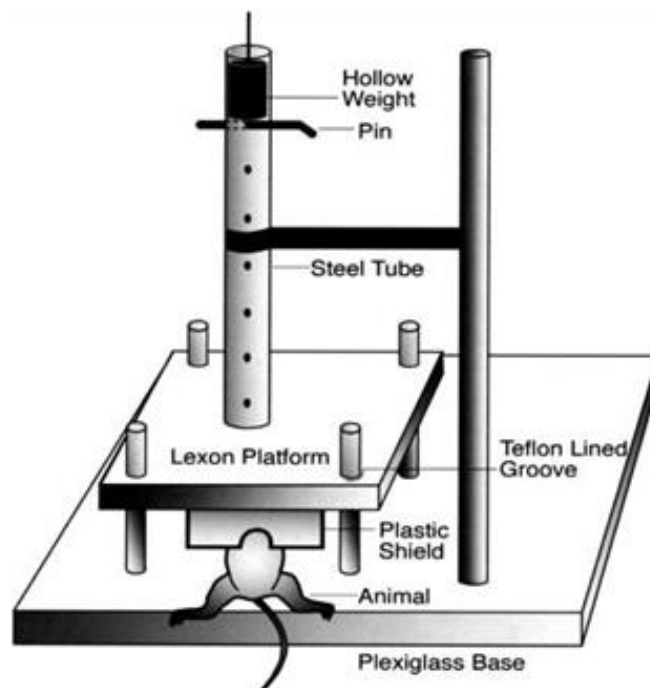


Figure 1. Pulmonary contusion induction method

Table 1. Group Division

Group	Remarks
Group 1	as many as 6 normal white rats
Group 2	as many as 6 white rats as negative controls were given aquadest 5 mL.
Group 3	as many as 6 white rats who received Astaxanthine 5 mg / kgBW
Group 4	as many as 6 white rats that get vitamin C 50 mg / kgBW and vitamin E 50 mg / kgBW
Group 5	as many as 6 white rats that get vitamin C 100 mg / kgBW and vitamin E 100 mg / kgBW
Group 6	as many as 6 white rats that received vitamin C 200 mg / kgBW and vitamin E 200 mg / kgBW

The process of lung evacuation in experimental animals is carried out by the step of making an incision in the midsternalis until the lung is exposed. Open the sternum bones of animals try carefully, not to damage lung tissue. Evacuation of experimental animal lungs, then enter into PBS with a volume of 10 x the volume of the brain to make paraffin block histological sedian.

Making histological preparations through four processes, namely dehydration, clearing embedding, and sectioning. The dehydration process is done in stages by using graded alcohol, starting from alcohol at a concentration of 70%, 80%, 95% up to the level of absolute alcohol (99%) and alcohol-toluene. The alcohol volume is ten times the tissue volume. At each concentration, tissue samples were inserted for 15 minutes, each concentration was repeated 3 times. The clearing process uses Xylol (Xylene) solution. The sample tissue is put into the xylol solution for 30 minutes until the sample tissue turns yellowish. Furthermore, the sample tissue is put into saturated paraffin-toluene for 12 hours, then put into liquid paraffin, then embedding. Cutting the sample tissue is done by rotary sectioning.

The next step is to conduct an immunohistochemistry examination of Caspase-3, and conduct an assessment of caspase-3 activity with Digital Image Analysis.

3. Results

This research was conducted in June - July 2017 at the Animal House and Biomolecular Laboratory of the Faculty of Medicine, Universitas Sriwijaya, Indonesia. Data were analyzed for normality using the Shapiro-Wilk test

To see the achievement of pulmonary contusions, TNF α levels were measured before and after induction. Induction is carried out by dropping a cylindrical load (400 grams) from a certain height (50 cm) through a vertical stainless steel tube.

Table 2. Test for normality of TNF α levels before and after pulmonary contusion induction

Variable	Treatment group(n	Before Induction	P
	= 6 per group)		
TNF α	Before Induction	17,22 \pm 0,407	0,523
	After Induction	98,3 \pm 1,819	0,869

With the Shapiro-Wilk normality test, it is obtained the probability value of both variables > 0.05 , which means the data distribution is normal, because the data distribution is normal, so to see the effect of pulmonary contusion induction on TNF α levels, paired-sample t test is used (Paired-Sample T Test).

Table 3. Effects of Pulmonary Contusion Induction on TNF Levels α

Variable	Before Induction	After Induction	Change	p
TNF α	17,22 \pm 0,407	98,3 \pm 1,819	81,08 \pm 2,187	0,000

From the statistical analysis, the probability value of TNF $\alpha < 0.05$. This shows that there are differences in the mean TNF α levels before and after induction, where an increase in TNF α levels is 4x times the initial level. It can be concluded that the pulmonary contusion condition has been reached.

To see the effect of giving vitamin C and E, caspase 3 expression was measured before and after administration of vitamins C and E. Previous data normality tests were done using Saphiro Wilk obtained the probability of all variables before treatment < 0.05 which means the data distribution is not normal, so to determine the effect of giving vitamin C and E all groups used an analysis with the Wilcoxon test.

Table 4. Test Caspase 3 Expression Normality Before and After Treatment

Variable	Average \pm SD	P
Caspase 3 Expression Before	20,17 \pm 18,22	0,000
Caspase 3 Expression After		
Aquadest	56,57 \pm 1,616	0,924
Astaxanthin 5mg/kgBW	44,97 \pm 1,373	0,603
VCE 50mg/kgBW	32,02 \pm 0,366	0,807
VCE 100mg/kgBW	47,83 \pm 1,218	0,275
VCE 200mg/kgBW	61,33 \pm 1,375	0,932

From Saphiro Wilk's analysis, the probability of all variables before treatment < 0.05 , which means the data distribution is not normal, so to find out the effect of giving vitamin C and E all groups used an analysis using the Wilcoxon test.

Table 5. Effects of Provision of Vitamins C and E on Caspase Expression

Group of rats	After Treatment	Change	Percentage	P
Aquadest	56,57 ± 1,616	36,4 ± 18,22	Up 180%	0,046
Astaxanthin	44,97 ± 1,373	24,8 ± 18,29	Up 122,9%	0,046
VCE 50 mg/kgBW	32,02 ± 0,366	11,85 ± 18,23	Up 58,75%	0,345
VCE 100 mg/kgBW	47,83 ± 1,218	27,67 ± 18,25	Up 136,8%	0,046
VCE 200 mg/kgBW	61,33 ± 1,375	41,17 ± 18,27	Up 204,1%	0,028

Table 5 shows the mean expression of caspase 3 for each group before and after administration of vitamins C and E. Caspase 3 expression before and after administration of vitamins C and E were then analyzed by the Wilcoxon test, obtained probabilities values of the aquadest, astaxanthin, vitamin C and E groups of 50 mg / kg and vitamin C and E 100 mg / kg < 0.05. This shows that there are differences in the mean expression of caspase 3 giving vitamins C and E in the aquadest group, astaxanthin, vitamin C and E of 50 mg/kg body weight and vitamin C and E 100 mg/kg body weight where there is an increase in expression of caspase 3 by 180% after administration of aquadest, an increase in amount to 122.9% after administration of astaxanthin, an increase of 136.8% after administering vitamin C and E doses of 100 mg/kgBW and an increase of 204.1% after administering vitamin C and E doses of 200 mg/kgBW.

However, in the 50 mg/kgBW vitamin C and E group, a probability value of 0.345 (> 0.05) was obtained so that it was concluded that there was no difference in caspase 3 expression of vitamin C and E administration after 50 mg/kgBW vitamin C and E after administration of 50 mg/kgBW, which occurred increased expression of caspase 3 by 58.75% after administering vitamin C and E doses of 50 mg / kg but not significant. The increase in

caspace 3 expression was greatest in the group giving vitamins C and E at a dose of 200 mg/kgBW and the lowest in the group giving vitamins C and E at a dose of 50 mg/kgBW.

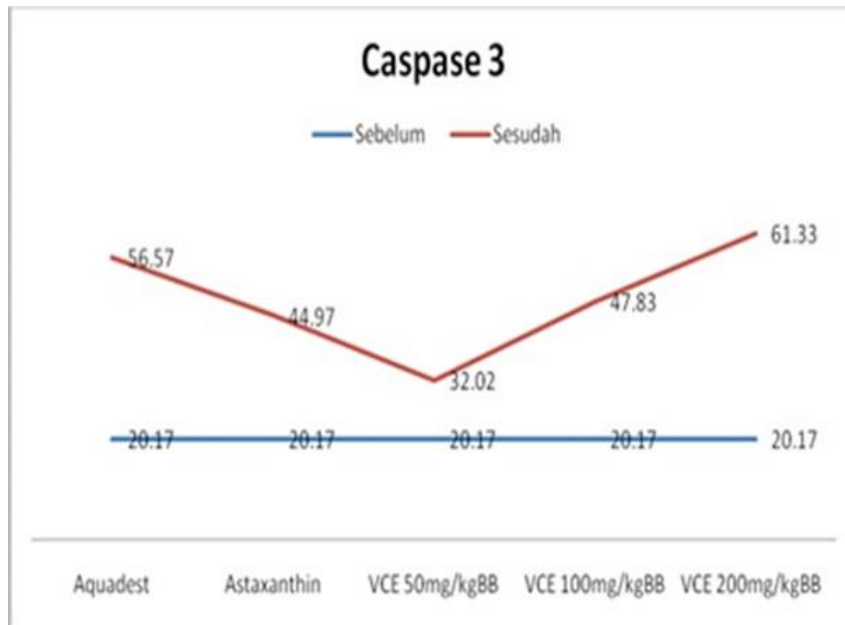


Figure 2. Effect of Giving Astaxanthin and Vitamins C and E on Caspace 3 Expression

Caspase 3 expression after treatment of all groups was measured then averaged and tabulated next caspace 3 expression after compared to each group. The mean caspace 3 expression after treatment can be seen in table 6.

Table 6. Average Caspace Expressions 3 after the Treatment

Group of rats	Caspase Expressions 3 After the Treatment
Aquadest	56,57 ± 1,616
Astaxanthin	44,97 ± 1,373
VCE 50 mg/kgBW	32,02 ± 0,366
VCE 100 mg/kgBW	47,83 ± 1,218
VCE 200 mg/kgBW	61,33 ± 1,375

The mean caspase 3 expression after treatment for all groups was between (32.02 ± 0.366) and (61.33 ± 1.375). With the Shapiro-Wilk normality test, the probability value of caspase 3 expression after the treatment of all groups > 0.05 , which means the data distribution of each group is normal, because the data distribution is normal then to see the average comparison of caspase 3 expressions between groups using the Independent T test (Independent T-test). The results of the analysis can be seen in table 7.

Table 7. Comparison of Caspase 3 Expressions between Groups

Group	Average \pm SD	Group	Average \pm SD	p value
Aquadest	$56,57 \pm 1,616$	Astaxanthin	$44,97 \pm 1,373$	0,000
		VCE 50 mg/kgBW	$32,02 \pm 0,366$	0,000
		VCE 100 mg/kgBW	$47,83 \pm 1,218$	0,000
		VCE 200 mg/kgBW	$61,33 \pm 1,375$	0,000
Astaxanthin	$44,97 \pm 1,373$	VCE 50 mg/kgBW	$32,02 \pm 0,366$	0,000
		VCE 100 mg/kgBW	$47,83 \pm 1,218$	0,063
		VCE 200 mg/kgBW	$61,33 \pm 1,375$	0,000
VCE 50mg/kgBW	$32,02 \pm 0,366$	VCE 100 mg/kgBW	$47,83 \pm 1,218$	0,000
		VCE 200 mg/kgBW	$61,33 \pm 1,375$	0,000
VCE 100mg/kgBW	$47,83 \pm 1,218$	VCE 200 mg/kgBW	$61,33 \pm 1,375$	0,000

From the results of the *Independent T test*, the probability value between the positive control group (Astaxanthin) with the negative control group (aquadest) and the group giving vitamins C and E all doses <0.05 (Table 7). Thus it can be concluded that the mean difference

in caspase 3 expression between the positive control group and the negative control group (aquadest) and the group giving vitamins C and E all doses. The mean caspase 3 expression of the astaxanthin group was greater than the 50 mg/kg body weight vitamin C and E dose group but smaller than the negative control group, the vitamin C and E administration group 100 mg / kg body weight and 200 mg/kg body weight.

After a bivariate test, caspase 3 expression was then analyzed by multivariate test with Oneway ANOVA. From the *Oneway ANOVA test* results obtained a probability value of 0,000 ($p < 0.05$). Thus there is a significant difference in mean caspase 3 expression between all groups.

Before conducting the Post Hoc confirmation test we first do a homogeneity test to see which test we will use in the Post Hoc analysis, by using a homogeneity test (*Levene's test*) a probability value of 0.347 ($p > 0.05$) means that the caspase 3 expression of all groups is homogeneous (variance) so that it is continued with the Post Hoc confirmation test using the Tukey Test. From the *Post Hoc* test results obtained probability values between the positive control group (astaxanthin) with the negative control group and the group giving vitamins C and E all doses < 0.05 . Thus it can be concluded that the mean difference in caspase 3 expression between the positive control group (astaxanthin) with the negative control group and the administration of vitamins C and E at all doses.

4. Discussion

The research subjects in this study were male Wistar strain white rats selected as experimental animals. The selection of these experimental animals is based on the fact that these animals are used for previous laboratory research so that the required data or information is easily obtained, besides the animals are available in standard strains so that a uniform genetic background is obtained, so that for the same treatment, each animal will provide the same response. Finally, the selection of these animals is also based on the closeness of the characteristics or traits under study with humans. On this consideration, researchers chose white rats because the required data is easily obtained, available in standard strains of the *Wistar strain*, and has similarities with humans.

Pulmonary contusions occur because of the rapid deceleration mechanism in the

collision process between the chest wall and the object. The condition of contusion in this study was assessed by measuring TNF α levels. In this study the results showed a significant increase ($p = 0,000$) TNF α levels by 4x times the initial levels after induction of pulmonary contusion so that it can be concluded that the condition of pulmonary contusion has been reached.

The inflammatory response to the pulmonary contusion will induce cell damage and increase apoptosis / necrosis of pulmonary epithelial cells, as well as damage the integrity of the alveoli- capillary membrane. In conditions where pulmonary contusions occur, activation of the inflammatory response will occur, which will then be followed by the production of free radicals. Increased free radicals (oxidants), will be followed by an increase in the secretion of endogenous antioxidant enzymes. If there is hyper-saturation of endogenous antioxidant enzymes in the body due to the inflammatory and oxidant responses that are formed very much, then the body has no defense against oxidants, which will lead to mitochondrial stress, which will then activate cell apoptotic signals mediated by Caspase-3.⁵

In this study, caspase 3 expression was examined before and after treatment. The treatment given is a combination of vitamins C and E in various doses in the treatment group and the administration of Astaxanthin 5mg / kgBW in the positive control group and administration of aquadest in the negative control group. With statistical analysis the results showed a significant increase in expression of caspase 3 by 180% after administration of aquadest, 122.9% after administration of astaxanthin, 136.8% after administration of vitamins C and E doses of 100 mg / kg and of 204.1% after administration of vitamin C and E dose is 200mg / kgBW. Meanwhile, the 50mg / kgBW vitamin C and E group gave an increase in caspase 3 expression of 58.75% but it was not significant. The increase in caspase 3 expression was greatest in the group giving vitamins C and E at a dose of 200mg / kgBW and the lowest in the group giving vitamins C and E at a dose of 50mg / kgBW.

Astaxanthin is an antioxidant which is a group of natural pigments from carotenoids. Astaxanthin can prevent apoptotic cell death in mice due to oxidative stress and increased ROS through the MEK pathway. Astaxanthin can also deactivate caspase 3 so that cell death does not occur. This is not in accordance with the results of this study where the increase in caspase 3 still occurs significantly after administration of astaxanthin 5mg / kgBW.

Vitamin C is a class of antioxidant vitamins that are able to ward off various extracellular free radicals.⁶ Vitamin C is an antioxidant that plays an important role in helping maintain cell health. Vitamin C works as a coenzyme and in certain circumstances is a reducing agent and antioxidant.^{7,8} While vitamin E is the main antioxidant in all cellular membranes, and protects unsaturated fatty acids against oxidation events. As an antioxidant in the body, vitamin E acts as a scavenger (catcher) of free radicals that enter the body or are formed in the body from normal metabolic processes.

In this study the administration of vitamins C and E at a dose of 50 mg/kg body weight can be said to be effective in reducing lung cell death in pulmonary contusion because even though there is an increase in caspase 3 expression after administration of vitamin C and E at a dose of 50 mg/kg body weight, it is not significant.

Based on the bivariate and multivariate analysis, it was concluded that the mean difference in caspase 3 expression between the positive control group (astaxanthin) with the negative control group and the vitamin C and E groups with all doses. Although there is a significant difference in caspase 3 expression between the positive control group of astaxanthin and the vitamin C and E groups at a dose of 50 mg/kgBW, it can be concluded that the administration of vitamin C and E doses of 50 mg/kgBW is more effective in reducing pulmonary cell death in pulmonary contusion than astaxanthin due to the astaxanthin group vitamins C and E have less caspase 3 expression than astaxanthin.

The addition of doses of vitamins C and E ranging from 50 mg/kgBW to 100 mg/kgBW and 200 mg/kgBW apparently did not increase its effectiveness, seen from the expression of caspase 3 which should decrease even more with increasing doses. This shows that the administration of vitamins C and E in reducing cell death in pulmonary contusions is Flat dose Curve that is effective.

5. Conclusion

Giving antioxidant combination of vitamin C and vitamin E as an effective adjunct therapy to reduce lung cell death in pulmonary contusions of white Wistar rats (*Rattus norvegicus*). The administration of Vitamin C and E at a dose of 50 mg/kgBW was effective in reducing the expression of caspase-3 in the pulmonary Wistar white mouse lung cells.

6. References

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