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THE POTENTIALITY OF ß- CAROTENE AS FREE RADICAL SCAVENGER: A STUDY OF THE RAISE OF SUPEROXIDE PRODUCTION IN MITOCHONDRIAL LYMPHOCYTE

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Abstract

This is a study on carotenoid extracted from 'Minyak Buah Merah' (*Pandanus conoideus*) in therapy simulation using high dose of β-carotene on two lymphocytes—that of a healthy subject and a leukemia patient. Superoxide production in mithocondrial lymphocyte as a result of β-carotene exposure was examined using a flow cytometer. The test finds that superoxide (a specific mitochondrial free radical) increases easier and faster in leukemic lymphocytes than in normal lymphocytes. The results of the study support previous meta-analysis studies that high dosages of β-carotene and vitamin E supplements as antioxidant should be discouraged because it increases all-cause mortality.

Keywords: antioxidant, β-carotene, free radical, superoxide, lymphocyte

1. Introduction

Controversies regarding the rich source of β-carotene in *Pandanus conoideus* (red papua fruit) has been much talked about. In correlation to that, many believe that *pandanus conoideus* is a potential source of income for the country. What we would like to highlight in this paper is, the dicussion should be shifted onto a more important point, that is the need to find an extraction technique for the fruit that is efficient and effective enough. This is more crucial since a high dose use of *pandanus conoideus* has been assumed of making a sick patient worse.

Pandanus conoideus oil contains high concentration of vitamin E and β-carotene and widely sold as antioxidant (free radical scavanger). Lay people, specifically people with sickness, consume *Pandanus conoideus* oil in the hope to restore their health.

According to a meta-analysis done by Miller III ER (2004) [1] since 1966 to August 2004 and Vivekananthan [2], vitamin E and β-carotene are capable to increase all-cause of death. Therefore, it might provide an opposite effect to what people expect when they comsume it to cure sickness. Its potential toxicity should be given closer attention and if its potential toxicity verified, then its consumption as well as its dispension should be handled more carefully.

Before, a high dose of this supplement was not thought of to be able to bring any hazard, although some researches showing that increased mortality rate is associated with high dose β-carotene had already started to emerge [2]. Mc Gills [3] found that in a healthy subject supplemented with β-carotene, leukocyte superoxide dismutase (SOD) activity decreased significantly ($p < 0.05$) at 30 and 60 days compared to baseline. The concentration of glutathione peroxidase serum also decreased significantly ($p < 0.05$) in humans. Similarly, Li et al. [4] revealed the effects of Cu/Zn SOD on strain injury has induced oxidative damage to the skeletal muscle. Low SOD defence (stress oxidative condition) caused serious injured cell to cell apoptosis or death [5]. Mitochondrial superoxide is generated as a by-product of oxidative phosphorylation. (Figure 1). In otherwise tightly coupled electron transport chain where approximately 1-3% of mitochondrial oxygen consumed is incompletely reduced, those *leaky* electrons can quickly interact with molecular oxygen to form superoxide anion, the predominant reactive oxygen species (ROS) in mitochondria. The increase of cellular superoxide production has been implicated in physical, chemical, biological and metabolic injury.

Based on the literature study on the potential toxicity of β-carotene and vitamin E to increase all-cause mortality, this study aims to verify that *Pandanus conoideus* oil, which contains high concentration of vitamin E and β-

carotene, has the potential to increase all-cause mortality and prove Miller's and Vivekananrhan's meta-analysis. β-carotene acts as free radical scavenger, but in many cases where humans consumpt it too much, it can acts also as predominant reactive oxygen species (ROS) that are dangerous and induce reactive nitrogen species (RNS).

To note, the raise of cellular superoxide production has been implicated in cardiovascular diseases, including hypertension, atherosclerosis, and diabetes-associated vascular injuries. This study measured the change in mitochondrial superoxide production in blood samples when exposed to β-carotene. The reaserch uses a computerized fluorescence microscope equipped with a flow part (flow cytometer), that is routinely used for measuring CD4/CD8 (HIV AIDS). This equipment can be used as long as the subject observed acts as an fluorophore that produce luminosity. The output of this study is a method or protocol in detecting chemical, superoxide and organelle structures, especially mitochondria which creates the most energy.

2. Methods

The study requires two blood samples, that of a healthy volunteer and of a leukimic patient. RPMI is used as culture medium to maintain the blood cells. The two samples are exposed to β-carotene that derives from two sources: pure β-carotene from B-Merck (later on refered to as "'pure'-carotene") and caretonoid from *Pandanus conoideus* (later on refered to as "'non-pure'-carotene"). The analysis later on uses post-hoc method.

In Indonesia, β-carotene contained in *Pandanus conoideus* oil in one of the products in market is 700 ppm. The concentration of carotenoid in this oil is rechecked by extracting the matter and then measuring it with HPLC. The product listed information of total carotenoid 12,000 ppm, total tocopherol (vitamin E) 11,000 ppm, alpha tocopherol 500 ppm, oleic acid 58%, linoleic acid 8.8%, linolenic acid 7.8%, and decanoat 2.0%.

To help with visualization during the test, the blood samples are given MitoTrackerGreen (MTG) and MitoSoxRed (MSR). These dyes are used since MSR mitochondrial superoxide indicator is a fluorgenic dye for highly selective detection of superoxide in mitocondrial living cells. The β-carotene exposure is done in a number of time frames and is later on measured in order to find a Mean Fluorescence Intensity of MTG and MSR.

Preceeding this study, a preliminary study was conducted to find the appropriate time span for exposing β-carotene to the blood sample using spermatozoa cells. Mitochondria visualization with MitoTrackerGreen (MTG) with β-carotene concentration of control, 2, 8, and 24 ppm were done at 2, 36, 48, and 96 hours. There is a decreasing visualization of MTG with high dose of 8 and 24 ppm. Even at 2 ppm, the visualization was low and spermatozoa began to die. This initial test shows that the main tests shoud be conducted with standard fluorescence microscope and flow cytometer at β-carotene concentration of control, 2, and 4 ppm between 2 and 24 hours.

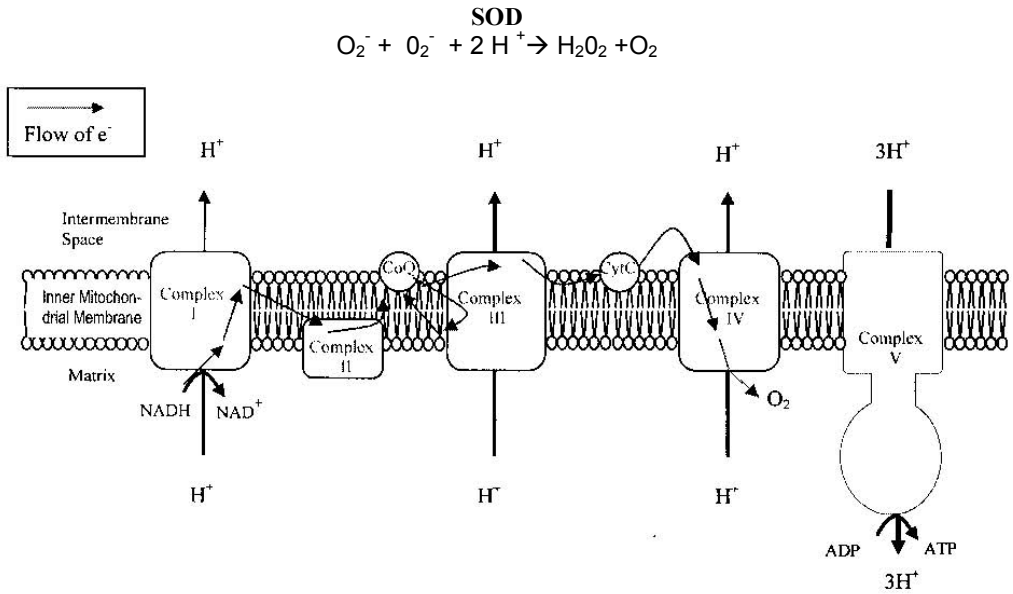


Figure 1. Electron Transport in Oxidative Phosphorylation

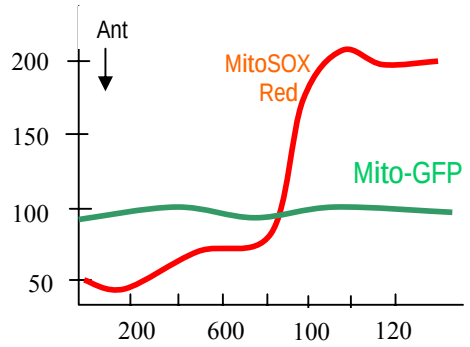


Figure 2. Representative Tracings of Mito-GFP (Green Fluorescent Protein) and MSR Fluorescence in Response to Antimycin A (AntA) 2 µM Showing an Increase on MSR Fluorescence in Stimulated Cells (Modified from Mukhopadhyay, 2007 [6])

Time sequences were 200, 400, 600, 800, 1000, 1200, 1400 seconds Mukhopadhyay et al. [6] studied a simple quantitative detection of mitochondrial superoxide production in living cells. Superoxide in this study was measured by flow cytometry and confocal microscope. Complex III inhibitor Antimycin A 20 µM time-dependently increases mitochondrial-derived superoxide generation measured by flow cytometry (Figure 2). Based on the finding of Mukhopadhyay's study, mean fluorescence intensity was measure after 5, 10, 20, and 40 minutes after exposure to β-carotene.

Mean fluorescence intensity of MTG (488/516nm) and MitoSoxRed-MSR (510/580nm) was measured with the back-ground of CD3PE (Em 578nm) and CD3-FITC (Em 522 nm). Gating was done with the help of CD 45 PE-Cy5.5 (Em 694nm) for control Positive SSC-H pan leukocyte and CD3-PE and CD3-FITC as a pan T lymphocyte marker

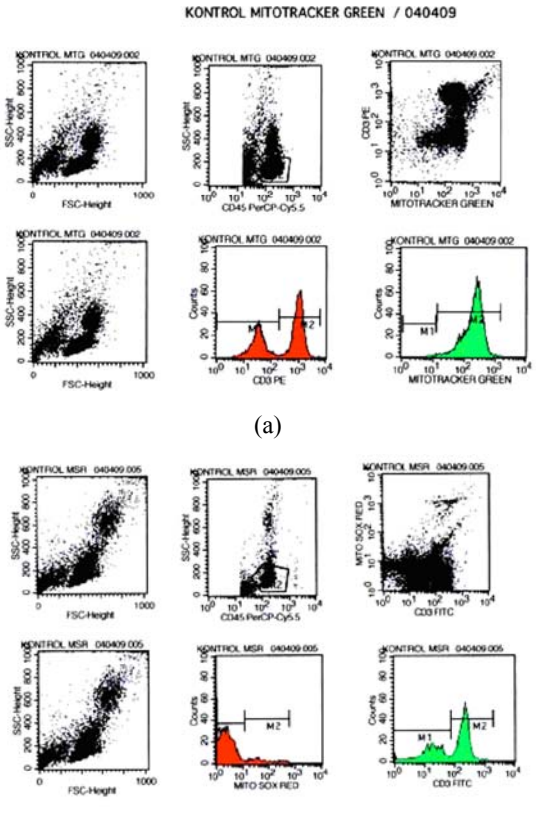
3. Results and Discussion

The mean superoxide (dyed with MSR) for total intensity of Ficoll total normal T lymphocyte (CD3-FITC) exposed to a 2 ppm dose of β-carotene is increasing along with time, reaching a peak at 20 minutes (Table 1). Mean total intensity for mitochondrial dyed with MSR is higher at 4 ppm than in 2 ppm. The β-carotene used in this phase is 'pure' β-carotene extracted from carrot by Merck.

Meanwhile, superoxide (MSR) of normal leucocytes with CD45PerCP-Cy5.5 exposed to a 2 ppm of pure β-carotene in 5 minutes (Figure 4) vs. 40 minutes shows an increase of B lymphocytes superoxide (see upper left quadrant) in 40 minutes. In leukemia patient, the cells were much brighter in T and non T lymphocyte than in normal subject as what can be seen in Figure 6.

Table 1. Mean Total Intensity of MSR in Normal T Lymphocyte (CD3-FITC)

B-Merck (minutes)	2 ppm
Control	38,31
5	56,03
10	78,19
20	188,34
40	158,99



(a)

(b)

Figure 3. Flow Cytometric Measurement of (a) Mitochondria (MTG) and (b) Superoxide (MSR) in Control Normal Lymphocytes

Tabel 2. Mean Total Intensity MTG in Normal Lymphocyte

MTG	C	2ppm	4ppm
Tot	232	299	320
M1	9.8	4	*
M2		299	320

M2 and Total (Fig. 3) in 4 ppm (vs. 2 ppm) and 40 minutes is higher than shorter time 2 ppm exposure of *Pandanus conoideus*. The mean total intensity of superoxide by MSR for both pure beta-carotene (B-Merck) and carotenoid (*Pandanus conoideus*) reaches a peak at 20 minutes (Table 4).

Tabel 3. Mean Total Intensity Superoxide (MSR) in Therapheutic Dose of 2 ppm Pure β-Carotene (Merck) 2 ppm with 5-10-20-40 Minutes in Normal Lymphocytes

MSR	Tot	M1	M2
C	4.79	2.38	64.0
5’	5.52	2.42	64.0
10’	7.55	2.96	60.2
20’	7.41	2.40	53.6
40’	10.1	2.61	67.9

Tabel 4. Mean Total Intensity of MSR at a Therapeutic Dose of Pure β-Carotene (Merck) vs. Carotenoid (*Pandanus conoideus*) with a 5-10-20-40 Minute Time Administrated Sequence on Leukemia Patient

2ppm	B-Merck	K-*Pandanus conoideus*
C	31,84	31,84
5’	13,45	18,16
10’	23,52	9,09
20’	46,58	192,57
40’	29,14	19,58

Table 5. Superoxide in T Lymphocytes (UR Upper Right) Quadrant of a Normal Subject

	MI	B/Tot	T/Tot	B+T/Tot	UR/T-L
5’	56.03	0.17	17.71	47.92	29.31
40’	155.99	0.71	15.07	75.58	45.21

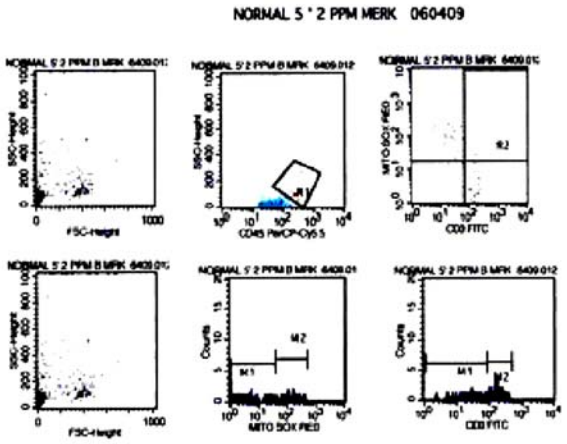


Figure 4. Normal Subject, 2 ppm Merck 5 Minutes

Table 6. Mean Intensity of Superoxide (MSR) in Upper Right Area (%MSR+CD3+)/Total CD3+ in based on the Concetration β-carotene and Time Exposure on a Normal Subject

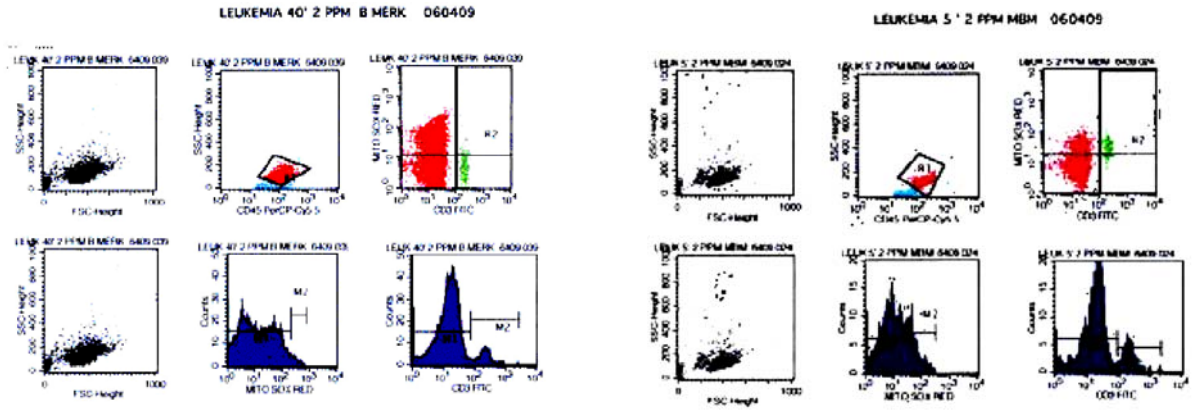
Normal	Time	MI	%MSR+/ total	%MSR+/ lymphocytes	%CD3+/ lymphocytes	%MSR+CD3+/ total CD3+
2 ppm	0	38.31	4.19	58.11	77.8	74.69
	5	56.03	0.17	17.71	60.42	29.31
	10	78.19	0.14	9.33	45.33	20.58
	20	188.34	0.25	13.81	23.75	58.15
	40	155.99	0.71	15.07	33.33	45.21
4 ppm	0	38.31	4.19	58.11	77.8	74.69
	5	80.69	0.19	25.33	50.66	50.00
	10	77.38	0.13	10.16	43.75	23.22
	20	196.18	0.16	6.32	13.04	48.47
	40	127.06	0.54	10.17	29.19	34.84
8 ppm	0	38.31	4.19	58.11	77.8	74.69
	5	71.55	0.19	22.09	50	44.18
	10	56.09	0.15	13.16	50.88	25.86
	20	157.27	0.19	6.11	16.08	38.00
	40	150	0.61	11.34	24.72	45.87

There is a higher superoxide production in upper right quadrant (high MSR T lymphocyte) in 40 minutes than 5 minutes of normal lymphocytes (Table 5). Also, a higher production of superoxide was found in leukemia lymphocytes with 2 ppm *Pandanus conoideus* after 5 minutes (69.17%) than B-Merck (11.32 %) (Table 8). Similar finding was not found in normal lymphocytes/ normal subject (Table 6).

There is a higher production of superoxide in a 2 ppm concentration, pure β-carotene with 40 minutes of exposure and non pure β-carotene from *Pandanus conoideus* of 5 minutes exposure in leukemic lymphocytes/ unhealthy patient (leukemia), shows by the upper right quadrant (UR) that appeares to be brighter (Figure 5b). It means that MSR in T lymphocyte was produced more. In non T lymphocyte

(left area), superoxide was produced highly as well. However, not every non T cells has produce high superoxide (as observed in lower left quadrant).

In the leukemic patient, 40 minutes and 2 ppm concentration exposure of *Pandanus conoideus* gives a percentage of MSR in T cells/ total T cell that is lower (21.92%) than the peak occured in 20 minutes exposure (100%) (Table 8). Pure β-carotene from B-Merck at 20 minutes of exposure only gives 31.16% (vs. 100% in *Pandanus conoideus*).



(a) (b)

Figure 5. Leukemia (a) 40 Minutes 2 ppm B Merck and (b) Leukemia 5 Minutes 2 ppm *Pandanus conoideus* (Brighter in UR Quadrant)

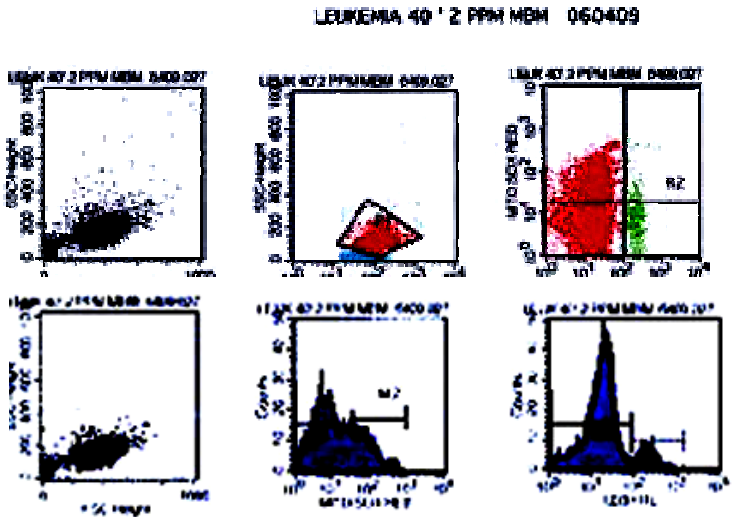


Figure 6. Leukemia 2 ppm *Pandanus conoideus* 40 Minutes

Table 7. Superoxide in T Lymphocytes (UR-Upper Right) Quadrant from Normal Subject

	MI	B/Tot	T/Tot	B+T/Tot	UR/T-L
5’	18.16	1.73	7.56	30.25	69.17
20’	192.57	0.37	18.5	97.5	100

Tabel 8. Mean Intensity of Superoxide (MSR) in Leukemic Subject with Pure β-carotene (B-Merck) and Non-Pure β-Carotene (*Pandanus conoideus* (Carotenoid)) of 2 ppm

MERCK					
Time	MI	% MSR+/ total	% MSR+/ Lymphocyte	%CD3+/ Lymphocyte	%MSR+CD3+/ total CD3+
0’	31.84	5.92	10.82	11.97	90.39
5’	13.45	0.18	1.54	13.6	11.32
10’	23.52	0.12	0.65	7.09	9.17
20’	46.58	0.38	2.82	9.05	31.16
40’	29.14	0.99	1.61	7.13	22.58
PANDANUS CONOIDEUS					
Time	MI	% MSR+/ total	% MSR+/ Lymphocyte	%CD3+/ Lymphocyte	%MSR+CD3+/ total CD3+
0’	31.84	5.92	10.82	11.97	90.39
5’	18.16	1.73	7.56	10.93	69.17
10’	9.09	0.13	0.61	9.96	6.12
20’	192.57	0.37	18.5	18.5	100.00
40’	19.58	0.96	1.71	7.8	21.92

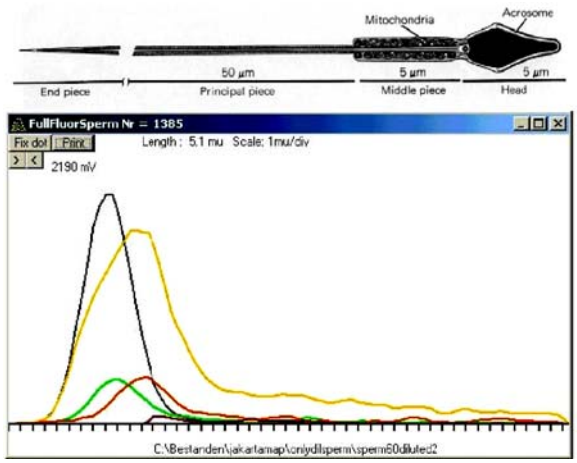


Figure 7. a) Scheme of Middle Piece of Spermatozoon; b) Seen with MTG by Flow Cytpmeter Analysis with Cytocluse was Checked by Standard Fluorescence Microscope as Middle Piece and Tail

MTG measurement using flow cytometer has shown a mean total intensity in carotenoid exposure from *Pandanus conoideus* oil. This is illustrated through the visualization caught by standard fluorescence microscope according to certain doses and time exposure. Even the 2 ppm therapeutic dose resulted into an increase of mean for total intensity of MSR as mitochondrial superoxide indicator specific mitochondrial free radical on 10-20 minutes earlier than the normally 40 minutes. This study explains and confirms Miller’s[1] meta-analysis and Vivekananthan’s study [2] that the high dose of β-carotene supplement and tocopherol increases all cause mortality. β-carotene as an antioxidant acts like two sides of the coin, it can be useful as well as harmful. β-carotene presents plentiful in *Pandanus conoideus* and can be an asset for world’s natural resources. However, β-carotene has an association with NO, iNOS, p53, Treg and lymphocyte proliferation. It is also a pathway to the increase of all cause mortality. It begins to increase p53 and with the high iNOS, it causes p53 mutation and becomes the cause for lung, colorectal and other carcinoma effects of high dose of β-carotene. Niedbala [7] has revealed the relation of NO, Treg via p53. Superoxide dismutase (SOD) opposes ROS formation in leukemia to program cell death, as confirmed by Maraldi [8]. Although Nitric oxide (NO) can induce apoptosis in a variety of cell types, a functional electron transport chain is required for nitric oxide-induced apoptosis during hypoxia [9]. Cytotoxicity as a results of NO/Superoxide will determine the impact of apoptotic cell death and survival of it has long been known [10].

6. Conclusions & Recommendations

These studies are consistent with the meta-analysis study saying high dose β–carotene increases all-cause mortality. This is supported by the total mean intensity of MSR in time sequence 5, 10, 20 to 40 minutes found by this study. In a leukemic patient, there was already decrease of superoxide in minute 5 and 10, increasing at 20 minutes (peak) and drastically decreases at 40 minutes. This could represent a failure of mitochondria (~dead mitochondria). Clinical experiment using β-carotene should be documented. Furthermore, the extraction of β-carotene, especially in large amount for industrial scope is a challenge. Therefore, Red Papua Oil, rich of β-carotene, should be proportionally handled and can be exported as a world β- carotene

source, but its potential toxicity has to be given closer attention.

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