Astaxanthin Improves Erythrocyte Sedimentation Rate (ESR), Malondialdehyde (MDA), 8-Hydroxydeoxyguanosine (8-OH-Dg) Levels, And Semen Quality In Human Sperm

Dian Nurmawati, Aucky Hinting, Sudjarwo

Abstract: Astaxanthin (3,3'-dihydroxy-4,4'-diketo-β,β'-carotene), which is originated from Haematococcus pluvialis has the highest antioxidant activity compared to vitamin C, vitamin E, coenzyme Q10, lipoic acid and other carotenoid groups. Antioxidant supplementation is the treatment of choice for treating idiopathic infertility in males. The purpose of this study was to determine the effect of astaxanthin on Erythrocyte Sedimentation Rate (ESR), Malondialdehyde (MDA) level, 8-Hydroxydeoxyguanosine (8-OH-dG) level, and semen quality in 25 infertile males in one hospital in Surabaya. Astaxanthin soft capsules in a single dose of 8 mg per day were given to 19 infertile males for 30 days, while 6 males were given with placebo for 30 days. ESR, MDA, 8-OHdG and semen quality tests were performed before and after astaxanthin and/or placebo administration. Results revealed significant differences (p<0.05) before and after the administration of astaxanthin on ESR, MDA, 8-OH-dG levels and semen quality (concentration, morphology and motility). Astaxanthin can significantly improve ESR, MDA, 8-OH-dG and semen quality in infertile males.

Keywords: Astaxanthin, MDA, ESR, 8-OH-dG, semen quality, male infertility, antioxidant

1 INTRODUCTION

The prevalence of infertility throughout the world has increased over time and has reached around 15% [1,2], the contribution of male factors to infertility by 36.8% [3] and causes in idiopathic men by 31.7% [4]. Oxidative stress (OS) causes 30%-80% of cases of infertility in males by reducing spermatozoa motility and suppressing fertility potential [5,6,7]. OS occurs because of the excessive production of Reactive Oxygen Species (ROS) so it can not be neutralized by antioxidants in the seminal plasma. ROS acts as an oxidizing agent which may cause oxidative damage to membrane lipids, proteins, and DNA [8]. In cases of idiopathic infertility, patients are advised to undergo ROS testing, one of which is by determining the levels of malondialdehyde (MDA), the final product of lipid peroxidation in human spermatozoa [9]. Measurement of MDA levels is an indirect measurement of free radical activity as an indicator of oxidative stress that can be done by visible spectrophotometer methods [5]. DNA oxidation produces 8-hydroxydeoxyguanosine (8-OHdG) compound as a marker of DNA damage [10] whose levels can be determined with an ELISA kit. ESR reflects changes in plasma protein that occur in acute or chronic infections that can affect sperm motility [11]. ROS causes a decrease in semen quality in terms of concentration, morphology and motility of spermatozoa [12]. Idiopathic infertility in males requires antioxidant supplementation which is proven to improve semen quality parameters [13].

Astaxanthin is an effective oral antioxidant supplement for the treatment of male infertility because it can improve one or more semen quality parameters [1,14]. Astaxanthin (3,3'-dihydroxy-4,4'-diketo-β,β'-carotene) is a carotenoid group compound that is isolated from the green algae Haematococcus pluvialis and has the highest antioxidant activity [15]. Astaxanthin activity is 40 times stronger than the carotene group, has an effectiveness of 500-1000 times better than vitamin E in preventing lipid peroxidation in vivo, 6000 times better than vitamin C, 800 times better than Coenzyme Q10, 560 times better than green tea extract, and 75 times better than lipoic acid [16,17,18]. This study aimed to determine the effect of the administration of astaxanthin in improving Erythrocyte Sedimentation Rate (ESR), MDA level, 8-OH-dG level, and semen quality in infertile males compared to placebo. The semen quality parameters observed were color, odor, pH, volume, viscosity, concentration, morphology and motility of spermatozoa, which were compared between conditions before and after therapy using astaxanthin.

2 METHODS

2.1 Study design and settings

This study was an experimental study. This study involved 25 married males who were infertility patients as participants. The participants were obtained from the Andrology Clinic, Dr. Soetomo Hospital, Surabaya. Each participant had signed an informed consent containing a statement of consent to undergo treatment and examination without coercion. The characteristics of the participants is shown in Table 1.

2.1 Number of the participants determination

The sample size or number of the participants was calculated using single population proportion formula with the following assumptions: p = prevalence of male infertile couples with idiopathic causes in the world 15% and in...
Indonesia is 1.11% [1,2,3,4,53], 95% confidence interval, and 5% margin of error (d).

\[ n = \left( \frac{Z_{\alpha/2}}{d} \right)^2 \frac{1 - p}{p} = \left( \frac{1.96}{0.05} \right)^2 \frac{(0.001665)(0.998335)}{(0.05)^2} = 2.55424 \]

Based on the formula above, obtained a sample size that must be examined at least three people. The selection of participants should use a simple random sampling procedure, that is, participants chosen directly randomly from a list whom meet the requirements to be participants. In research in the community this procedure is almost impossible to do so it is modified using the “design effect” so that to obtain the required precision the number of samples is twice the size [19,20,21]. Thus, the number of participants for the placebo group was 6 people or twice from the minimum number of samples while for the sample group numbered 19 people.

2.2 Treatment of the participants
The participants were divided into two groups, 19 in treatment group, and 6 in placebo group. Each participant in the treatment group received 8 mg of astaxanthin soft capsules per day in a single dose for 30 days, and the placebo group received 30 mg of CMC-Na capsules daily for 30 days. Astaxanthin used in this study were from the green algae Haematococcus pluvialis, in the form of 4 mg Na capsules.

2.3 Materials
Materials used were distilled water (Brataco), EDTA, NaCl 0.85%, Eosin B 0.5% solution in aquadest (Merck) and negrosin 10% solution in aquadest (Merck), oil immersion (Merck), methanol (Merck), safranin 0.1% in water (Merck), phosphate buffer with pH 6.8 (Sigma), 0.25 gram violet crystal (Sigma), acetate buffer pH 2.4 (Merck), NaHCO₃ (Sigma), 35% formalin (Merck), concentrated gentian violet liquid (Merck), 4 mg Astaxanthinsof capsules, and 30 mg CMC-Na capsules.

2.4 Instruments
Glass (Pyrex), Westergreen tube, Eliason pipette, centrifuge (Eppendorf 5403), multi-channel pipette (bio Rad, USA), hemocytometer (Perkin Elmer 9800), pH meter (Eutech), incubator (Forma Scientific), waterbath (Sorvall), microwave (National 800 W; IEC-705), vortex (Maxi Mix II type 37,600), phase contrast microscope (Nikon), centrifuge (Eppendorf 5403), water bath and HP 8452A Diode Array Spectrophotometer, Quick DNA Universal Kit, Zymo Research Brand Miniprep Kit catalogue no.: D4068 & D4069, and ELISA Kit (ab201734).

2.5 Collection of sperm
Sperm was preceded by a period of sexual abstinence (no discharge of sperm in any way) for 3-5 days. Sperm collection was done by masturbation and ejaculated into a clean, dry, wide-mouthed and closed container, ensuring all ejaculate was accommodated and the temperature was maintained between 20°C-40°C [22].

2.6 Determination of Erythrocyte Sedimentation Rate (ESR)
The ESR values in this study were determined using the modified Westergreen method, using EDTA blood samples diluted with 0.85% NaCl 1:4 then put in a Westergreen tube allowed to stand upright for 1 hour at a temperature of 18°-25°C [23].

2.7 Determination of Malondialdehyde (MDA) level

Procedure for determining MDA levels
As much as 0.5 mL sperm was added with 1.0 ml 30% TCA and centrifuged at 2500 rpm for 5 minutes. The supernatant was pipetted as much as 0.7 ml, 200 µl of 1% Na Thiobarbiturate was added, then 1 N HCl to 10.0 ml was added, then incubated for 135 minutes on a water bath. The uptake was observed with the spectrophotometer method at a wavelength of 532 nm [5].

The making of MDA standard solution.
MDA (50.4 mg) was mixed with distilled water to 100.0 ml (504.0 µg/ml), pipetted 1.0 ml added with distilled water to 10.0 ml (50.4 µg/ml). MDA (50.4 µg/ml) in pipettes of 6; 10; 12; 20; 30; 50; then 70 and 125 µl were added with 1.0 ml of 30% TCA and 200 µl of 1% Na Thiobarbiturate, incubated for 135 minutes on a water bath and the absorption was observed with a spectrophotometer at a wavelength of 532 nm [5].

2.8 Determination of 8-Hydroxydeoxyguanosine (8-OH-dG) level
Determination of 8-OH-dG concentration using ELISA kit (ab201734).

2.9 Analysis of sperm quality
According to procedure of WHO (2010), the analysis of sperm quality was performed on sperm color, odor, pH, volume, viscosity, concentration, morphology and motility at the time before and after astaxanthin or placebo administration.

2.10 Statistical analysis
Data were analyzed with correlation tests (Spearman Rank Correlation), comparison tests (Mann Whitney U Test & Wilcoxon Sign Rank Test) and cut-off points. Receiver Operating Characteristic (ROC) analysis was performed using IBM SPSS Statistics with significance level of 0.05. ROC analysis was used to determine cut-off value of factors that have correlations and significant comparison test results in motility.

3 RESULTS

3.1 Characteristics of the participants
Characteristics of the participants are listed in Table 1. Of the 25 participants, the average age was 25.48±6.6651 years, length of marriage was 3.93±4.7000 years, and the number of children was 0.41±0.7703. Participants with primary infertility indication were 72% and secondary infertility was 28%.

Table 1. Profile of the participants (N = 25)

<table>
<thead>
<tr>
<th>Biodata</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>25.48±6.6651 years old</td>
</tr>
<tr>
<td>Occupation</td>
<td></td>
</tr>
<tr>
<td>- Entrepreneur</td>
<td>12 (48%) persons</td>
</tr>
<tr>
<td>- Online driver</td>
<td>6 (24%) persons</td>
</tr>
<tr>
<td>- Government employee</td>
<td>5 (20%) persons</td>
</tr>
<tr>
<td>- No formal job</td>
<td>2 (8%) persons</td>
</tr>
<tr>
<td>Length of marriage</td>
<td>3.93±4.7000 years</td>
</tr>
<tr>
<td>Number of children</td>
<td>0.41±0.7703 children</td>
</tr>
<tr>
<td>Infertility status</td>
<td></td>
</tr>
<tr>
<td>- Primary infertility</td>
<td>18 (72%) persons</td>
</tr>
<tr>
<td>- Secondary infertility</td>
<td>7 (28%) persons</td>
</tr>
</tbody>
</table>

3.2 Erythrocyte Sedimentation Rate (ESR)
ESR observations in both groups showed that before treatment there were no significant differences (p>0.05), but after treatment there were significant differences between placebo and sample groups (p<0.05). ESR before and after treatment in placebo group did not show significant differences (p>0.05). ESR before and after Astaxanthin administration in the sample showed significant differences (p<0.05). ESR before and after treatment in the total group (placebo group and sample group) showed significant differences (p<0.05). The comparison of ESR before and after treatment in placebo and sample groups is as shown in Figure 1.

3.3 Level of Malondialdehyde (MDA)
Figure 3 shows the MDA levels before and after treatment in placebo and samples group. Observation of MDA concentrations in both groups showed that before treatment there was no significant difference, but after treatment there were significant differences between placebo and astaxanthin groups. The MDA before and after treatment in placebo group did not show significant differences. The sample group showed significant differences before and after Astaxanthin administration. All groups (placebo and sample) showed significant differences before and after treatment. Figure 4 shows MDA cut-off point on spermatozoa motility. ROC analysis on MDA showed of 0.662, cut-off value <1.98, sensitivity value of 100% and specificity of 35.3%. This shows that if the MDA value <1.98, the prediction of normal motility level (>40) is 100%. If the MDA value is >1.98, the prediction of abnormal motility level (<40) is 35.5%.
MDA level showed a negative correlation with spermatozoa motility, in an equation of $y=-2.1018x + 40.08$, which means that the higher the absorbance ($y$), the lower the MDA concentration level ($x$) with $R^2 = 0.0389$.

**3.4 Level of 8-Hydroxydeoxyguanosine (8-OH-dG)**

Figure 5 shows the 8-OH-dG level before and after treatment in placebo and sample groups. Observation of 8-OH-dG concentration in both groups showed that before treatment there was no significant difference, but after treatment there were significant differences between placebo and astaxanthin groups. Level of 8-OH-dG before and after treatment in placebo group did not show significant differences. The sample group showed significant differences before and after administration of Astaxanthin. In all groups (placebo and sample) there were significant differences before and after treatment.

ROC analysis on 8-OH-dG (Figure 6) showed AUC value of 0.662, cut-off value $<0.476$, sensitivity value of 81.3% and specificity of 50%. This shows that if the 8-OH-dG value $<0.476$, prediction of normal motility level (>40) is 81.3%. If the 8-OH-dG value $>0.476$, prediction of abnormal motility level (<40) is 50%.

**3.5 Semen quality**

Diagnosis for male infertility generally uses semen quality analysis with reference to the WHO 2010 provisions which include color and odor, pH, volume, viscosity, concentration, morphology and motility. The results of before-after effects of astaxanthin administration on the parameters of semen quality in the treatment groups and placebo are shown in Table 2. In both groups before and after treatment, seminal color and odor was normal. The pH of the semen was in normal pH range of 7.2-7.8.
4 DISCUSSION

The quality of semen correlates with age. The higher the age, the lower the nitric oxide concentration. This is related to erectile dysfunction in older men so that sperm quality will decrease and the number of fertilized embryos also decreases [24]. In this study, the average age was under 40 years, so it was not included in Advanced Paternal Age (APA) category which had a significant impact on infertility [25]. The ESR, before administration of astaxanthin was higher than normal, which indicated the presence of inflammatory process. Whereas, after being given with astaxanthin, the ESR number was less than normal, which indicated an improvement. Research data showed that astaxanthin could significantly reduce ESR to 6.58±5.843 from 25.16±4.678. Another research conducted in the US [40], Norway [41] and Pakistan [42,43]. A retrospective study in Brazil show that sperm volume is decreased in older males who do not produce sperm at all. This decrease in sperm volume can be caused by a disturbance in the process of spermatogenesis, for example high testicular temperatures or smoking [45,46]. Morphological changes in spermatozoa affects sperm quality so DNA is fragmented and carries a high mutagenic potential. Oocytes result in hypermutability, genomic instability, and infertility [35]. Spermatozoa must be protected by antioxidants in the cytoplasm and seminal plasma. The cytoplasm of spermatozoa is very small (20 mm³) and collected in the mid-piece of spermatozoa, not distributed evenly so that it relies on antioxidant protection in seminal plasma [36]. Antioxidant therapy is needed because the levels of antioxidants in seminal plasma are not able to compensate for increased ROS production [37,38]. One of administered oral antioxidants is astaxanthin. The results showed that oral antioxidant supplements were effective for the treatment of male infertility by increasing one or more sperm parameters [1,14]. The mechanism of sperm damage mediated by ROS is hypothesized as lipid peroxidation, a process in which free radicals interact with polyunsaturated lipids from cell membranes to form bioactive compounds that affect the integrity of lipid bilayer and DNA structure, thereby disrupting fertilization, embryo formation, and conception failure [39]. There were no significant differences pH of the semen in the two groups before and after placebo and astaxanthin. Similar results were reported in studies conducted in the US [40], Norway [41] and Pakistan [42,43].

Table 2. Parameters of semen quality in the placebo and treatment groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Placebo group</th>
<th>Treatment group</th>
<th>Comparability test (α 0.05)</th>
<th>Comparability test (α 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Color</td>
<td>Normal</td>
<td>Normal</td>
<td>p&gt;0.05</td>
<td>Normal</td>
</tr>
<tr>
<td>Odor</td>
<td>Normal</td>
<td>Normal</td>
<td>-</td>
<td>Normal</td>
</tr>
<tr>
<td>pH</td>
<td>7.2±7.2</td>
<td>7.2±7.2</td>
<td>p&lt;0.05</td>
<td>7.2±7.2</td>
</tr>
<tr>
<td>Volume (mL)</td>
<td>0.37±0.725</td>
<td>0.37±0.725</td>
<td>P&lt;0.05</td>
<td>0.37±0.725</td>
</tr>
<tr>
<td>Viscosity (cm)</td>
<td>2.00±0.00</td>
<td>2.00±0.00</td>
<td>P&lt;0.05</td>
<td>2.00±0.00</td>
</tr>
<tr>
<td>Concentration (x10^6/mL)</td>
<td>67.33±56.48</td>
<td>67.33±56.48</td>
<td>P&lt;0.05</td>
<td>67.33±56.48</td>
</tr>
<tr>
<td>Morphology (%)</td>
<td>3.33±3.5</td>
<td>3.33±3.5</td>
<td>P&lt;0.05</td>
<td>3.33±3.5</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>24.17±11.43</td>
<td>24.17±11.43</td>
<td>P&lt;0.05</td>
<td>24.17±11.43</td>
</tr>
</tbody>
</table>

4 DISCUSSION

The quality of semen correlates with age. The higher the age, the lower the nitric oxide concentration. This is related to erectile dysfunction in older men so that sperm quality will decrease and the number of fertilized embryos also decreases [24]. In this study, the average age was under 40 years, so it was not included in Advanced Paternal Age (APA) category which had a significant impact on infertility [25]. The ESR, before administration of astaxanthin was higher than normal, which indicated the presence of inflammatory process. Whereas, after being given with astaxanthin, the ESR number was less than normal, which indicated an improvement. Research data showed that astaxanthin could significantly reduce ESR to 6.58±5.843 mm/h (<15 mm/h). The role of astaxanthin as an anti-inflammatory was first discovered by Kurashige. Rats receiving astaxanthin showed significant reduction in inflammation compared with vitamin E or control [26]. Astaxanthan can suppress the expression of inflammatory cytokines (eg, NF-k, TNF-α and IL-1), improve apoptosis with anti-inflammatory effects depending on the dose [27,28,29,30]. High MDA levels result in decreased spermatozoa motility. This is influenced by lipid peroxidation level, which indirectly indicates high numbers of free radicals [31], which increases membrane permeability, cellular enzyme inactivation, DNA structure damage and cell apoptosis, resulting in decreased motility [32]. Astaxanthin protects the body from oxidative damage through two mechanisms, i.e. neutralizing oxygen singlets and lipid peroxidation [33, 34]. Astaxanthin has keto and hydroxyl groups, so it can be hydrophilic and lipophilic. As a result, astaxanthin is more effective against lipid peroxidation in cell membranes. A long astaxanthin structure increases rigidity and mechanical strength. This is what distinguishes astaxanthin from other antioxidants [18]. So astaxanthin is able to protect the body against lipid peroxidation and oxidation damage by LDL cholesterol, cell membranes, cells, and tissues [34,17]. Spermatozoa are cells that are very sensitive to oxidation. Marker of DNA damage caused by oxidation is the 8-OH-dG because guanine is a nucleic base that is prone to oxidation [2]. The production of 8-OHdG limits the repair capacity of spermatozoa so DNA is fragmented and carries a high mutagenic potential. Oocytes result in hypermutability, genomic instability, and infertility [35]. Spermatozoa must be protected by antioxidants in the cytoplasm and seminal plasma. The cytoplasm of spermatozoa is very small (20 mm³) and collected in the mid-piece of spermatozoa, not distributed evenly so that it relies on antioxidant protection in seminal plasma [36]. Antioxidant therapy is needed because the levels of antioxidants in seminal plasma are not able to compensate for increased ROS production [37,38]. One of administered oral antioxidants is astaxanthin. The results showed that oral antioxidant supplements were effective for the treatment of male infertility by increasing one or more sperm parameters [1,14]. The mechanism of sperm damage mediated by ROS is hypothesized as lipid peroxidation, a process in which free radicals interact with polyunsaturated lipids from cell membranes to form bioactive compounds that affect the integrity of lipid bilayer and DNA structure, thereby disrupting fertilization, embryo formation, and conception failure [39]. There were no significant differences pH of the semen in the two groups before and after placebo and astaxanthin. Similar results were reported in studies conducted in the US [40], Norway [41] and Pakistan [42,43]. A retrospective study in Brazil show that sperm volume is the only parameter that decreases with age [44]. The lower concentration of spermatozoa, the more fertile a person is, but rarely are there males who do not produce sperm at all. This decrease in sperm concentration can be caused by a disturbance in the process of spermatogenesis, for example high testicular temperatures or smoking [45,46]. Morphological changes in spermatozoa affects sperm maturity so that it also affects motility and viability. [47, 46]. There is a correlation between normal forms of morphology with pregnancy rates in vivo and in vitro [48]. Spermatozoa motility is a significant influencing factor and is a major
cause of infertility [35, 49, 50]. Decreased spermatozoa motility is partly due to OS [51]. Astaxanthin is a natural carotenoid class needed to maintain cell membrane integrity and spermatogenesis regulation that affects spermatozoa motility and results in male infertility [52,53]. The effect of astaxanthin is revealed through several mechanisms, such as electron scavenger through conjugated double bonds and inhibition of lipid peroxidation [54,55]. Recent studies about the antioxidant activity of astaxanthin found that intraperitoneal injection of astaxanthin increased sperm motility, number and viability by reducing lipid peroxidation and increasing antioxidant defenses [53].

5 CONCLUSION
Astaxanthin in a single dose of 8 mg per day consumed for 30 days can improve ESR, lipid peroxidation, DNA oxidation, concentration, morphology and motility of human spermatozoa.

6 ACKNOWLEDGMENT
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