THE PROTECTIVE EFFECTS OF THE ORGANIC SELENIUM (HIGH-SELENIUM YEAST) ON THE OXIDATIVE STRESS CAUSED BY METHANDIENONE ON THE BRAIN OF MALE RABBITS

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Abstract

The aim of this study is to investigate the protective role of the organic selenium (high-selenium yeast) in adult male rabbits treated with Methandienone and combination with selenium. A total of 20 intact male rabbits were divided into four equal groups: control group receiving distal water, (T1) receiving Methandienone (oral dose of 0.35 mg / kg.),(T2) receiving high-selenium yeast (3 µg/ kg orally) and (T3) receiving both methandienone and selenium. A significant decrease in GSH with an increase in SOD activity were found and a significant increase in the oxidative markers MDA and PCC in serum (0.97+0.021) and in brain tissue (1.01+0.058) in T1 compared with other groups (control, T2 and T3). On the other hand, T3 group shows an increase in serum GSH and a decrease in SOD, MDA and PCC in serum and brain tissue. These changes are significant compared to AAS group (T1). Moreover, there is a significant increase in reverse transcription (mRNA) of Nrf2, TSPO and CYP19A1 genes in AAS group (T1) as compared with other groups (T2 and T3). In addition, we showed a significant decrease in levels Nrf2, TSPO, and CYP19A1 mRNA in the selenium-yeast group compared to the AAS group (T1). In conclusion, these results showed that organic selenium (high selenium yeast) has a positive effect (protective role) against oxidative damage to the brain caused by AAS.

Keywords: Methandienone, selenium, PCC, Nrf2, TSPO, and CYP19A1.

Introduction

Anabolic androgenic steroids (AAS) are synthetic derivatives of the male sex hormone testosterone, developed to increase bioavailability and reduce adverse androgenic properties, while maximizing anabolic effects. Nowadays, AAS are clinically used in hormone replacement therapies, including hypogonadism and aging and to treat muscle wasting due to cancer(S. M. Choi & Lee, 2015). In this experiment, we used a compound of the third class AAS (Methandienone), which is alkylated at C17. Since alklylation delays metabolism in the liver, this AAS group is orally active (Basaria et al., 2001). It is used in treating postmenopausal osteoporosis and pituitary deficiency dwarfism (Llewellyn, 2011). Methandienone is still produced today, but usually in countries that have strict rules for prescription drugs and companies that still prefer to serve the underground sports market.

Overuse of AASs causes serious side effects that involve the cardiovascular system, mood disorders, the reproductive system and the liver (van Amsterdam et al., 2010). At present, neurobiological mechanisms and sites of action of AASs are unclear. In vitro, low AAS concentrations increase the death of excitotoxic neurons (Orlando et al., 2010). In normal male volunteers, high doses of AAS cause cognitive impairment (Dalry et al., 2003). The neurotoxicity induced by AASs can be complicated by the induction of excitotoxicity effect. This phenomenon occurs after a massive release of glutamate. However, over activation of NMDA and AMPA receptors by glutamate results in mitochondrial dysfunction, redox imbalance and pro-apoptotic pathways (Prentice et al., 2015). AASs have been shown to cause a wide range of toxic effects in the brain, such as direct action on GABA receptor, an excessive increases in cytosolic Ca²⁺, calcium overload, ATP depletion, neuronal oxidative stress and cell death (M Vicenico et al., 2011; Oberlander et al., 2012; Basile et al., 2013). The objective of this experiment is to determine the AAS-induce oxidative stress in brain and prophylactic role of organic selenium to reduce this effect.

Little is known about the role of AASs in the regulation of oxidative status in the brain. Oxidative stress is characterized by an imbalance between the production of reactive oxygen species (ROS), reactive nitrogen species (RNS) and their elimination (Birben et al., 2012). Protein carbonyl content (PCC), which is the most widely used marker of oxidative protein modification (Handayani et al., 2019). Nuclear factor erythroid 2 related factor 2 (Nrf2) a sensory regulator of redox homeostasis, Under normal conditions, Nrf2 is detected at very low levels in the cell cytoplasm due to constant degradation of the proteosomes, Nrf2 levels increase with a quick response to a changing intracellular environment, which allows it to be transported into the inner nucleus and increases the expression of these protective genes (more than 100 genes) in order to maintain redox homeostasis in mammalian cells (Tonelli et al., 2018). Translocator Protein (TSPO) is a protein expressed at very low levels in the brain, this protein is found in the outer membrane of mitochondria, but with damage or inflammation of the brain, its expression is enhanced in areas of the brain that are specific for damage. TSPO is mainly expressed in two types of cells, microglia and astrocytes (Loth, 2018).

Aromatase (CYP19A1) is the enzyme responsible for all endogenous 17β-estradiol (E2) production in both females and males. Importantly, it expressed in the brain as peripheral organs (i.e., gonads and adipose tissue) (Hara et al., 2015). The expression of CYP19A1 correlates with neuroprotection. Indeed, weak neurodegenerative stimuli cause severe neuro degeneration when the aromatase is
genetically or pharmacologically inhibited (Garcia-Segura et al., 2003).

The second partition of this research, we used selenium (selenium-enriched yeast) as protection against AAS-induced oxidative stress in brain. Selenium has been found in many oxidative stress in brain. Selenium has been found in many
(selenium-enriched yeast) as protection against AAS-induced microchondria, the plasma membrane and DNA from the oxidative damage of ROS (Zoidis et al., 2018). Imam et al. (1999) showed the role of selenium in improving against methamphetamine-induced neurotoxicity. Moreover, The positive responses obtained during selenium therapy for neurodegenerative diseases are providing evidence of the important role of reactive oxygen species and oxidative stress in pathological processes (Santamaria et al., 2003; Dominia et al., 2016).

Materials and Methods

Animals and experimental design

We used 20 male adult rabbits (8-12 weeks old; body weight 820-1050 g). The animals were divided into four equal groups as follows: Control took 1 ml / kg of distal water orally, T1 received an oral dose of 0.35mg/kg B.Wt of Methandienone provided by Black dragon pharma (Thailand), T2 was treated with 3 µl / Kg body weight of Se-yeast provided by 21 Century® (USA), T3 received both Methandienone and Se-yeast orally (0.35mg/kg and 3 µl/kg respectively). All animals received treatment every day for 60 days by oral administration. After the experiment, animals were anesthetized by double dose anesthesia and blood samples were taken for biochemical analysis and brain tissues for RNA isolation.

Biochemical analyses:

Table 1.1 : Primer names and their sequence

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2 (Nuclear factor erythroid 2 related factor 2)</td>
<td>F: CCCACAACGGTITGCGCATCAC R: TGGCGATTCTTTGCGGTCT</td>
</tr>
<tr>
<td>TSPO (Translocater protein)</td>
<td>F: GTGGACCTCTGCTCCTCAC R: ACGGGTGAAGGGTACAGGC</td>
</tr>
<tr>
<td>CYP19A1 (Aromatase enzyme)</td>
<td>F: GGAAGAATGCATCGACTTGAGTT R: GGCCCAAACAAATGGT</td>
</tr>
<tr>
<td>GAPDH (glyceraldehyde-3-phosphate dehydrogenase)</td>
<td>F: ACCGCCATGTAAAGGGTACAGGC R: AGGATGCCGGTGACTCAATC</td>
</tr>
</tbody>
</table>

R: Reverse
F: Forward

Statistical Analysis:

Data have been analyzed statistically using (SPSS) version 24. Statistical analysis of data was conducted on the basis of (One-way) Analysis of Variance (ANOVA) utilizing a least significant differences (LSD) as portrayed by (Larsen et al., 1973). A level of p<0.05 was defined as statistically significant.

Results

The effect of Methandienone, Selenium and both on the oxidative markers

According to the results which are illustrated in table and figure (1). There was a significant decrease (p > 0.05) in serum GSH concentration in T1 group when compared with other groups. There was a significant increase (p < 0.05) in serum MDA concentration in T1 when compared with control, T2 and T3 groups. At the same time the T1 group (AAS) showed a significant increase (p<0.05) of serum SOD activity as compared with other groups. Also, we observed a significant increase (p<0.05) of SOD in T3 (Metha. + Se-yeast) compared to control and T2. The result also appeared a significant increase of protein carbonyl content (PCC) in serum and brain tissue of T1 as compared with other groups(control,T2 and T3). while, Metha. + Se-yeast group (T3) showed a significant increase (p<0.05) serum PCC concentration in comparable to control and Se-yeast groups.

Reduced Glutathione (GSH) was evaluated using a modified method (Burris et al., 2012). Malondialdehyde (MDA) Assay was determined by colorimetric method(TBARS)used by (Tateishi et al., 1987). Total-Superoxide Dismutase Activity (SOD) activity was determined as previously described by Fernandez et al. (2009) using SOD kit (Elabscience Biotech. Inc. china). Protein carbonyl content (PCC) in both serum and brain tissue was determined by colorimetric method (Levine et al., 2000)using Protein Carbonyl Colorimetric Assay Kit (Elabscience Bio.tech. Inc. china).

Gene expression analysis

Total RNA was isolated using the magnetic nanosorbent kit ((RealBest Extraction 100, Vector-Best, Novosibirsk, Russia)) according to the protocol of the manufacturer. The RNA integrity and purity were measured using a spectrophotometer . Afterwards, isolated RNA was then reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Synthol, Russia) according to the manufacturer’s instructions. as a template for the real-time (RT) polymerase chain reaction (PCR) (EvaGreenRT-PCR kit; Synthol, Russia) with specific primers. All primers were obtained from Bioneer (Korea). The primer sequences are shown in the Table (1.1) below. The expression of these genes (genes of interest) was analysed, and GAPDH was used as a reference housekeeping gene. For RT-PCR, start Exicycler™ 96 Real-Time Quantitative Thermal Block instrument and loaded the following Program was used with the following PCR program as follows: reverse transcription step (50 °C,1hour), PCR initial activation step (95 °C, 5 min), two steps cycling (95 °C, 20 s and 60 °C, 45 s respectively), repeated 45 times. Melting curve analysis was performed to check the specificity of PCR products. The delta-delta CT method was used for relative quantification of expression of particular gene as described elsewhere (Livak & Schmittgen, 2001).
Table 1: The oxidative stress markers (GSH, MDA, SOD and PCC) in serum and PCC activity in brain tissue of different groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameter</th>
<th>Control</th>
<th>Methandienone (T1)</th>
<th>Se-yeast (T2)</th>
<th>Metha. + Se-yeast (T3)</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSH (µmol/l)</td>
<td>1.56±0.057 A</td>
<td>0.60±0.020 C</td>
<td>1.11±0.072 B</td>
<td>1.21±0.130 B</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>MDA (µmol/l)</td>
<td>0.14±0.005 B</td>
<td>1.28±0.018 A</td>
<td>0.12±0.003 C</td>
<td>0.20±0.011 B</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>SOD Activity (U/ml)</td>
<td>19.62±0.288 C</td>
<td>34.11±0.360 A</td>
<td>19.37±0.389 C</td>
<td>28.14±0.349 B</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td>PCC (serum) (nmol/mgprot)</td>
<td>0.52±0.023 C</td>
<td>0.97±0.021 A</td>
<td>0.25±0.011 D</td>
<td>0.73±0.145 B</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>PCC (brain tissue) (nmol/mgprot)</td>
<td>0.40±0.057 B</td>
<td>1.01±0.058 A</td>
<td>0.43±0.033 B</td>
<td>0.46±0.033 B</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Value express as mean ± SE. Number of animals per each group (5). The different capital letters refer significant differences between groups within one row at (P≤0.05).

Control group received 1 ml / kg of distal water orally for 60 days. T1: Animals received an oral dose of 0.35 mg / kg of Methandienone for 60 days. T2: selenium was treated with 3 µl / Kg body weight of selenium (High Selenium yeast). for 60 days. T3: The combined group received both Methandienone and selenium-yeast orally (0.35 mg / kg and 3 µl / Kg body weight, respectively). for 60 days.

**The effect of Methandienone, Selenium and both on the Relative genes expression**

The results of relative gene expression in (Nrf2) gene appeared clear difference in fold change of gene expression levels between control and treated groups. Where, T1 group (AAS) up regulation at (19.46±0.384), T2 group (Se-yeast) showed low change at (6.10±0.378), T3 group (Metha. + Se-yeast) showed up regulated of expression of TSPO gene as compared with other groups (T2 and T3). On other side, the T3 group (Metha. + Se-yeast) appeared up regulated of expression of TSPO gene as compared with T2 group (Se-yeast).
In the same table and figure, we demonstrated a high level of reverse transcription of Cyp19A1 gene in T1 group compared with T2 and T3 groups. Also, we see increased mRNA level in T3 group compared to T2. The statistical analysis of relative gene expression (Nrf2, TSPO, and CYP19A1) found significant differences in AAS (methan) group compared with other groups at level $P \leq 0.05$.

### Table 2: The relative gene expression analysis of Nrf2, TSPO, and CYP19A1 gene in brain of different groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Methandienone (T1)</th>
<th>Se-yeast (T2)</th>
<th>Metha. + Se-yeast (T3)</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold change ($2^{\Delta \Delta CT}$) of Nrf2 gene</td>
<td>1.00±0.00</td>
<td>19.46±0.384</td>
<td>6.10±0.378</td>
<td>14.20±0.152</td>
<td>0.9</td>
</tr>
<tr>
<td>Fold change ($2^{\Delta \Delta CT}$) of TSPO gene</td>
<td>1.00±0.00</td>
<td>22.88±0.646</td>
<td>4.10±0.208</td>
<td>6.41±0.145</td>
<td>1.13</td>
</tr>
<tr>
<td>Fold change ($2^{\Delta \Delta CT}$) of CYP19A1 gene</td>
<td>1.00±0.00</td>
<td>6.20±0.141</td>
<td>2.11±0.106</td>
<td>3.59±0.095</td>
<td>0.32</td>
</tr>
</tbody>
</table>

The different capital letters refer significant differences between groups within one row at ($P \leq 0.05$) control group received 1 ml/kg of distal water orally for 60 days. T1: Animals received an oral dose of 0.35 mg/kg of Methandienone for 60 days. T2: selenium was treated with 3 μl/Kg body weight of selenium (High Selenium yeast) for 60 days. T3: The combined group received both Methandienone and selenium-yeast orally (0.35 mg/kg and 3 μl/Kg body weight, respectively). for 60 days.

Nrf2 (Nuclear factor erythroid 2-related factor 2), TSPO (Translocator Protein), Cyp19A1 (Aromatase).

**Fig. 2**: The variation in genes expression (Nrf2, TSPO, and CYP19A1) in brain of different groups.

**Discussion**

The oxidative stress state increases the oxidation of GSH to the binary sulfurous form of GSSG by inhibiting the pentose phosphate shunt pathway, which determines the NADPH production necessary for the activity of the glutathione reductase enzyme to recreate its oxidized form GSH (Hansen et al., 2019). A decrease in GSH leads to an increase production in ROS due to AAS-induced oxidative stress.

In addition to depletion of GSH, lipid peroxidation (damage in cell compartments) occurs during the administration of AAS. Thus, measuring serum MDA levels
provides a convenient indicator of lipid peroxidation and is a non-invasive oxidative stress biomarker (Janicka et al., 2010). In this study, MDA increased significantly after administration of AAS alone and plus with Se-yeast compared to control.

This result is consistent with Camilletti-Moirón (2016), which reported a significant increase in plasma MDA concentration and the harmful effects caused by the administration of AASs during the oxidation of lipids and proteins in the kidneys.

The importance of SOD activity comes from its high effectiveness as an antioxidant as it blunts cascading reactions caused by superoxide radical, thus it is considered a major antioxidant and the first defense (Buettner, 2011). Androgen testosterone has been reported to increase the production of ROS and oxidative effect in cardiomyocytes by transmitting signals through the androgen receptor (AR) through genomic and non-genomic mechanisms, activated androgen receptor translocates in the nucleus and increases expression of prooxidant enzymes genes (e.g. NAD (P) H oxidase cyclooxygenase 2 (COX-2) and xanthine oxidase) which, in turn, enhances the activation of pro-oxidant enzymes and elevate production ROS by changing the function of mitochondria (Cruz-Topete et al., 2020). This explains the significant increase activity of SOD after administration of AAS in male rabbits. In addition, ROS leads to protein damage, measured by protein carbonyl contents (PCC), direct damage to proteins or a chemical change in their amino acids as a result of oxidative stress can increase PCC, protein oxidation leads to the loss of sulfhydryl groups and changes in the resonant structure of amino acids that change the function of the protein and therefore body integrity (Parvez & Raisuddin, 2005).

The present study showed that treatment with AAS leads to a rise in PCC in brain tissue. Turillazzi et al. (2016) mentioned the metabolic activation of androgen (testosterone) derivatives leads to the release of free and caused oxidative effect that observed in all areas of the brain caused by prolonged administration of AAS (nandrolone). Secondary reaction with product of lipid peroxidation (e.g. HNE) with certain amino acid residues that cause elevated of protein oxidation (Hall et al., 2016). As well as, the classical intracellular androgen receptor via, AASs can exert apoptotic effect through a non-genomic pathway (M Vicencio et al., 2011).

On the other hand, we use the ameliorative effect of selenium-enriched yeast on AAS-inducing oxidative stress that caused improvement of antioxidant defense and reduction of oxidant markers. The type of Se supplementation used in this study was Se-Yeast, which is a spray dried Se yeast formulation (Saccharomyces cerevisiae) and a common form of Se used for supplementation of the dietary intake (Griffiths et al., 2006; Aremmt et al., 2019). The organic Se content in Se-Yeast is equal to or greater than 98% of the total Se content (Griffiths et al., 2006). El-Demerdash & Nasr (2014) reported that selenium is crucial in several enzymes with physiological antioxidant properties, including GPx and thioredoxin reductase. GPx scavenges 

\[ \text{H}_2\text{O}_2 \] and lipid hydroperoxides, using reducing equivalents from glutathione and protecting membrane lipids and macromolecules from oxidative damage (Zoidis et al., 2018).
(Rupprecht et al., 2010). For example, estrogens. In neurons, estrogens prevent the death of neurons by raising the endogenous synthesis of anti-apoptotic molecules (Meda et al., 2000).

Because aromatase expression (CYP19A1) correlates with neuro protection. In fact, weak neurodegenerative stimuli cause strong neuro degeneration(inflammation when the enzyme is genetically or pharmacologically inhibited (Garcia-Segura et al., 2003), possibly because aromatase is used for local production of neuroprotective estrogens (Pareto et al., 2013). Garcia-Segura et al. (1999) found that although astrocytes usually do not express aromatase, enzyme expression in these glial cells is induced by various types of brain damage.

The results obtained indicate the vital role of local formation of astroglial estrogen in brain restoration. Thus, we observed increased regulation of CYP19A1 (aromatase) in the AAS group to reduce the adverse effects of AASs on the brain. In addition, the function of TSPO for steroidogenesis in the brain. On other side, AAS can also be aromatized to estrogens and interface with both estrogen receptor alpha and beta (ERα, ERβ).

On the other side, we observed that the protective role of Se (T3 group) is manifested in a significant decrease in molecular bioactive markers involved in the level of expression of the Nrf2, TSPO, and CYP19A1 genes in brain tissue.The recommended daily dose – 55 µg/day – is based on the amount needed to maximize the activity of the selenium-dependant master antioxidant enzyme glutathione, selenium also binds to the sulfur-containing amino acid cysteine and forms antioxidant selenoproteins in the body, selenoproteins carry selenium to the tissues, reduce inflammation, support a function of immune system and thyroid gland (Rocourt & Cheng, 2013). Wrobel et al. (2013) explained in his study the main effect of different selenium compounds such as synthetic L-selenomethionine, inorganic sodium selenite, and organic high-selenium yeast on the development of metastatic brain lesions have shown that the selenium-enriched yeast formulation resulted in a higher survival rate and in decreased tumor growth as compared to controls.

Another study shows the comparative effects of two kinds of selenium supplement selenomethionine and high-selenium yeast supplements has showed a decrease in oxidative stress biomarkers after supplementing with the organic high-selenium yeast compared to selenomethionine supplementation (Richie et al., 2014). Thus, Se is very important for your brain health. Low Se levels are linked to poor cognition, memory problems, and low neurotransmitter levels in the brain. Alzheimer’s patients had only 60% of the brain selenium levels of healthy people in one study (Cardoso et al., 2013). So that in this study we found the Se-yeast reduction of damages induction by AAS.

References


