

## Selenium Enriched Yeast Modifies the Effects of Methandienone in Male Rabbits on the HPA Axis and Adrenal Gland Oxidative Stress

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### Abstract

The aim of this study was to investigate the changes caused by anabolic androgenic steroids (methandienone) in the hypothalamic adrenal axis (HPA) and oxidative status in adrenal glands and a protective role by using organic selenium (high-selenium yeast) in adult male rabbits which treated with Methandienone in combination with selenium. A total of 20 intact male rabbits were divided into four equal groups (n = 5 for each group): a control group receiving distilled water, the AAS group (T1) receiving Methandienone (oral dose of 0.35 mg / kg.B.W.), the selenium group (T2) receiving high-selenium yeast (3 µg/ kg B.W orally) and the combination group (T3) getting both methandienone and selenium. The dosing every day for 60 days. The hormones β-endorphin, ACTH and cortisone were measured in serum during two periods, 30 and 60 days of the experiment and the expression levels of Nuclear factor erythroid-2 and related factor-2 (Nrf2) and Translocator protein (TSPO) genes were measured in adrenal gland. a significant increase in beta-endorphin and ACTH and a decrease in cortisone were found in both 30 and 60 day periods in the AAS group (T1) compared with another groups (control, T2 and T3). On the other hand, T3 group shows a decrease in serum β-Endorphin and ACTH and no significant changes in cortisone in both periods. These changes were compared with to AAS group. Moreover, there is a significant increased in revers transcription (mRNA) of Nrf2 factor and TSPO genes in AAS group(T1) as compared with other groups (T2 and T3). In addition, we observed a significant decrease in levels of Nrf2 and TSPO 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 adrenal gland caused by AAS with no effect on the adrenal steroidogenesis, which are affected by AAS.

**Keywords:** Anabolic androgenic steroids, Methandienone, Selenium, Rabbit.

الدور التعديلي للسلينيوم المحضر عن طريق الخمائر على تأثيرات الميثاندينون في ذكور الأرانب على محور HPA والإجهاد التأكسدي للغدة الكظرية.

### الخلاصة

الهدف من هذه الدراسة هو التحقيق في التغييرات التي تسببها الستيرويدات الابتنائية-الاندروجينية (methandienone) على المحور الوطائي-النخامي-الكظري (HPA) hypothalamic-pituitary-adrenal والحالة التأكسدية في الغدة الكظرية، وكذلك الدور الوقائي الذي يلعبه السلينيوم العضوي (السلينيوم المحضر بواسطة الخمائر) في ذكور الأرانب المعاملة بالميثاندينون. استخدم 20 ذكراً من ذكور الأرانب البيض قسّمت إلى 4 مجاميع اعتماداً على وزن الجسم: مجموعة سيطرة جرعت بماء المقطر، (T1) مجموعة AAS عوملت بالميثاندينون عن طريق الفم وجرعة 0.35 ملغرام/كغم، (T2) مجموعة السلينيوم عوملت بجرعة 3 مايكروليتر/كغم من السلينيوم عن طريق الفم، (T3) مجموعة الوقاية والتي عوملت بالميثاندينون والسلينيوم معا. واستمر تجريع الحيوانات لمدة 60 يوم. تم تقدير الهرمونات بيتا اندورفين ACTH، والكورتيزول في المصل في اليوم 30 و 60 من التجربة، ومستويات التعبير الجيني للجينات (Nrf2) و (TSPO) فقد تم تقديرها في الغدة الكظرية. اظهرت النتائج زيادة معنوية في مستوى بيتا إندورفين و ACTH ونقص في الكورتيزون في كلا الفترتين (30 و 60 يوم) في مجموعة الستيرويدات الابتنائية (T1) مقارنة بالمجموعات الأخرى (السيطرة، T2 و T3) من ناحية أخرى ظهر في مجموعة T3 انخفاضاً في مستوى بيتا إندورفين و ACTH مع عدم وجود تغييرات معنوية في مستوى الكورتيزون في كلا الفترتين هذه التغييرات الحاصلة مقارنة بمجموعة AAS على ذلك، هناك زيادة معنوية في مستويات التعبير الجيني (mRNA) لكلا الجينين Nrf2 و TSPO في مجموعة AAS (T1) مقارنة بالمجموعات الأخرى (T2 و T3) بالإضافة إلى ذلك، لاحظنا انخفاضاً معنوياً في مستويات mRNA لكلا الجينين Nrf2 و TSPO في مجموعة خميرة السلينيوم مقارنة بمجموعة AAS (T1) يستنتج من هذه الدراسة أن للسلينيوم العضوي (المحضر بواسطة الخمائر) تأثير إيجابي (دور وقائي) ضد الضرر التأكسدي للغدة الكظرية الناتج عن الستيرويدات الابتنائية-الاندروجينية وليس له تأثير على تكوين الستيرويد الكظري الذي يتأثر بـ AAS.

## Introduction

Anabolic- androgenic steroids (AASs) are synthetic derivatives of testosterone. For decades, it has been used to treat short stature condition, burns, wasting signs, osteoporosis, and severe anemia (1). In the current study used methandienone, commercially known (dianabol), which is a third class of AAS characterized by the addition of an alkyl group to 17 carbon atoms (2).

AASs are addicted primarily in the status of vigorous exercise and for the purposes of building muscle mass as opposed to euphoria induced by this drug. Androgenic steroids also modulates the hypothalamic–pituitary–adrenal (HPA) axis and changes to stress hormone and endorphin release that may be increase the reinforcing value of exercise (3). The activation of the adrenal axis after exposure to stressful conditions provides an adaptive response through a cascade of hormonal secretion of physiological and behavioral changes aimed at maintaining homeostasis in the body (4). After exposure to stress, corticotropin releasing hormone (CRH) secretes and stimulates an increase in the expression of the proopiomelanocortin (POMC) gene in the pituitary gland, which is subsequently translated into peptides such as adrenocorticotrophic hormone (ACTH) and beta endorphin (5). Then ACTH activates the adrenal gland to initiate a stress response, beta endorphin reduces the stress response, at least in part, by inhibiting of CRH secretion (6). Beta endorphin is a morphine-like action substance secreted by the hypothalamus and pituitary gland. it's a released in excessive quantities in response to pain, severe exercise and depression (7).

A little information about effect of AAS on adrenal steroidogenesis and oxidative status, thus this study focusing on HPA axis and molecular bioactive markers, which involved Nrf2 and TSPO genes in adrenal tissues. AAS can compete for binding to glucocorticoid receptors, and this competition has been reported to induce anabolic effects by reducing glucocorticoid-induced

catabolism (8).

The transcription factor (Nrf2) is an important protective factor in the cell against the negative effects associated with oxidative stress, which regulates the detoxifying and antioxidant protective genes expression in the cell by attaching to the antioxidant response element (ARE) in the nucleus and increased expression of its target genes (9). Translocator protein (TSPO) is a transmembrane protein that resides in the outer membrane of mitochondria and is expressed primarily in steroid tissues and the brain, It has the function of translocating cholesterol from the outer to the inner mitochondrial membrane, which is stimulated by steroidogenesis, and TSPO expression is increased with brain damage and neurodegeneration, so consider a sensitive biomarker of brain function, As well as, it contributed in redox homeostasis (10).

The adrenal glands have high levels of antioxidants such as vitamin C, E and glutathione, as well as antioxidant enzymes such as GPX and SOD (11). So that, we used in the second partition of this study selenium (selenium enrich yeast) as a protective role against AAS administration, as well as its consider a precursor of selenoproteins which have important antioxidant activity and protect mitochondria from ROS oxidative damage (12). The aim of this study was to investigate the effect of AAS on adrenal function and its protection with organic selenium.

## Material and methods

### Animals and experimental design

20 male New Zealand rabbits weighing 850-1100 g and aged 2.5 to 3.5 months were used in this experiment. The animals were kept in cages in an air-conditioned room (23-26 ° C) at the animal house of the College of Veterinary Medicine, University of Baghdad. Four equal groups the animal were divided as follows: the control received 1 ml / kg of distilled water orally, T1 received an oral dose of 0.35 mg / kg B.Wt methandienone (AAS) from Black dragon pharma (Thailand). T2 was treated with 3 µl / kg body

weight of Se-yeast supplied by 21st Century® (USA). T3 received methandienone and Se-yeast orally (0.35 mg / kg and 3 µl / kg, respectively). All animals were treated every day for 60 days by oral administration. Blood samples were taken by cardiac puncture for each rabbit after 30 and 60 days of the experiment for hormonal analysis. At the end of the experiment, the animals were anesthetized with double anesthesia and adrenal tissues were taken for RNA isolation.

#### Hormonal assays

Serum β-endorphin (pg / ml), ACTH and cortisone concentrations were determined using available commercially of ELISA kit for each hormone (Elabscience Biotech Co., Ltd., China) according to the manufacturer's instructions.

#### Gene expression analysis

Total isolated RNA extracted using a set of magnetic nanosorbents (RealBest Extraction 100, Vector-Best, Novosibirsk, Russia) in according with the manufacturer's informations. The integrity and purity of the RNA was measured using a spectrophotometer. Then, the isolated RNA was returned back into DNA (cDNA) using M-MLV reverse transcriptase (Synthol, Russia) according to the manufacturer's informations. As a matrix for real-time polymerase chain reaction (PCR) (EvaGreenRT-PCR kit; Syntol, Russia) with specific primers. All primers were made from Bioneer (Korea). The primer sequences are shown in table (1) below. The expression of these genes (genes under study) was analyzed and GAPDH was used as a reference helper gene. For qRT-PCR, run Exicycler™ 96 in real time for the quantitative thermoblock and load the following program used with the following PCR program: reverse transcription step (50 ° C, 1 hour), primary PCR activation step (95 ° C, 5 min), two-stage cyclicality (95 ° C, 20 s and 60 ° C, 45 s, respectively), is repeated 45 times. A melting curve analysis was performed to check the specificity of the PCR products. The delta-delta CT method was used to relative quantify the expression of a specific gene, as described

elsewhere (13).

Table (1): Primer names and their sequence

Primer	Sequence	
Nrf2 (Nuclear factor erythroid 2 and related factor 2)	F	CCCACACAAGGTTTCGGC ATCAC
	R	TGGCGATTCTCTGGCG TCT
TSPO (Translocator protein)	F	GTGGACCTCCTGCTCCT CAC
	R	ACGCCATGTAAGGGTAG AGC
	R	GGGCCCAAACCAAAT GGT
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	F	ATGCCCCCATGTTTGTG ATG
	R	AGGATGCGTTGCTGACA ATC

R: Reverse

F: Forward

#### Statistical Analysis:

Data have been analyzed statistically using (SPSS) program version 24. Statistical analysis of data was conducted on the basis of (One and two way) Analysis of Variance (ANOVA) using a least significant differences (LSD) as portrayed by (14).

#### Results and discussion

##### The effect of Methandienone, Selenium and both on blood serum concentrations of β-endorphin, ACTH and Cortisone.

According to the results which are illustrated

in table (2). In the T1 group, there was a significant increase ( $p < 0.05$ ) in serum concentrations of B-endorphin and ACTH compared with other groups. At the same time, we observed a significant increase in the level of these hormones after 60 days of administration of Methandienone compared to 30 days. There was a significant decrease ( $p > 0.05$ ) in blood serum Cortisone levels in T1 when compared with

control and T2 groups. At the same time the T1 group (AAS) showed a significant decrease ( $p > 0.05$ ) of serum Cortisone after 60 day of methandienone administration compared to 30 days. At the same time, we represent the protective group (T3), shows a significant decrease changes in the level of B-endorphin and ACTH compared to T1 group.

**Table (2):** The hormonal analysis (B-endorphin, ACTH and cortisone) in the rabbits blood serum of different groups during 30 and 60 days of the experiment.

Parameters	Time (day)	Groups				LSD
		Control	Methandienone (T1)	Se-yeast (T2)	Metha. + Se-yeast (T3)	
B-endorphin (pg/ml)	30	54.70±1.83 C a	91.50±1.81 A b	54.53±2.23 C a	82.86±1.43 B a	4.62
	60	53.23±1.12 D a	98.33±1.19 A a	58.00±1.61 C a	87.16±0.20 B a	
ACTH (pg/ml)	30	51.10±0.700 C a	59.63±0.66 A b	49.66±0.26 C a	54.40±0.58 B b	2.92
	60	52.80±0.37 C a	63.96±1.96 A a	52.30±0.95 C a	58.10±1.13 B a	
Cortisone (nmol/L)	30	11.60±0.68 A a	6.70±0.26 C a	9.66±0.43 B a	6.16±0.17 C a	1.40
	60	11.80±0.57 A a	5.23±0.20 C b	9.60±0.43 B a	5.46±0.24 C a	

Values express as mean ± SE, Number of animals per each group (5).

The different capital letters refer significant differences between groups within one row at ( $P \leq 0.05$ )

The different small letters refer significant differences between times within one column for each hormone at ( $P \leq 0.05$ ).

The results from this study show that administration of AASs causes an increase in beta endorphin and ACTH, while cortisone levels are lower.  $\beta$ -Endorphin regulates the activity of Adrenal axis (HPA-axis) through  $\mu$ -opioid receptor mediated inhibition (15). The precursor of  $\beta$ -Endorphin synthesis is POMC, which consider a precursor protein for many peptide hormones such as ACTH, melanocyte stimulating hormone (MSH) and beta endorphin, POMC transcription is sensitive to stimulation of stress hormones such as corticotropin-releasing hormone (CRH) (16). AAS submitted at high doses stimulates the release of endorphin in the brain of rodent (17), change the expression levels of delta, kappa and muopioid receptors in the hypothalamic nuclei (18) and increase the opioid peptides metabolism (19). Earlier studies reported an increase in corticosterone levels after AASs administration (20,21) and an increased elimination half-life of cortisol by associated AASs (22).

Hildebrandt *et al* in (2014) mention the effect of AAS enhancement by the endocrine stimulation, where AAS users in the cycle have elevated  $\beta$ -endorphin and ACTH levels, but lower cortisol levels when compared to strong exercise (3). This result may be an enhancement of the adaptive increase in androgens observed in response to competition, where basal testosterone and certain mood states interact to regulate the activation of the HPA axis after social competition (23).

Hildebrandt and Greif (2013) suggests a relationship between AAS and increase opioids like action by androgenic steroids can increase the enhancing value of exercise (24). On the other hand, a temporary decrease in cortisol levels caused by AAS treatment has also been observed in horses after exercise (25). The conclusion of the present study indicated that  $\beta$ -endorphin and ACTH levels and a decrease in cortisone levels may be the effect of AAS on adrenal steroidogenesis (cortisone).

### **The effect of Methandienone, Selenium and both on the Relative genes expression in adrenal gland.**

The results expression of relative gene in gene (Nrf2) showed a difference in fold change in gene expression levels compared to other treated groups, which are presented in the table (3) and in the figure (1). Where, T1 group (AAS) up regulation at  $(2.53 \pm 0.185)$ , T2 group (Se-yeast) showed low change at  $(0.51 \pm 0.143)$ , T3 group (Metha. + Se-yeast) showed up regulation at  $(1.74 \pm 0.194)$  relative to T2 and control group that is equal to one fold change of gene expression levels (table 3 and figure 1). Moreover, we observed a significant increase of mRNA of TSPO gene in T1 group (methan.) as compared with other groups (T2 and T3). On other side, the T3 group (Metha. + Se-yeast) appear up regulated of expression of TSPO gene as compared with T2 group (Se-yeast).

Statistical analysis of the relative expression of the Nrf2 and TSPO genes revealed significant differences in the AAS (methane) group compared with the other groups at the  $P \leq 0.05$  level.

**Table (3):** The gene expression analysis of Nrf2 and TSPO genes in adrenal gland of different groups

Groups Parameters	Control	Methandien one (T1)	Se-yeast (T2)	Metha. + Se- yeast (T3)	LSD
Fold change ( $2^{-\Delta\Delta CT}$ ) of Nrf2 gene	1.00±0.00 0 C	2.53±0.185 A	0.51±0.14 3 C	1.74±0.194 B	0.49
Fold change ( $2^{-\Delta\Delta CT}$ ) of TSPO gene	1.00±0.00 0 C	6.12±0.117 A	1.20±0.09 8 C	2.14±0.029 B	0.25

Values express as mean ± SE, Number of animals per each group (5).

The different capital letters refer significant differences between groups within one row at ( $P \leq 0.05$ )

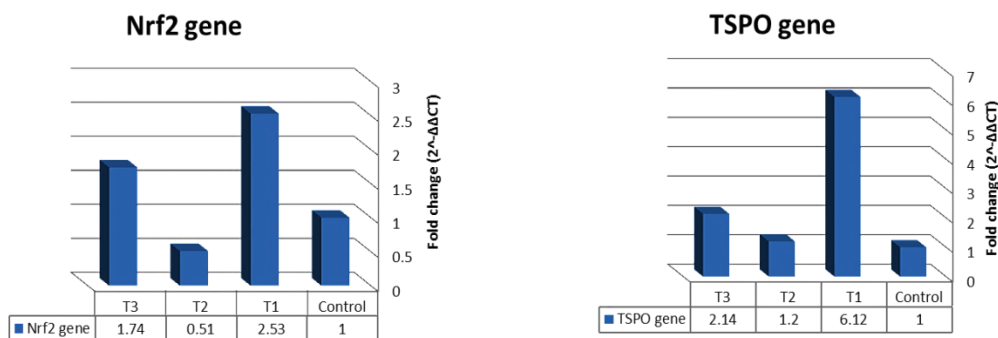


Figure (1). The variation in genes expression Nrf2 and TSPO genes in adrenal gland of different groups

In the present study found that the AAS-induce oxidative stress reflected by clearly increase in the expressions of Nrf2 and TSPO genes in adrenal tissues.

Nrf2 is a transcription regulatory factor that plays a key functional role in the regulation of intracellular redox signaling (26). Free radical such as reactive oxygen and nitrogen species are produced by the electron transport chain in the mitochondria during oxidative phosphorylation (27). As in other cells, ROS are produced from this source in adrenal cells (11). In addition, the adrenal cells contain cytochrome P450 enzymes catalyze the oxidation of metabolic intermediates

in the steroidogenic pathway, thereby generating additional free radicals (28).

This study observe hyperactivity of HPA axis due to a decrease in serum cortisone levels after AAS administration, which causes the detection of ROS production and an increase in AAS-induced mineralocorticoid steroidogenesis (29). All this explains the increased regulation of the Nrf2 gene.

In the present study, it was found that AAS-induces oxidative stress, which showed an increase in the expression of the TSPO gene in adrenal tissues.

TSPO is a regulatory transmembrane protein

located predominantly on the outer mitochondrial membrane (30). The endogenous ligand for TSPO in steroid tissue is Cholesterol, cholesterol-binding amino acid consensus (CRAC) motif that can bind to cholesterol has been identified in the translocator protein in C-terminal region (31).

The steroidogenesis begins with the enzymatic cleavage of the cholesterol side chain to form pregnenolone consider the first steroid in mitochondria (32). For the translocation of cholesterol from cytoplasm into mitochondria, only two proteins does this function: 1) channel modified-like translocator protein (TSPO) (33). peripheral benzodiazepine receptor (PBR), as previously known, which is found in the external mitochondrial membrane; 2) steroidogenic acute regulatory protein (Star) (34), these channel or proteins translocated the cholesterol inside the mitochondria. This study shows the up-regulation of TSPO gene expression in adrenal tissues after administration of AAS compared to other groups. In addition, there is a decrease in the synthesis of cortisone. Patt *et al.* (2020) show that the effect of testosterone on bovine adrenal cells, stimulating receptor of angiotensin, as well as aldosterone synthesis *in vivo* and inhibition of adrenal corticosteroid synthesis (29), as suggested for AAS, should lead to negative feedback mediated by the HPA axis activation, ultimately leads to adrenal hyperplasia. This explains the increased mRNA levels of TSPO levels due to increased mineralocorticoid synthesis.

On the other partition, we used organic selenium (yeast high in selenium) as a protective role against AAS, which is present in the T3 group. We observed an ameliorate effect due to a decrease in reverse transcription of the Nrf2 and TSPO genes compared to the AAS (T1) group. The antioxidant defense mechanism in adrenal tissue has specific properties, including high levels of non-enzymatic antioxidants such as vitamin C, E and glutathione, as well as antioxidant enzymes such as SOD and GPX are highly active in the adrenal glands (11).

In this study, it was suggested that higher ROS

levels following AAS administration due to electron leak by the cytochrome P450 enzymes renders steroidogenic tissues acutely vulnerable to redox imbalance and oxidative stress in adrenal disorders by AAS-induced oxidative stress that cause decreased cortisone that observed in this study that causes the feedback regulation (HPA axis) to cortisone biosynthesis so that when administration of selenium, we observed improvement in cortisone synthesis. El-Demerdash and Nasr (2014) reported that selenium has a crucial function in several enzymes with physiological antioxidant properties, including GPx and thioredoxin reductase (35).

### Conclusion

From this study, we concluded that androgenic steroids have an adverse effect on adrenal function and selenium reduces this effect, but not for a long time.

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