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Alpha Lipoic acid role on pituitary testicular function in mice treated with Nitrofurantoin or Methotrexate

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Abstract
This experiment was designed to investigate the antioxidative role of alpha lipoic acid against the reproductive adverse effect of some drugs. Thirty adult male swiss albino mice were divided in to six groups. Two of them were treated with Nitrofurantoin (NFT) or Methotrexate (MTX). Three groups were supplemented with (10mg\kg B.wt) Alpha Lipoic acid (ALA) either alone or with NFT or MTX. The sixth group was administered with distilled water as a control. The results reveal an increase in LH, FSH and Testosteron with a decrease in MDA concentration in serum of mice supplemented with ALA. Moreover, these groups showed a significant decrease in the percentage of dead and abnormal sperms with an increase in sperm motility and concentration. It was concluded that ALA supplementation could protect the male reproductive system against deleterious effects of NFT or MTX through its antioxidant role.

Keywords: Male infertility, Nitrofurantoin, Methotrexate, Alpha Lipoic Acid

Introduction
Male infertility is a relatively common medical condition affecting up to 12% of male globally [1]. Disorders of male infertility can arise from dysfunction of the hypothalamic–pituitary region, from the testes themselves, or from post-testicular problems. In general [2]. Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage [3]. Involvement of oxidative stress and reactive oxygen species (ROS) are reported in a wide range of diseases. Many illnesses caused by inflammation may in fact be induced by oxidative stress**,** a clear link between oxidative stress and fertility status has been established [4]. Several drugs are known to impair semen parameters, leading to temporary or persistent infertility. Thus, the effects of conventional systemic therapies for men of reproductive age are common concerns in the clinical setting. Methotrexate (MTX) is a cytotoxic agent used to treat malignancies and a variety of non-malignant etiologies [5]. MTX affects to male reproduction transiently influences male fertility and sperm DNA integrity. Animal studies clearly indicate an adverse effect of MTX on spermatogenesis, primarily by affecting the germ cells [6]. Cytotoxic action may occur at the level of sperm development or direct injury to sperm after crossing the blood testes barrier (BTB) ultimately causing sperm death and oligospermia or dysfunctional sperm, potentially resulting in impaired fertility [7]. From the other hand, Microbial infections have been reported to reduce sperm viability. Staphylococcus aurous is the most prevalent Gram positive organism, while Escherichia coli is the most prevalent Gram negative organism isolated from the semen of males with primary infertility [8]. These microorganisms. Also lead to deterioration of spermatogenesis, loss of sperm function and/or blockage of the seminal tract [9]. Moreover, some drugs like Nitrofurantoin (NFT) which is used for treatment of urinary tract infection (UTI) has the same effect [10]. Gram-negative organisms cause approximately 90% of uncomplicated UTI, [11]**.**NFT at therapeutic doses can cause mitochondrial dysfunction, especially through inhibition of this complex. Thus, it seems that the toxicity of NFT can be partly mediated by mitochondrial complexes inhibition [12]. Recently, adverse effects of NFT had been reported on male reproductive system and testicular tissue of mice [13]. This effect was mainly attributed to the oxidative stress of NFT. Alpha Lipoic Acid (ALA), or thioctic acid, is a sulfur-containing fatty acid compound that acts as a co-enzyme and an antioxidant.

It is called a "universal antioxidant" because of its potent ability to neutralize a wide range of free radicals (single oxygen, superoxides, peroxynitrite and hydroxyal radicals) Recent studies suggests that alpha lipoic acid may improve situations in the body involving free radicals, such as heart disease and cancer, and other disorders associated with inflammation and aging. The uses and benefits of alpha lipoic acid are numerous, making it an important nutrient for maintaining and regaining health in the 21st century [14]. Not only is alpha lipoic acid able to neutralize free radicals, it is also able to recycle or regenerate several other important antioxidants, including vitamin C and glutathione [15]. Lipoic acid complex with lysine, called lipoamide, functions as an essential co-factor in the mitochondrial dehydrogenase complexes that catalyze the oxidative decarboxylation of α-keto acids [16]. Recently. Protective role of alpha lipoic acid against Metabolic Disorders and cardiovascular dysfunction induced by Ovariectomy has been studied [17].

2. Materials and Methods

2.1. Experimental animal and management

This experiment was carried out at the University of Baghdad from the period January-April/2016. Thirty adult male Swiss albino mice were 8-10 weeks old and 28-30gm B.wt. Animals have free access to water and standard pelletes of freshly prepared ration. They were kept under suitable environmental conditions of 20-25 ċ in an air conditioned room and photoperiod of 12 hours daily. The animals were kept for at least 2 weeks for acclimatization before beginning the experiment [18].

2.2. Experimental Design

Thirty animal were divided in to six groups and were treated as a follows: Group one (G1) received distilled water as control.G2 were received daily oral dose of 10mg/kg B. wt ALA [19] G3 were treated with oral dose of 200mg/kg B. wt daily NFT [13] G4 were received NFT and ALA. G5 were treated with 2.5 mg/kg B. wt. MTX for 3 time a week [20] G6 were treated with MTX and ALA. The drugs were given by oral gavage and administration was lasted for six weeks. ALA was given 30 minutes before meals whereas, NFT and NTX were given after meals.

Blood sample collection

At the end of the experiment, blood was obtained via cardiac puncture from each mouse using disposable insulin syringes. Samples were placed into Eppendorf tubes and allowed to clot. Serum was isolated after centrifugation at a speed of 3000 rpm for 10 minutes. LH estimation was performed by using a sandwich immune detection method as described by [21].I-CHROMA TM FSH is based on immunoassay system using antigen antibody interaction and fluorescence technology [22]. Testosteron assay was determined according to [23] by solid phase enzyme –linked immunosorbent assay (ELISA) based on the competition principal. The method reported by [24] was used for determination of MDA concentration in serum.

2.4. Semen Evaluation

After blood collection all animals were Sacrificed by over dose anesthesia by cardiac air injection then animals were dissected and the caudal of epididymus of each side were isolated and embedded in one ml of phosphate buffer saline at 37 ºC in a Petridish. Then, the tail of epididymus was cut into small sections by microsurgical scissors, to perform the microscopical examination on sperm evaluations [24]. Viability, morphologically abnormal sperms and motility were evaluated according to Bearden [25]. The method of sperm concentration was estimated according to Lenz [26].

2.5. Statistical analysis

All data were subject to Statistical Analysis System –version 9. (SAS) one-way ANOVA with least significant differences (LSD) were conducted [27]

3. Results and Discussion

3.1. Effect of ALA administration on serum LH, FSH, Testosteron and MDA concentration in mice treated with Nitrofurantoin or Methotrexate.

The data represented in Table -1- indicated a significant increase in serum LH, FSH, Testosteron level in groups supplemented with 10 mg/Kg B.wt. ALA for 6 weeks. On the other hand, groups of mice that received Nitrofurantoin or Methotrexate (G3 and G4) showed no change in LH concentration coincided with high level of FSH and low level of testosterone as compared with the control group. However, administration of ALA with MTX or NFT (G4 and G6) induce an increase in serum FSH and testosterone concentration. From the other hand, MDA concentration in serum shows a higher level in the group treated with NFT or MTX alone, which are 4.22 and 6.21 respectively. Supplementation of ALA alone to groups of mice or to that treated with NFT or MTX induces a significant decrease in MDA concentration compared with the non supplemented groups.

The exact mode of action of Nitrofurantoin is not completely understood, though, it is mainly known to inhibit a number of bacterial and somatic enzymes that participate in bacterial carbohydrate metabolism at some points in the Krebs cycle as well as interfering with cell wall synthesis [15]. Metabolically, under aerobic condition, the reduction of Nitrofurantoin stimulate consumption of oxygen and the production of superoxide anion free radical and hydrogen peroxide. This may result in cytotoxicity, Localized injury and sperm deformity, which is likely to be caused by an inability of testicular cells to use carbohydrates and oxygen [29]. However 3 mg/kg B.wt. of of Nitrofurantoin have been shown to cause maturation arrest in the testis which inversely decreases testosterone level accompanied by testicular damage with hemorrhage [13]. This could be attributed mainly to the oxidative stress of NFT which is documented by high levels of MDA in the present study. Moreover the direct effect of NFT and MTX on testes and the resulting decrease in testosterone synthesis may inversely affect the gonadotropin secretion from the pituitary gland. This explain the significant increase in plasma FSH with a non-significant increase in LH level reduced plasma testosterone level in the group received MTX suggest an enzymatic defect in the gonadal steroid synthesis. Antiestrogenic and antiprogestational activity of MTX [30]. correlates with the low levels of G6PD in the female reproductive tissues. It is certainly possible that G6PD levels may be low due to the inhibition of G6PD catalysis by MTX resulting in NADPH blockage. NADPH is vital for the synthesis of cholesterol an important constituent of steroidogenesis [31]. Methotrexate (MTX) is a chemotherapeutic agent causing defective oogenesis and spermatogenesis, MTX caused in significant increases in MDA levels (an important marker of lipid peroxidation). Free radicals generate the lipid peroxidation process in an organism. Malondialdehyde (MDA) is one of the final

products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes overproduction of MDA. Malondialdehyde level is commonly known as a marker of oxidative stress and the antioxidant status in cancerous patients [32].

However, treatment with alpha lipoic acid in mice alone or with NFT or with MTX was able to maintain near normal spermatogenesis and increase testosterone and gonadotropins concentration. This proves the direct protective role of ALA on the cellular level. Unlike other antioxidant ALA has the ablity to neutralize free radical with in aqueous and lipid regious of the cell, as well as in intracellular and extracellular environment. Morover it act as coenzyme in the energy cycle (citric acid cycle)of the cell and play a vital role in the mitochondrial electron transport reaction required for metabolizing glucose to ATP **[33]**. It had been reported that treatment of rats which exposed to pesticides with ALA showed an improvement in the seminiferous tubules, spermatogenic germ cells and decrease the fibrosis between testis tissues [34]. This documented the powerful antioxidant action of ALA which is clear by decreasing the MDA concentration in the current experiment.

3.2. Effect of ALA on Viability, abnormality, motility and concentration of sperms in mice treated with Nitrofurantoin or Methotrexate.

Table -2-concering the value of semen evaluation for the experimental groups. Mice treated with Nitrofurantoin or Methotrexate (G3 and G5) shows a high percentage of abnormal and dead sperms comparing with untreated groups (G1 and G2). On the other hand, supplementation of ALA in both treated groups (G4 and G6) induce a significant decrease in the dead and abnormal sperms present compared with non supplemented groups (G3 and G5). However, these results coincided with a significant decrease in sperms motility and count in the treated group. ALA administration increases the motility and concentration of sperms when given alone or with the drugs.

Oxidative stress due to excessive production of reactive oxygen species (ROS) has been associated with the defective sperm function and infertility [35]. Reactive oxygen species are known to affect cellular lipids, proteins and DNA. Oxidative stress to the sperm DNA can have profound implications for normal embryonic development and long-term health of progeny **[36]**. High cytochrome c levels in seminal plasma suggest significant mitochondrial damage by high ROS in the infertile male. Release of series of such proteins from mitochondrial inner space is likely to accelerate the process of apoptosis, possibly leading to DNA damage.Spermatozoa are rich in mitochondria [37]. Our results demonstrate a clear adverse effect on sperm viability and motility induce by NFT or MTX treatment. This was accompanied by decreasing their number and increasing the percentage of sperm abnormality. The oxidative stress adversely affects sperm function by altering membrane fluidity, permeability, and impairing sperm functional competence; to assess the membrane damage, the level of MDA, the final marker of lipid peroxidation, can be considered. MDA may be considered as a marker of semen quality. Many studies reporting correlations between MDA levels and semen parameters [38]. When the level of ROS exceeds above the normal level, it could produce damage to spermatozoa by increasing lipid peroxidation (LPO), DNA damage. These effects have been associated to affect the physiology of spermatozoa and fertility status [39]. Sperm mobility is largely dependent upon three major factors - regulation, structural integrity and energy supply. Regulation of movements is controlled at the mid-piece, particularly the flagellar and principal area. Each of these locations handles a unique function of sperm movement. The flagellar mid-piece controls the activation of motility, while the principal mid-piece handles hyperactivation [40]. The unsaturated lipid content and saturated protein channels in the mid-piece usually make it the first choice for free radical attack. Addition of ALA into the extender media allows the antioxidant (ALA) to protect these components by creating a shield surrounding the mid-piece (aqueous layer) and within the structure itself (lipid layer) [41]. The ability of ALA to create a robust shield on the cell membrane, along with the liquid that surrounds the sperm indirectly, enhance the ability of the sperm to tolerate higher volumes of free radical attack. This ability will, in turn, indirectly reduce the formation of deep pores and cracks on the sperm surface, thus ensuring structural integrity. The rate of sperm movement is largely dependent on the availability of its energy supply. Due to this, normal active sperm usually have a very active functioning mitochondria, which in turn generates high quantities of free radicals as a by-product. To ensure constant generation of ATP, external and internal structural integrity of the organelle must be maintained **[42]**. Since the membrane wall and the various compartments of the organelle are high in lipid content, addition of ALA would protect these structures from the ever-increasing free radical species, which are a by-product of the Krebs cycle. Sperm mitochondria ability is also dependent on the availability of ATP-based enzymes. Addition of ALA is thought to have assisted in the metabolism of oxidative decarboxylation by acting as a co-enzyme [43]. The increase in oxidative decarboxylation would increase cytochrome C concentration and thus directly increase the mitochondria's membrane potential, improving regulation of mitochondria function and its biogenesis [44].

Researcher call alpha lipoic acid the universal antioxidant, because it can neutralize a wide variety of free radical. Thus, we could conclude that supplementation of ALA could protect the male reproductive system against antibacterial and Anticancer drugs therapy and hence maintain fertility.

Table 1: Effect of ALA administration on serum LH, FSH, Testoserone and MDA concentration in mice treated with Nitrofurantoin or Methotrexate.

Group / parameter	G1 Control	G2 10mg/kg B.wt ALA	G3 3mg/kg B.wt NFT	G4 NFT + ALA	G5 2.5mg / kg B.wt MTX	G6 MTX + ALA	LSD
Serum LH Concentration (mlu/ml)	1.33 b ± 0.04	1.55 a ± 0.03	1.24 b ± 0.02	1.26 b ± 0.06	1.27 b ± 0.03	1.29 b ± 0.05	0.1567
Serum FSH Concentration (mlu/ml)	3.59 e ± 0.26	13.20 a ± 0.42	10.86 c ± 0.35	11.82 b ± 0.32	8.37 d ± 0.20	12.24 ab ± 0.30	0.8781

Serum Testoserone Concentration (pg/ml)	1.72 d ± 0.03	6.54 a ± 0.25	1.32 e ± 0.03	3.47 b ± 0.04	1.16 e ± 0.02	2.10 c ± 0.02	0.3320
Serum MDA Concentration M mol / L)(	2.08 d ± 0.02	1.09 e ± 0.02	4.22 b ± 0.16	2.16 d ± 0.03	6.21 a ± 0.17	3.46 c ± 0.08	0.3741

Values denote the mean of 5 adults mice ± SE.different small letters mean significant ($P \leq 0.05$) between groups

Table 2: Effect of ALA administration on viability, abnormality, motility and concentration of sperms in mice treated with Nitrofurantoin or Methotrexate

Group / parameter	G1 Control	G2 10mg/kg B.W ALA	G3 3mg/kg B.W NFT	G4 NFT + ALA	G5 2.5mg / kg B.W MTX	G6 MTX + ALA	LSD
Dead sperms (%)	13.26 de ± 0.92	10.56 e ± 0.53	43.70 b ± 2.41	14.58 d ± 0.85	78.66 a ± 1.78	25.38 c ± 1.21	3.5864
Abnormal sperms (%)	14.70 e ± 0.86	8.58 f ± 0.83	28.32 c ± 1.18	19.42 d ± 0.81	78.18 a ± 1.87	60.18 b ± 1.69	3.1785
Motile sperms (%)	74.28 a ± 1.74	76.32 a ± 1.76	55.26 c ± 2.28	66.28 b ± 1.72	21.22 d ± 1.24	50.24 c ± 2.31	5.5870
Sperms count ($nX10^7$)	2.644 c ± 8.78	3.544 a ± 11.88	1.060 e ± 8.89	2.938 b ± 8.64	0.924 e ± 13.58	1.768 d ± 8.43	0.1420

Values denote the mean of 5 adults mice ± SE. different small letters mean significant ($P \leq 0.05$) between groups

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