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THE PROTECTIVE ROLE OF CAROB EXTRACT IN PRESERVING THE MORPHOLOGY AND FUNCTION OF SPERM AND ENHANCING ANTIOXIDANT LEVELS IN TESTICULAR TISSUE EXPOSED TO METHOTREXATE TOXICITY

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Abstract: This study was designed to investigate the protective effect of the aqueous extract of carob fruit (Prosopis farcta) against oxidative damage in the testes of male albino rats induced by the drug methotrexate (MTX). It also aimed to examine its potential antioxidant activity and free radical scavenging ability. The study involved 20 male laboratory albino rats of the Sprague Dawley strain, weighing between 170-200 grams. They were divided into four groups, each containing 5 rats. The study was conducted at the animal house of the College of Veterinary Medicine, University of Tikrit, and the experiment lasted for 28 days under standard laboratory conditions. Several physiological and biochemical variables were measured, including oxidative stress parameters such as malondialdehyde (MDA), which is one of the products of oxidation. Additionally, the enzymes superoxide dismutase (SOD) and glutathione (GSH) were measured as antioxidants at the testicular tissue level. The groups treated with MTX showed a significant decrease ($P \leq 0.05$) in the level of glutathione (GSH), while there was a significant increase ($P \leq 0.05$) in the levels of malondialdehyde (MDA) and superoxide dismutase (SOD) compared to the control group. Animals exposed to oxidative stress induced by MTX for 28 days and treated with the aqueous extract of P.FARCTA fruit showed a significant decrease ($P \leq 0.05$) in the elevated levels of MDA. Additionally, there was a significant increase ($P \leq 0.05$) in the activity levels of the antioxidants SOD and GSH compared to the MTX group at the tissue extract level. Histological sections of the sperm showed deformities, including head fusion, tail coiling, and midpiece twisting. However, a significant improvement in sperm deformities was observed in the groups treated with the aqueous extract of P.FARCTA fruit.The results of the current study suggest that the aqueous extract of P.FARCTAfruit plays a crucial role in improving the physiological, biochemical, and histological variables induced by MTX administration in testicular tissue. This supports its protective, therapeutic, and antioxidant roles, making it potentially useful in preventing and mitigating disorders caused by free radicals and reactive oxygen species.

Key words: methotrexate. prospois farcta . Antioxidant. Sperms Parameters. Abonormalaty Sperms.

Introduction

Methotrexate (MTX) is a folic acid antagonist widely used in chemotherapy and as an immunosuppressant in the treatment of autoimmune diseases. It is the most recommended and commonly prescribed disease-modifying drug for the treatment of rheumatoid arthritis [1]Methotrexate is also commonly used as chemotherapy for a variety of malignancies. Patients with psoriasis, systemic lupus erythematosus, Crohn's disease, vasculitis, and a variety of other connective tissue diseases can also benefit from this drug[2].

Methotrexate is absorbed via human reduced folate carriers and is converted into methotrexate-polyglutamate within the cell. Both MTX and methotrexate-polyglutamate inhibit the enzyme dihydrofolate reductase, which catalyzes the conversion of dihydrofolate to tetrahydrofolate, the active form of folic acid[3]. One of the significant side effects of methotrexate treatment is its impact on the liver, known as hepatotoxicity. Studies over many years have discovered that the development of hepatotoxicity is caused by a disruption in the cellular antioxidant defense mechanism. When the antioxidant defense mechanism is impaired, active oxygen free radicals increase and cause oxidative damage in hepatic cells. It is believed that combining an antioxidant with methotrexate may reduce these side[3]. Natural antioxidants are widely found in foods and medicinal plants. These compounds, especially polyphenols and carotenoids, exhibit a variety of beneficial biological effects, such as anti-inflammatory properties, anti-aging effects, prevention of atherosclerosis, and anti-cancer activities. Effectively extracting and evaluating antioxidants from medicinal plants is crucial, as it allows for the exploration of potential antioxidant sources and enhances their application in functional foods, medicines, and food additives [4]

Prosopis farcta is a small thorny shrub, ranging in height from 30 to 80 cm, and belongs to the legume family Mimosodeeae[5]. Used in traditional medicine, numerous studies have demonstrated the therapeutic activity of this plant, including its anti-tumor effects, antioxidant capacity, anti-parasitic activity, and antimicrobial properties[6]. The therapeutic activity of this plant may be linked to its constituents, such as lectins, the flavonoid compound quercetin, and the alkaloid compound tryptamine[7]. Prosopis farcta contains important active compounds such as tannins, saponins, carotenoids, flavonoids, and glycosides. Flavonoid compounds isolated from the aqueous extract of the carob plant's fruit reduce total cholesterol, triglycerides, and malondialdehyde levels, and act as a strong antioxidant. This plant can often be used in medical applications[8].

Materials and Methods:**

Methotrexate (MTX) treatment was determined using the effective dose method to calculate the oral dose based on the human equivalent dose. The dose used for treatment was 0.1 mg/150 g according to[9]. The animals were administered the aqueous extract of carob fruit at a dose of 200 mg/kg body weight, based on previous studies and research [10]

****Animals Used in the Study**:**

The animals were dosed on September 18, 2023, and the study lasted for 30 days. Twenty male Albino rats, aged 15-16 weeks and weighing between 170-200 grams, were obtained from the College of Veterinary Medicine at the University of Tikrit. The rats were housed in the animal facility of the College of Veterinary Medicine, University of Tikrit. They were placed in plastic cages with metal mesh covers and lined with clean wood shavings, which were replaced every four days. The cages were kept in a room with a temperature of 22±3°C and a lighting cycle of 12 hours dark and 12 hours light. The animals were allowed to acclimate for one week before dosing and were provided with water and manufactured feed throughout the experiment

****Design of the Experiment**:**

The animals were randomly divided into four groups, each consisting of 5 animals with similar weights. All groups were dosed for 28 days as detailed below:

* .1Group 1: Control Group** - This group received regular drinking water.

* .Group 2: MTX Group** - This group received a dose of MTX at 0.1 mg/100 g body weight via oral gavage.

* .Group 3: MTX + Carob Extract *prosopis farcta*- This group received MTX at 0.1 mg/100 g body weight along with the aqueous extract of carob fruit at 200 mg/kg body weight via oral gavage.

*Group 4: Carob Extract *prosopis farcta* ** - This group received the aqueous extract of carob fruit at 200 mg/kg body weight via oral gavage.

****Animal Dissection****:

After the experiment concluded, the animals fasted for 24 hours and were then anesthetized using chloroform. Following anesthesia, the abdominal cavity was opened to extract the required organs (testes). The testes were placed in a buffer solution for tissue homogenization using a homogenizer to measure antioxidants (SOD, GSH, MDA) at the tissue level.

****Semen Collection****:

The epididymis was separated from the testes and placed in a glass Petri dish. The epididymis was cut into small pieces using a sharp scalpel, and normal saline solution was added according to the method of[11]. The mixture was continuously stirred using a wood stick (toothpick) to obtain the semen. A portion of the semen was extracted using a medical syringe and used for direct examination of sperm parameters.

****Preparation of Sperm Smears****:

The epididymis was isolated from the testes and finely chopped. The epididymal tail was minced in 2 ml of 0.9% physiological saline solution. A drop was taken and placed on a glass slide, then stained with Eosin-Nigrosin stain and spread thinly on the slide. It was allowed to dry, and then the slide was washed with water to remove the excess stain. The slide was left to dry completely for the observation of normal and abnormal sperm morphology under a light microscope. Dead sperm appeared with stained heads using Eosin, while Nigrosin served as the background for the glass slide.

****Results and Discussion****:

Statistical analysis of the results in the current study (Table 1-1) revealed a significant decrease at a probability level ($P \leq 0.05$) in the activity of the enzyme glutathione (GSH) in the group treated with methotrexate (0.2790 ± 11.6080) compared to the control group (0.1167 ± 11.9313). Additionally, there was a significant increase at a probability level ($P \leq 0.05$) in the concentration of SOD in the group treated with methotrexate (8.20 ± 68.80) compared to the control group (7.74 ± 56.48). Moreover, there was a significant increase at a probability level ($P \leq 0.05$) in the concentration of MDA in the group treated with methotrexate (15.89 ± 158.33) compared to the control group (11.15 ± 131.91) The results of the current study demonstrate the preventive role of the aqueous extract of carob fruit in reducing the negative effects of MTX treatment and normalizing the concentration of MDA. It was found that the group treated with the aqueous extract of carob fruit significantly decreased (11.54 ± 129.70) compared to the control group. These results are consistent with a study conducted by Almalki, R. S. (2024), which showed that MTX treatment significantly increases the level of malondialdehyde (MDA) while reducing the levels of GSH in liver tissues, contributing to oxidative stress and the formation of lipid peroxides.

The results of the current study were also consistent with the study conducted by Khawaja, G., & El-Orfali, Y. (2024), where MDA levels were significantly elevated with MTX treatment alone. These findings align with those of Abraham et al. (2010), who demonstrated that MTX treatment led to a 100% increase in SOD activity in the testes compared to the control. However, the results showed that the group treated with the aqueous extract of carob fruit alongside MTX treatment significantly decreased compared to the MTX-treated group alone in terms of antioxidants (GSH, MDA) and significantly increased in terms of superoxide dismutase (SOD). Nevertheless, conflicting results were observed in studies conducted by[12], where SOD

activity decreased in the liver of mice after a high dose of methotrexate. This discrepancy may be attributed to differences in the duration or intensity of the dose between studies.

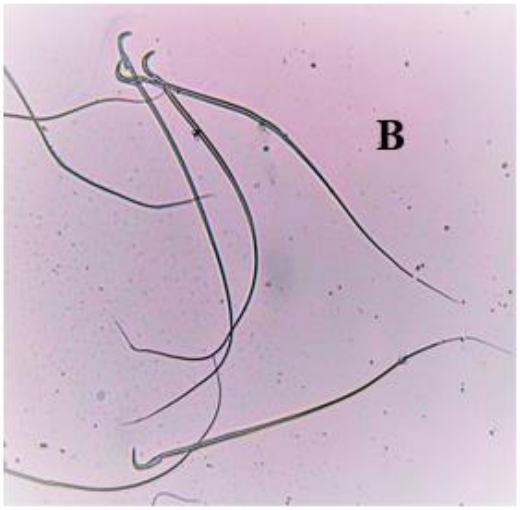
The results of the current study indicated a significant increase in the levels of antioxidants SOD and GSH, and a significant decrease in the level of MDA in the blood serum of rats treated with methotrexate and carob fruit extract (Table 1). These findings are consistent with[13], who demonstrated that parts of the carob plant contain a high concentration of quercetin, which acts as an excellent antioxidant to inhibit and eliminate free radicals, protect cells from any oxidative stress including reactive oxygen species (ROS) Moreover, the study conducted by (Mahmud Ahmed et al. 2021) showed that a moderate dose of carob fruit extract significantly reduced the level of MDA in the group of rats exposed to oxidative stress compared to the control group, and increased antioxidants compared to oxidative stress induced by cyanamide.

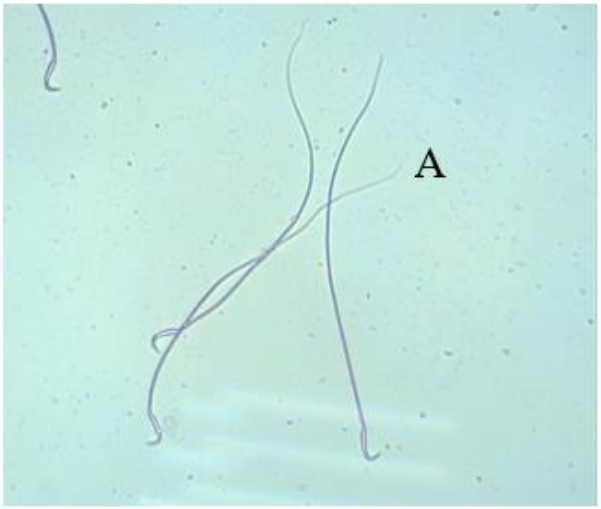
Study of Sperms Parameters :

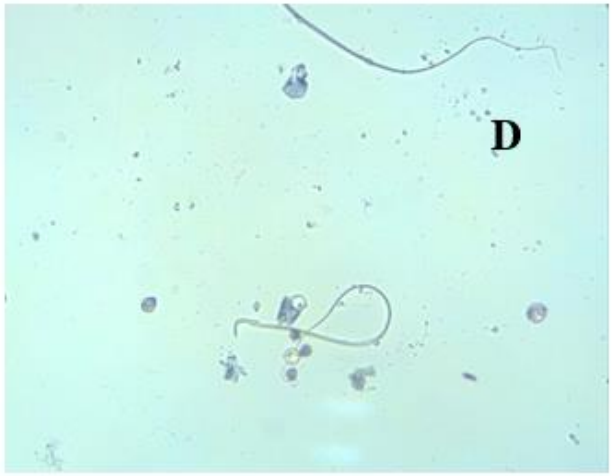
The results of the current study, through microscopic examination of rat sperm in the control group and the group treated with carob fruit aqueous extract, showed the natural morphology of sperm in terms of the head, midpiece, and tail, as depicted in Figures 4-9,10). This was in comparison to the groups treated with MTX drug, where microscopic examination of rat sperm revealed deformities in the majority of sperm. These deformities varied, including tails with curled morphology, distortion in the midpiece with swelling and twisting, as well as observed bending and deformities in the head, as shown in Figures (4-11,12,13,14,15,16.) It can be concluded that the sperm abnormalities caused by MTX are a result of physiological, toxicological, and genetic alterations in the DNA, leading to an increase in the number of abnormal spermatozoa [14]exposed mice to weekly injections of methotrexate. They reported a decrease in sperm count and an increase in sperm head deformities. It is known that MTX administration causes reproductive damage, including decreased testicular and epididymal weights, decreased sperm count in the epididymis, and infertility Previous studies have shown that different doses of MTX accelerate the process of programmed cell death through oxidative stress and may cause harmful effects on sperm formation, leading to infertility [15] Oxidative stress develops due to an imbalance between reactive oxygen species and the antioxidant system. Excessive amounts of reactive oxygen species stimulate the production of abnormal sperm with coiled tail deformities and infertility[16]. Several studies have shown that antioxidants can play a positive role in improving sperm quality. They work to prevent oxidative damage to sperm cells, thereby helping to increase sperm count and reduce mortality rates. Additionally, they contribute to enhancing sperm motility by maintaining their integrity and effectiveness[2]. Moreover, carob tree pods contain high levels of vitamin C[17]). Thanks to its content of vitamins C and E, carob extract plays a significant role in sperm health by regulating its maturation process. It also increases the level of testosterone hormone. The reason for this increase is attributed to its high levels of vitamin E, which plays a vital role in preserving the cells responsible for testosterone production. Testosterone is essential for sperm formation and protection against oxidative damage[12]. The vitamin E present in carob helps prevent free radicals from extracting hydrogen atoms from antioxidants instead of unsaturated fatty acids. This effect leads to the disruption of the free radical chain reactions, resulting in a significant decrease in free radical reactions and protection of sperm membranes[18].

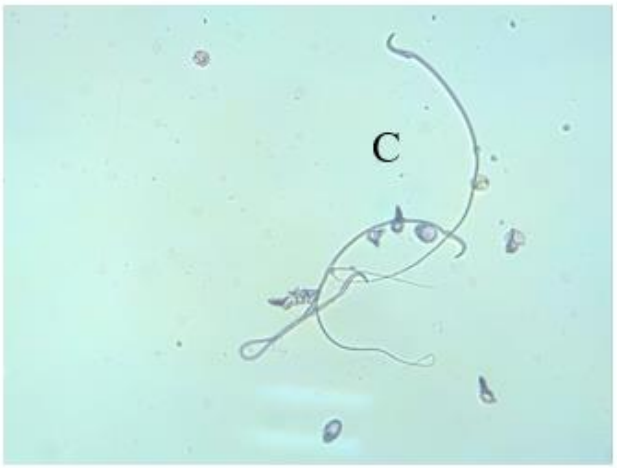
Table 1: Levels of Antioxidants

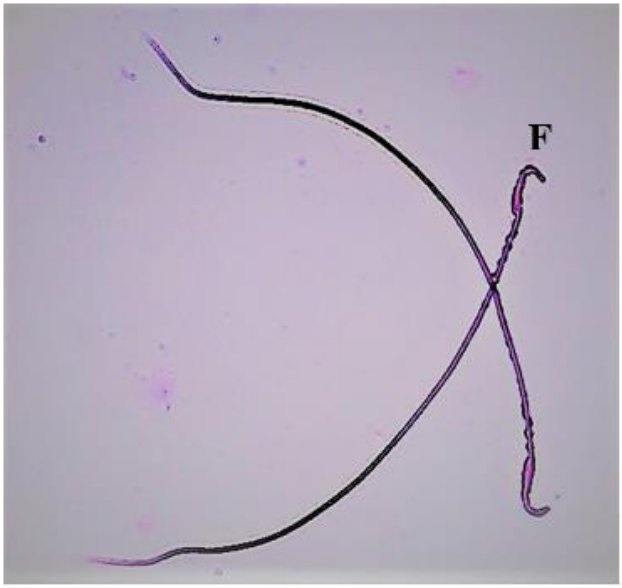
Group	GSH (Mean ± SD)	SOD (Mean ± SD)	MDA (Mean ± SD)
Control Group	**11.9313 ± 0.1167**	**56.48 ± 7.74**	**131.91 ± 11.15**
MTX Treated Group	**11.6080 ± 0.2790**	**68.80 ± 8.20**	**158.33 ± 15.89**
MTX + Carob Extract	**11.7200 ± 0.1800**	**57.30 ± 7.80**	**135.20 ± 11.70**
Carob Extract Only	**11.8900 ± 0.1500**	**56.90 ± 7.60**	**132.00 ± 11.40**

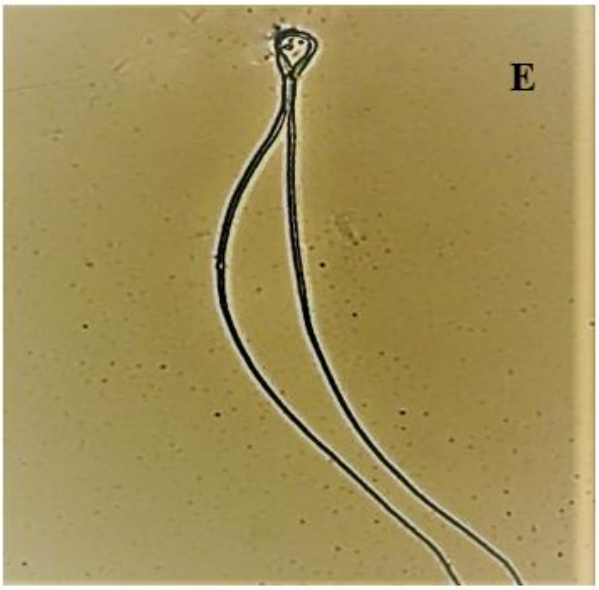

B

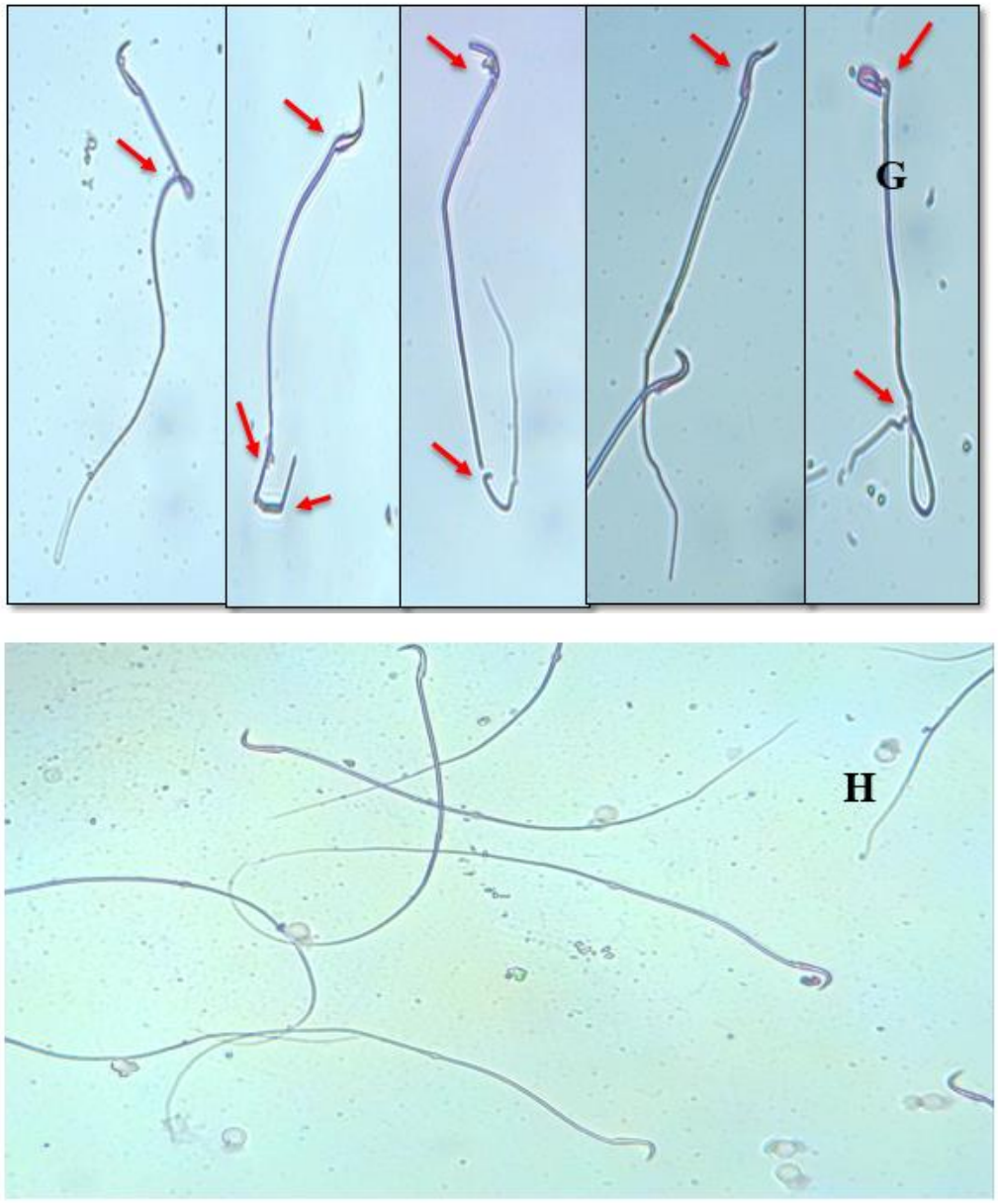

A


D


C


F


E



(Images .A, B)Sperm of the control group showing normal morphology (Image.C, D)Sperm of the methotrexate-treated group showing tail coiling deformities (.Image. E) Sperm of the methotrexate-treated group showing head fusion deformity.(Image F)Sperm of the methotrexate-treated group showing midpiece coiling deformity.(Image. G)Sperm of the methotrexate-treated group showing deformities in the head, tail, and midpiece(.Image . H) Sperm of the group treated with the aqueous extract of P.F. and MTX showing normal morphology.

Conclusions:

Histological sections of the sperm showed deformities, including head fusion, tail coiling, and midpiece twisting. However, a significant improvement in sperm deformities was observed in the groups treated with the aqueous extract of *P.FARCTA* fruit.The results of the current study suggest that the aqueous extract of *P.FARCTA*fruit plays a crucial role in improving the physiological, biochemical, and histological variables induced by MTX administration in testicular tissue. This supports its protective, therapeutic, and antioxidant

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