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Evaluation of effect of aqueous and ethanolic extracts of *Tribulus terrestris*, *Phoenix dactylifera* and *Nasturtium officinale* on reproduction in male mice treated with theobromine

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Abstract:

Background

Tribulus terrestris (TT), *Phoenix dactilyfera* (Pd) and *Nasturtium officinale* (No) were believed to have fertility-enhancing effects and given by herbalists in Baghdad to men who need to increase their fertility capacity

Objectives

To investigate the possibility that aqueous and ethanolic extracts of the plants mixture can counteract the adverse effect of theobromine on reproductive system of male mic

Materials and Methods:

One hundred and twenty mature male mice were injected intraperitoneally with 250 mg/kg/day of theobromine for four weeks. Then the males were treated intraperitonially with different dose levels of aqueous and ethanolic extracts of a mixture of the three plants for four weeks to evaluate their effects on body weight, reproductive organs weight, sperm parameters, hormones level and reproductive performance.

Results

Both extracts showed a significant enhancement in all parameters studied. Almost all these results showed a dose dependent pattern with the ethanolic extract being the more effective than aqueous extract in all parameters.

Conclusions

Mixture of the three plants seems to counteract the action of theobromine on the reproductive system of male mice through enhancing the concentration and motility of spermatozoa, increasing epididymis and seminal vesicles weight which are an indicator of testosterone production which brought about the stimulation of all male reproductive organs.

Key words: Theobromine, *Tribulus terrestris*, *Phoenix dactilyfera*, *Nasturtium officinale*..

Introduction

Theobromine, 2,6-Dihydroxy-3,7-dimethylpurine,3,7-Dimethylxanthine($C_7H_8N_4O_2$) with a molecular weight of 180.16, also known as xantheose, is a bitter alkaloid of the cacao plant found in chocolate, as well as in a number of other foods, including leaves of the tea plant, and the cola or cola nut. Theobromine caused vacuolation within the Sertoli cell, abnormally shaped spermatids, and failed release of late spermatids in rats (1). It also exhibits both antioxidant and prooxidant properties (2).

Tribulus terrestris, Pd and No are plants of future. These plants were believed to have fertility-enhancing effects and given by herbalists in Baghdad to men who need to increase their reproductive performance. The aqueous extract of TT given to mature male mice showed a

significant enhancement in sperm concentration, motility percent and grade of motility(3) and in mature female mice,it cause superovulation (4) and increase the number of growing follicles (5).

Tribulus terrestris is an annual herb found throughout India, Pakistan, Srilanka, China, Japan and Iraq (6) .It is common in the forest zone sttepe and desert of Iraq (Sulaimania, Mosul, Al-Anbar, Hilla and Baghdad). The traditional uses of this plant include treatment of sexual impotency, kidney problem and as cough remedy (7, 8).

The pollen grains of date palm have been used by the Egyptian to improve fertility in women. Experimentally, date extracts have been shown to increase sperm count in guinea pigs and to enhance spermatogenesis (9). Some of the beneficial effects of No may be due to a general stimulation of metabolism and the nervous system, including autonomous regulation (10). In German pediatric medicine, watercress is used as a disinfectant drug for antibacterial action in the treatment of lower urinary tract infections (11). The fresh herb is used in naturopathy as a blood purifier (12, 13). The present study was designed to evaluate the effects of mixture of these three plants on reproductive organs weight, sperm parameters, hormones level and reproductive performance of male mice previously treated with theobromine.

Materials and Methods

Animals: Swiss white male mice (23-27g body weight and 9 weeks of age) used in this study were raised and housed at the animal facility of the Institute of Embryo Researches and Infertility Treatment, Al-Nahrain University. Mice were maintained at 23±2°C and were subjected to 12-h light-dark cycle, housed in standard cages, fed on standard laboratory food and had free access to water *ad libitum*.

Plant materials and preparation of extracts: The three plants, TT (aerial parts), Pd (pollen grain) and No (seeds), were collected from Baghdad and identified by the Iraqi National Herbarium staff. The herbs were grounded to a fine powder in an electrical grinder .The three plants were mixed in a proportion of: TT (40%), Pd (30%) and No (30%). The aqueous and ethanolic extracts were prepared as follow: Preparation of the aqueous extract: Fifteen gm of the dry powder were dissolved with 150 ml cold distilled water in a closed vessel and allow to stand for 24hrs, shaking occasionally. Strain, press the marc and mix the liquids obtained. Extraction is performed by repeated maceration with agitation. The crude extracts were filtered and the filtrates were then evaporated to dryness in an oven below 50 °C, the yield was 13%.The gummy residue dissolved in adequate amount of distilled water and stored in a labeled sterile screw capped bottle at -20° C until use.

Preparation of the ethanolic extract: Fifteen gm of the dry powder were dissolved with 150 ml 96% ethanol, repeated maceration with agitation done, press the marc and repeated extraction. The crude extracts were filtered, and the filtrates were then evaporated to dryness in an oven below 50 °C, the yield was 18%. The gummy residue was dissolved in adequate amount of olive oil and stored in a labeled sterile screw capped bottles at -20°C until use.

Experimental protocol: One hundred and twenty mature male mice were given theobromine (this preparation was obtained as a powder 98% purity from Fluka company, product No. 88304) injection 250 mg/kg/day for 4 weeks. By the end of treatment, the animals divided into two equal batches, the first one given aqueous extract and the other given ethanolic extract. Three groups of 20 animals each were used for each extract. The first group (G1) is the control group given normal saline or olive oil injection (0.5) ml daily and the other two groups (G2 and G3) injected intraperitonially with 150 and 300mg/kg/day of the aqueous or ethanolic extract respectively for a period of 4 weeks.In both treatments, the body weight was recorded once a week. Ten animals from each group were killed at the end of the treatment to asses the reproductive organs weight, the sperm parameters (14) and hormonal levels (FSH, LH and testosterone) (15). Five males from each group were mated with untreated females at a proportion of one male/three females to evaluate the reproductive performance of the males at the end of the treatment period.

Statistical analysis: Computerized statistical analysis was performed using the SPSS (Statistical Package of Social Sciences) version 10 under windows XP-2000(Inc, Chicago, IL,USA) computer soft ware and the use of excel program. Values reported are means ±SE .Experimental results were statistically analyzed using the t-test, with P values less than 0.05 considered significant (16).

Results

Effects of the aqueous and ethanolic extracts of plants mixture on body weight, testes, epididymis and seminal vesicles weight are shown in table (1). A significant ($P<0.05$) increase was found in the body weight of G2 and G3 treated with both extracts as compared with the control group G1. The testes, epididymis and seminal vesicles weight were also significantly ($P<0.05$) higher in G2 of both extracts in comparison with the control group G1 except the testicular weight in G2 of the ethanolic extract in which the difference is highly significant ($P<0.01$) in comparison with the control group G1. The weight of the testes, epididymis and seminal vesicles in G3 of both extracts were significantly ($P<0.01$) higher than in the controls. Table (2) showing the effects of ip injection of the aqueous and ethanolic extracts of the mixture on the sperm concentration, percent motility and percent of abnormal morphology. A highly significant ($P<0.01$) increase in G2 and G3 with respect to the concentration and motility of sperm and a highly significant ($P<0.01$) decrease in

abnormal sperm morphology of both extracts was observed compared with G1. The effect of the two extracts on hormonal levels are shown in table (3). From these results its obvious that there were a highly significant (P<0.01) increase in levels of all hormones in G2 and G3 of both extracts except the testosterone level of G2 of the aqueous extract and FSH level of G3 of the aqueous extract which showed a significant (P<0.05) increase compared with the control group G1. Mating of the treated males with untreated females showed a highly significant (P<0.01) increase in the percent of pregnancy, the litter size and a highly significant (P<0.01) decrease in gestation period of groups G2 and G3 of both extracts compared to the control group G1, table (4).

Table 1: Effects of IP injection of the aqueous and ethanolic extracts of herbal mixture on body weight (gm), testes, epididymis and seminal vesicles weight (mg/100 gm body weight).

Group / Treatment	Parameters	G_1	G_2	G_3
Aqueous extract (4 wks)	Body wt.	25.1±0.36	26.8±0.20*	26.8±0.69*
	Testes wt.	1116.5±0.40	1128.9±1.15*	1221.3±1.21*
	Epididymis wt.	80.8±0.49	83.3±0.39	90.2±0.16**
	Seminal vesicle wt.	351.8 ±0.55	361.2± 0.77*	369.2±0.80**
Ethanolic extract (4 wks)	Body wt.	25.1±0.56	26.9±0.20*	26.9±0.70*
	Testes wt.	1116.0±0.49	1199.9±1.15**	1221.3±1.21**
	Epididymis wt.	81.8±0.49	89.3±0.39*	93.1±0.16**
	Seminal vesicle wt.	351.8± 0.55	381.3± 0.77*	399.1±0.80**

Values = mean ± SE. n= 10 males
P<0.05 in comparison (G1).
**P<0.01 in comparison (G1).

Table 2: Effects of IP injection of the aqueous & ethanolic extracts of herbal mixtureon sperm parameters (concentration, motility and morphology of sperm).

Group / Treatment	Parameters	G_1	G_2	G_3
Aqueous extract (4 wks)	Conc./ml	Zero	$18 \times 10^6 \pm 3.9$**	$39 \times 10 \pm^6 1.87$**
	Motile %	Zero	64±2.74**	81±2.24**
	Abnormal morphol %	Zero	39±2.95**	28±2.51**
Ethanolic extract (4 wks)	Conc./ml	Zero	$28 \times 10^6 \pm 3.9$**	$39 \times 10^6 \pm 1.87$ **
	Motile %	Zero	67±2.74**	89±2.24**
	Abnormal morphol. %	Zero	29±2. 95**	20±2.51**

Values= mean ± SE. n= 10 males
*P<0.05 in comparison with (G1).
**P<0.01 in comparison with (G1).

Table 3: Effects of IP injection of the aqueous & ethanolic extracts of herbal mixture on hormonal levels (LH, FSH, testosterone)

Group / Treatment	Parameters	G_1	G_2	G_3
Aqueous extract (4 wks)	LH mIU/ml	0.64±0.02	1.32±0.01**	1.39±0.08**
	FSH mIU/ml	0.75±0.01	1.49±0.01**	1.59±0.02*
	Testosterone pg/ml	1.5±0.09	2.9±0.08*	3.84±0.05**
Ethanolic extract (4 wks)	LH mIU/ml	0.63±0.01	1.39±0.08**	1.48±0.09**
	FSH mIU/ml	0.74±0.02	1.58±0.01**	1.63±0.02**
	Testosterone pg/ml	1.49±0.09	3.1±0.58**	3.94±0.95**

values= mean ± SE. n= 10 males
*P<0.05 in comparison with (G1).
* *P<0. 01 in comparison with (G1).

Table 4: Effects of IP injection of the aqueous & ethanolic extracts of herbal mixture on reproductive performance.

Group / Treatment	Parameters	G_1	G_2	G_3
Aqueous extract	Pregnancy %	Zero	40%**	60%**
	Gestation period (day)	Zero	21.4±0.63**	20.4±0.63**
	No. of fetus	Zero	3..33±0.90**	4.4±0.74**
Alcoholic extract	Pregnancy %	Zero	50%**	63%**
	Gestation period (day)	Zero	23.4±0.63**	20.4±0.63**
	No. of fetus	Zero	3.67±0..90**	5.0±0.74**

values= mean ± SE. n= 10 females
**P<0.01 in comparison with (G1)

Discussion

Theobromine has an effect on Sertoli cell function through increasing inhibin secretion which causes a decrease in FSH level, which is an important factor in spermatogenesis. The FSH decrease causes a decrease in concentration, motility and normal morphology of the spermatozoa. Theobromine also has a negative effect on Leydig cells and resulted in decreased level of LH secretion (17).

Results of the present study showed a significant increase in the body weight in all groups treated with the aqueous and ethanolic extracts of the plants mixture which may be due to regulation of fat and carbohydrate metabolism and improvement of appetite, digestion and assimilation (18). A positive relationship between the increase in body weight and testicular weight was reported (19). In addition to that the results also indicated an increase in epididymal and seminal vesicles weight which are an indicator of testosterone production which brought about the stimulation of all male reproductive organs (20). Treatment of the animals injected with theobromine by the aqueous and ethanolic extracts of the mixture of TT, Pd, and No plants showed a significant improvement in the sperm concentration and motility. These results agree with the reports traced in the literature concerning the activity of TT& Pd on animals and human (21-23).

The presence of vitamins C, E, A and B in the mixture may act as a potent antioxidant which may protects sperm membrane against lipid peroxidation, lowering the percentage of dead sperm and maintain normal sperm morphology. Consequently, the plants mixture contains Ca, Mg, Mn, Na and K ions (24). These minerals especially Ca is known to inhibit the enzyme phosphate diesterase which prevents cAMP degeneration and increasing sperm motility and sperm hyperactivation (25). Moreover, the presence of zinc in the mixture also leads to improvement in sperm count, motility and morphology percent (26). The improvement in sperm motility may also have been brought about by increasing intracellular cAMP which is known to be a very important factor in stimulating sperm motility (27).

As concerning the hormonal levels, protodioscin (steroidal saponin) which is present in the mixture leads to an increase in LH and DHEA levels which resulted in the stimulation of spermatogenesis and sperm concentration (21, 28). Tribulus terrestris may increase fertility by a direct action on Sertoli and germinal cells thus improving spermatogenesis and libido by increasing LH which activates the production of testosterone from the Leydig cells by increasing androgen receptor sensitivity or by stimulating the enzyme 5-alpha reductase which increases the conversion of testosterone into dihydro testosterone (29, 30).

Improvement of libido and sexual behavior parameters are due to the increase in testosterone and other possible reasons such as the hypotensive effect of TT(9) and also due to increasing endothelial nitric oxide with direct smooth muscle relaxant effects of TT. Such effect resulted in increasing blood flow into the corpus cavernosa .All these may lead to increase in the concentration and motility of sperm and in some cases increased volume of ejaculation (8). These results may also explain the significant increase in the number of pregnancies and litter size.

From the present results it can be concluded that the adverse effects caused by the theobromine treatment were significantly improved by the ip injection of the aqueous and ethanolic extracts of the mixture of the three plants suggesting that this route can be used as well as the oral route.

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