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Infertility

Effects of *Carthamus tinctorius* on Semen Quality and Gonadal Hormone Levels in Partially Sterile Male Rats

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Purpose: Traditional herbal medicine is just one of the many different approaches using plants in the remedy of diseases. *Carthamus tinctorius* (CT) or safflower is a popular plant that is used for coloring and flavoring in food industries. The effect of CT on spermatogenesis and sperm parameters has been reported in traditional medicine but has not yet been confirmed scientifically. Therefore, this study was designed to determine the effects of CT on spermatogenesis and the male reproductive system in an animal model.

Materials and Methods: Sixty male rats were divided into five groups. Four groups were injected with 5 mg/kg of busulfan as a model of partial infertility. Then, the experimental groups were treated with 10 mg/kg, 25 mg/kg, or 50 mg/kg of CT extract for 35 days. The control was treated with busulfan (infertile control) or distilled water only. After this period, the animals were sacrificed and blood samples were taken for hormonal assay. The semen was collected from the epididymis and the reproductive organs were assessed. Sperm count and motility were measured and smears were prepared for assessment of the other parameters.

Results: The results indicated that the percentage of sperm with good morphology, motility, and count increased significantly in the group treated with 10 mg/kg CT (p=0.002, p=0.03, and p= 0.00001, respectively). The effects on hormonal changes and genital organ weights were also positive.

Conclusions: It is probable that the CT extract affects spermatogenesis and as a result sperm quality. Further studies are needed.

Key Words: *Carthamus tinctorius; Gonadal hormones; Rats; Semen analysis*

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INTRODUCTION

Herbal medicine is a complementary therapy that uses plants to treat disease. Many well-established medicines come from plants, for example, aspirin from willow bark and digoxin from foxglove [1]. Traditional herbal medicine is just one of the many different approaches to the use of plants as remedies [2]. Today, herbal remedies are a common therapy. *Ginkgo biloba* is used for mental clarity and to delay the onset of Alzheimer's disease [3]. Medicinal plants serve as a therapeutic alternative or safer choice, or in some cases, as the only effective treatment. It is common among people of different cultures and areas to believe that particular local plants can cure their medical problems both cheaply and safely [4]. Many think that chemical and synthetic medicinal drugs have more side effects and cost more than do their plant counterparts.

A large number of these plants and their extracts have shown beneficial effects, including antioxidant, immunomodulatory, anti-inflammatory, and anti-cancer effects [5-8].

Carthamus tinctorius (CT), or safflower, is a plant species that is widely used both in the food industry (as an additive, flavoring, etc) and in the herbal industry. The main chemical composition of safflower is oil, protein, and carthamin pigment. The oil components of safflower are com-

posed of the fatty acid α-linoleic acid [9]. Reproductive medicine is another area of medicine that uses herbal medicines and that has undergone tremendous changes over the past decade [4,10,11].

Increasing concern has been expressed about the declining sperm count of humans over the past few decades. This may be due to exposure to environmental or chemical substances, which can lead to infertility and health problems [12].

A male factor is involved in one half of all infertile couples. Ideally, evaluation of the infertile male should result in identification of the specific abnormality responsible for the infertility. Infertility in male patients is treated with specific therapy, including herbal remedies, in an attempt to improve the semen parameters [13].

There is no proven effective medical therapy for idiopathic oligoazoospermia, and the synthetic drugs that have been tried have proven to be costly and not always available. Therefore, abundant and cheaper therapeutic methods with minimal risk potential are needed [14]. The present study was designed to study the probable effects of an herbal remedy on the male reproductive system and spermatogenesis. The aim of this study was to investigate the effects of safflower extracts on sperm parameters, hormones, and reproductive organs in male Sprague-Dawley rats treated with busulfan as an infertile or subfertile animal model.

MATERIALS AND METHODS

1. Animal selection

In this study, 60 male Sprague-Dawley rats aged more than 2 months old with body weights ranging from 250 to 350 g were randomly selected from the university animal facility. The rats were divided into five groups of 12 animals each.

All rats were acclimated to the laboratory condition for 1 week before the experiment. During the period of the study, they were maintained in a controlled temperature (22°C to 24°C) environment with a regular light and dark period (12 hours light, 12 hours dark) and with free access to food and water. At the beginning of the experiment, the animals were weighed. In four groups of animals, a single intraperitoneal injection of 5 mg/kg/body weight of busulfan (B2635, Sigma-Aldrich Co., St. Louis, MO, USA) was given. The purpose of the busulfan injection in this study was to create a partially infertile or subfertile animal model. After the injection of busulfan, three experimental groups received 10, 25, or 50 mg/kg/day safflower extract, respectively, through a feeding needle for 35 days. The normal control group received distilled water only for 35 days, and the infertile control model group received 5 mg/kg busulfan only.

2. Processing of the extract

Dried safflower flowers were prepared from traditional medicine centers. After identification and specification (Voucher No. 93) at the Department of Botany at the School of Plant Sciences of the university, the dried plant material was ground and stored for use in this study. Then, the powder from the dried safflower was placed in a sieve in a Soxhlet apparatus and mixed with distilled water for a period of time.

After concentration with the rotator apparatus, the extract was dried at room temperature; its volume was measured, and it was stored at 4°C until use. Then it was dissolved in distilled water to prepare solutions of 10, 25, and 50 mg/kg to be used as treatment doses.

3. Animal assessment

After 35 days of administrating the extract or distilled water to the experimental and control groups, the animals were sacrificed under deep anesthesia.

The animals were dissected and blood was taken from the heart immediately for hormonal assay. Then the testis and epididymis were carefully dissected out and trimmed of all fat and blood. The epididymis was removed and the sperm in its caudal part was compressed to the distal part and squeezed in a defined volume of HBSS for assessment of sperm parameters. The left testis, epididymis, and seminal vesicle were removed and after washing in normal saline, their weight and volumes were measured.

After a 5-minute incubation of the sperm in HBSS, a drop of it was loaded under the hemocytometer and sperm counting was done. Then another drop was prepared for sperm motility evaluation and some drops of the sperm solution were used for smear preparation and dried at room temperature. These slides were used to assess morphology and DNA denaturation.

4. Semen analyses

Sperm motility: In each slide, 10 fields were randomly selected. In each field, 10 sperm were assessed; motile, semimotile, and nonmotile sperm cells were counted; and the mean percentage of all slides was calculated. These data were used for the statistical analysis.

Sperm morphology: For assessment of sperm morphology, the sperm smears were stained with eosin and 10 fields in each slide were randomly selected. For every field, the percentage of the sperm with normal morphology was counted, and the mean percentage of all the slides was calculated and recorded.

Acridin orange staining: Acridine orange is a fluorescent stain for distinguishing double-stranded (normal) from single-stranded (abnormal) DNA. If the stain reacts to double-stranded DNA, it emits a green fluorescent color; in reaction to single-stranded DNA, it shows a red color [15].

In this experiment, the percentage of the sperm with normal DNA (green) and abnormal DNA (red) can be distinguished and recorded. The numbers of normal and abnormal sperm cells in the 10 fields were counted cells and the percentage of each group in each slide was calculated.

TABLE 1. Sperm count, motility, morphology, and testosterone level in the three groups treated with CT extract compared with the two control groups

Group	Count (10^6/ml)	Morphology (%)	Motility (%)	Testosterone (ng/ml)
Control (G1)	35.5±8.21	84±0.07	76±0.17	0.87±0.8
Busulfan (G2)	24±5.76	75±0.07	58±0.24	0.74±0.28
10 mg/kg CT (G3)	45.55±8.57[a]	90±0.072[b]	78±0.075[c]	1.11±0.72
25 mg/kg CT (G4)	37.87±8.32	68±0.11	23±0.09	0.75±0.51
50 mg/kg CT (G5)	17.62±5.52	43±0.25	18±0.17	0.75±0.25

Values are presented as mean±SD.
CT, *Carthamus tinctorius* extract.
[a]:Significantly different from the busulfan group (p=0.00001), [b]:Significantly different from the busulfan group (p=0.002), [c]:Significantly different from the busulfan group (p=0.03).

5. Statistical analysis

The data were given as means±standard deviations. Multiple comparisons among groups were performed by one-way analysis of variance. Student's t-test was performed to compare the two groups. Values of $p < 0.05$ were considered statistically significant.

RESULTS

As the results of this study show, there were evident differences between the normal control (group 1) and the busulfan infertile or subfertile (group 2) and three experimental groups treated with 10, 25, and 50 mg/kg CT extract (groups 3, 4, and 5, respectively).

As shown in Table 1, the sperm count was 24×10^6/ml in the subfertile busulfan group, 35.5×10^6/ml in the control, and 45.55×10^6/ml in the group treated with 10 mg/kg CT extract. The difference between the subfertile group and the group treated with 10 mg/kg CT was significant (p=0.00001). The higher doses of the extract did not improve the sperm count, and in fact it decreased to 17×10^6/ml with the 50-mg/kg dose.

The percentage of motile sperm was 78% in the 10-mg/kg treated group and 58% in the subfertile busulfan group, which was a significant difference (p=0.03). Motility dropped to 18% in the group treated with 50 mg/kg, which was much lower in comparison with the other groups.

The mean percentage of sperm with good morphology was 90% in the 10-mg/kg group and 75% in the subfertile group, and the difference was significant (p=0.002). The sperm with good morphology in the higher dose or 50-mg/kg group was 43%, a decrease to around half of the value of the 10-mg/kg group. As these results indicated, all of the sperm parameters were improved at the 10-mg/kg dose and the effects of the CT extract at this dose were statistically significant and higher in comparison with the subfertile group.

Although the testosterone level increased from 0.74 ng/ml in the subfertile group to 1.1 ng/ml in the 10-mg/kg group, the difference was not statistically significant. The testis weight results showed that mean testis weight was 0.96 g in the subfertile group and 1.18 g in the 10-mg/kg group (Table 2). The p-value of this increasing weight was 0.005 and statistically significant. The mean testis volume in the subfertile group was 0.89 ml, whereas in the 10-mg/kg group was 1.16 ml, which was a significant improvement (p=0.003).

TABLE 2. Testis weight and volume in the three groups treated with CT extract compared with the two control groups

Group	Testis weight (g)	Testis volume (ml)
Control (G1)	1.40±0.25	1.3±0.27
Busulfan (G2)	0.96±0.11	0.89±0.17
10 mg/kg CT (G3)	1.18±0.18[a]	1.16±0.15[b]
25 mg/kg CT (G4)	1.15±0.037	1.12±0.03
50 mg/kg CT (G5)	0.78±0.01	0.73±0.02

Values are presented as mean±SD.
CT, *Carthamus tinctorius* extract.
[a]:Significantly different from the busulfan group (p=0.005), [b]:Significantly different from the busulfan group (p=0.003).

As indicated in Table 3, the epididymis weight in the subfertile group was 0.58 g, whereas in the 10-mg/kg group it was 0.72 g, which was a significant difference (p=0.0005). The epididymis volume in the busulfan-treated group was 0.56 ml and in the 10-mg/kg group it was 0.66 ml, which was a significant difference (p=0.003).

Seminal vesicle weight was 1.26 g in the subfertile group and 1.56 g in the 10-mg/kg group, which was a significant difference (p=0.001). The volume of the seminal vesicle changed from 1.06 ml in the busulfan group to 1.52 in the 10-mg/kg group (p=0.0006).

By use of the acridine orange stain, the percentage of normal sperm cells regarding the presence of double-stranded DNA was assessed. In all groups in this study, no significant changes were noted compared with the control.

These results suggest that the safflower extract changed the sperm parameters, reproductive organs, and hormone levels of male rats positively.

DISCUSSION

In addition to its use as a food additive and in coloring, CT

TABLE 3. Epididymis and seminal vesicle weight and volume in the three groups treated with CT extract compared with the two control groups

Group	Epidymis weight	Epidymis volume	SV weight	SV volume
Control (G1)	0.58±0.03	0.55±0.02	1.4±0.15	1.11±0.27
Busulfan (G2)	0.58±0.08	0.56±0.06	1.26±0.19	1.06±0.17
10 mg/kg (G3)	0.72±0.04[a]	0.66±0.04[b]	1.56±0.14[c]	1.52±0.10[d]
25 mg/kg (G4)	0.69±0.05	0.60±0.06	1.44±0.14	1.42±0.18
50 mg/kg (G5)	0.68±0.03	0.60±0.04	1.43±0.17	1.37±0.15

Values are presented as mean±SD.
CT, *Carthamus tinctorius* extract; SV, seminal vesicle.
[a]:Significantly different from the busulfan group (p=0.0005), [b]:Significantly different from the busulfan group (p=0.003), [c]:Significantly different from the busulfan group (p=0.001), [d]:Significantly different from the busulfan group (p=0.0006).

or safflower has immense medicinal and therapeutic properties. The petals of CT contain many components, such as carthamin, gamma linolenic, and flavonoids (luteolin-acetyl-glucoside and quercetin-acetyl-glucoside); these two flavonoid components have potent antioxidative activities against lipid peroxidation [16].

Dried CT petals, which are used in folk medicine, have been shown to invigorate the blood circulation, break up blood stasis, promote menstruation, and relieve inflammation [17]. Neuroprotective effects of carthamin and the polyphenol components of CT have also been reported [18].

In the present study, we investigated the effects of the safflower extract on sperm parameters, reproductive organs, and testosterone hormone in male rats treated with busulfan as a subfertile animal model. For this purpose, the animals were injected with a sublethal dose of busulfan [19]. Busulfan is a bifunctional alkylating chemotherapeutic and cytostatic agent that can remove the endogenous germ cells from the testis of the treated animal, thus creating a subfertile model [20,21].

Our results showed that all the sperm parameters and reproductive organs were recovered after treatment with safflower extract. Therefore, the components of the extract of this plant can help to improve spermatogenesis and consequently promote fertility. Moreover, the results of the present study showed that the sperm count was increased about two-fold in comparison with the busulfan group (45.55×10^6/ml and 24×10^6/ml, respectively).

The sperm count was 35.5×10^6/ml in the normal control group, which was less than that in the treated extract group. It seems that the isoflavone, carthamin, and polyphenol components of safflower increased spermatogenesis and the sperm count in this study. These effects may be due to increasing blood circulation and the antioxidant activity of the safflower extract [17,18].

Although the increase in the testosterone level was not statistically significant, it changed from 0.74 ng/ml in the busulfan group to 1.11 ng/ml in the 10-mg/kg group; it seems that the safflower extract could affect spermatogenesis through hormonal changes. As Zirkin reported, testosterone is necessary for spermatogenesis, and high concentrations of this hormone can lead to negative feedback mechanisms [21].

Sperm motility and morphology also improved after treatment with safflower extract. Sperm motility, which declined to 58% in the busulfan group, was increased to 78% in the 10-mg/kg group; the morphology also increased from 75% in the busulfan-treated group to 90% in the 10-mg/kg groups. These results showed that the extract not only could promote sperm motility and morphology but also could improve sperm quality. The improvement of the sperm parameters was most effective at the dose of 10 mg/kg of safflower; at that dose, the sperm count was almost doubled in comparison with the busulfan control group. This effectiveness appeared to be different at various doses; at the higher doses of 25 mg/kg and 50 mg/kg, the CT extract actually decreased sperm parameters, sometimes to less than half of the 10-mg/kg dose. This suggests that there may be a negative feedback phenomenon at higher doses. This negative feedback can depend on the effects of high levels of hormone and of extract on the hypothalamo-hypophysial axis [22-24].

Therefore, the higher concentrations of the extract, such as 25 and 50 mg/kg, may have an adverse effect on these parameters as the result of factors in the extract or as a result of the concentration of testosterone, which can affect the hypothalamo-hypophysial axis.

The acridine orange staining for DNA integrity showed no significant differences between the control, busulfan, and experimental groups. This result may have been due to the chromatin DNA structure, the role of which in spermatogenesis and mitosis is very critical and which is one of the key molecules for cell division. Therefore, it can be concluded that after the DNA was improved in all the groups, new sperm generation with normal sperm chromatin occurred.

As the results of the study on testis weight and volume showed, the testis weight was significantly increased in comparison with the busulfan group (0.96 g in the busulfan group and 1.18 g in the 10-mg/kg group). The testis volume was increased from 0.89 ml in the busulfan group to 1.16 ml in the 10-mg/kg group. As the data for sperm parameters showed, the sperm count was increased significantly and this occurred in parallel with the increase in the weight and

volume of the testis. As reported previously, safflower can promote the blood circulation and has antioxidant activity [17,18]; therefore, the higher sperm count could be due to the increased spermatogenesis through blood circulation in the testes, which would lead to gains in weight and volume in the testes.

Epididymis weight and volume also increased significantly; the weight changed from 0.58 g in the busulfan subfertile group to 0.72 g in the 10-mg/kg group, which was significantly different (p=0.0005). The volume of the epididymis increased from 0.56 ml in the busulfan group to 0.66 ml in the 10-mg/kg group, which was also significant (p=0.003). Increases in the weight and volume must be dependent on the improvement in spermatogenesis caused by the safflower extract; testes send more sperm to the epididymis, which of course will make it heavier and larger in weight and volume.

The weight of the seminal vesicle was 1.26 g in the busulfan group and 1.56 g in the 10-mg/kg extract-treated group. The volume of the seminal vesicle changed from 1.06 ml in the busulfan group to 1.52 ml in the 10-mg/kg groups. These changes in both weight and volume of the seminal vesicle were significant (p=0.001 and p=0.0006, respectively).

As previous studies have reported, the seminal vesicle and its secretion impact fertility and spermatogenesis [25,26]; therefore, the increased weight of this organ as the result of treatment with the CT extract could help the sperm parameters.

CONCLUSIONS

As the results indicated, busulfan treatment reduced all the values assessed in this study, and most of them had still not recovered to the level of the control group at 35 days after administration. Treatment with the CT extract, however, had positive effects on the values assessed. In conclusion, CT and its flavonoid antioxidant components along with increased blood circulation in the reproductive system could improve fertility. However, the mechanism of this effect needs to be investigated in future studies.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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