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Tribulus terrestris Extract Improves Human Sperm Parameters In Vitro

Sara Khaleghi, MSc[1], Mitra Bakhtiari, PhD[1,2], Atefeh Asadmobini, MSc[1], and Farzane Esmaeili, BSc[1]

Abstract

Objective. The object of present study was to investigate the effects of direct addition of *Tribulus terrestris* extract on human sperm parameters. *Design.* Semen specimens from 40 healthy men volunteers were divided into 4 groups: one group received no treatment (control group) while the others were incubated with 20, 40, and 50 μg/mL of *T terrestris* extract (experimental groups). Motility, viability, and DNA fragmentation were assessed in all groups. *Results.* The incubation of human semen with 40 and 50 μg/mL of *T terrestris* extract significantly enhanced total sperm motility, number of progressive motile spermatozoa, and curvilinear velocity over 60 to 120 minutes' holding time ($P < .05$ or $P < < .01$). Furthermore, viability was significantly enhanced by using *T terrestris* extract ($P < .01$). *Conclusions.* In vitro addition of the *T terrestris* extract to human sperm could affect male fertility capacity.

Keywords

Tribulus terrestris, human sperm, motility, viability, DNA fragmentation

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Infertility is described as failure to conceive after one year of intercourse without any prevention,[1] approximately 8% to 12% of couples worldwide are infertile.[1] Many factors are involved in the process of conception that affects both men and women,[2] whereas 40% to 50% of infertility cases are the results of male infertility.[2] The initial step in assessment of male factor infertility is the semen quality, which is commonly evaluated by sperm concentration, morphology, and motility via semen analysis.[3]

The ability of sperm to move properly toward an oocyte is described as sperm motility.[4] It is known that sperm motility is an important factor in evaluation of semen quality.[4] Insufficient sperm motility is considered as a one of the most important causes of subfertility or infertility.[4] The fertilization capacity of sperm is not only dependent on motility but also on other parameters such as viability,[5] and sperm DNA fragmentation is increasingly being recognized as important factors of this.[6]

In recent years, in view of beneficial effects of botanical preparations as medicine, the use of spices and herbs has been gradually increasing in developing countries.[7] Although for male infertility improvement, numerous medicinal plants have been investigated, but only a few plants were traditionally used to treat this problem. Use of herbal antioxidants, has been gaining attention in several earlier reports.[8-10] Many medicinal plants have high antioxidant potential.[11] For instance, the plant species such as *Tribulus terrestris* have been tested for development of the natural antioxidant formulations in the areas of medicine and nutrition.[2,12]

Tribulus terrestris L belonging to the family of Zygophyllaceae has been successfully used in Europe and Asia to treat sexual dysfunctions.[2] This plant is composed of several biologically active compounds such as steroids, saponins, flavonoids, alkaloids, unsaturated fatty acids, vitamins, tannins, and so on.[13] The main active components of *T terrestris* are saponins from furostanol type that are termed protodioscin[14,15] These compounds have been extensively used for treatment of various diseases, such as urinary[16] and cardiovascular disorder.[17] Several studies have been reported that, administration of *T terrestris* extract in human and animals improves libido and spermatogenesis.[18,19]

Previous reports indicated that this plant has a positive effect on the fertility potential of oliguzoospermia patients and reproductive parameters and sperm quality in human, sexual

[1] Medical Biology Research Center, Kermanshah University of medical sciences, Kermanshah, Iran
[2] Fertility & Infertility Research Center, Kermanshah University of Medical Sciences, Sorkheh Lizheh, Kermanshah, Iran

Corresponding Author:
Mitra Bakhtiari, PhD, Fertility & Infertility Research Center, Kermanshah University of Medical Sciences, Sorkheh Lizheh, Kermanshah 4614749146, Iran.
Email: mbakhtiari@kums.ac.ir

activity, spermatogenesis, and erection of experimental animals such as rat, ram, and rabbit.[12,13,20-24] Zheleva-Dimitrova et al[10] demonstrated that regard to high antioxidant and inhibited lipid peroxidation activity of *T terrestris*, this plant could be benefit in infertility therapy.[10] Adaay and Mattar[25] reported the extract of *T terrestris* increased sperm concentration and motility and decreased abnormal morphology in mice. There are no data concerning the effect of *T terrestris* extract on human sperm parameters. Therefore, this study aimed at investigating the effect of *T terrestris* extract on human sperm parameters in vitro.

Material and Methods

Plant material

Tribulus terrestris was collected from the local vegetable markets in Kermanshah in April-May, 2013. The plant material was identified with the help of experts in Department of Agriculture, Razi University, Kermanshah, Iran.

Fresh plant material was cleaned and shed dried at 25°C. Then, the dried material was ground with a blender. The powder was kept in nylon bags in a freezer (−20°C) before starting the experiments.

Solvent Extraction

The plant powder was thoroughly mixed with distilled water using a stirrer for 24 hours. Then, the mixture was filtered and centrifuged for 15 minutes at 5000 × *g*. The supernatant was collected and the solvent was evaporated under reduced pressure at 37°C. In the experimental setup using human spermatozoa, the stock solution was mixed in Ham's-F10 (Sigma), resulting in final concentrations of 20, 40, and 50 μg/mL. Ham's-F10 without *T terrestris* extract served as a control agent.

Semen Samples Collection

This study was carried out at Infertility Treatment Research Center, Motazedi Hospital, Kermanshah, Iran. Healthy fertile men (n = 40) aged 20 to 35 years (average 27.8 years) were enrolled. Written informed consent was obtained from the participants before recruitment. Consent forms and protocols were approved by Ethics Committee of Kermanshah University of Medical Sciences.

Semen samples were collected by masturbation into sterile container after 3 days of abstinence and kept at 37°C immediately prior to the examination for liquefying. The nonliquefied samples were checked at 20-minute intervals until they were liquefied. Microscopic analysis was conducted according to World Health Organization (WHO) manual. All the routine semen parameters were consistent with the normal ranges according to WHO guidelines.[26-28] Exclusion criteria included abnormal semen analysis according to the WHO criteria,[29] sexual transmitted disease (HIV, syphilis, and hepatitis B), or genital tract infection diseases.

Sperm Processing

Semen specimens were preprocessed by swim-up optimization technique to obtain highly motile spermatozoa. They were then diluted with the Ham's F-10 medium (Sigma) to obtain a sperm suspension with a concentration of 50 × 10^6/mL. The percentage of progressively motile spermatozoa (level a + b) and sperm motility should be higher than 45% and 80%, respectively.

In Vitro Incubation of Spermatozoa With Tribulus terrestris *Extract*

The sperms of each sample were divided into 4 groups. For group 1 (control), Ham's F-10 medium (0 drug concentration) was added. For groups 2, 3, and 4, Ham's F-10 medium with *T terrestris* extract at doses of 20, 40, and 50 μg/mL were added, respectively. The parameters recorded for each sample were measured after 15, 30, 60, and 120 minutes of incubation at 37°C, 5% CO_2, and humidity 90%.

Sperm Concentration and Motility Analysis

Semen quality analysis was performed using the computer-assisted semen analysis (CASA) version 12 IVOS (Hamilton Thorne Biosciences). For automatic analysis, 5 μL semen samples were dropped on the sperm analysis chamber. Using CASA, at least 10 fields were evaluated regarding sperm concentration, sperm motility, and different sperm motion variables, including percentage of progressive motility (percentage of A + B level of spermatozoa) and movement characteristics such as curvilinear velocity (VCL) and straight line velocity (VSL).

After 0 seconds and once in every 15, 30, 60, and 120 minutes of incubation, the sperm motility parameters were estimated in 10 randomly chosen fields using the CASA system.

Sperm Viability Analysis

Eosin B staining[30] was carried out to assess sperm viability. Four drops of semen were mixed thoroughly with one drop of 1% eosin B and mixed well. Immediately, a drop of the mixture was placed on a clean glass slide and allowed to be air dried. The prepared slide was examined that 100 cells per sample were determined. Pink-stained dead sperms were differentiated from unstained live sperm, and their numbers were recorded. The percentage of the live spermatozoa was calculated triplicately.

Determination of DNA Fragmentation

For all the experimental and control groups 30 μL of samples were mixed with70 μL of 1% low-melting point aqueous agarose (to obtain a 0.7% final agarose concentration) at 37°C. Aliquots of 50 mL of the mixture were pipetted onto a precoated glass slide with 0.65% standard agarose dried at 80°C and covered with a coverslip. Then, slides were left to be solidified at 4°C for 4 minutes. The coverslips were removed slowly, then the slides were immediately immersed horizontally in fresh acid denaturation solution (0.08 N HCl) at 22°C for 7 minutes in a dark space. The slides were transferred to a tray with neutralizing and lysing solution No. 1 (0.4 M Tris, 0.8 M dithiothretol [DTT], 1% sodium dodecyl sulfate [SDS], and 50 mM ethylenediamine tetra-acetic acid [EDTA] with pH of 7.5) at room temperature for 10 minutes. All the slides were incubated in neutralizing and lysing solution No. 2 (0.4 M Tris, 2 M NaCl, and 1% SDS with pH of 7.5) at room temperature for 5 minutes. This stage was followed by washing in Tris-borate-EDTA buffer (0.09 M Tris-borate and 0.002 M EDTA with pH of 7.5) for 2 minutes, dehydrating in sequential 70%, 90%, and 100% ethanol baths (each for 2 minutes), and air drying. The cells were stained with Wright stain (1:1 in phosphate buffered saline)

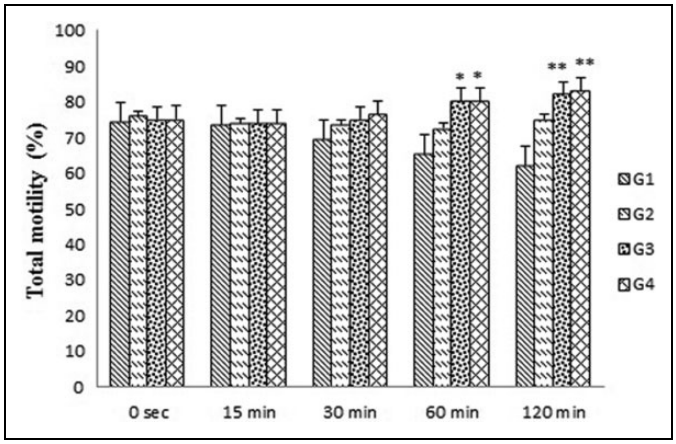

Figure 1. Effects of *Tribulus terrestris* extract on total motile spermatozoa in human sperm at different concentrations in vitro. Values = mean ± standard error of the mean (SEM), N = 10. *$P < .05$, **$P < .01$.

(Merck) for 10 minutes. Then the slides were studied with light microscopy under 100× magnification.[31]

Statistical Analysis

The results of experiments are expressed using mean ± standard error of mean. Differences between two groups were compared by using Student's t test, and statistical comparisons between experimental groups were made by analysis of variance and Tukey's post test, as offered by GraphPad InStat version 3.0 (GraphPad Software Inc, La Jolla, CA). Statistical significance was determined as $P < .05$.

Results

All semen samples were examined using light microscopy and classified as normal according to WHO guidelines.[32] Aliquots of all the experimental groups were incubated for 120 minutes with 20, 40, and 50 μg/mL concentration of *T terrestris* extract and untreated semen under the same conditions was used as a control agent.

Effects of Tribulus terrestris *Extract on Sperm Motion Parameters*

Sperm motility was assessed in all the treated and untreated groups. The sperm motility was evaluated during counting all motile and immotile spermatozoa in 10 randomly chosen fields using a CASA system. The effect of *T terrestris* extract on the total motility of sperm in all the experimental and controlling groups are reported in Figure 1. This figure demonstrated that the incubation of human semen with 20 μg/mL of *T terrestris* extract (group 2) for all holding times (0, 15, 30, 60, and 120 minutes) had no effect on total sperm motility compared with the control. When the concentration of *T terrestris* extract was elevated to 40 and 50 μg/mL (groups 3 and 4, respectively), the total sperm motility had no increase at 0 to 30-minute holding times, but was significantly was enhanced over 60- to 120-minute holding times ($P < .01$) compared with the control group (Figure 1).

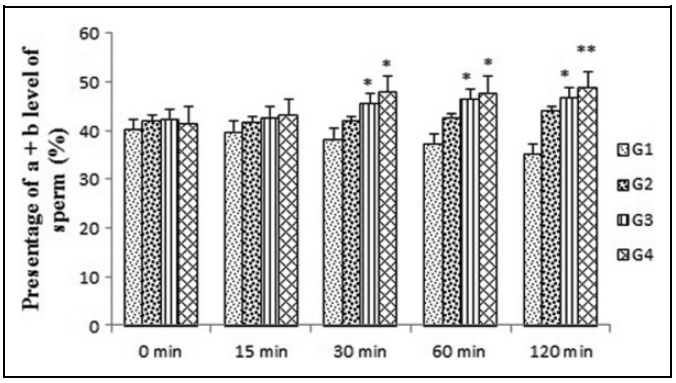

Figure 2. Effects of *Tribulus terrestris* extract on number of progressive motile spermatozoa in human sperm at different concentrations in vitro. Values = mean ± standard error of the mean (SEM), N = 10. *$P < .05$, **$P < .01$.

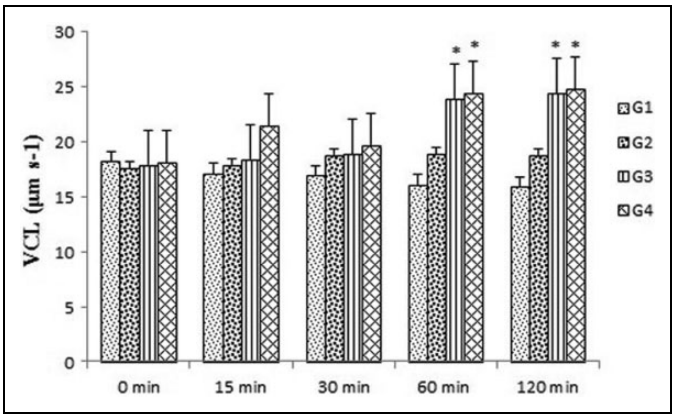

Figure 3. Effects of *Tribulus terrestris* extract on curvilinear velocity (VCL) in human sperm at different concentrations in vitro. Values = mean ± standard error of the mean (SEM), N = 10. *$P < .05$.

Tribulus terrestris extract could consistently increase the number of progressive motile spermatozoa over 30- to 120-minute holding times in groups 3 and 4 compared with the control group ($P < .05$ and $P < .01$, respectively) (Figure 2). In groups 3 and 4, the VCL was significantly enhanced by this extracts for 60 minutes and remained higher until 120 minutes in groups 3 and 4 compared with the control group ($P < .05$) (Figure 3). The VSL was not significantly changed in all experimental groups after exposure to the drug for 0 to 120-minute holding times (Figure 4).

Effect of Tribulus terrestris *Extract on Spermatozoa Viability (Live/Dead Ratio)*

The effect of *T terrestris* extract on the viability of sperm are reported in Figure 5. This figure demonstrated that the incubation of human semen with 20 μg/mL (group 2) of *T terrestris* extract for all the holding times (0, 15, 30, 60, and 120 minutes) had no effect on the viability of sperm versus the controlling group. Effect of *T terrestris* extract on the viability of sperm was insignificantly changed from 0 to 60 minutes' exposure time to

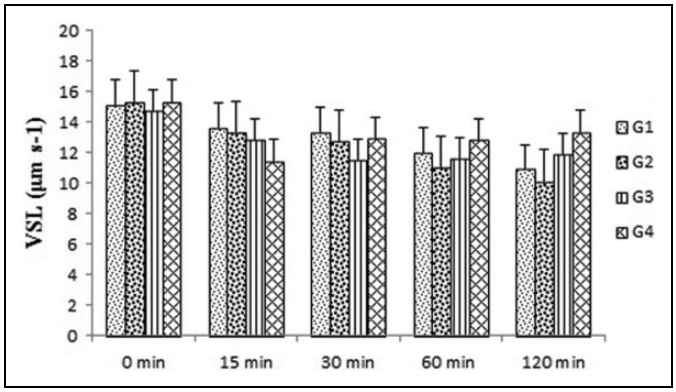

Figure 4. Effects of *Tribulus terrestris* extracts on straight line velocity (VSL) in human sperm at different concentrations in vitro. Values = mean ± standard error of the mean (SEM), N = 10.

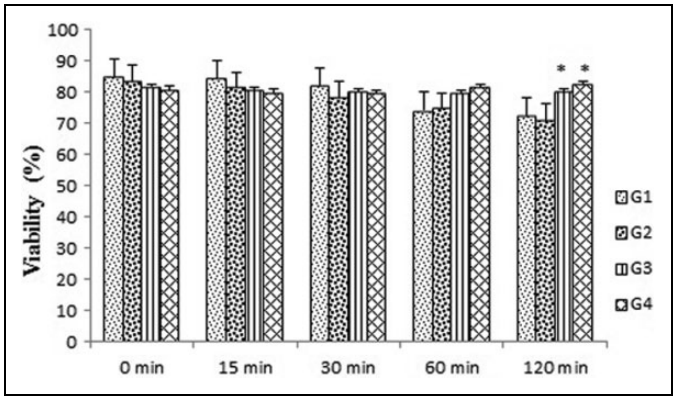

Figure 5. Effects of *Tribulus terrestris* extract on viability of human sperm at different concentrations in vitro. Values = mean ± standard error of the mean (SEM), N = 10. *$P < .05$.

Table 1. Effect of *Tribulus terrestris* Extracts on DNA Fragmentation in Human Sperm at Different Concentrations In Vitro.[a]

Group	n	Concentration ($\times 10^6$/mL)	DNA Fragmentation	*P*
Group 1	10	108.66 ± 3.8	11.43 ± 1.5	Nonsignificant
Group 2	10	102.80 ± 2.7	12.25 ± 1.3	Nonsignificant
Group 3	10	105.30 ± 3.3	12.00 ± 0.7	Nonsignificant
Group 4	10	104.40 ± 4.1	12.30 ± 0.6	Nonsignificant

[a]Values are presented as mean ± standard error of the mean.

the drug at dose of 40 and 50 µg/mL (groups 3 and 4), whereas the viability of sperm was considerably increased at doses of 40 and 50 µg/mL of *T terrestris* extract versus the controlling ($P < .05$) after 120 minutes' holding time (Figure 5).

Sperm Chromatin Dispersion Test Values

In order to determine the DNA fragmentation levels in semen samples the sperm chromatin dispersion test was performed. The results of sperm chromatin dispersion tests in all experimental groups indicated that *T terrestris* extract had no significant effect on DNA fragmentation versus the control group (Table 1).

Discussion

The results of present study indicated that *T terrestris* extract has a considerable effect on the motility and viability of human sperm. However, the extract of *T terrestris* as a supplementation had no effect on DNA fragmentation of human sperm in vitro.

In procedures such as intrauterine insemination, in vitro fertilization, and gamete intra fallopian transfer, washed spermatozoa is used.[33] In many cases of infertility, the problem encountered is poor sperm motility.[33] Advanced techniques for optimizing sperm function in these procedures are of evident value.[33]

Many herbal medicines have been reported to enhance sperm motility. Apparently, the studies about the effect of *T terrestris* extract on human sperm motility in vitro have not been reported to the best of our knowledge. Previous studies on the in vivo effect of *T terrestris* extract on sperm motility showed that this extract could significantly increase the sperm motility in mice.[25,33]

The results of the present work demonstrated that *T terrestris* extract had considerable effect on improvement of total motile spermatozoa and enhancing the progressive motility due to significantly increase in VCL motility significantly after 60 to 120 minutes of treatment. The results also showed that this extract had no effect on the VSL in all the experiments. The velocity, one of the important sperm motility parameters, had a significant predictive value in male fertility.[33] The threshold value for evaluation of sperm motility was known as VCL > 25 µm and was used as special and independent parameter in estimating male fertility by CASA system.[34] Recent reports showed that improved VCL, rather than VSL, can be correlated with advanced rates of in vitro fertilization.[35] Because mean linearity VSL/VCL, LIN had no increase with a little increase in VCL.

Although the mechanisms of *T terrestris* extract for improving sperm motility are unknown, Nassar et al[36] suggested that a significant stimulatory effect on human sperm motility might have a relation with the trace elements, especially Ca^{2+} that is known in *T terrestris* extract. Ca^{2+} could inhibit the enzyme phosphate diesterase, which prevents cyclic adenosine monophosphate degradation and also enhances the sperm motility.[37] Zinc, another trace elements in this extract, leads to improve the sperm motility because of its involvement in protein synthesis and nuclear chromatin stabilization.[16] Furthermore, free radicals have an important role on male infertility as the antioxidants could prevent their harmful effects on the sperm.[38] It has been reported that antioxidants have the greatest effect on sperm motility.[38] *T terrestris* extract contains total polyphenols, which have a wide class of components such as phenolic acids and flavonols. These components are highly correlated with antioxidant activity.[39,40] So this extract with antioxidant

compounds has an antioxidant activity, which could be another effective mechanism on human sperm motility improvement.

A useful analysis of sperm is viability test, which is an important factor for in vivo and in vitro male fertilization capacity. There is no previous report on the effect of *T terrestris* extract for evaluation of the human sperm viability.

The results of this investigation demonstrated that *T terrestris* extract significantly increased the human sperm viability after 120 minutes of incubation at doses of 40 and 50 μg/mL in vitro. This activity might be through the same effective mechanism of this extract on the sperm motility. Oxidative stress is harmful to sperm function and impairs male fertility by affecting the sperm viability.[41] High concentrations of NO could affect the sperm viability by cytotoxic effect that is probably mediated by oxidative stress and lipid peroxidation of sperm membranes.

Another important factor for male infertility is the negative effect of sperm DNA fragmentation. The effect of *T terrestris* extract on sperm DNA fragmentation was investigated and no change in sperm DNA fragmentation was shown during exposure to the *T terrestris* extract after 120-minute holding time. This result was similar to a previous investigation about the effect of crude extract of *Polygala tenuifolia* Willd on sperm DNA fragmentation.[42] Lower DNA fragmentation levels in processed semen samples have to be regarded as an effect of the sperm preparation process.[43,44]

Collectively, the present results showed that the effect of *T terrestris* extract on human sperm parameters at the dependent doses significantly increased the motility and viability, while it had no positive effect on DNA fragmentation. Nevertheless, further studies are necessary to investigate fertilization capacity of sperms and quality of embryos after exposure to *T terrestris* extract.

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Author Contributions

All authors contributed to the design, execution, analysis, and write up of this article.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

The project received approval from the Department of Agriculture, Razi University, Kermanshah, Iran.

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