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Effect of carob *(Ceratonia Siliqua)* kibble extract on the kinematic parameters of human frozen-thawed sperm: A preliminary study

Mahmoud Heidari [1*], Fereshteh Dardmeh [2], Hiva Alipour [2], Nasrinsadat Azami [1*]

1. Department of Biology, Faculty of Science, Islamic Azad University, Gorgan Branch, Gorgan, Iran
2. Laboratory of Regenerative Medicine, Department of Health Science and Technology, Aalborg University, Aalborg, Denmark

* Correspondence: Mahmoud Heidari and Nasrinsadat Azami. Department of Biology, Faculty of Science, Islamic Azad University, Gorgan Branch, Gorgan, Iran. Tel: +989112691256; Email: mahmoud.heidari@iau.ac.ir and Na.azami@iau.ac.ir

Abstract

Background: Semen quality and related parameters correlate directly with fertilization, consequently assisting reproductive technology outcomes. Traditional studies on carob (*Ceratonia siliqua*) have demonstrated its effect on male fertility potential via the reductive effect on reactive oxygen species. This study aimed to investigate the effect of carob kibble extract on sperm motility.

Methods: The extract was made using acetone as a solvent, followed by vacuum evaporation and filtration. Following thawing, each of the forty human semen samples was divided into four groups and exposed to concentrations of 0.0 (Control), 0.05, 0.2, and 0.8 mg/ml of the extract. Percentages of progressive motile, non-progressive motile, and immotile sperms, as well as other kinematic parameters, were assessed by computer-aided sperm analysis immediately after exposure to the concentrations (T0) and one hour later (T1). Data were analyzed by repeated measure analysis of variance and paired sample t-student tests using SPSS software. The level of $p< 0.05$ was considered statistically significant.

Results: No significant difference was found between groups at T0 or T1 values. However, a comparison of matched doses at T0 and T1 indicated that lower doses 0.05 and 0.2 mg/ml could significantly ($p<0.05$) inhibit natural decline in motility.

Conclusion: Adding lower doses of carob kibble extract on a thawing medium could have a supportive effect on sperm motility. However, adding the extract to a vitrification solution before a freezing process, as well as oral intake of the extract seems to have more efficiency than would be a subject for further studies.

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Highlights

What is current knowledge?

Supplementation of freezing/thawing media with C. siliqua extract improves sperm quality.

What is new here?

Lower doses of carob kibble extract on a thawing medium could have more supportive effect on sperm motility.

Introduction

The World Health Organization (WHO) refers to infertility as: "A disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (1). Estimatedly, 15% of couples are globally affected by infertility at some point (2). Approximately male factors account for 20-30% of infertile cases (3), and semen volume, sperm concentration, motility, and morphology are known as the most common markers of sperm quality (4).

Different conditions, including chemotherapy, surgical infertility treatment, viral infections, and assisted reproductive techniques (ART), are indications for the cryopreservation of human semen samples (5). However, the main issue is the post-cryopreservation decline of sperm quality (6). A study assessing the effect of cryopreservation and thawing on human sperm parameters demonstrated a reduced total number of motile spermatozoa from 63% to 30% and an increased total number of immotile spermatozoa from 24% to 64% following cryopreservation (7).

DNA damage has also been confirmed to be induced post-cryopreservation, possibly due to increased oxidative stress (8). Therefore, different studies have been conducted to improve sperm recovery by changing the freezing medium or using a specific supplement such as Tempol and Trolox as an antioxidant agent (9-11). Tempol, as a nitroxide compound, imitates superoxide dismutase anti-ROS activity, and Trolox, as a derivative of vitamin E, can protect the cells against reactive oxygen species (ROS) in the cell culture system (10,11).

In some cases, undergoing ART, retaining the number of motile spermatozoa following sperm thawing would greatly influence intrauterine insemination (IUI) outcome, the first approach and cost-effective procedure in infertility clinics (12,13). Besides, alternative and complementary medicine, in which various herbs and supplements are used, is an approach that some couples with low fertility seek for treating their problem (14,15). Amongst, carob (Ceratonia siliqua), as an evergreen tree that grows in the Middle East, North and South Africa, the Mediterranean zone, and other Mediterranean-like regions such as Mexico and Chile, has been considered to be effective in sperm motility (16,17). Detecting high levels of polyphenolic antioxidants in carob kibble has shed light on using carob pulp extract in traditional practices to improve fertility potential (18). The antioxidative properties and supportive effects of carob products have been used in several medical and animal fields (19-22). It has been shown that supplementation of freezing/thawing media with C. siliqua extract improves sperm quality but not the kinematic parameters of normozoospermic samples (23). However, despite the common use of the carob kibble extract to increase fertility potential in traditional medicine, no previous study has assessed its effect on human sperm motility and kinematic parameters following thawing. Improvement of sperm motility by carob could provide not only a cheap, natural, and accessible treatment option for idiopathic asthenozoospermia but also for preserving sperm motility during an IUI procedure. Therefore, this study aimed to investigate the potential effect of carob kibble extracts on sperm motility characteristics of frozen-thawed asthenozoospermic samples in vitro.

Methods

In this study, the extraction of carob extract was performed according to the methods of a previous study evaluating the efficacy of different solvents for the recovery of polyphenols from carob kibble (24). The extract used in the study was derived from deseeded and dried carob kibbles, chopped into tiny pieces ($< 2\ mm^3$). Approximately 10 g of the chopped kibbles were immersed into 50 ml of acetone (80%) as solvent inside a round-bottomed flask. The flask was spun for 20 min on a rotary evaporator without vacuum at $t \leqslant 30^\circ$ C. Next, a filtration process was performed in which the extract ran through a paper filter twice before the extract was merged and concentrated under vacuum at $t \leqslant 40^\circ$ C. Finally, the concentrated extract (45.3 mg) was brought up to 25 ml with 80% methanol (MeOH).

On the day of the study, the warm carob extract was diluted with culture media to create 0.05, 0.2, and 0.8 mg/ml concentrations of carob extract (23). Forty normozoospermic cryopreserved human semen samples were acquired from CRYOS International sperm bank (Aarhus, Denmark) and used following the Declaration of Helsinki. The consent form has been signed by donors at the company. The samples were treated through a thawing procedure done at room

temperature. Briefly, an equal amount of HEPES buffered Hams-F10 culture medium was added to the sample at room temperature, and the suspension was centrifuged for 5 min at 1300 rpm. The supernatant was discarded, and each sample was then divided into four parts to make different extract concentrations, including 0.0 (Control), 0.05, 0.2, and 0.8 mg/ml.

Motility measures of semen samples, including progressive motile (PM), non-progressive motile (NPM), immotile sperm (IM), and other kinematic parameters, were assessed immediately after exposure to the extract (T0). Motility measures of samples were repeated one hour later (T1) to evaluate the possible effect of carob extract in ruling out natural motility decline in vitro.

In this study, the motility module of the Sperm Class Analyzer (SCA, Version 6.1; Microptic S.L., Barcelona, Spain) computer-aided sperm analysis (CASA) system was used to assess sperm motility and detailed kinematic parameters and categorize the sperm into progressive motile, non-progressive motile and immotile groups according to the WHO criteria (25). The SCA CASA system consisted of a Nikon Eclipse 50i (Nikon, Japan) coupled with a computer running the SCA software through a firewire Basler (Sca987, Basler, Germany) camera.

The motility assessments were performed at two different points in time: adding the extract immediately after thawing (T0) and one hour later (T1). Samples were thoroughly mixed to a homogeneous state before being placed on 10 µm chamber slides (Leja, Netherlands) and analyzed by the SCA to determine the following kinematic parameters: curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity index (LIN), straightness index (STR).

Statistical analysis

The data were checked for normality using the Kolmogorov-Smirnov test. Repeated measures analysis of the variance (ANOVA) and paired t-student tests were used in the SPSS statistical software (Ver. 25, IBM, USA). P value<0.05 was considered significant.

Results

The figures present significant differences in some values of the assessed motility parameters (Figures 1-4). As Figure 1 shows, no significant difference was in progressive motility between groups not only at T0 but also at T1. However, a comparison of match doses showed significant (p<0.05) progressive motility decline in control and dose 0.8 mg/ml groups of T1 compared to those of T0. In other words, doses 0.05 and 0.2 mg/ml could inhibit motility decline at T1 compared to T0. As shown in Figure 2, no significant difference was found in non-progressive motility not only between groups at T0 and T1 but also between matched doses. Figure 3 shows a natural increase of immotile sperm percent one hour after thawing (T1). No significant change exists between the control and experimental groups at T0 as well as T1. However, a comparison of matched doses of T0 and T1 shows a significant decrease (p< 0.05) for dose 0.8 mg/ml of T1 compared to that of T0, while there was no significant difference in other groups (Control, 0.05, and 0.2 mg/ml). Figure 4 shows no significant difference in all kinematic parameters, including curvilinear velocity (4A), straight-line velocity (4B), average path velocity (4C), linearity (4D), and straightness (4E) not only between control and experimental groups at T0 and T1 but also between matched doses.

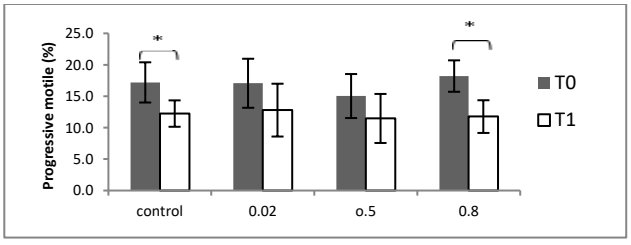

Figure 1. Effects of different doses of carob extract on progressive motility. No significant difference was between the control and experimental groups at T0 and T1. However, significant differences (P<0.05) were between the matched control and dose 0.8 mg/ml.

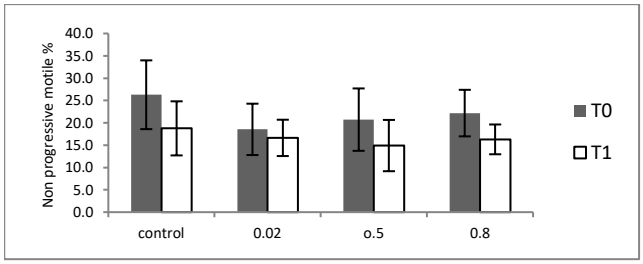

Figure 2. Effects of different doses of carob extract on non-progressive motility. No significant difference was made not only between the control and experimental groups at T0 and T1 but also between T0 and T1 matched groups.

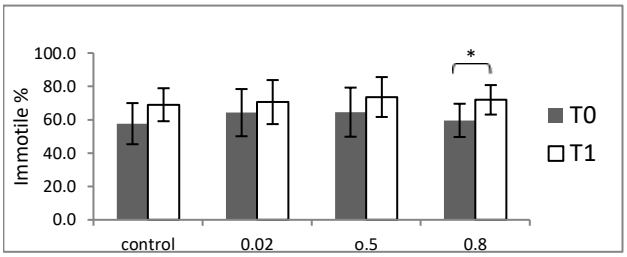

Figure 3. Effects of different doses of carob extract on immotile sperm. No significant difference was found between the control and experimental groups at T0 and T1. However, a significant difference (P<0.05) was observed between the matched group of dose 0.8 mg/ml.

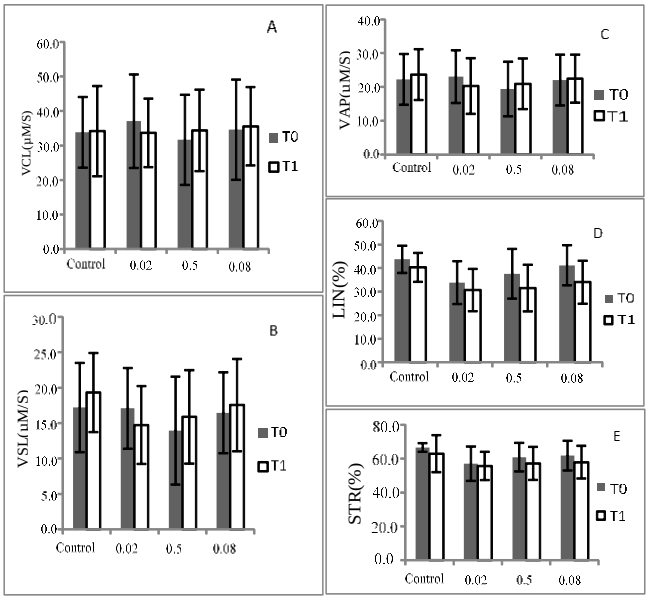

Figure 4. Effects of different doses of carob extract on the kinematic parameters including VCL: curvilinear velocity (4A); VSL: straight line velocity, (4B); VAP: average path velocity, (4C); LIN: linearity index, (4D); STR: straightness index, (4E). No significant difference was made not only between the control and experimental groups at T0 and T1 but also between matched groups of T0 and T1.

Discussion

Mammalian spermatozoa have been shown to produce reactive oxygen species (ROS) in the form of hydroxyl radical (OH.), hydrogen peroxide (H_2O_2), the superoxide anion (O_2−.), and hypochlorite radical (OHCl.) during incubation and presence of oxygen (26). A clear correlation between reduced sperm motility and elevated levels of ROS has been shown (27), and antioxidants are known to neutralize excess levels of ROS, inhibiting the deteriorating effect of oxidative stress on sperm motility (28,29).

Since sperms naturally express a decline in motility with time during the assessment of semen samples, the effect of carob extract as a source of polyphenol antioxidants on sperm motility was primarily studied by comparing the different experimental groups to the control group immediately after the addition of the extract (T0) and one hour later (T1). Demonstratively, intraperitoneal intake of carob extract for 35 days could exert a supportive effect on sperm quality in infertile mouse models (17). Undoubtedly, polyphenols are considered the most potent components of antioxidant activity in plants owing to their framework and capability of donating H+ to radicals produced during lipid peroxidation (30,31). CASA assessments in the present study regarding motility and other kinematic parameters demonstrated that although a higher dose (0.8 mg/ml) of carob extract exerted a supportive effect for sperm progressive motility at T0. However, lower doses of carob extract could inhibit the natural decline of progressive motility after one hour elapsed time. Previously, it has been shown that a lower dose of carob seed extract has more efficiency than a higher dose in ram sperm parameters during the freeze-thawing process (19). Faramarzi et al. also showed a supporting role for carob extract in sperm quality of frozen human normozoospermic samples following thawing (23). Further, the same studies demonstrated that consuming food rich in antioxidants improves sperm motility by reducing oxidative stress (21,32,33). Since antioxidative polyphenols have been confirmed to exist in abundance in the carob fruit (17,18), more efficiency was expected to be observed by carob extract on sperm motility one hour after sperm thawing.

An explanation that a higher dose (0.8 mg/ml) could not keep progressive motility percent elevated or inhibit the speed of the natural decline of sperm motility at T1 could be related to the notion that excess amounts of antioxidants can be physiologically damaging for cells by disrupting the redox balance, a phenomenon known as the double-edged sword of antioxidant (34). Since sperm motility is known to be dependent on the energy supply, excess amounts of carob products as an antioxidant source increased the energy consumption of the spermatozoa, as well as several consequences, including earlier burnout of energy sources, pH and osmotic fluctuation of culture media, inhibition of redox enzymes and interfering antioxidant capacity balance of spermatozoa (19,35). Consequently, the increased rate of energy consumption might then cause faster depletion of their energy sources, leading to a more significant reduction of progressive motile sperm in this study. During spermatogenesis, a high replication rate was confirmed to generate free radicals, causing more damage due to increased mitochondrial oxygen consumption (30), which in this case could be reflected by a more rapid reduction of progressive motile in dose 0.8mg/ml.

Regarding valuable sperm kinematic and velocity parameters taken automatically by the CASA system, including VCL, VSL, VAP, LIN, and STR, no significant change was observed not only at T0 but also at T1. Hence, since carob fruit extract was added to the culture medium right after thawing human semen in this study, adding carob extract to the vitrification medium can be suggested, as a previous study demonstrated that adding carob seed extract into the freezing solution has a beneficial effect on the ram sperm during freeze-thawing (19).

Conclusion

Considering the traditional importance of carob fruit as a source of antioxidants in male fertility potential, the results of the present study indicated that a thawing medium supplemented with carob kibble extract may improve some sperm parameters in lower doses. However, achieving the goal of using plant antioxidant ingredients as a supplement in sperm freezing and thawing media in infertility treatment clinics requires more detailed investigations, but it will not be far from the mind.

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Ethical statement

It has been mentioned at material and method.

Conflicts of interest

The authors declared no conflict of interest in this study.

Author contributions

Mahmoud heidari and Hiva Alipour designed the experiment and wrote the manuscript. Nasrinsadat Azami provided the kibbles and prepared the extract. Fereshteh Dardmeh analyzed the data.

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