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The effects of purslane (*Portulaca oleracea*) and fennel (*Foeniculum vulgare Mill*) hydroalcoholic extracts on the functional parameters of human spermatozoa after vitrification

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Objective: Reactive oxygen species (ROS) are produced during cryopreservation of human sperm and impair sperm function. Antioxidant compounds, such as fennel and purslane, reduce the damaging effects of ROS. This study aimed to evaluate motility parameters, plasma membrane integrity (PMI), mitochondrial membrane potential (MMP), intracellular ROS, and DNA damage to determine the optimum concentrations of hydroalcoholic extracts of fennel and purslane for human spermatozoa cryopreservation.
Methods: Twenty human sperm samples were used and divided into seven equal groups consisting of fennel hydroalcoholic extract (5, 10, and 15 mg/L), purslane hydroalcoholic extract (25, 50, and 100 mg/L), and no additive.
Results: Supplementation of 25 mg/L and 50 mg/L purslane extract and 10 mg/L fennel extract in cryopreservation extender significantly increased the motility and PMI of sperm with a significant reduction in intracellular ROS compared to control groups ($p<0.05$). A 50 mg/L concentration of purslane extract elevated progressive motility and MMP compared to the control group ($p<0.05$). No significant differences were seen for motion patterns and DNA damage of frozen-thawed human sperm in extender containing these extracts.
Conclusion: The results showed that supplementation of 50 mg/L purslane extract and 10 mg/L fennel extract in semen cryopreservation extender has the potential to decrease intracellular ROS and subsequently elevate the motility and PMI of human sperm.

Keywords: Fennel; Human sperm; Purslane; Vitrification

Introduction

Previous studies have investigated the effect of using different antioxidants in sperm cryopreservation extenders for animal and human species to reduce cryopreservation damage [1-5]. Despite the significant scientific evidence published on the effects of synthetic antioxidants to improve sperm parameter functions, the use of less toxic medicinal plants with antioxidant properties and bioactive compounds has also received much attention from researchers [6-8]. Previous studies have reported improvements in the quality of cryopreserved sperm cells with the addition of plant extracts such as fennel (*Foeniculum vulgare*), rosemary, and green tea to the cryopreservation medium [7,9-11]. Portulaca olerace, also known as purslane, is an edible green herb that can be used raw or cooked [12]. This medicinal plant was named a "global panacea" by the World Health Organization (WHO) [13]. Purslane contains compounds such as flavonoids, terpenoids, phenolic acids, alkaloids, saponins, omega-3 fatty acids, carotene, vitamins, glutathione, and melatonin [14-16]. Some of the biologically active compounds existing in this plant have ex-

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hibited antibacterial, anti-inflammatory, and antioxidant activities [17,18]. Previous research has attributed the antioxidant effect of purslane to three phenolic alkaloids (oleracein A, B, and E), which lead to free radical scavenging and prevent lipid peroxidation in rats [19]. In Nigeria, the plant has been used by traditional medicine practitioners to control infertility in women. In southern Nigeria, in the Niger Delta region, the leaves of *P. oleracea* are used by women to enhance fertility. In eastern Nigeria, the aerial parts of the plants are crushed to extract juice, which is taken with or without raw egg for the purpose of improving fertility in both males and females [20]. Many *in vitro* and *in vivo* studies have documented the antioxidant and protective effects of different extracts of purslane against oxidative stress [21]. The main compounds of purslane (phenols and flavonoids) might be responsible for its antioxidant effects [21]. In other studies, purslane extract increased activity levels of superoxide dismutase (SOD) and decreased levels of malondialdehyde (MDA) in an ulcerative colitis mouse model [22]. *F. vulgare* mill, also known as fennel, is an aromatic Mediterranean plant that has received much attention in traditional medicine [23,24]. This medicinal plant has diuretic, analgesic, anti-inflammatory, antipyretic, and antioxidant activity [25,26]. The therapeutic properties of fennel can be attributed to the presence of volatile compounds such as phenols and flavonoids in fennel extract. Among other compounds identified in this plant are terpenes, terpenoids, coumarin, anethole, and fenchone [27-29]. Fennel is an important source of antioxidants. It can significantly increase the activity of SOD and catalase. Malo et al. [10] showed that fennel decreased MDA levels in boar semen cryopreservation, thereby inhibiting lipid peroxidation. The cryopreservation of spermatozoa is widely used in infertility treatments [30]. In artificial insemination, sperm cryopreservation and its timely use have helped to increase the fertility rate of infertile couples, though still not as successfully as artificial insemination with fresh sperm [31]. The process of semen cryopreservation can lead to cell death and may reduce the rate of fertility post artificial insemination by triggering oxidative stress reactions [32,33]. Sperm viability is reduced under the influence of ice crystals formed by cryopreservation [34]. One method of sperm cryopreservation, vitrification, is capable of reducing the damage caused by conventional cryopreservation methods by reducing intracellular ice crystals. Vitrification can also be implemented in less time and at less expense than other methods [35,36]. During the cryopreservation process, reactive oxygen species (ROS) are produced whose products can increase lipid peroxidation [37]. Sperm is normally protected by antioxidant systems existing in its cytoplasm and cell membrane [30], but this protection reduces during cryopreservation and sperm are largely deprived of antioxidant protection. This imbalance between ROS and the sperm antioxidant system leads to cryopreservation damage and affects sperm characteristics such as motility, plasma membrane integrity (PMI), mitochondrial membrane potential (MMP), and DNA structure [38,39]. Although several studies have investigated the effect of fennel and purslane extracts on the quality of sperm, no study has yet compared the antioxidant effect of the extracts of these two plants on human functional parameters during cryopreservation. Therefore, the aim of this study was to compare the antioxidant effect of hydroalcoholic extracts of fennel and purslane on sperm motility, PMI, MMP, DNA damage, and intracellular ROS parameters during cryopreservation.

Methods

This study was approved by the Ethics Committee of Tehran University of Medical Sciences and informed consent was obtained from participants.

1. Preparation of hydroalcoholic extracts of fennel and purslane

Fennel seeds and fresh purslane wild plants were prepared by a plant classification expert from the local medicinal plants market and identified by a pharmacognosy expert. Maceration was used to prepare the hydroalcoholic extract of fennel seeds. In this method, the seeds were first crushed into powder by an electric grinder for 6 minutes. Then, 100 g of the seed powder was mixed with 500 mL of 70% ethanol and placed at room temperature for 48 hours. During this period, the resulting suspension mixture was stirred intermittently. The materials were filtered using Whatman No. 1 filter paper. The solution was concentrated under vacuum conditions on a rotary operator, and then put in an oven at 40 °C for 48 hours to be dried. The prepared powder was kept at 4 °C until use [40]. The dried powder was dissolved in distilled water in order to obtain the required concentration of the extract [41].

To prepare hydroalcoholic purslane extract, the method described by Azimi et al. [38] was used. In this method, the plant was first crushed into powder using an electric mill for 10 minutes, then 100 g of the plant powder was mixed with 800 mL of methanol and the resulting suspension was kept at room temperature for 72 hours. The materials were then passed through a piece of fabric and the resulting filtered solution was concentrated using the vacuum system of a rotary operator. Finally, the solution was put in an oven at 30 °C for 48 hours to be dried and the prepared powder was kept at 4 °C until use [38]. The dried powder was dissolved in distilled water in order to obtain the required concentration of the extract [38].

2. Subject and semen collection

Our research was carried out in the embryology laboratory at the

Research Center of Tehran University of Medical Sciences. A total of 20 normal sperm samples were obtained from Aban Infertility Center from February 2020 to April 2020 and used for this study. Study samples were collected from patients referred to the Infertility Center who had obtained samples by masturbation after 4–6 days of sexual abstinence. Sperm parameters were assessed according to the WHO (2010) guidelines. Sperm motility and concentration were assessed using the CASA system sperm class analyzer version 5.1 (Microptic). The criteria for inclusion of each sample were: progressive motility of 70%, volume of 2–6 mL, and concentration greater than 1×10^9 sperm/mL.

3. Vitrification and warming procedure

A micro-droplet technique was used to freeze sperm samples [42]. For cryopreservation, the spermatozoa samples were first added to the human tubal fluid (HTF; Sigma) solution. The resulting solution was then diluted with 5% human serum albumin (HSA; Sigma) and 0.5 mol/L of sucrose. The solution was then divided into seven equal parts for the experimental groups and fennel extract (5, 10, and 15 mg/L) or purslane extract (25, 50, and 100 mg/L) was added to each group, respectively. Finally, this suspension (at 30 µL/drop) was incubated in a nitrogen tank for 1 week. In the thawing phase, 5 mL of HTF solution was heated at 37 °C for 2 hours. In this phase, samples and –1% HSA were immersed in HTF solution. The resulting suspension was then incubated at 37 °C and 5% CO_2 for 5 minutes. Finally, samples were centrifuged at 500 ×*g* for 5 minutes and suspended in 50 µL of HTF [39].

4. Assessment of sperm motion characteristics

First, 10 µL of a sperm sample was placed on a Makler slide and the details were examined using the CASA system. The investigated parameters included motility (%), progressive motility (%), average path velocity (VAP; µm/sec), curvilinear velocity (VCL; µm/sec), linearity (LIN; %), straight-line velocity (VSL; µm/sec), straightness (STR; %), wobble (WOB; %), amplitude lateral head displacement (ALH; %), and beat cross frequency (BCF; %). Finally, five microscopic fields for 400 spermatozoa were selected to be assessed.

5. Assessment of sperm PMI

To determine the PMI, the hypo-osmotic swelling (HOS) test was used. To this purpose, 500 µL of a sperm sample was mixed with 500 µL of HOS solution (1.35 g of fructose [Merck], 0.73 of g sodium citrate [Merck], and 100 mL of distilled water; osmolality ~190 mOsmol/kg) at 37 °C for 30 minutes. A total volume of 10 µL of the suspension was placed on a dry slide. Finally, evaluations were performed using phase contrast microscopy (Olympus BX20) [43].

6. Assessment of mitochondrial membrane potential

Lipophilic cationic dye, JC-1 (T4069, Sigma-Aldrich), was used to investigate the MMP. During this phase, samples were centrifuged (500 ×*g* for 5 minutes) and then dissolved in phosphate-buffered saline (PBS) up to a concentration of 1×10^6 sperm/mL. After that, 1 µg of JC-1 dye was added to 1 mL of sample solution. Finally, samples were evaluated using flow cytometry techniques with orange (FL1, 585 nm) and red fluorescence (FL2, 530 nm) [44].

7. Assessment of DNA damage

Acridine orange (AO) fluorescence was used to investigate DNA damage. After centrifugation (500 ×*g* for 5 minutes), samples were added to Tris-null-EDTA buffer solution (1 mmol of EDTA, 10 mmol of Tris, and 0.15 mol of NaCl). The resulting solution was mixed with 400 µL of detergent acid and 1.2 mL of AO solution. The final assessment was performed using the flow cytometry technique for intact chromatin (FL1, 500–530 nm) and altered chromatin (FL2, 620 nm) [43].

8. Assessment of intracellular ROS

Dihydroethidium solution was used to evaluate the intracellular ROS. The reaction between dihydroethidium and anion superoxide leads to its oxidation. Then, it intercalates into DNA and emits red fluorescence. Samples, each with a concentration of 1×10^6 sperm/mL, were suspended with PBS solution, and 10 µL of dihydroethidium solution (Sigma-Aldrich Co.) was then added to the suspension and incubated at 25 °C for 20 minutes. Finally, red fluorescence (FL1, 525–625 nm) was assessed using the flow cytometry technique [45].

9. Flow cytometric analysis

The flow cytometric analysis was conducted using FACS Calibur (BD Immunocytometry Systems) with excitement by an argon laser at 488 nm. After eliminating debris, 10,000 spermatozoa were evaluated using flow cytometry (Flowing Software version 2.5.1; Cell Imaging and Cytometry Core).

10. Statistical analysis

The Kolmogorov-Smirnov test was used to confirm the normality of data distribution. Additionally, the differences between experimental groups were assessed using analysis of variance and the Tukey test. Data were analyzed using IBM SPSS ver. 20.0 (IBM Corp.). The results were shown as mean ± standard error, and $p < 0.05$ was considered to indicate statistical significance.

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Table 1. Motility parameters of post-thawed human spermatozoa supplemented with different concentrations of fennel and purslane hydroalcoholic extracts

Group	Control	Purslane			Fennel		
		25 mg/L	50 mg/L	100 mg/L	5 mg/L	10 mg/L	15 mg/L
Motility (%)	60.14 ± 1.59[c),d)]	67.43 ± 1.99[a),b)]	71.13 ± 1.42[a)]	57.40 ± 2.48[d)]	64.95 ± 1.59[b),c)]	66.75 ± 1.90[a),b)]	58.92 ± 1.50[d)]
Progressive motility (%)	31.73 ± 1.40[b),c)]	35.89 ± 1.43[a),b)]	37.30 ± 1.15[a)]	30.30 ± 1.65[c)]	33.87 ± 1.12[a),b),c)]	35.36 ± 1.29[a),b)]	29.93 ± 1.30[c)]
VCL (μm/sec)	48.90 ± 0.80	51.35 ± 0.72	52.62 ± 0.98	48.19 ± 0.81	50.13 ± 0.79	50.46 ± 0.85	47.99 ± 0.81
VSL (μm/sec)	27.25 ± 0.55	28.31 ± 0.55	28.88 ± 0.56	26.23 ± 0.51	28.10 ± 0.71	27.78 ± 0.52	27.06 ± 0.62
VAP (μm/sec)	33.68 ± 0.66	34.52 ± 0.81	35.95 ± 0.64	32.44 ± 0.62	34.41 ± 0.66	34.37 ± 0.61	32.62 ± 0.75
LIN (%)	55.60 ± 0.67	54.83 ± 0.62	55.20 ± 0.61	54.31 ± 0.69	55.63 ± 0.66	55.32 ± 0.67	56.23 ± 0.61
STR (%)	82.30 ± 0.37	82.05 ± 0.34	81.75 ± 0.50	82.03 ± 0.39	82.36 ± 0.45	82.25 ± 0.45	83.31 ± 0.45
WOB (%)	69.81 ± 0.66	69.06 ± 0.66	69.77 ± 0.56	68.35 ± 0.68	69.33 ± 0.65	69.51 ± 0.71	69.74 ± 0.59
ALH (%)	6.45 ± 0.05	6.55 ± 0.05	6.61 ± 0.06	6.50 ± 0.05	6.46 ± 0.07	6.52 ± 0.05	6.41 ± 0.05
BCF (%)	9.84 ± 0.08	9.82 ± 0.08	9.86 ± 0.06	9.96 ± 0.07	9.95 ± 0.04	9.85 ± 0.04	9.75 ± 0.04

Values are presented as mean±standard error.
VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude lateral head displacement; BCF, beat cross frequency.
[a),b),c),d)]Means with different letters in the same row demonstrate significant differences ($p<0.05$).

Table 2. PMI, MMP, DNA damage, and intracellular ROS of post-thawed human spermatozoa supplemented with different concentrations of fennel and purslane hydroalcoholic extracts

Group	Control	Purslane			Fennel			*p*-value
		25 mg/L	50 mg/L	100 mg/L	5 mg/L	10 mg/L	15 mg/L	
PMI	65.15 ± 0.99[c)]	69.74 ± 1.05[b)]	74.96 ± 0.83[a)]	68.30 ± 0.84[b),c)]	68.34 ± 1.45[b),c)]	69.59 ± 1.35[b)]	65.09 ± 0.95[c)]	< 0.05[e)]
MMP	9.12 ± 0.18[b),c)]	45.85 ± 2.17[a),b),c)]	49.53 ± 1.89[a)]	44.26 ± 2.50[a),b),c)]	44.97 ± 2.07[a),b),c)]	48.50 ± 2.86[a),b)]	41.45 ± 2.31[c)]	> 0.05[f)]
DNA damage	91.80 ± 2.03	7.95 ± 0.15	7.90 ± 0.16	8.49 ± 0.18	8.91 ± 0.16	8.71 ± 0.16	9.22 ± 0.19	< 0.05[e)]
ROS (intracellular)	41.85 ± 1.20[a),b)]	75.76 ± 1.98[c),d)]	72.35 ± 1.78[d)]	79.40 ± 1.52[b),c)]	79.14 ± 1.61[b),c)]	75.10 ± 1.45[c),d)]	85.32 ± 0.85[a)]	< 0.05[e)]

Values are presented as mean±standard error.
PMI, plasma membrane integrity; MMP, mitochondrial membrane potential; DNA damage; ROS, reactive oxygen species.
[a),b),c),d)]Means with different letters in the same column demonstrate significant differences; [e)]$p<0.05$; [f)]No significant difference.

Results

The effects of fennel and purslane extracts on the motility of cryopreserved-thawed human sperm are shown in Table 1. Cryopreservation extender supplementation with 25 mg/L and 50 mg/L purslane extract and 10 mg/L fennel extract improved sperm speed compared to the cryopreserved control group ($p < 0.05$). In addition, 50 mg/L purslane extract had the highest progressive motility, which was statistically significant compared to the cryopreservation control group ($p < 0.05$). The motility characteristics (VCL, VSL, VAP, LIN, BCF, ALH, STR, and WOB) showed no significant difference in the presence of both extracts compared to the cryopreserved control group ($p \geq 0.05$). Human sperm cryopreservation extender supplemented with 25 mg/L and 50 mg/L purslane extract and 10 mg/L fennel extract significantly increased PMI in sperm and cause a significant reduction in ROS compared to the cryopreserved control group ($p < 0.05$). Additionally, MMP increased with the addition of 50 mg/L purslane extract to the cryopreservation extender ($p < 0.05$). No significant difference was observed in sperm DNA damage between groups ($p \geq 0.05$) (Table 2).

Discussion

The main aim of this study was to assess the protective effects of two plant extracts on sperm functional parameters in a vitrification process. The analysis demonstrated that sperm speed as well as PMI and ROS production improved with 25 mg/L and 50 mg/L extracts of purslane and the 10 mg/L fennel extract. The highest increase in motility was observed with the 50 g/mL purslane extract, which also increased MMP. Sperm DNA damage and motility characteristics did not show significant relationships with the different doses of purslane and fennel in the vitrification groups.

Mammalian sperm is prone to the effects of increased intracellular ROS and lipid peroxidation products due to low levels of antioxidants

in the cytoplasm and high levels of saturated fatty acids in the plasma membrane. The presence of these products can disrupt the normal function of sperm. Though small amounts of ROS are required for sperm physiological activity, high amounts of ROS are toxic to sperm. Anion superoxide, the main source of ROS in sperm, is converted to hydrogen peroxide spontaneously or through the action of SOD. The results of our study are in agreement with a previous report on boar sperm, indicating that the presence of fennel extract causes a reduction in ROS levels compared to the control group. Moreover, Malo et al. [10] showed that fennel inhibits 5-lipoxygenase activity due to its antioxidant function. Fennel is also capable of stopping the release of the peroxidative chain reaction. Previous research has shown that purslane extract causes an increase in the activity of SOD [22]. Purslane extract can thus be effective in converting anion superoxide to hydrogen peroxide and thereby reducing intracellular ROS products. Therefore, the observation of a decrease in intracellular superoxide anion in this study can probably be attributed to the increased enzymatic capacity of antioxidants, which leads to the removal of ROS within sperm cells. Sperm mitochondria are the main site of ROS production due to their oxidative phosphorylation activity [46]. Koohestanidehaghi et al. [39] showed a negative relationship between intracellular ROS in human sperm and mitochondrial membrane activity after sample thawing. An increase in sperm MMP in the presence of purslane extract can be related to the high potential of this extract to reduce and eliminate intracellular ROS, and consequently protect the activity of the mitochondrial membrane. Sperm motility is one of the main characteristics of successful fertility. The findings of this study with regard to sperm motility align with those of previous studies in which fennel and purslane extracts increased the motility of boar and goat sperm post thawing [38,10]. Research evidence shows that phenols and flavonoids, the main components of purslane extract, are mainly responsible for its antioxidant and healing activity [19]. Previous studies have shown that goat and rat semen cryopreservation, supplemented with 50 mg/L purslane extract and 100 mg/kg and 200 mg/kg fennel extract significantly improved sperm motility [38,41,47] and Asadmobini et al. [30] showed the beneficial effects of fennel and purslane extracts, finding that they increased rat and human sperm motility [30,41]. Damage to the sperm axoneme structure and the reduction of adenosine triphosphate caused by ROS products are among the primary reasons for sperm motility reduction [48]. Several studies have shown a negative relationship between high levels of ROS and sperm motility in different animal species and humans [39,43,49]. In this study, superoxide anion levels were decreased in the 50 mg/L and 25 mg/L purslane extracts and 10 mg/L fennel extracts, mainly because of the increase in human sperm motility post-thawing. Additionally, motility characteristics (BCF, ALH, STR and VCL, VSL, VAP, LIN, and WOB) showed no significant differences between the experimental groups containing fennel and purslane extracts at all concentrations and the control group. The result of this part of our study is supported by the study of Topraggaleh et al. [49] on buffalo sperm in which the sperm motility characteristics were not different after an antioxidant was added to the cryopreservation extender. In addition to sperm motility, sperm fertility and survival post-thawing can be affected by PMI [50]. Malo et al. [10] reported that fennel extract in cryopreservation extender reduced MDA formation in the thawed sperm compared to the control group, indicating that this antioxidant is capable of inhibiting lipid peroxidation by inhibiting 5-lipoxygenase. Additionally, Azimi et al. [38] showed that the presence of purslane extract reduced the amount of MDA and lipid peroxidation. However, reports have suggested that the antioxidant abilities of plants depend on their phenolic compounds [51]. Perhaps the reduction of superoxide anion through antioxidant extract supplementation was the main reason for plasma membrane protection against ROS. In addition to the mentioned parameters, a healthy sperm chromatin structure is necessary for successful fertility and fetal growth [52]. In our study, cryopreservation supplementation with fennel and purslane extracts showed no significant effect on preventing DNA damage in thawed cryopreserved sperm. This finding might be attributable to the higher concentration of sperm chromatin during puberty, which promotes stability of the sperm DNA structure and protects against the ROS produced during cryopreservation, as well as the resultant DNA damage.

Supplementation of fennel and purslane extracts at concentrations of 10 mg/L and 50 mg/L, respectively, reduced intracellular ROS during cryopreservation and thawing of sperm, contributing to an increase in sperm motility and PMI compared to the control group. This study also emphasized the improvement of progressive motility and MMP with the addition of 50 mg/L purslane extract to cryopreservation extender. The addition of fennel and purslane extracts to cryopreservation extender had no significant negative effect on human sperm motility characteristics or DNA damage compared to the control group. Therefore, we recommend the addition of 10 mg/L fennel and 50 mg/L purslane extracts to cryopreservation extender to improve human sperm quality post-thawing, although further research is recommended to improve the *in vitro* and *in vivo* fertility rates of thawed cryopreserved human sperm using fennel and purslane extracts.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Conceptualization: MT, YK. Data curation: KL, FG. Formal analysis: KL. Methodology: YK, KL. Project administration: YK. Visualization: KL. Writing–original draft: KL. Writing–review & editing: KL, YK.

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