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# Effect of purslane (*Portulaca oleracea L.*) extract in the extender on cryopreserved ram sperm quality

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**Abstract** The use of an appropriate extender is crucial in mitigating the risks of freezing and thawing damage to sperm. Therefore, this study investigated the effect of purslane extract in a freezing extender on qualitative parameters of ram sperm. Semen samples were collected from five rams using an artificial vagina. The mixed semen sample was divided into five aliquots and diluted in extenders (1:20 ratio) containing different concentrations of purslane extract (0, 50, 100, 150, and 200 µg/mL). Sperm velocity, membrane integrity and activity, morphology, lipid peroxidation rate, and sperm DNA fragmentation were assessed before and after cryopreservation. Before freezing, there were no significant differences (P>0.05) in motility, viability, functionality, and morphology of sperm between treatments. However, the freezing-thawing process significantly decreased sperm motility parameters in all treatments. This detrimental impact of freezing was most pronounced (P≤0.05) in the treatment containing 200 µg/mL purslane extract, with total motility reduced to 18.12%. However, the viability, functionality, and morphology of sperm after the freezing-thawing process were not affected by treatments. The treatment without a supplement of purslane extract had the highest amount of MDA (110.37 nmol/mL) and the lowest percentage of sperm with healthy DNA (28.50%). This research indicated that including purslane extract in the freezing diluent for ram sperm enhanced both DNA integrity and sperm motility after cryopreservation.

**Keywords:** extender, freezing, purslane, ram, spermatozoa

## Introduction

The key factor in the effective use of artificial insemination technology lies in the acquisition of healthy and fertile frozen sperm (Aisen et al., 2002). The process of freezing minimizes sperm metabolism, consequently enhancing its longevity after preservation (Hartwig et al., 2012). Nonetheless, this freezing procedure imposes both physical and chemical stress on the sperm membrane, which ultimately compromises its viability and fertility. The resultant damages encompass cold shock, the formation of ice crystals, oxidative stress, membrane integrity issues (Watson and Martin, 1975), and DNA fragmentation (Baumber et al., 2003; Awda et al., 2009). Oxidative stress induces lipid peroxidation in the sperm cell membrane which causes DNA fragmentation in the nucleus and mitochondria, resulting in apoptosis (Takeshima et al., 2021).

An effective approach to combat oxidative stress is the addition of antioxidants to diluted semen. Several plant extracts are economical and natural sources of antioxidants and bioactive compounds that can effectively preserve and enhance sperm viability during semen storage (Mehdipour et al., 2016; Ros-Santaella and Pintus, 2021; Nasiri et al., 2022). Purslane (*Portulaca oleracea L.*) is one of the richest

green plant sources of omega-3 fatty acids, antioxidants such as α-tocopherol, ascorbic acid, and glutathione (Uddin et al., 2014; Montoya-García et al., 2023) as well as flavonoids (kaempferol, quercetin, and apigenin), vitamins A, C, and E, minerals, glutathione, coenzyme Q10, and melatonin (Naeem and Khan, 2013; Aljarari and Alamoudi, 2020). These compounds collectively contribute to the antioxidant properties and free radical scavenging activities of purslane (Yang et al., 2009), which can effectively maintain sperm quality after the freezing-thawing process (Kojo, 2004; Masoudi et al., 2019; Silvestre et al., 2021).

Purslane extract was shown to be advantageous for cryopreservation of goat sperm, where the total motility, viability, mitochondrial activity, and DNA integrity of sperm in the treatment containing purslane extract were higher than those in the control group (Azimi et al., 2020). Variations exist among species regarding the sensitivity of their sperm to the freezing process (Holt, 2000). Furthermore, to the best of our knowledge, there has been no research on the effects of purslane extract on the cryopreservation of ram sperm. Hence, this study aimed to assess the influence of purslane extract in the extender on the quality of cryopreserved ram sperm.

## Materials and methods

### *Preparation of purslane extract*

Purslane plants were harvested from the agricultural fields in Ahwaz region (Khuzestan province, Iran) washed thoroughly, air-dried, and then ground into a fine powder using a mill. To obtain the ethanolic extract, 40 g of purslane powder were mixed with 400 mL of 70 % ethanol and incubated at 35 °C for 48 h in a shaking incubator set at 150 rpm. The resulting mixture was then centrifuged at 1500 g, and the supernatant was filtered through Whatman filter paper (No. 1). The ethanol solvent was evaporated using a freeze-dryer for 24 h, and the resulting dried extract was stored at 4 °C (Gorran et al., 2015).

### *Semen collection and processing*

Semen samples were collected twice a week for 4 weeks from five mature Lori rams (3-5 years old) using an artificial vagina. Immediately after collection, the samples were transferred to the physiology laboratory in a flask containing water at 35 °C and placed in an incubator at 35 °C. For processing, semen samples were selected based on the following criteria: semen volume of 0.5-5 mL; progressive motility ≥80%, sperm concentration >2.0 × $10^9$ sperm/mL, and abnormal sperm forms <10%. To account for individual ram variation, the selected samples were pooled and divided into five equal aliquots. Each aliquot was then diluted in one of the five extenders, with a final concentration of 100 × $10^6$ sperm/mL. Purslane extract was added to each extender in varying amounts (0, 50, 100, 150, and 200 µg/mL) to prepare five extenders. The base extender was composed of 2.7 g Tris, 1.0 g fructose, 1.4 g citric acid, and 1.0 g soy lecithin (w/v) dissolved in 100 mL redistilled water, with a pH of 7.2 and osmolality of 320 mmol/kg (Forouzanfar et al., 2010). The diluted semen was gradually cooled to 5 °C over three h, with glycerol slowly added to reach a final concentration of 5% in the extenders during the cooling process. The cooled samples were then aspirated into 0.25 mL straws, placed in liquid nitrogen vapor (5 cm above the liquid nitrogen surface) for 12 min, and then plunged directly into liquid nitrogen for storage. Before analysis, the straws were thawed in a water bath at 37 °C for 30 s (Najafi et al., 2013).

### *Evaluation of sperm kinetic parameters*

Sperm motility parameters were analyzed using a computer-assisted sperm motility analysis system (HFT CASA-Houshmand Fanavar-Tehran-Iran) (Mogheiseh et al., 2022). The CASA was adjusted for ram sperm analysis, as shown in Table 1. In this case, sperm droplets (10 µL) were loaded between the chambers of glass slides (Microscopic Slides, ISOLAB, 20 µm) and covered with a cover glass. The prepared slides were then analyzed by the CASA software to assess different motion parameters including motility and velocity parameters such as total motility (TM, %), progressive motility (PM, %), linearity (LIN, %), curvilinear velocity (VCL, µm/s), straight linear velocity (VSL, µm/s), the amplitude of lateral head displacement (ALH, µm), straightness (STR, %), average path velocity (VAP, µm/s) and beat cross frequency (BCF, Hz).

**Table 1.** Setup for computer-assisted analysis (CASA) of ram sperm kinetics

| Parameters | Set value |
|---|---|
| Calssifier | WHO 2010 |
| Calibration | 20 × |
| Frame rate | 50 |
| Chamber depth | 20 mk |
| Magnification | 200 × |
| Threshold | 89 |
| Minimum contrast | 60 Pixels |
| Minimum VCL mean | 15 µm/s |
| Minimum STR mean | 0.7 µm/s |
| Minimum ALH mean | 0.3 µm/s |

### *The integrity of sperm plasma membrane*

Eosin-nigrosin staining was used to evaluate the integrity of the plasma membrane (Benmoula et al., 2018). To prepare the stain, 1.67 g of eosin Y and 2.9 g of sodium citrate were dissolved in 100 mL of distilled water with gentle heating. Subsequently, 10 g of nigrosin dye was added to the solution and boiled. The dye solution was then cooled to room temperature (20 °C) and filtered using a 0.2 µm filter to remove any particles. For sample evaluation, 10 mL of sperm suspension were placed on a clean, warm slide and gently mixed with an equal volume of the stain. The slides were then observed

under a microscope at 400× magnification. At least 200 sperm were counted on each slide, and the percentage of stained (dead) and unstained (live) sperm was calculated (Figure 1).

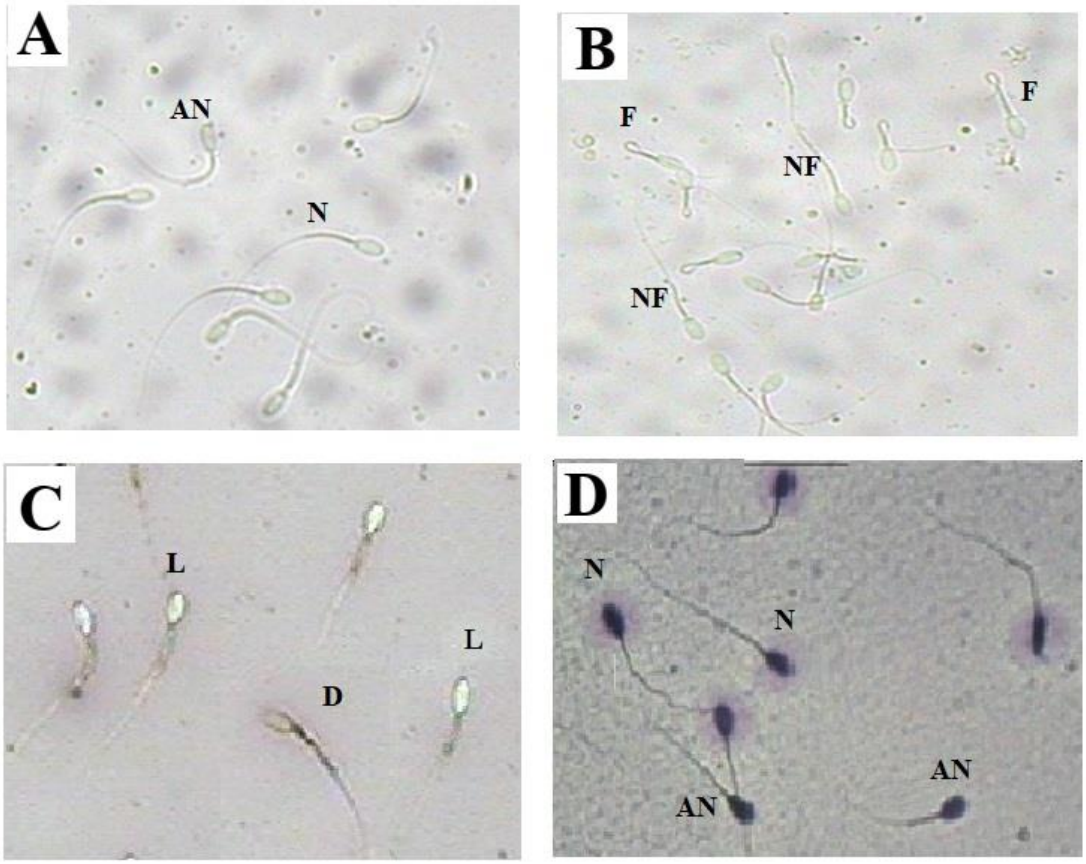


**Figure 1.** Detection of the sperm morphology (A), functionality of plasma membrane (B), integrity of plasma membrane (C) and sperm DNA fragmentation (D). N; normal, AN; abnormal, F; functional, NF; non-functional, L; live, D; dead, N; sperm with normal DNA, and AN; sperm with fragmented DNA.

*The functionality of sperm plasma membrane*

The functionality of the sperm plasma membrane was evaluated using a hypoosmotic swelling (HOS) test (Revell and Mrode, 1994). To conduct the test, 0.9 g of fructose and 0.49 g of sodium citrate were dissolved in 100 mL distilled water. To begin, 30 µL of semen sample was mixed with the hypoosmotic solution to reach an osmotic pressure of 100 mmol/kg. The mixture was then incubated at 37 °C for 30 min, after which 10 µL of the mixture was placed on a glass slide and sperm plasma membrane functionality was assessed by a light microscope at 1000× magnification. At least 200 spermatozoa in five different fields were examined on each slide. The proportion of spermatozoa with swollen and bent tails, indicating functional membranes, was then counted.

*Sperm morphology*

Hancock's solution was used to evaluate sperm morphology (Schäfer and Holzmann, 2000). The solution consisted of 62.5 mL formalin (37%), 150 mL saline, 150 mL PBS buffer, and 500 mL redistilled water. To record sperm abnormalities, a 20 µL semen sample was mixed with 2 mL Hancock solution at 37 °C. Then a 10 µL aliquot was placed on a preheated slide (37 °C), covered with a coverslip, and examined using a phase-contrast microscope (×1000 magnification). A total of 200 sperm from each sample were examined and counted to calculate the percentage of total abnormal sperm.

*The integrity of sperm DNA*

The integrity of sperm DNA was evaluated using the sperm DNA fragmentation assay kit (SDFA® kit; Ravan Saze, Tehran, Iran) according to the manufacturer's instructions. The procedure entails denaturing the sperm chromatin and DNA in a microgel solution by acidic treatment. Subsequently, the DNA strands are dispersed around the sperm head by removing chromatin protein, resulting in a halo around the sperm head when stained (indicating sperm with intact DNA). Conversely, sperm DNA damage results in the lack of halos around the sperm head (indicating sperm with fragmented DNA) (Fernández et al., 2005).

### *Lipid peroxidation rate*

The concentration of malondialdehyde (MDA) was measured using thiobarbituric acid (TBA). One mL of each semen sample was mixed with one mL ethylenediaminetetraacetic acid (EDTA), one mL butylated hydroxytoluene (BHT), and two mL trichloroacetic acid (TCA). The suspension was then centrifuged at 1200 *g* for 15 min, and the supernatant was separated. One mL of the supernatant was mixed with one mL TBA and incubated in a water bath at 95 °C for 20 min. After cooling to room temperature for 30 min, the absorbance at 532 nm was measured using a spectrophotometer (Esterbauer and Cheeseman, 1990).

### *Statistical analysis*

This study was conducted using a completely randomized design with seven replications. Each replication involved the assessment of sperm parameters using three straws. Initially, the normality and homogeneity of variance of the data were assessed using the Shapiro–Wilk's test and Levene Median test, respectively. Following the verification of data distribution normality and the homogeneity of variance, the data were analyzed using the General Linear Model procedure in SAS statistical software (SAS 9.1; 2002; SAS Institute, Cary, NC). Statistical significance among treatments was considered at the P≤0.05 level using the Tukey's test.

## Results

There were no differences in sperm quality parameters between treatments after the equilibration period and before cryopreservation (*P*>0.05; Table 2). The freezing-thawing process significantly reduced sperm motility parameters in all treatments. The detrimental effect of freezing was highest (P≤0.05) in the treatment containing 200 µg/mL purslane extract (total motility 18.12 %) (Table 3).

The viability, functionality, and morphology of sperm after the freezing-thawing process were not affected by treatments (Table 2). The MDA concentration in the control extender (110.37 nmol/mL) was higher than treatments containing purslane extract (P≤0.05). Additionally, the treatment without purslane extract had the lowest percentage of sperm with DNA integrity at 28.50% (P≤0.05) (Table 4).

**Table 2.** Effects of purslane addition to the extender on motility, viability, functionality, and morphology of ram sperm before cryopreservation

| | Treatment | | | | | |
|---|---|---|---|---|---|---|
| Parameter | Pur-0 | Pur-50 | Pur-100 | Pur-150 | Pur-200 | SEM |
| PM (%) | 54.41 | 53.05 | 59.48 | 56.92 | 46.58 | 5.42 |
| TM (%) | 73.26 | 73.27 | 82.00 | 76.23 | 61.74 | 11.51 |
| Lin (%) | 25.70 | 25.24 | 27.53 | 26.55 | 25.28 | 2.75 |
| VSL (µm/s) | 10.45 | 11.82 | 14.05 | 13.15 | 10.99 | 3.57 |
| STR (%) | 44.85 | 45.41 | 51.47 | 49.34 | 44.08 | 7.00 |
| VCL (µm/s) | 32.61 | 37.50 | 44.98 | 41.08 | 29.11 | 11.83 |
| VAP (µm/s) | 16.57 | 18.44 | 21.47 | 19.57 | 14.25 | 4.87 |
| ALH (µm) | 1.78 | 1.95 | 2.39 | 2.13 | 1.44 | 0.53 |
| BCF (Hz) | 3.54 | 3.39 | 3.48 | 3.35 | 0.98 | 1.05 |
| Live sperm (%) | 50.79 | 59.32 | 64.17 | 63.29 | 64.66 | 6.00 |
| Functional sperm (%) | 59.80 | 63.16 | 66.17 | 64.56 | 62.93 | 3.26 |
| Normal sperm (%) | 69.01 | 66.02 | 66.82 | 72.00 | 70.66 | 4.65 |

SEM: standard error of the mean, Pur-0, Pur-50, Pur-100, Pur-150, and Pur-200 represent semen extenders containing 0, 50, 100, 150, and 200 µg/mL purslane extract, respectively. TM: total motility, PM: progressive motility, LIN: linearity, VCL: curvilinear velocity, VSL: straight linear velocity, ALH: the amplitude of lateral head displacement, STR: straightness, VAP: average path velocity, and BCF: beat cross frequency.

## Discussion

The findings of the present research revealed that freezing and thawing negatively impacted sperm motility parameters. The treatment with 200 µg/mL of purslane extract exhibited a detrimental effect on motility, resulting in a total motility of 18.12% (Table 3).

The inefficacy of most doses of purslane extract in improvement of motility vs. control is inconsistent with the findings of Azimi et al. (2020) and Khalil et al. (2023) who found that the addition of purslane extract to sperm extenders enhanced the sperm motility. In the latter studies, improved sperm parameters in extenders with purslane extract were due to enhanced antioxidant status, as shown by higher TAC and lower $H_2O_2$ levels (Khalil et al., 2023). However, Torkamanpari et al. (2023) found no differences in sperm motility with fennel and purslane extracts compared to the control (Torkamanpari et al., 2023). These discrepancies could be due to various factors, such as product formulation, concnetration of in the extender, base extender type, semen composition variations among species, sperm sensitivity, and extract compositions (Reis et al., 2023).

Treatments did not significantly influence the integrity, cell membrane functionality, or the proportion of normal sperm post-freezing. This is in contrast to a previous study where purslane extract was shown to improve sperm viability and mitochondrial activity (Azimi et al., 2020). Additionally, Khalil et al. (2023) found that using 50 and 100 µg/mL purslane leaf extract in the

extender enhanced goat sperm viability and membrane integrity post-thawing (Khalil et al., 2023).

In this study, the control treatment exhibited the highest MDA concentration (110.37 nmol/mL) and the lowest sperm DNA integrity (28.50 %). This is consistent with the findings of Azimi et al. (2020) regarding goat sperm, indicating that the inclusion of purslane extract resulted in a reduction in reactive oxygen species (ROS) compared to the control group.

**Table 3.** Effects of purslane addition to the extender on the viability, functionality, and morphology of frozen-thawed ram sperm

| Parameter | Treatment | | | | | SEM |
|---|---|---|---|---|---|---|
| | Pur-0 | Pur-50 | Pur-100 | Pur-150 | Pur-200 | |
| PM (%) | 19.26 | 19.67 | 23.99 | 22.74 | 15.39 | 3.14 |
| TM (%) | 23.37 $^{ab}$ | 24.78 $^{ab}$ | 29.76 $^{a}$ | 27.15 $^{ab}$ | 18.12 $^{b}$ | 3.69 |
| Lin (%) | 11.15 | 10.61 | 13.41 | 12.49 | 8.71 | 1.85 |
| VSL (µm/s) | 3.03 | 3.00 | 3.77 | 3.72 | 2.58 | 0.58 |
| STR (%) | 15.27 | 16.43 | 20.47 | 18.75 | 12.85 | 2.54 |
| VCL (µm/s) | 8.02 | 8.38 | 10.24 | 9.13 | 6.10 | 1.45 |
| VAP (µm/s) | 4.45 | 4.47 | 5.50 | 5.15 | 3.49 | 0.78 |
| ALH (µm) | 0.54 | 0.58 | 0.69 | 0.61 | 0.42 | 0.08 |
| BCF (Hz) | 0.53 | 0.55 | 0.58 | 0.55 | 0.33 | 0.13 |
| Live sperm (%) | 22.55 | 24.73 | 24.12 | 21.81 | 32.13 | 6.20 |
| Functional sperm (%) | 41.30 | 43.07 | 44.86 | 40.56 | 43.20 | 6.28 |
| Normal sperm (%) | 56.09 | 46.44 | 48.13 | 55.95 | 54.71 | 4.94 |

SEM: standard error of the mean. Pur-0, Pur-50, Pur-100, Pur-150, and Pur-200 are values of 0, 50, 100, 150, and 200 µg/mL Purslane extract in the extender, respectively. TM: total motility, PM: progressive motility, LIN: linearity, VCL: curvilinear velocity, VSL: straight linear velocity, ALH: the amplitude of lateral head displacement, STR: straightness, VAP: average path velocity, and BCF: beat cross frequency. a,b: Within rows, mean values with common superscript (s) are not different (P>0.05; Tukey's test).

**Table 4.** Lipid peroxidation rate and integrity of DNA in frozen-thawed ram sperm

| Parameter | Treatment | | | | | SEM |
|---|---|---|---|---|---|---|
| | Pur-0 | Pur-50 | Pur-100 | Pur-150 | Pur-200 | |
| MDA (nmol/mL) | 110.37 $^{a}$ | 24.71 $^{b}$ | 17.90 $^{b}$ | 22.46 $^{b}$ | 22.58 $^{b}$ | 6.16 |
| Sperm with normal DNA (%) | 28.50 $^{b}$ | 69.60 $^{a}$ | 73.43 $^{a}$ | 71.27 $^{a}$ | 69.20 $^{a}$ | 6.24 |

SEM: standard error of the mean, Pur-0, Pur-50, Pur-100, Pur-150, and Pur-200 are values of 0, 50, 100, 150, and 200 µg/mL Purslane extract in the extender, respectively. a,b: Within rows, mean values with common superscript (s) are not different (P>0.05; Tukey's test).

Sperm freezing contributes to increased lipid peroxidation and the formation of ROS (Büyükleblebici et al., 2014). Low level of MDA could potentially improve the quality of frozen sperm (Pei et al., 2018). The inadequate protective features of DNA make the sperm nucleus particularly vulnerable to the detrimental effects of free radicals and lipid peroxidation. Consequently, an increased concentration of reactive oxygen species can lead to the destruction of DNA bases, the cross-linking of proteins, and overall DNA degradation (Aitken et al., 2014).

In this study, the lowest percentage of sperm with intact DNA after the freezing process was recorded in the control extender. This finding supported the findings of Azimi et al. (2020), who showed that sperm DNA integrity was improved in the presence of purslane extract. In contrast, Torkamanpari et al. (2023) reported no significant differences in DNA damage in frozen human sperm in the extender containing hydroalcoholic extracts of purslane and fennel. Sperm cells with fragmented DNA are linked to the activation of caspase, dysfunction of the mitochondrial membrane, and an increase in membrane permeability (Martin et al., 2004).

There is a negative relationship between the percrntage of sperm containing fragmented DNA and important reproductive parameters (Simon et al., 2010). The positive impact of purslane extract on the preservation of sperm DNA integrity can be attributed to its rich composition of antioxidants (Aljarari and Alamoudi, 2020) which enhance the antioxidant capabilities and free radical scavenging potential of purslane (Yang et al., 2009).

## Conclusions

In summary, the results of this study indicated that adding purslane extract to the freezing extender improved the integrity of ram sperm DNA and decreased the lipid peroxidation rate. These observations may lead to improvement in ram fertility, but further investigations through field fertility trials are needed to confirm this hypothesis.

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## Declaration of interest

The authors declare no conflicts of interest.

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