Novel additive for sperm cryopreservation media: Holotheria parva coelomic cavity extract protects human spermatozoa against oxidative stress—A pilot study

Novel additive for sperm cryopreservation media: Holotheria parva coelomic cavity extract protects human spermatozoa against oxidative stress—A pilot study.

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Abstract

Cryopreservation is the most effective method for preserving semen for a long period of time. However, during the freeze–thaw process, production of reactive oxygen species (ROS) leads to a steep reduction in sperm fertility indices. In this study, we tested the effects of the extract of the coelomic cavity of five Holotheria parva, a marine organism rich in antioxidants, for its ROS-scavenging activity and cryoprotective effects on oxidative stress. Using a total of 50 semen samples, our results demonstrated that doses of 250 and 500 µg/ml of H. parva coelomic cavity extract significantly increased sperm vitality as compared to the control ($p < .05$). The addition of 250 µg/ml of the extract exerted a significant positive effect on sperm motility. Moreover, sperm DNA damage and ROS production were significantly reduced at extract concentrations of 250 and 500 µg/ml ($p < .05$). To the best of our knowledge, the results of this study represent the first demonstration of the possibility of improving sperm parameters and reducing ROS production and DNA damage by supplementing sperm freezing media with H. parva coelomic extract. Our results suggested that H. parva coelomic extract could be useful for improving the fertilising ability of frozen-thawed human semen.

1. Introduction

Cryopreservation is the most effective method for preserving semen for a long period of time (Bahadur et al., 2002). Sperm cryopreservation provides the opportunity of the preservation of male fertility through sperm banks. With the guarantee of maintaining semen in such a sperm bank, men undergoing chemotherapy, radiotherapy and testicular surgery or with ejaculatory failure have the opportunity to father a child using cryopreserved semen by means of artificial insemination (AI) technologies (Bucak et al., 2007; Meseguer et al., 2006; Williams, 2010).

The cryopreservation process induces cryo-shock and osmotic stress, which in turn enhance the rate at which reactive oxygen species (ROS) are produced (Agarwal, Saleh, & Bedaiwy, 2003). It has been shown that high levels of ROS in semen are negatively correlated with several sperm fertility parameters (Agarwal, Ikemoto, & Loughlin, 1994; Agarwal et al., 2003). Sperm generate ROS through two main mechanisms: the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system present in the plasma membrane (Aitken, Buckingham, & West, 1992), and NADH-dependent oxidoreductase in the mitochondria (Agarwal et al., 2003). Some studies also reported a role for leucocytospermia in excessive seminal ROS production (Agarwal et al., 2003; Aggarwal, Puri, Dada, & Saurabh, 2015). Regardless of the mechanisms through which ROS are produced, if excessively

61 available, these highly reactive compounds ultimately reduce sperm motility, viability and induce
62 DNA fragmentation (Agarwal, Virk, Ong, & du Plessis, 2014; Opuwari & Henkel, 2016).

63 In the context of human reproduction, in order to decrease ROS production, many studies suggested
64 the likely benefits of administering some synthetic or natural antioxidants including vitamin E,
65 melatonin, curcumin, glutathione and cysteine (Champroux, Torres-Carreira, Gharagozloo, Drevet,
66 & Kocer, 2016; Galli et al., 2012; Lobo, Patil, Phatak, & Chandra, 2010; Marzony, Ghanei, & Panahi,
67 2016; Sabeti, Pourmasumi, Rahiminia, Akyash, & Talebi, 2016; Sen & Chakraborty, 2011).
68 However, very few studies have determined the efficacy of extracts from marine organisms in
69 reducing ROS production. Recently, Sobhani et al. reported antioxidant effects of brown Algae
70 Sargassum on sperm parameters (Sobhani, Eftekhaari, Shahrzad, Natami, & Fallahi, 2015a). Sea
71 cucumbers, belonging to the class Holothuroidea, are marine invertebrates inhabiting both benthic
72 areas and deep seas across the world (Pishehvarzad, Yousefzadi, Kamrani, Moini Zanjani, & Ali
73 Ahmadi, 2014; Seydi et al., 2015). They have long been used as a food source and traditional medicine
74 in Asian and Middle Eastern communities. Sea cucumbers have an impressive profile of valuable
75 vitamins such as thiamine, riboflavin, niacin and vitamin A, and minerals including calcium,
76 magnesium, iron and zinc (Esmat, Said, Soliman, El-Masry, & Badiea, 2013; Pishehvarzad et al.,
77 2014).

78 A number of unique biological and pharmacological activities have been ascribed to various species
79 of sea cucumbers, including anti-cancer, anti-angiogenic, anti-hypertension, anti-inflammatory,
80 antioxidant, antithrombotic and wound healing properties (Seydi et al., 2015). Therapeutic and
81 medicinal benefits of sea cucumbers, including H. parva, can be linked to the presence of a wide array
82 of bioactive agents. This includes triterpene glycosides (saponins), chondroitin sulphates,
83 glycosaminoglycan (GAGs), sulphated polysaccharides, sterols (glycosides and sulphates),
84 phenolics, cerebrosides, lectins, peptides, glycoprotein, glycosphingolipids and essential fatty acids
85 (de Melo et al., 2014; Myron, Siddiquee, & Al Azad, 2014; Wijesinghe, Jeon, Ramasamy, Wahid, &
86 Vairappan, 2013; Yang, Wang, Jiang, & Lv, 2015).

87 Considering that numerous marine organisms are currently investigated in order to find novel
88 compounds suitable for medicinal use (Qeshmi, Homaei, Fernandes, & Javadpour, 2018; Sharifian,
89 Homaei, Hemmati, Luwor, & Khajeh, 2018; Sharifian, Homaei, Kamrani, Etzerodt, & Patel, 2019),
90 the aim of the present study was to test the effects of the addition of an extract of the coelomic cavity
91 from H. parva as a cryoprotectant on oxidative stress levels and human semen parameters after
92 thawing (morphology, motility, viability, DNA fragmentation).

2. Materials and Methods

Ethical clearance was obtained from the Institutional Review Board of Hormozgan University of Medical Sciences. The study was conducted in accordance with the Declaration of Helsinki on Biomedical Research Involving Human Subjects. A total of 63 healthy male participants that signed inform consent provided semen samples for analysis, of which 13 were excluded leaving a total of 50 participants for analysis.

2.1 Extract preparation

Five specimens of Holothouria parva were caught in the May month from Bandar Abbas, State of Hormozgan, Iran, and immediately transported to our laboratory at Hormozgan University where they were freshly prepared. All the different body parts (gonads, respiratory branch, coelom cavity and body wall) were removed from the adhering meninges and blood, and the grey matter was removed by gross dissection. 3 mg of each different body part tissue was resuspended in 3 ml of 50 mM phosphate buffer at pH 7.5. The suspension was subjected to sonication for 15 s with 40-s pauses for 10 min by a SYCLON Ultra Sonic Cell SKL950-IIDN. Cell debris was discarded by centrifugation at 15,000 g at 4°C for 20 min. The supernatant was immediately stored at −20°C until use.

2.2 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The ROS-scavenging activity of different parts of the body of H. parva (gonads, respiratory branch, coelomic cavity and body wall) was evaluated according to the method of Yamaguchi, Takamura, Matoba, and Terao (1998). In brief, 1 ml of DPPH (Sigma-Aldrich) solution (0.1 mmol/L, in 95% ethanol (v/v)) was incubated with various concentrations of the extract from the different body parts. The mixture was shaken, incubated for 20 min at room temperature, and the absorbance was read at 517 nm against a blank using a Perkin Elmer Lambda 850 UV/VIS Spectrometer. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and calculated using the following equation:

$$\text{DDPH scavenging effect (\% inhibition)} = \left[\frac{(A_0 - A_1)}{A_0} \times 100\right].$$

A_0 is the absorbance of the control reaction and A_1 the absorbance in presence of the extract samples. The parameter for the evaluation of DPPH method is the IC50 value (inhibition concentration at 50%), which indicates the concentration of antioxidant that causes 50% loss of the DPPH activity. The analyses were performed in triplicate.

2.3 Collection of semen samples

Out of 50 patients enrolled in the study, a total number of 50 semen samples were collected from healthy individuals attending the IVF clinic of Dr. Khashavi, Bandar Abbas, Iran, with the median age of 30 (Range from 25 to 35) were included for analysis. Healthy male participants were recruited from the IVF clinic at Bandar Abbas, Iran. Men with a medical history of varicocele, renal disease, hepatic disease, haematological disease, hormonal disorders, genetic disorders, erectile dysfunction, infection and testicular trauma were not accepted in the study. Semen samples for experimentation were required to fulfilled the standard criteria of the World Health Organization (WHO, 2010; sperm count ≥15 million/ml, total motility ≥40%, normal sperm morphology ≥4%, seminal volume ≥1.5 ml, pH ≥7.2, normal appearance and viscosity, and maximum liquefaction time of 1 hr at room temperature).

Semen samples were obtained by masturbation into a clean, wide-mouthed nontoxic plastic container. The containers were kept at ambient temperature, between 20°C, to avoid large changes in temperature that may affect samples. Semen samples were liquefied in an incubator at 37°C for semen liquefaction.

2.4 Cryopreservation and thawing protocols

Sperm preparation for cryopreservation was performed using a simple washing procedure, where an equal volume of the semen sample and human tubal fluid (HTF containing 5% albumin) were centrifuged at 448 g for 10 min. The supernatant was then discarded, and the same volume of HTF was added to the remaining pellet and centrifuged again at 448 g for 10 min. The resulting pellet was then resuspended with HTF, and the same volume of a commercial sperm freezing medium (HEPES containing 10% albumin) was slowly added. Semen aliquots were divided in the experimental groups: control group received no extract, while the experimental groups were treated with different concentrations of the H. parva coelom cavity extract. Samples were loaded in straws, sealed and frozen at −179°C in liquid nitrogen vapour phase for 10 min, then immediately transferred to a liquid nitrogen tank and stored for one week. Samples were thawed at 25°C ± 2°C for 10 to 30 min.

2.5 Sperm analysis by Computer Assisted Sperm Analysis (CASA) system

Sperm quality analysis was performed using the Sperm Analysis System IVOS (Hamilton Thorne Biosciences). Twenty µl of semen was placed on a clean slide and observed under a microscope.

Sperm motility and motility parameters were recorded, specifically grade A: percentage of spermatozoa with fast forward motility (>40 μm/s VCL and LIN ≥60%); grade B: percentage of spermatozoa with slow forward motility (20 ≤ VCL ≤40 μm/s); grade C: percentage of spermatozoa with nonprogressive motility (>40 μm/s VCL and LIN < 60%); and grade D: percentage of immotile spermatozoa (<20 μm/s).

Morphology was determined using the Diff-Quick Staining Technique. Semen samples were centrifuged for 5 min at 448 g, an aliquot of the homogenised sediment smeared on a slide, air-died and fixed for 15–20 s and then mixed with the first and second staining solution. Slides were then washed with distilled water and air-dried. Normal or abnormal sperm morphology was evaluated using the 100× lens and immersion oil by the CASA system.

2.6 Sperm vitality assessment

Sperm vitality was assessed using the eosin staining. Thirty microlitre of semen sample were well mixed with 100 μl of a solution containing 0.5 g of eosin Y (Sigma) in 100 ml of 0.9% NaCl and then left for 30 s. Subsequently, one droplet of this suspension was transferred to a labelled slide where it was smeared by sliding a coverslip in front of it. The smears were air-dried, and slides were evaluated under a light microscope scoring at least 200 spermatozoa per sample. The percentage of live spermatozoa was obtained by identifying the number of stained (dead) and unstained (alive) cells. If the stain was limited to only a part of the neck region, and the rest of the head area was unstained, this was considered a ‘leaky neck membrane’, not a sign of cell death and total membrane disintegration. Therefore, these cells were considered as alive.

2.7 Sperm chromatin dispersion test

The Halosperm kit (Halotech DNA) was used to analyse the status of DNA fragmentation in spermatozoa. Semen was mixed with low melting point agarose, pipetted onto a pre-coated glass slide with 0.65% of standard agarose, covered with a coverslip, and left to solidify at 4°C. Coverslips were then carefully removed and the samples were denatured with 0.08 mol/L HCl for 7 min and were neutralised for 25 min with the neutralisation solution provided by the kit. Slides were then washed in distilled water, dehydrated through an ethanol series (70%, 90%, 100%; 2 min each at room temperature) and air-dried. The cells were stained with Wright solution for 10 min, washed with water, air-dried, mounted with Eukitt Mounting Medium and finally observed under a light microscope scoring at least 300 spermatozoa. Spermatozoa without DNA fragmentation show halos

of dispersed DNA, which can be big or medium, whereas those sperm nuclei with fragmented DNA produce either small halos or no halos at all (**Figure 1**).

2.8 Oxidative stress assessment

Oxidative stress levels were assessed using the Oxisperm kit® (Halotech DNA) that measures an excess of superoxide anions. Based on the kit protocol, tubes containing the reactive gel (RG) were placed in a 900 W microwave for 1 min for liquefaction. Afterwards, the temperature was reduced to 37°C. The RG gel was then mixed with the semen samples and incubated at 37°C for 45 min. After incubation, the colour of the sediment was compared with the standard colours of the kit manual, which varies based on superoxide anion concentration from pale pink to dark purple at four levels of N1, N2, N3 and N4.

2.9 Statistical analysis

All statistical analyses were performed using SPSS 21.0 (SPSS), a statistical software package. Results are expressed as the mean ± SEM, and one-way analysis of variance was determined by Tukey's post hoc test to determine significant differences for all parameters across all groups. A p value of $<.05$ was considered to be statistically significant.

3. Results

3.1 DPPH radical scavenging activity

In order to test which part of the body of H. parva displays the highest radical scavenging activity, samples from varying body parts (gonads, respiratory branch, coelom cavity, body wall) were tested. As shown in **Figure 2**, the extract from the coelom cavity displayed a greater efficacy in comparison with other parts ($p < .001$).

3.2 Sperm motility

Coelom cavity extract of H. parva at a concentration of 250 µg/ml significantly ($p < .001$) increased sperm motility after thawing (**Figure 3**). In addition, at concentrations of 25, 50, 100, 500 and 750 µg/ml the extract had no ($p > .05$) effect on sperm motility as compared to the cryopreserved control group. However, treatment with 1,000 µg/ml of the extract induced a significant decrease in sperm motility compared to the cryopreserved control group ($p < .01$).

3.3 Normal morphology and sperm vitality

Normal sperm morphology of thawed semen was not affected by any concentration of the H. parva extract (**Figure 4**). Although there was an increase in the percentage of normal morphology at 250 µg/ml, this effect was not significant.

Holotheria parva extract at 250 and 500 µg/ml significantly increased sperm vitality as compared to cryopreserved control samples ($p < .05$; **Figure 5**). At higher concentrations, sperm viability decreased in a dose-dependent manner.

3.4 DNA fragmentation

The result for the determination of sperm DNA fragmentation is reported in **Table 1**. The cryopreserved control group showed the highest percentage of score 4 and 5 sperm indicating DNA damage. The groups treated with 250 and 500 µg/ml of extract showed a significant decrease in the percentages of DNA-damaged sperm, compared to the cryopreserved control group.

3.5 Oxidative stress

Assessment of oxidative stress (OS) by Oxisperm showed that cryopreserved control samples displayed significantly lower levels of N1 scores (lowest degree of ROS production) compared to samples treated with 250 and 500 µg/ml respectively (**Table 2**). Conversely, cryopreserved control samples displayed significantly higher levels of N4 scores (highest degree of oxidative stress) compared to samples treated with 250 and 500 µg/ml ($p < .001$; **Table 2**). The frequency of N3 scores also showed significant differences ($p < .05$) between control and treatment groups. Analysis between treated groups (250 and 500 µg/ml) indicated that 250 (µg/ml) had much more efficacy in reduction OS during the cryopreservation procedure ($p < .05$; **Table 2**).

4. Discussion

ROS have been known for decades to be a detrimental factor in many physiological and pathological processes. This includes a strong correlation between ROS production and cancer, cardiovascular disease, diabetic neuropathy and infertility (Iqbal, Andrabi, Riaz, Durrani, & Ahmad, 2016; Motlagh et al., 2014; Sarıözkan et al., 2015). Macleod first reported that ROS production arose in spermatozoa and that this increase in the partial pressure of oxygen would reduce sperm motility (MacLeod, 1943b). Aitken, Clarkson, and Fishel (1989) stated a possible physiological role for ROS at low levels in different physiological processes (Aitken et al., 1989). Low and controlled generation of ROS plays

a physiological role during capacitation and acquisition of sperm fertilising ability. However, oxidative stress generated by an excess of ROS induces adverse effects on sperm plasma membrane, DNA and physiological processes, leading to cell death (Aitken, 2017b).

While references to sperm cryopreservation date back as far as the 1600s (Sherman, 1964), it was not until the development of artificial insemination (AI) in the late 1950s and early 1960s when the dairy industry needed longer-term storage methods for bull spermatozoa, that sperm cryopreservation became a major area of scientific investigation (Walters, Benson, Woods, & Critser, 2009). Similarly, the need for cryopreservation of human spermatozoa arose with the advent and propagation of assisted reproductive techniques. However, despite many advances made in the field of cryopreservation, increases in ROS production continue to reduce overall sperm function.

Our results indicate that the freeze–thaw processes of semen lead to increased ROS production and reduce sperm parameters confirming the cryopathogenic role of oxidative stress during cryopreservation, thus confirming results of previous reports in this regard (Agarwal, Gupta, & Sharma, 2005; Agarwal et al., 2014; Saleh & Agarwal, 2002). Many studies have been performed demonstrating the protective effect of antioxidants during sperm cryopreservation (Amidi, Pazhohan, Nashtaei, Khodarahmian, & Nekoonam, 2016; Taylor, Roberts, Sanders, & Burton, 2009). However, to our knowledge, no data are present in the literature on the biological activity of H. parva extracts on human freeze-thawed spermatozoa. In the present study, the extract from the coelom cavity (the body part that displayed the higher radical scavenging activity) of H. parva was added to semen cryopreservation medium in order to evaluate its ability in reducing oxidative stress and improving post-thaw sperm parameters. Significant differences in the radical scavenging activity between the different body parts of the sea cucumber were observed with the coelomic cavity showing the highest activity. This is most probably due to markedly higher concentrations of antioxidants in this body part. Yet, the specific nature and concentration of antioxidants in the different body parts are not yet know and subject to further research.

Holotheria parva extract supplementation significantly decreased the oxidative stress at concentrations of 250 and 500 µg/ml and exerted a significant positive effect on sperm post-thaw motility at 250 µg/ml. Sperm viability improved significantly at 250 and 500 µg/ml respectively. Our results agree well with Sobhani et al. (2015b) who showed that brown algae (Sargassum sp.) extracts could reduce the amount of ROS improving frozen human sperm parameters. A difference between our study and that of Sobhani et al. (2015b) was the buffer chosen for extracts. In their study, methanol, which has been shown to extensively damage spermatozoa, was used for the Sargassum

extract preparation. We chose to use the much more sperm-friendly phosphate-buffered saline (PBS) technique in our preparations.

In our study, the coelom cavity extract was obtained from the H. parva digestive tract. According to the H. parva diet, digestive cells are continuously in contact with marine micro-nutrient that contain oxidants and antioxidants (John Aitken, Clarkson, & Fishel, 1989). ROS can also be suppressed by metal binding proteins, endogenous and exogenous antioxidants (Aitken, 2017a; MacLeod, 1943a; Sobhani et al., 2015b). H. parva coelom extract contains vitamins, co-enzymes as well as superoxide dismutase, glutathione reductase and catalase (Ghanbari, 2018). Hence, it seems that unlike former studies that inhibited only one pathway, H. parva extract could be able to restrain action on all three pathways. Therefore, we speculate that the improvement in sperm motility and viability observed after adding H. parva coelom cavity extract to the cryopreservation medium could be due to antioxidant protection of the spermatozoa from changes caused by ROS.

We showed that ROS production induced by cryopreservation led to sperm DNA damage, which is in line with the findings of Agarwal et al. (2003). These authors observed that teratozoospermic patients showed higher percentage of DNA-damaged spermatozoa caused by higher ROS levels compared to patients with lower ROS levels. ROS damages DNA by binding and sharing unstable electrons in the outer orbit (Bae, Oh, Rhee, & Do Yoo, 2011; de Lamirande & O'Flaherty, 2008). This ROS-induced DNA damage can be overcome, if antioxidants scavenge these electrons (Agarwal & Said, 2005; Blokhina, Virolainen, & Fagerstedt, 2003; de Lamirande & O'Flaherty, 2008). Interestingly, we observed that supplementation of cryopreservation medium with H. parva extract at the concentration of 250 and 500 µg/ml was able to reduce oxidative stress and significantly improved post-thaw DNA integrity.

In conclusion, the present study demonstrated that an extract of H. parva can preserve spermatozoa against cryo-damage by reducing ROS production suggesting a possible activity in improving the fertilising ability of frozen-thawed of semen. The nature of the bioactive compounds as well as the mechanism behind these findings are currently under investigation.

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Conflict of Interest

The authors declare no conflict of interest regarding the publication of this paper.

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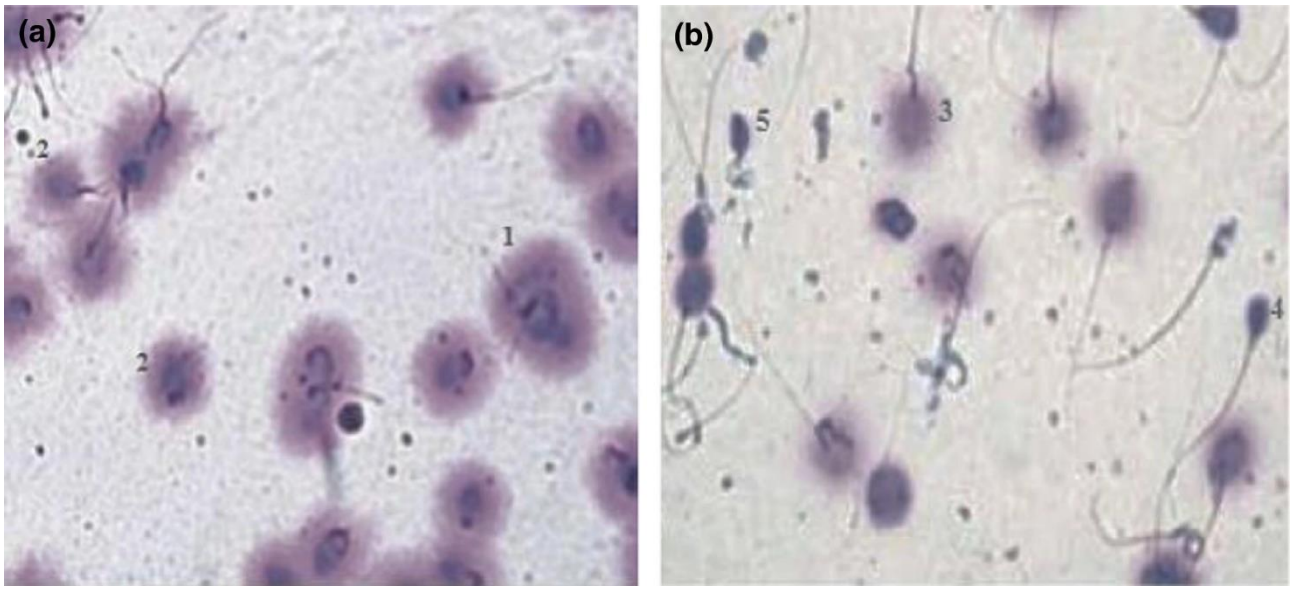


Figure 1

Representative micrographs of sperm DNA fragmentation assayed by sperm chromatin dispersion test. (a) 250 µg/ml group, (b) control group. Score 1 showed no fragmentation, 2 low, 3 moderate and 4, 5 indicative highest DNA damage. Value and number of damaged spermatozoa significantly decreased in the groups which received treatment

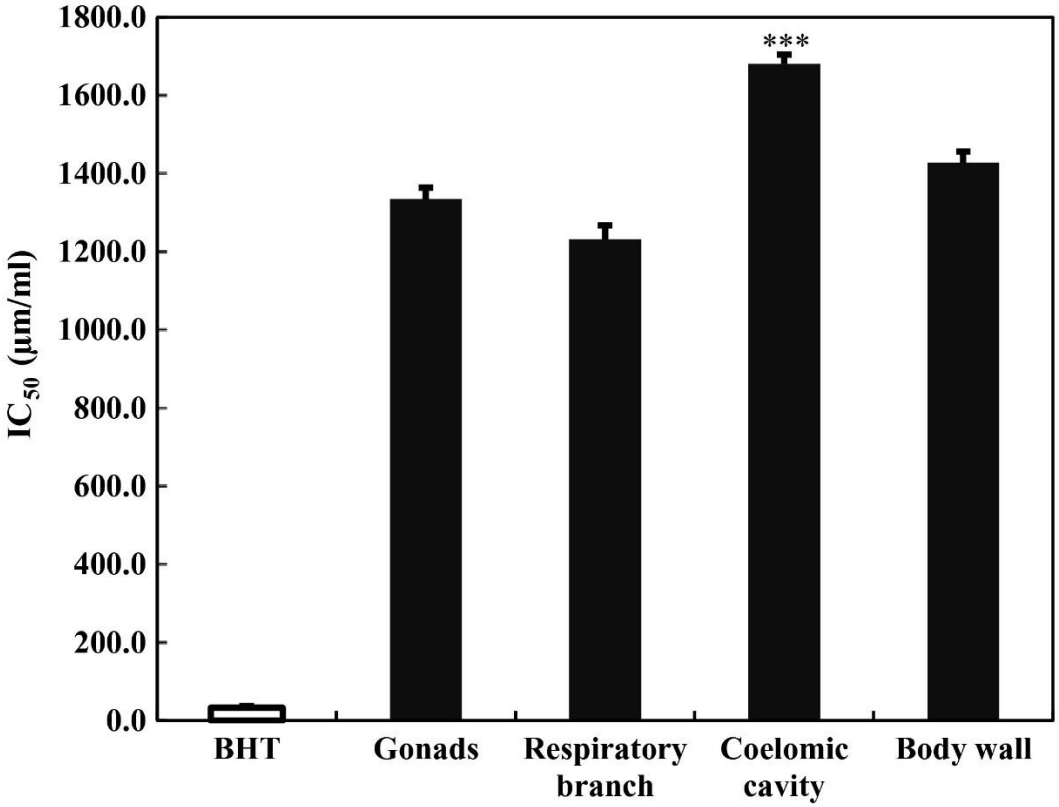
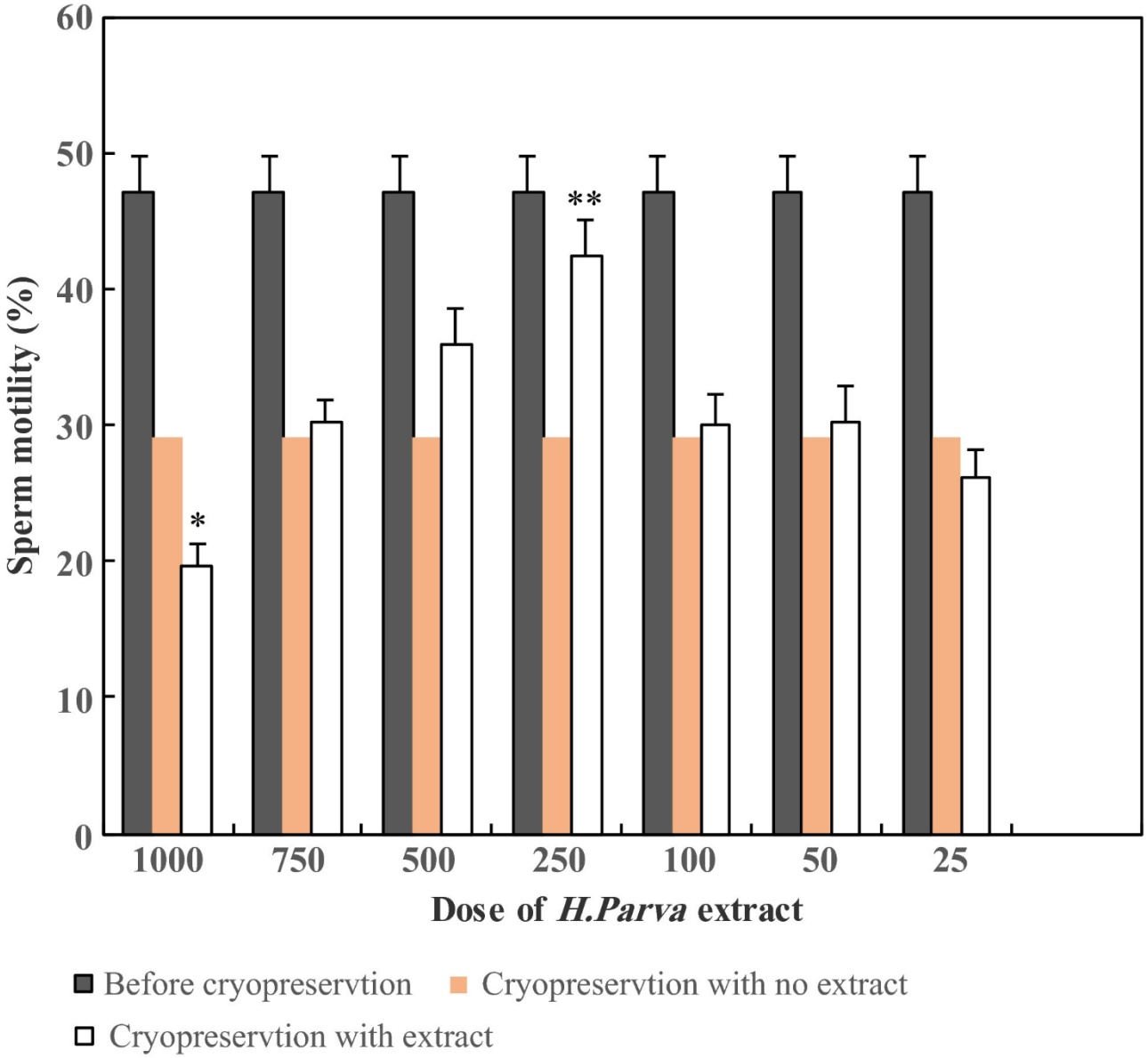


Figure 2

Radical scavenging activity in H. parva extract obtained from different body parts (gonads, respiratory branch, coelomic cavity, body wall). The extract from the coelomic cavity had significantly higher efficacy in comparison with other parts ($p < .001$)



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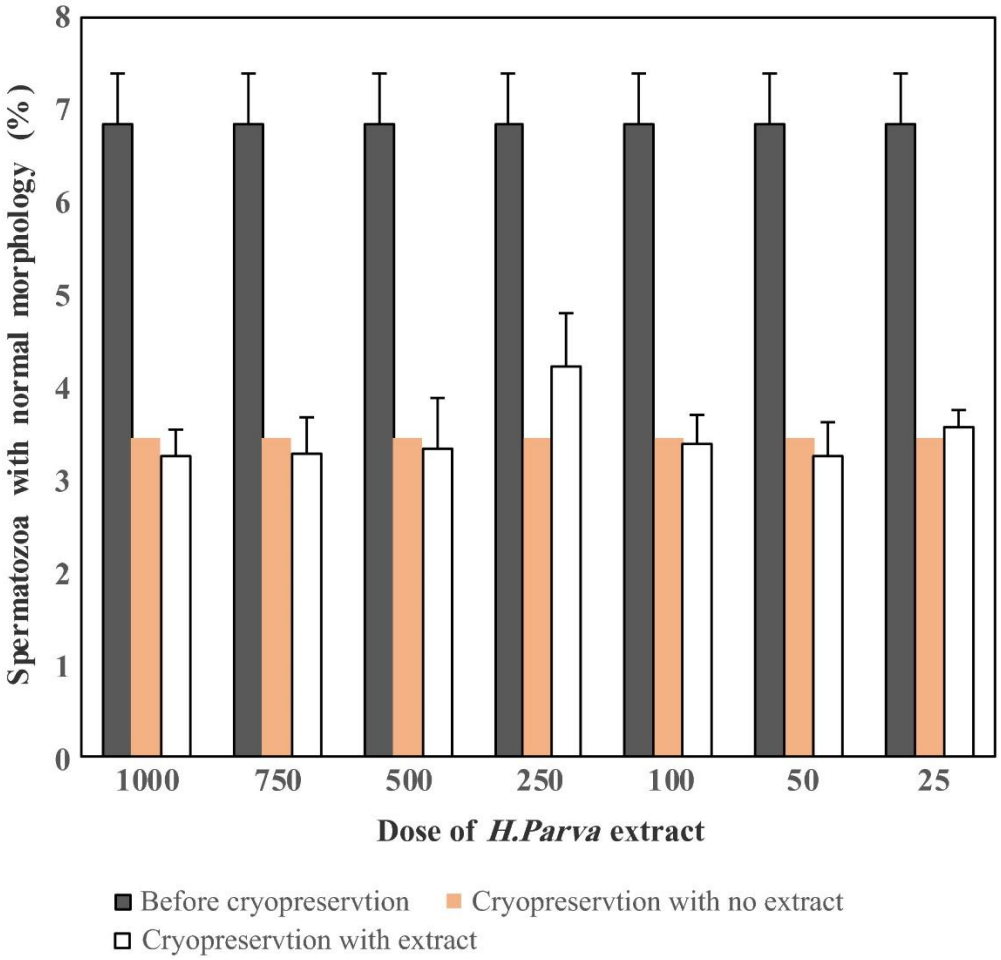
456

457 Figure 3

458 Effect of different concentrations of H. parva coelomic cavity extract on sperm motility. * and **
459 indicate significant differences ($p < .01$ and $p < .001$ respectively) between cryopreserved treated
460 groups and cryopreserved control group



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462 Figure 4

463 Effect of different concentrations of H. parva coelomic cavity extract on the percentage of
464 spermatozoa with normal morphology

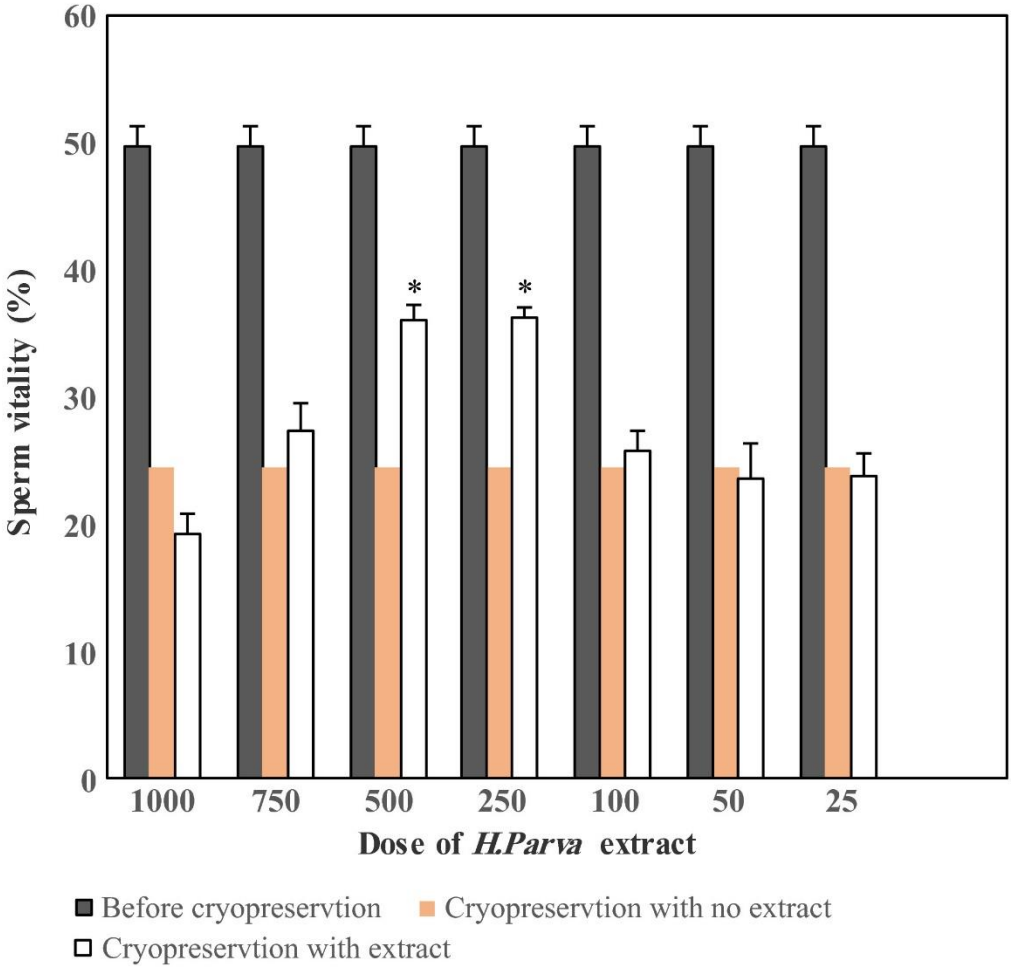

Figure 5

Effect of different concentrations of H. parva coelomic cavity extract on sperm vitality. * indicates a significant difference ($p < .05$) between cryopreserved treated groups and cryopreserved control group

Table 1. Sperm chromatin dispersion (SCD) data (mean ± *SEM*) after freezing spermatozoa in presence or absence of different concentrations of *H. parva* coelom cavity extract

Experimental groups	Rate of Dispersion									
	Score 1 (big halo)		Score2 (big/ moderate halo)		Score 3 (medium halo)		Score 4 (small halo)		Score 5 (no halo)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control	8	1.2	12	0.9	14	0.2	44	0.3	22	0.2
250 µg/ml of H. parva extract	46	0.3 *	32	0.2 *	14	0.2	4	1.1 *	6	0.3 *
500 µg/ml of H. parva extract	26	0.4 *	36	0.9 *	36	0.9	12	0.6 *	4	2.4 *

* Indicates significant differences ($p < .05$) between the treated and cryopreserved control group.

474 **Table 2**. Oxidative stress (OS) levels before and after cryopreservation of spermatozoa in presence
475 or absence of different concentrations of *H. parva* coelom cavity extract

476

Experimental groups	Rate of Oxidative Stress							
	N1		N2		N3		N4	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Before cryopreservation	44	0.3 *	28	0.2	20	0.9	8	1.0 *
Control	2	1.7	14	0.2	36	0.2 *	48	0.2
250 µg/ml of H. parva extract	36	0.2 *	38	0.4 *	20	0.1	6	1.6 *
500 µg/ml of H. parva extract	22	0.1 *	40	2.1 *	26	0.2	12	0.1 *

477 * Indicates a significant difference ($p < .05$) between the cryopreserved treated groups and the control group.