

Alma Mater Studiorum Università di Bologna
Archivio istituzionale della ricerca

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

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Khashavi Z., Homaei A., Koohnavard F., Kamrani E., Spinaci M., Luwor R.B., et al. (2020). Novel additive for sperm cryopreservation media: Holothuria parva coelomic cavity extract protects human spermatozoa against oxidative stress—A pilot study. ANDROLOGIA, 52(6), e13604-e13604 [10.1111/and.13604].

Availability:

This version is available at: <https://hdl.handle.net/11585/766604> since: 2021-07-08

Published:

DOI: <http://doi.org/10.1111/and.13604>

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).
When citing, please refer to the published version.

(Article begins on next page)

This is the final peer-reviewed accepted manuscript of:

Khashavi Z, Homaei A, Koohnavard F, et al.

Novel additive for sperm cryopreservation media: *Holothuria parva* coelomic cavity extract protects human spermatozoa against oxidative stress—A pilot study. *Andrologia*. 2020;52:e13604.

The final published version is available online at: <https://doi.org/10.1111/and.13604>

Rights / License:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

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

Zahra Khashavi¹ | Ahmad Homaei² | Fahimeh Koohnavard³ | Ehsan Kamrani^{2,4} |
Marcella Spinaci⁵ | Rodney B. Luwor⁶ | Mahsa Archang³ | Ashok Agarwal⁷ | Ralf Henkel^{7,8}

¹Infertility Therapy and IVF Center of Om-e-Leila Hospital, Bandar Abbas, Iran

²Department of Marine Biology, Faculty of Marine Science and Technology, University of Hormozgan, Bandar Abbas, Iran

³Hormozgan University of Medical Sciences, Bandar Abbas, Iran

⁴Department of Fisheries Science, Faculty of Marine Science and Technology, University of Hormozgan, Bandar Abbas, Iran

⁵Department of Veterinary Medical Sciences, University of Bologna, Bologna, Italy

⁶Department of Surgery, The Royal Melbourne Hospital, The University of Melbourne, Parkville, VIC, Australia

⁷American Center for Reproductive Medicine, Cleveland Clinic, Cleveland, OH, USA

⁸Department of Medical Biosciences, University of the Western Cape, Cape Town, South Africa

Correspondence

Ahmad Homaei, Department of Marine Biology, Faculty of Marine Science and Technology, University of Hormozgan, P.O.

Box 3995, Bandar Abbas, Iran.

Email: a.homaei@hormozgan.ac.ir,

a.homaei@gmail.com

Keywords

cryopreservation, *Holothuria parva*, ROS, Spermatozoa

30 Abstract

31 Cryopreservation is the most effective method for preserving semen for a long period of time.
32 However, during the freeze–thaw process, production of reactive oxygen species (ROS) leads to a
33 steep reduction in sperm fertility indices. In this study, we tested the effects of the extract of the
34 coelomic cavity of five *Holothuria parva*, a marine organism rich in antioxidants, for its ROS-
35 scavenging activity and cryoprotective effects on oxidative stress. Using a total of 50 semen samples,
36 our results demonstrated that doses of 250 and 500 µg/ml of *H. parva* coelomic cavity extract
37 significantly increased sperm vitality as compared to the control ($p < .05$). The addition of 250 µg/ml
38 of the extract exerted a significant positive effect on sperm motility. Moreover, sperm DNA damage
39 and ROS production were significantly reduced at extract concentrations of 250 and 500 µg/ml ($p <$
40 $.05$). To the best of our knowledge, the results of this study represent the first demonstration of the
41 possibility of improving sperm parameters and reducing ROS production and DNA damage by
42 supplementing sperm freezing media with *H. parva* coelomic extract. Our results suggested that *H.*
43 *parva* coelomic extract could be useful for improving the fertilising ability of frozen-thawed human
44 semen.

45 1. Introduction

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

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

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

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.

122 **2.3 Collection of semen samples**

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

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

137 **2.4 Cryopreservation and thawing protocols**

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

148 **2.5 Sperm analysis by Computer Assisted Sperm Analysis (CASA) system**

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

151 Sperm motility and motility parameters were recorded, specifically grade A: percentage of
152 spermatozoa with fast forward motility ($>40 \mu\text{m/s}$ VCL and $\text{LIN} \geq 60\%$); grade B: percentage of
153 spermatozoa with slow forward motility ($20 \leq \text{VCL} \leq 40 \mu\text{m/s}$); grade C: percentage of spermatozoa
154 with nonprogressive motility ($>40 \mu\text{m/s}$ VCL and $\text{LIN} < 60\%$); and grade D: percentage of immotile
155 spermatozoa ($<20 \mu\text{m/s}$).

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

161 **2.6 Sperm vitality assessment**

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

171 **2.7 Sperm chromatin dispersion test**

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

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

183 **2.8 Oxidative stress assessment**

184 Oxidative stress levels were assessed using the Oxisperm kit® (Halotech DNA) that measures an
185 excess of superoxide anions. Based on the kit protocol, tubes containing the reactive gel (RG) were
186 placed in a 900 W microwave for 1 min for liquefaction. Afterwards, the temperature was reduced to
187 37°C. The RG gel was then mixed with the semen samples and incubated at 37°C for 45 min. After
188 incubation, the colour of the sediment was compared with the standard colours of the kit manual,
189 which varies based on superoxide anion concentration from pale pink to dark purple at four levels of
190 N1, N2, N3 and N4.

191 **2.9 Statistical analysis**

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

196 **3. Results**

197 **3.1 DPPH radical scavenging activity**

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

202 **3.2 Sperm motility**

203 Coelom cavity extract of *H. parva* at a concentration of 250 $\mu\text{g/ml}$ significantly ($p < .001$) increased
204 sperm motility after thawing (**Figure 3**). In addition, at concentrations of 25, 50, 100, 500 and 750
205 $\mu\text{g/ml}$ the extract had no ($p > .05$) effect on sperm motility as compared to the cryopreserved control
206 group. However, treatment with 1,000 $\mu\text{g/ml}$ of the extract induced a significant decrease in sperm
207 motility compared to the cryopreserved control group ($p < .01$).

208 3.3 Normal morphology and sperm vitality

209 Normal sperm morphology of thawed semen was not affected by any concentration of the *H. parva*
210 extract (**Figure 4**). Although there was an increase in the percentage of normal morphology at 250
211 $\mu\text{g/ml}$, this effect was not significant.

212 *Holothuria parva* extract at 250 and 500 $\mu\text{g/ml}$ significantly increased sperm vitality as compared to
213 cryopreserved control samples ($p < .05$; **Figure 5**). At higher concentrations, sperm viability
214 decreased in a dose-dependent manner.

215 3.4 DNA fragmentation

216 The result for the determination of sperm DNA fragmentation is reported in **Table 1**. The
217 cryopreserved control group showed the highest percentage of score 4 and 5 sperm indicating DNA
218 damage. The groups treated with 250 and 500 $\mu\text{g/ml}$ of extract showed a significant decrease in the
219 percentages of DNA-damaged sperm, compared to the cryopreserved control group.

220 3.5 Oxidative stress

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

229 4. Discussion

230 ROS have been known for decades to be a detrimental factor in many physiological and pathological
231 processes. This includes a strong correlation between ROS production and cancer, cardiovascular
232 disease, diabetic neuropathy and infertility (Iqbal, Andrabi, Riaz, Durrani, & Ahmad, 2016; Motlagh
233 et al., 2014; Sariözkan et al., 2015). Macleod first reported that ROS production arose in spermatozoa
234 and that this increase in the partial pressure of oxygen would reduce sperm motility (MacLeod,
235 1943b). Aitken, Clarkson, and Fishel (1989) stated a possible physiological role for ROS at low levels
236 in different physiological processes (Aitken et al., 1989). Low and controlled generation of ROS plays

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

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

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

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

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

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

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

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

295 **Acknowledgements**

296 The authors wish to thank Morteza Salimi at the Student Research Committee, Hormozgan University
297 of Medical Sciences, Bandar Abbas and Dr. Samira Daniali and Prof. A. Elliasi and Mr Farzad
298 Shayanfar at the Department of Physiology, Shahid Beheshti University of Medical Sciences, Tehran,
299 Iran, as well as Omme Leila Hospital affairs, Bandar Abbas, Iran.

300 **Conflict of Interest**

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

302 **REFERENCES**

- 303 Agarwal, A., Gupta, S., & Sharma, R. K. (2005). Role of oxidative stress in female reproduction.
304 Reproductive Biology and Endocrinology, 3(1), 28.
- 305 Agarwal, A., Ikemoto, I., & Loughlin, K. (1994). Levels of reactive oxygen species before and after
306 sperm preparation: Comparison of swim-up and L4 filtration. *Archives of Andrology*, 32(3), 169–174.
307 <https://doi.org/10.3109/01485019408987783>
- 308 Agarwal, A., & Said, T. M. (2005). Oxidative stress, DNA damage and apoptosis in male infertility:
309 A clinical approach. *BJU International*, 95(4), 503–507. [https://doi.org/10.1111/j.1464-](https://doi.org/10.1111/j.1464-410X.2005.05328.x)
310 [410X.2005.05328.x](https://doi.org/10.1111/j.1464-410X.2005.05328.x)
- 311 Agarwal, A., Saleh, R. A., & Bedaiwy, M. A. (2003). Role of reactive oxygen species in the
312 pathophysiology of human reproduction. *Fertility and Sterility*, 79(4), 829–843.
313 [https://doi.org/10.1016/S0015-0282\(02\)04948-8](https://doi.org/10.1016/S0015-0282(02)04948-8)
- 314 Agarwal, A., Virk, G., Ong, C., & du Plessis, S. S. (2014). Effect of oxidative stress on male
315 reproduction. *The World Journal of Men's Health*, 32(1), 1–17.
316 <https://doi.org/10.5534/wjmh.2014.32.1.1>
- 317 Aggarwal, R., Puri, M., Dada, R., & Saurabh, G. (2015). Correlation between leukocytospermia and
318 oxidative stress in male partners of infertile couples with leukocytospermia. *International Journal of*
319 *Reproduction, Contraception, Obstetrics and Gynecology*, 4, 168–172. [https://doi.org/10.5455/2320-](https://doi.org/10.5455/2320-1770.ijrco.g2015.0230)
320 [1770.ijrco.g2015.0230](https://doi.org/10.5455/2320-1770.ijrco.g2015.0230)
- 321 Aitken, R. J. (2017a). Reactive oxygen species as mediators of sperm capacitation and pathological
322 damage. *Molecular Reproduction and Development*, 84(10), 1039–1052.
323 <https://doi.org/10.1002/mrd.22871>
- 324 Aitken, R. J., Buckingham, D. W., & West, K. M. (1992). Reactive oxygen species and human
325 spermatozoa: Analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent
326 chemiluminescence. *Journal of Cellular Physiology*, 151(3), 466–477.
327 <https://doi.org/10.1002/jcp.1041510305>
- 328 Aitken, R. J., Clarkson, J. S., & Fishel, S. (1989). Generation of reactive oxygen species, lipid
329 peroxidation, and human sperm function. *Biology of Reproduction*, 41(1), 183–197.
330 <https://doi.org/10.1095/biolr.eprod41.1.183>
- 331 Amidi, F., Pazhohan, A., Nashtaei, M. S., Khodarahmian, M., & Nekoonam, S. (2016). The role of
332 antioxidants in sperm freezing: A review. *Cell and Tissue Banking*, 17(4), 745–756.
333 <https://doi.org/10.1007/s10561-016-9566-5>
- 334 Bae, Y. S., Oh, H., Rhee, S. G., & Yoo, Y. D. (2011). Regulation of reactive oxygen species
335 generation in cell signaling. *Molecules and Cells*, 32(6), 491–509. [https://doi.org/10.1007/s10059-](https://doi.org/10.1007/s10059-011-0276-3)
336 [011-0276-3](https://doi.org/10.1007/s10059-011-0276-3)

337 Bahadur, G., Ling, K., Hart, R., Ralph, D., Wafa, R., Ashraf, A., ... Oyede, A. (2002). Semen quality
338 and cryopreservation in adolescent cancer patients. *Human Reproduction*, 17(12), 3157–3161.
339 <https://doi.org/10.1093/humrep/17.12.3157>

340 Blokhina, O., Virolainen, E., & Fagerstedt, K. V. (2003). Antioxidants, oxidative damage and oxygen
341 deprivation stress: A review. *Annals of Botany*, 91(2), 179–194. <https://doi.org/10.1093/aob/mcf118>

342 Bucak, M. N., Ateşşahin, A., Varışlı, Ö., Yüce, A., Tekin, N., & Akçay, A. (2007). The influence of
343 trehalose, taurine, cysteamine and hyaluronan on ram semen: Microscopic and oxidative stress
344 parameters after freeze–thawing process. *Theriogenology*, 67(5), 1060–1067.
345 <https://doi.org/10.1016/j.theriogenology.2006.12.004>

346 Champroux, A., Torres-Carreira, J., Gharagozloo, P., Drevet, J., & Kocer, A. (2016). Mammalian
347 sperm nuclear organization: Resiliencies and vulnerabilities. *Basic and Clinical Andrology*, 26(1),
348 17. <https://doi.org/10.1186/s12610-016-0044-5>

349 de Lamirande, E., & O’Flaherty, C. (2008). Sperm activation: Role of reactive oxygen species and
350 kinases. *Biochimica Et Biophysica Acta (BBA) - Proteins and Proteomics*, 1784(1), 106–115.
351 <https://doi.org/10.1016/j.bbapap.2007.08.024>

352 de Melo, A. A., Carneiro, R. F., de Melo Silva, W., Moura, R. D. M., Silva, G. C., de Sousa, O. V.,
353 ... Sampaio, A. H. (2014). HGA-2, a novel galactoside-binding lectin from the sea cucumber
354 *Holothuria grisea* binds to bacterial cells. *International Journal of Biological Macromolecules*, 64,
355 435–442. <https://doi.org/10.1016/j.ijbio mac.2013.12.035>

356 Esmat, A. Y., Said, M. M., Soliman, A. A., El-Masry, K. S., & Badiea, E. A. (2013). Bioactive
357 compounds, antioxidant potential, and hepatoprotective activity of sea cucumber (*Holothuria atra*)
358 against thioacetamide intoxication in rats. *Nutrition*, 29(1), 258–267.
359 <https://doi.org/10.1016/j.nut.2012.06.004>

360 Galli, F., Battistoni, A., Gambari, R., Pompella, A., Bragonzi, A., Pilolli, F., ... Cabrini, G. (2012).
361 Oxidative stress and antioxidant therapy in cystic fibrosis. *Biochimica Et Biophysica Acta (BBA) –*
362 *Molecular Basis of Disease*, 1822(5), 690–713. <https://doi.org/10.1016/j.bbadis.2011.12.012>

363 Ghanbari, R. (2018). Review on the bioactive peptides from marine sources: Indication for health
364 effects. *International Journal of Peptide Research and Therapeutics*, 25, 1–13.

365 Iqbal, S., Andrabi, S. M. H., Riaz, A., Durrani, A. Z., & Ahmad, N. (2016). Trehalose improves semen
366 antioxidant enzymes activity, postthaw quality, and fertility in Nili Ravi buffaloes (*Bubalus bubalis*).
367 *Theriogenology*, 85(5), 954–959. <https://doi.org/10.1016/j.theriogenology.2015.11.004>

368 John Aitken, R., Clarkson, J. S., & Fishel, S. (1989). Generation of reactive oxygen species, lipid
369 peroxidation, and human sperm function. *Biology of Reproduction*, 41(1), 183–197.
370 <https://doi.org/10.1095/biolreprod.41.1.183>

371 Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional
372 foods: Impact on human health. *Pharmacognosy Reviews*, 4(8), 118. <https://doi.org/10.4103/0973-7847.70902>

374 MacLeod, J. (1943a). The role of oxygen in the metabolism and motility of human spermatozoa.
375 *American Journal of Physiology-Legacy Content*, 138(3), 512–518.

376 Marzony, E. T., Ghanei, M., & Panahi, Y. (2016). Relationship of oxidative stress with male infertility
377 in sulfur mustard-exposed injuries. *Asian Pacific Journal of Reproduction*, 5(1), 1–9.
378 <https://doi.org/10.1016/j.apjr.2015.12.001>

379 Meseguer, M., Molina, N., García-Velasco, J. A., Remohí, J., Pellicer, A., & Garrido, N. (2006).
380 Sperm cryopreservation in oncological patients: A 14-year follow-up study. *Fertility and Sterility*,
381 85(3), 640–645. <https://doi.org/10.1016/j.fertn.stert.2005.08.022>

382 Motlagh, M. K., Sharafi, M., Zhandi, M., Mohammadi-Sangcheshmeh, A., Shakeri, M., Soleimani,
383 M., & Zeinoaldini, S. (2014). Antioxidant effect of rosemary (*Rosmarinus officinalis* L.) extract in
384 soybean lecithin-based semen extender following freeze–thawing process of ram sperm. *Cryobiology*,
385 69(2), 217–222.

386 Myron, P., Siddiquee, S., & Al Azad, S. (2014). Fucosylated chondroitin sulfate diversity in sea
387 cucumbers: A review. *Carbohydrate Polymers*, 112, 173–178. <https://doi.org/10.1016/j.carbp>
388 [ol.2014.05.091](https://doi.org/10.1016/j.carbp)

389 Opuwari, C. S., & Henkel, R. R. (2016). An update on oxidative damage to spermatozoa and oocytes.
390 *BioMed Research International*, 2016, 1–11. <https://doi.org/10.1155/2016/9540142>

391 Pishehvarzad, F., Yousefzadi, M., Kamrani, E., Moini Zanjani, T., & Ali Ahmadi, A. (2014).
392 Antioxidant activity of extracts of two species of Sea Cucumber *Holothuria parva* and *Holothuria*
393 *leucospilota* from the Persian Gulf, Iran. *Journal of Aquatic Ecology*, 4(1), 34–39.

394 Qeshmi, F. I., Homaei, A., Fernandes, P., & Javadpour, S. (2018). Marine microbial L-asparaginase:
395 Biochemistry, molecular approaches and applications in tumor therapy and in food industry.
396 *Microbiological Research*, 208, 99–112. <https://doi.org/10.1016/j.micres.2018.01.011>

397 Sabeti, P., Pourmasumi, S., Rahiminia, T., Akyash, F., & Talebi, A. R. (2016). Etiologies of sperm
398 oxidative stress. *International Journal of Reproductive Biomedicine*, 14(4), 231–240.

399 Saleh, R. A., & Agarwal, A. (2002). Oxidative stress and male infertility: From research bench to
400 clinical practice. *Journal of Andrology*, 23(6), 737–752.

401 Sariözkan, S., Tuncer, P., Büyükleblebici, S., Bucak, M., Cantürk, F., & Eken, A. (2015).
402 Antioxidative effects of cysteamine, hyaluronan and fetuin on post-thaw semen quality, DNA
403 integrity and oxidative stress parameters in the Brown Swiss bull. *Andrologia*, 47(2), 138–147.
404 <https://doi.org/10.1111/and.12236>

405 Sen, S., & Chakraborty, R. (2011). The role of antioxidants in human health. In S. Andreescu & M.
406 Hepel (Eds.), *Oxidative stress: Diagnostics, prevention, and therapy* (pp. 1–37). Washington, D.C.:
407 ACS Publications.

408 Seydi, E., Motallebi, A., Dastbaz, M., Dehghan, S., Salimi, A., Nazemi, M., & Pourahmad, J. (2015).
409 Selective toxicity of Persian Gulf Sea cucumber (*Holothuria parva*) and sponge (*Haliclona oculata*)
410 methanolic extracts on liver mitochondria isolated from an animal model of hepatocellular carcinoma.
411 *Hepatitis Monthly*, 15(12), e33073. <https://doi.org/10.5812/hepat.mon.33073>

412 Sharifian, S., Homaei, A., Hemmati, R., Luwor, R. B., & Khajeh, K. (2018). The emerging use of
413 bioluminescence in medical research. *Biomedicine & Pharmacotherapy*, 101, 74–86.
414 <https://doi.org/10.1016/j.biopha.2018.02.065>

415 Sharifian, S., Homaei, A., Kamrani, E., Etzerodt, T., & Patel, S. (2019). New insights on the marine
 416 cytochrome P450 enzymes and their biotechnological importance. *International Journal of*
 417 *Biological Macromolecules*, 142, 811–821.

418 Sherman, J. K. (1964). Dimethyl sulfoxide as a protective agent during freezing and thawing of
 419 human spermatozoa. *Proceedings of the Society for Experimental Biology and Medicine*, 117, 261–
 420 264.

421 Sobhani, A., Eftekhari, T. E., Shahrzad, M. E., Natami, M., & Fallahi, S. (2015a). Antioxidant
 422 effects of Brown Algae sargassum on sperm parameters: CONSORT-compliant article. *Medicine*,
 423 94(52), e1938. <https://doi.org/10.1097/MD.00000000000001938>

424 Taylor, K., Roberts, P., Sanders, K., & Burton, P. (2009). Effect of antioxidant supplementation of
 425 cryopreservation medium on post-thaw integrity of human spermatozoa. *Reproductive Biomedicine*
 426 *Online*, 18(2), 184–189. [https://doi.org/10.1016/S1472-6483\(10\)60254-4](https://doi.org/10.1016/S1472-6483(10)60254-4)

427 Walters, E. M., Benson, J. D., Woods, E. J., & Critser, J. K. (2009). The history of sperm
 428 cryopreservation. In A. A. Pacey (Ed.), *Sperm banking: Theory and practice* (pp. 1–17). Cambridge,
 429 UK: Cambridge University Press.

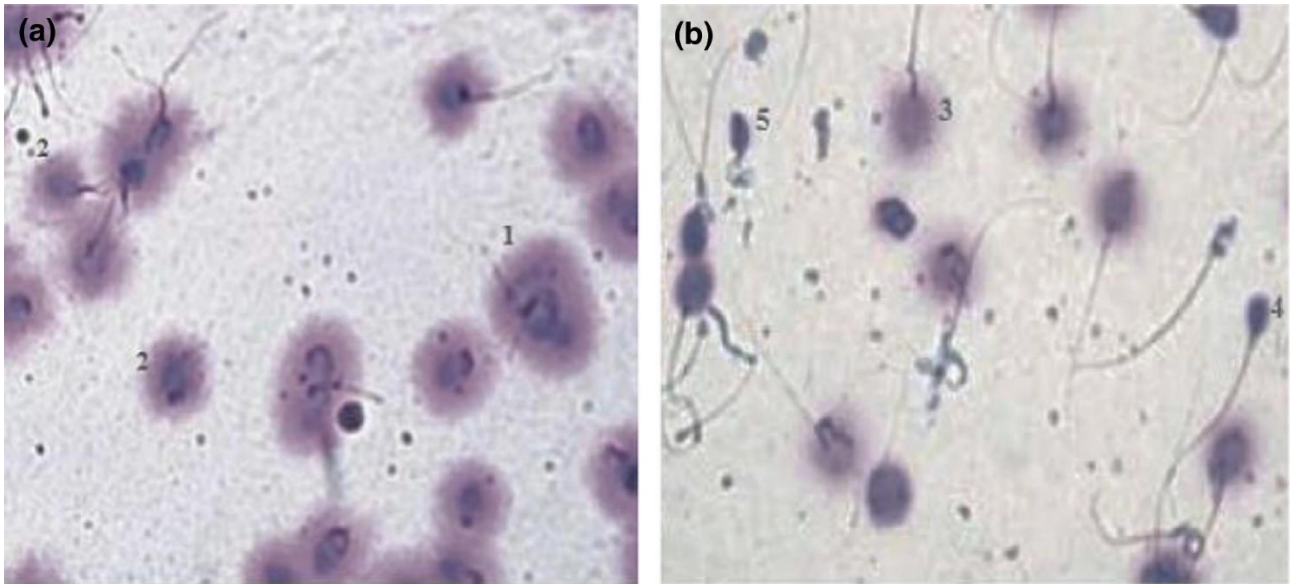
430 Wijesinghe, W., Jeon, Y. J., Ramasamy, P., Wahid, M. E. A., & Vairappan, C. S. (2013). Anticancer
 431 activity and mediation of apoptosis in human HL-60 leukaemia cells by edible sea cucumber
 432 (*Holothuria edulis*) extract. *Food Chemistry*, 139(1–4), 326–331. <https://doi.org/10.1016/j.foodchem.2013.01.058>

434 Williams, D. H. IV. (2010). Sperm banking and the cancer patient. *Therapeutic Advances in Urology*,
 435 2(1), 19–34. <https://doi.org/10.1177/1756287210368279>

436 World Health Organization (WHO) (2010). *WHO laboratory manual for the examination and*
 437 *processing of human semen* (5th ed.). Geneva, Switzerland: World Health Organization.

438 Yamaguchi, T., Takamura, H., Matoba, T., & Terao, J. (1998). HPLC method for evaluation of the
 439 free radical-scavenging activity of foods by using 1, 1-diphenyl-2-picrylhydrazyl. *Bioscience,*
 440 *Biotechnology, and Biochemistry*, 62(6), 1201–1204. <https://doi.org/10.1271/bbb.62.1201>

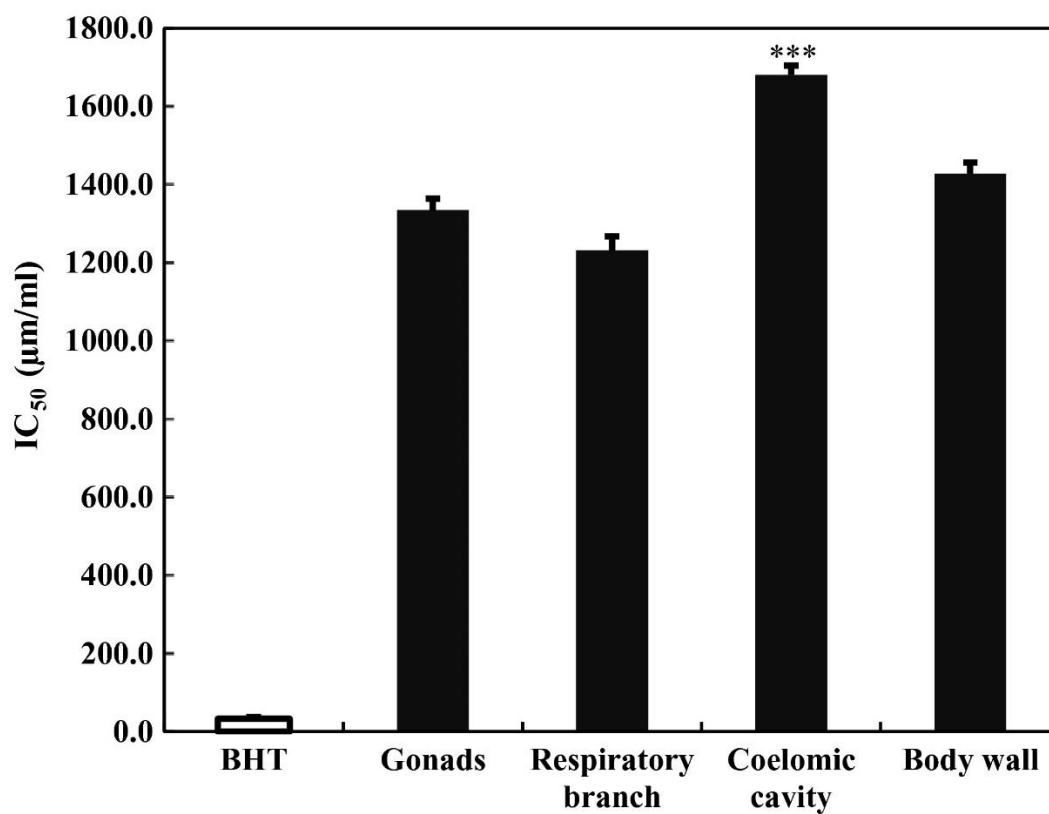
441 Yang, J., Wang, Y., Jiang, T., & Lv, Z. (2015). Novel branch patterns and anticoagulant activity of
 442 glycosaminoglycan from sea cucumber *Apostichopus japonicus*. *International Journal of Biological*
 443 *Macromolecules*, 72, 911–918. <https://doi.org/10.1016/j.ijbiomac.2014.10.010>



444

445 Figure 1

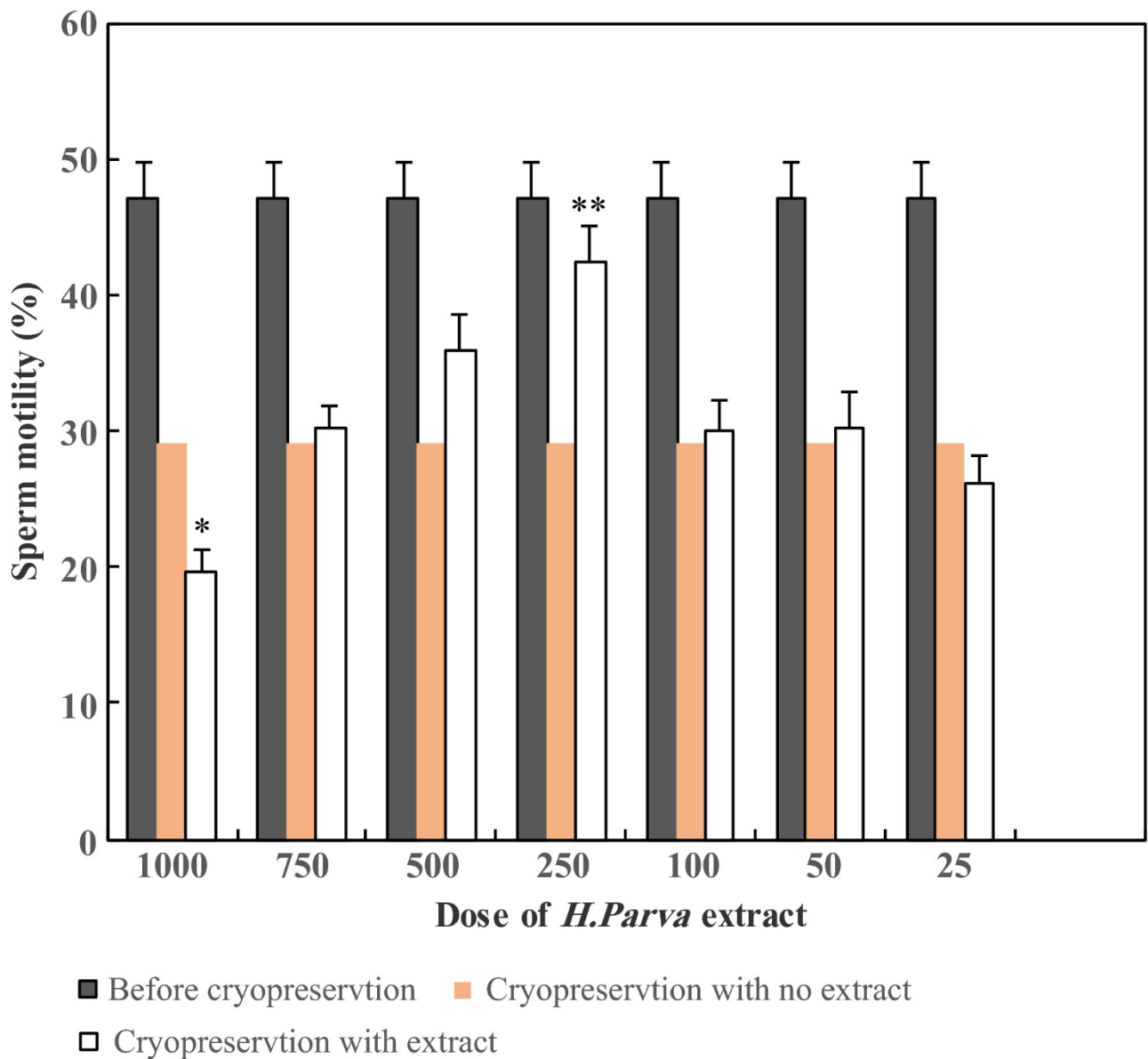
446 Representative micrographs of sperm DNA fragmentation assayed by sperm chromatin dispersion
447 test. (a) 250 µg/ml group, (b) control group. Score 1 showed no fragmentation, 2 low, 3 moderate and
448 4, 5 indicative highest DNA damage. Value and number of damaged spermatozoa significantly
449 decreased in the groups which received treatment



450

451 Figure 2

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

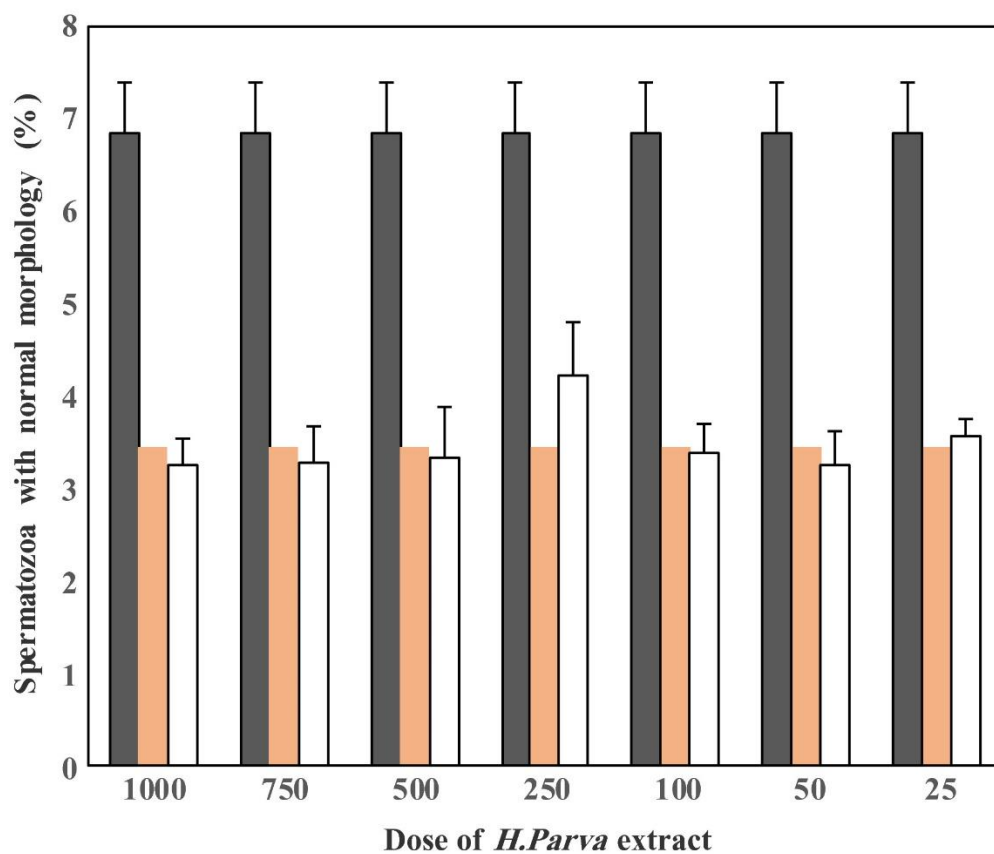


455

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

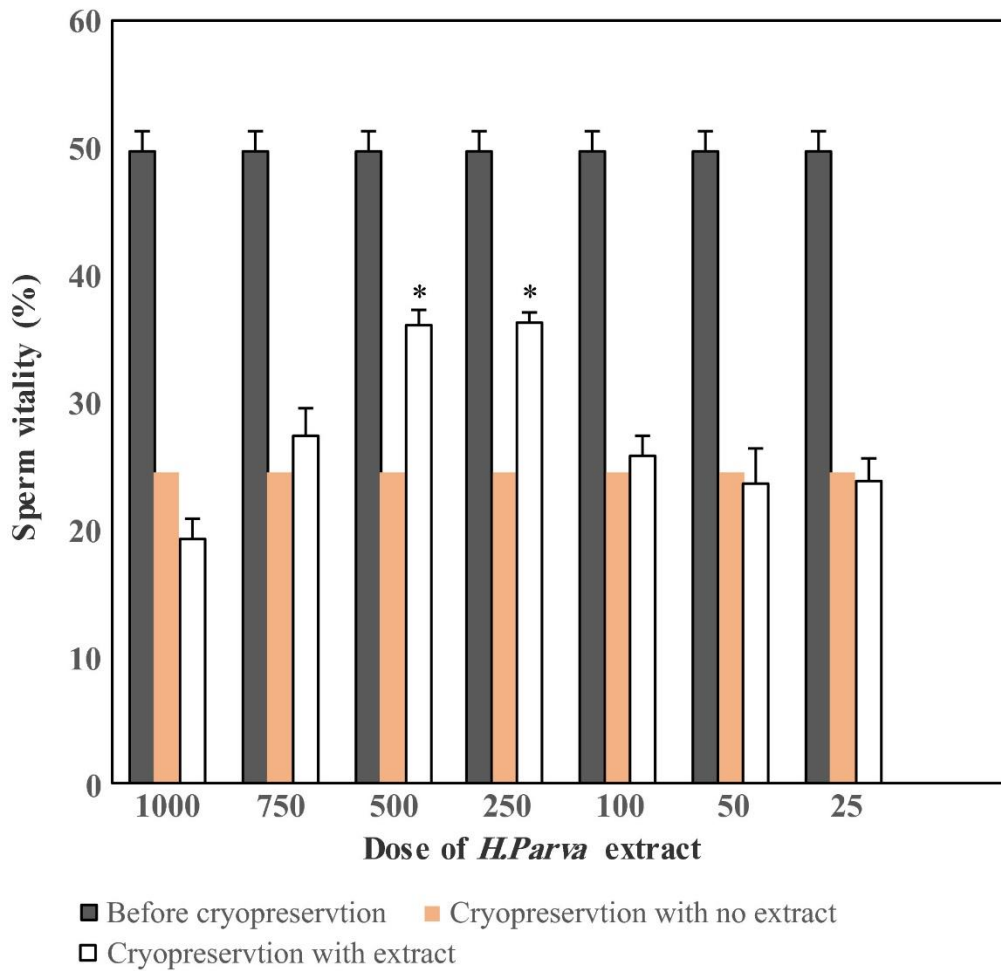


■ Before cryopreservtion ■ Cryopreservtion with no extract
 □ Cryopreservtion with extract

461

462 Figure 4

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



465

466 Figure 5

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

470 **Table 1.** Sperm chromatin dispersion (SCD) data (mean \pm SEM) after freezing spermatozoa in
 471 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 *

472 * Indicates significant differences (p < .05) between the treated and cryopreserved control group.
 473

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 <i>H. parva</i> extract	36	0.2 *	38	0.4 *	20	0.1	6	1.6 *
500 µg/ml of <i>H. parva</i> 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.