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

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

5  
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27 **Keywords**

28 cryopreservation, *Holothuria parva*, ROS, Spermatozoa

29

## 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).

## 93        **2. Materials and Methods**

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

### 99        **2.1 Extract preparation**

100       Five specimens of *Holothuria parva* were caught in the May month from Bandar Abbas, State of  
101       Hormozgan, Iran, and immediately transported to our laboratory at Hormozgan University where they  
102       were freshly prepared. All the different body parts (gonads, respiratory branch, coelom cavity and  
103       body wall) were removed from the adhering meninges and blood, and the grey matter was removed  
104       by gross dissection. 3 mg of each different body part tissue was resuspended in 3 ml of 50 mM  
105       phosphate buffer at pH 7.5. The suspension was subjected to sonication for 15 s with 40-s pauses for  
106       10 min by a SYCLON Ultra Sonic Cell SKL950-IIDN. Cell debris was discarded by centrifugation  
107       at 15,000 g at 4°C for 20 min. The supernatant was immediately stored at -20°C until use.

### 108       **2.2 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay**

109       The ROS-scavenging activity of different parts of the body of *H. parva* (gonads, respiratory branch,  
110       coelomic cavity and body wall) was evaluated according to the method of Yamaguchi, Takamura,  
111       Matoba, and Terao (1998). In brief, 1 ml of DPPH (Sigma-Aldrich) solution (0.1 mmol/L, in 95%  
112       ethanol (v/v)) was incubated with various concentrations of the extract from the different body parts.  
113       The mixture was shaken, incubated for 20 min at room temperature, and the absorbance was read at  
114       517 nm against a blank using a Perkin Elmer Lambda 850 UV/VIS Spectrometer. The radical  
115       scavenging activity was measured as a decrease in the absorbance of DPPH and calculated using the  
116       following equation:

$$117 \quad \text{DDPH scavenging effect (\% inhibition)} = \left[ \frac{(A_0 - A_1)}{A_0} \times 100 \right].$$

118        $A_0$  is the absorbance of the control reaction and  $A_1$  the absorbance in presence of the extract samples.  
119       The parameter for the evaluation of DPPH method is the IC50 value (inhibition concentration at  
120       50%), which indicates the concentration of antioxidant that causes 50% loss of the DPPH activity.  
121       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  $\geq 15$  million/ml, total motility  $\geq 40\%$ , normal sperm morphology  $\geq 4\%$ , seminal volume  $\geq 1.5$  ml,  
131 pH  $\geq 7.2$ , 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  $-179^{\circ}\text{C}$  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  $\mu\text{l}$  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-died  
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  $\mu\text{l}$  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 $^{\circ}\text{C}$ . 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.

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300 **Conflict of Interest**

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

302 **REFERENCES**

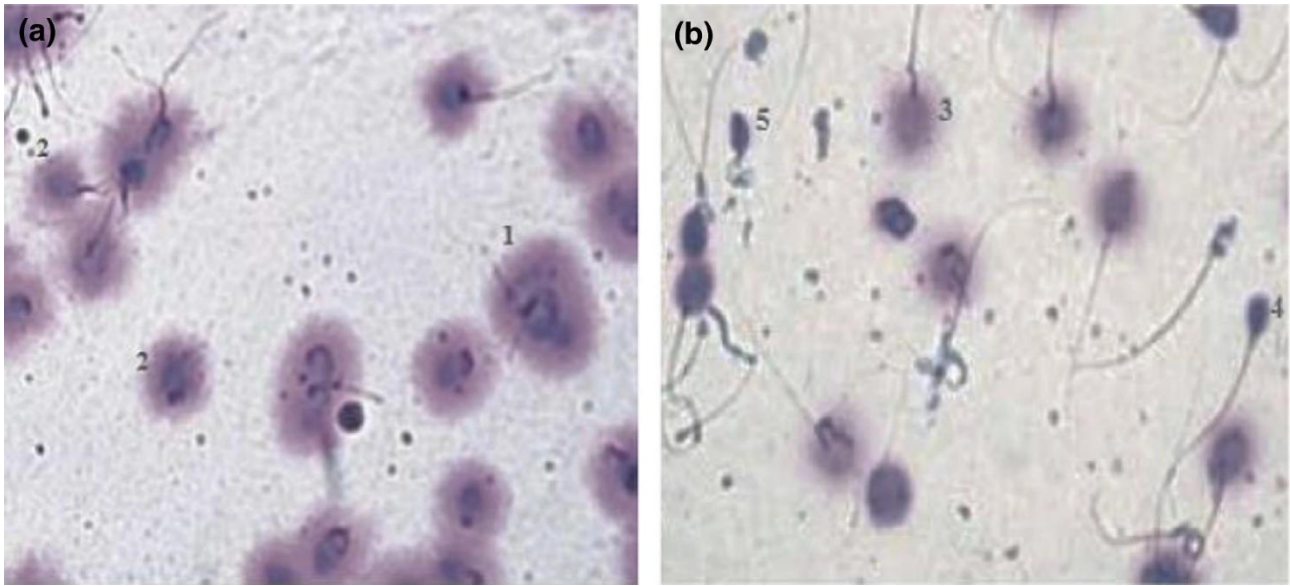
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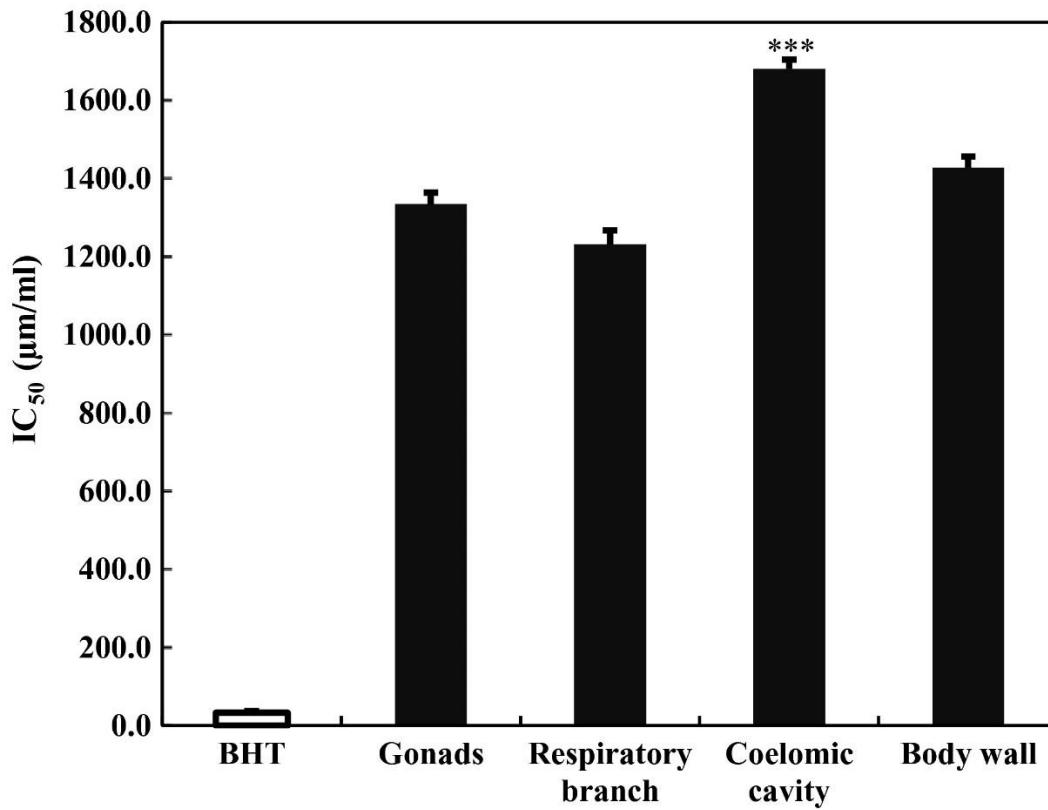




444

445 Figure 1

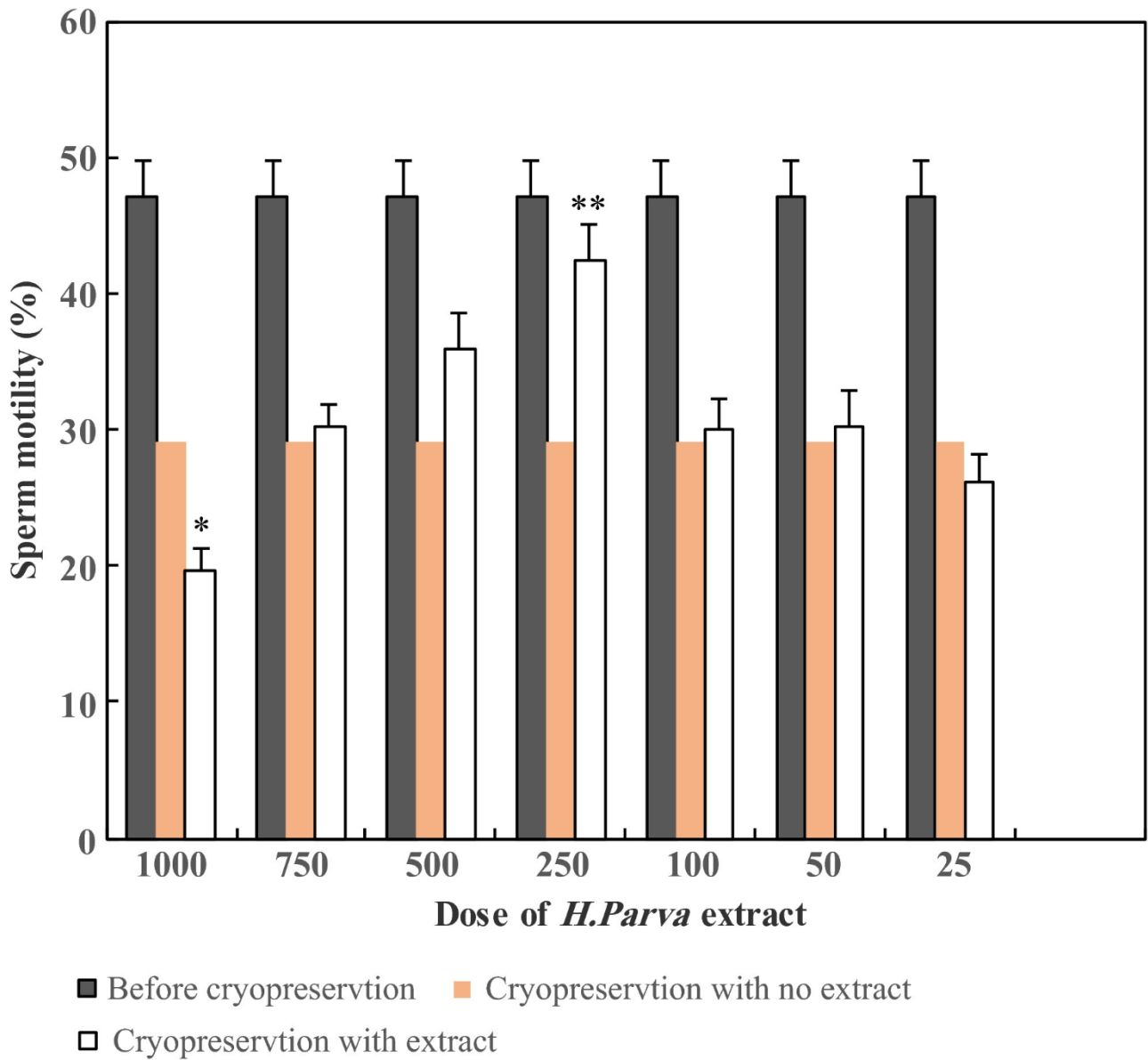
446 Representative micrographs of sperm DNA fragmentation assayed by sperm chromatin dispersion  
447 test. (a) 250 µg/ml group, (b) control group. Score 1 showed no fragmentation, 2 low, 3 moderate and  
448 4, 5 indicative highest DNA damage. Value and number of damaged spermatozoa significantly  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



450

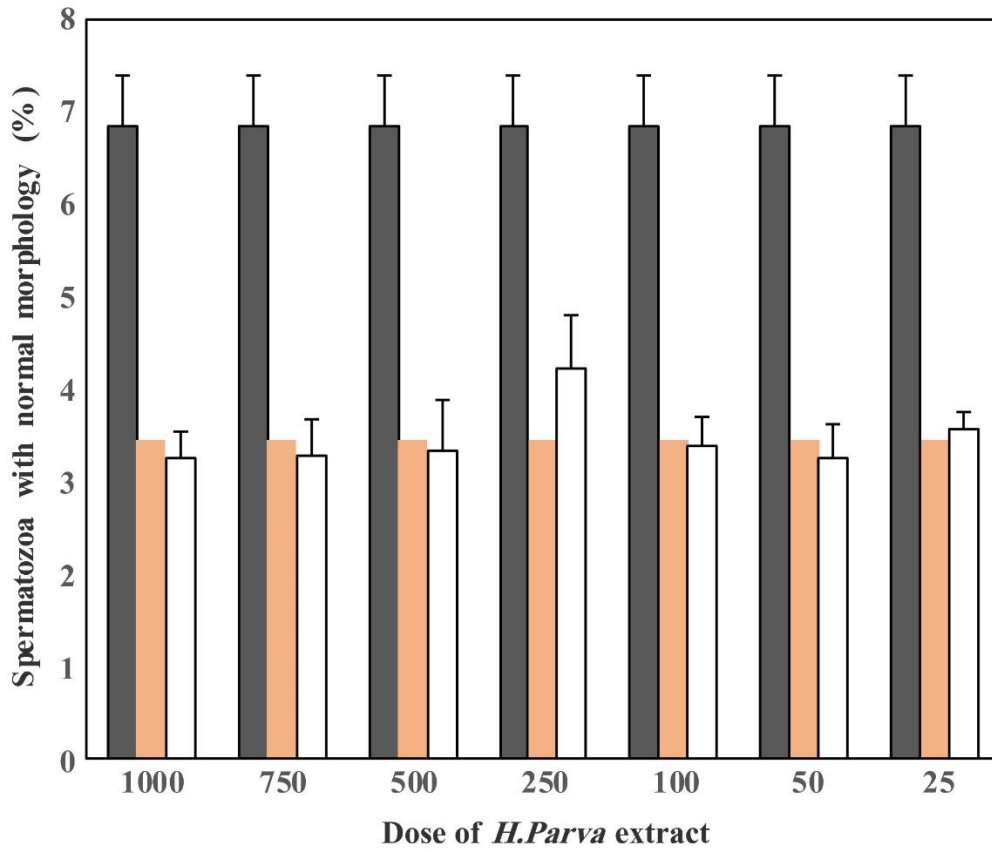
451 Figure 2

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 ( $p < .001$ )



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

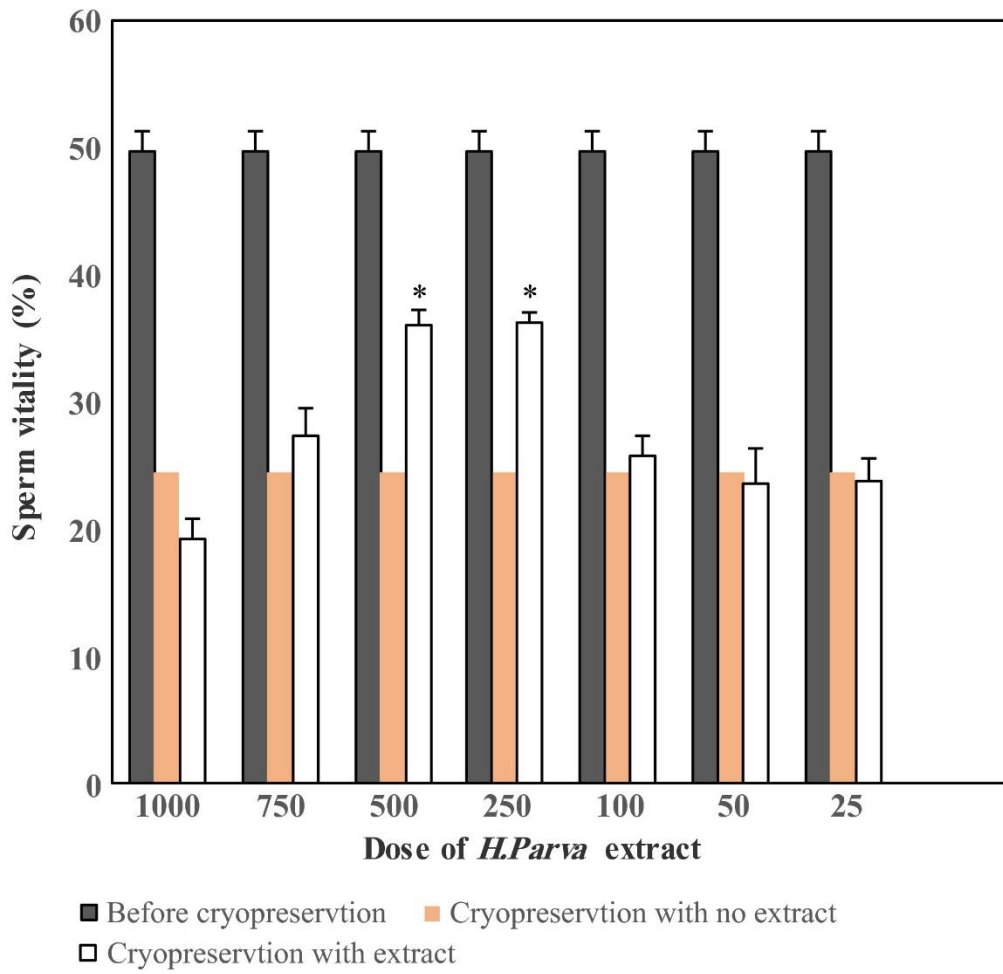


■ Before cryopreservtion    ■ Cryopreservtion with no extract  
 □ Cryopreservtion with extract

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 $\mu$ g/ml of <i>H. parva</i> extract	46	0.3 *	32	0.2 *	14	0.2	4	1.1 *	6	0.3 *
500 $\mu$ g/ml of <i>H. parva</i> 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.