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Biochemical changes of cryopreserved seminal plasma and spermatozoa of the giant grouper *Epinephelus lanceolatus* after preservation and transportation using dry-ice

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ABSTRACT

The present study aims to investigate the effects of exposure of the seminal plasma and spermatozoa of the giant grouper *Epinephelus lanceolatus* to dry ice (−79 °C) during transport on their quality. In all, 15 amino acid compounds were determined. The quantification of total proteins were measured using the Bradford method, and amino acid concentration were measured using the HPLC method. The cryopreserved seminal plasma was transferred from a liquid nitrogen tank to a styrofoam box filled with dry ice. Total protein and amino acids were measured after 24, 48, and 72 h. For comparative purposes, total protein and fifteen compound of amino acid were also measured. Both parameters were also measured after the cryopreserved seminal plasma were immersed in liquid nitrogen after 24 and 48 h exposed to dry ice. The results showed that the exposure of seminal plasma to dry ice for 24, 48 and 72 h during transportation or immersion back into the liquid nitrogen after 24 and 48 h does not change the total protein levels either in seminal plasma or spermatozoa. However, the level of each amino acid compound in the seminal plasma had significantly decreased.

Introduction

The cryopreservation is an important technique in the aquaculture industry especially for the hybrid fry production (Che-Zulkifli et al., 2020; Magnotti et al., 2018). Some male gametes of particular species are difficult to obtain (Contreras et al., 2019; Xin et al., 2019). The hybrid grouper (*Epinephelus lanceolatus* ♂ x *Epinephelus fuscoguttatus* ♀) becomes the most popular fish in the Malaysian marine aquaculture industry. In this case, the cryopreserved sperm is the solution for the lack of the *Epinephelus lanceolatus* gametes problem. To produce hybrid grouper fry, the cryopreserved sperm must be shipped from the cryopreservation center to the hatchery. The transportation of cryopreserved sperm using a liquid nitrogen tank is costly and packaging chilled fresh spermatozoa and transporting them in a styrofoam box is suggested. However, the quality of the cryopreserved sperm using a styrofoam box in the most cultured fishes is unknown. Proteomic approaches to male reproduction can provide new information on the functioning of the reproductive system (Ciereszko et al., 2017). Proteomic approaches were also studied by Om et al. (2013) to identify the vitellogenin protein of the giant grouper, *Epinephelus lanceolatus*. The total protein is an accepted parameter to measure the quality of sperm. Fatihah et al. (2011) studied the total protein in the sperm of spiny lobster and measured the biochemical changes after cryopreservation. The protein components in seminal plasma are adsorbed onto the surface of ejaculated sperm as it passes through the male or female reproductive tract (Muiño-Blanco et al., 2008).

The molecular weight of protein in the seminal plasma is a marker that indicates the potential of seminal plasma for cryopreservation. Cooling and thawing in cryopreservation damage the function of sperm protein (Corcini et al., 2012). Zhang et al. (2015) stated

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that the protein plays an important role in the maintenance of sperm motility after the freezing and thawing processes; those researchers also found an association between the heat shock protein 90 (HSP90) and the sperm quality such as motility, plasma membrane and acrosome integrity. Molecular studies on the HSP90 were performed by Chen et al. (2010). In bull spermatozoa, cooling and thawing influenced the level of sperm surface protein (P25b) (Lessard et al., 2000). The presence of protein bands in the seminal plasma is not associated with the quality of frozen sperm with different extenders (Goularte et al., 2014). However, the protein profile is correlated to the characteristics of ram sperm, and it can be used to measure the fertility and quality of the ram seminal plasma (Almadaly et al., 2016). The protein profile measures the two classes of protein (albumin and globulin). Albumin prevents fluid leakage from the blood vessels, and globulin plays an important role in the immune system. The accumulation of amino acids forms part of an organism's resistance to a cool shock (Bucak et al., 2009). Amino acids are an important component of the spermatozoa nuclei.

Glutamine prevents the denaturation of muscle protein in fish (Matsumoto and Noguchi, 1971). In human sperm, glutamine acts on an extracellular level to enhance post-thaw motility rates for potential fertilization (Renard et al., 1996). At high concentrations, alanine may affect the motility and viability of the sperm (Koskinen & Rapoport, 1989). Taurine, hypotaurine, and vitamins E and C are important antioxidants that are naturally present in the seminal plasma (Cabrita et al., 2011). In addition, taurine is a byproduct of amino acids cysteine and methionine. It is a semi-essential amino acid, which is not incorporated into proteins (Schuller-Levis & Park, 2003). The addition of taurine and hypotaurine to the freezing media during the sperm cryopreservation protocol of European sea bass increases the motility rate and reduces DNA damage (Martínez-Páramo et al., 2013). The function of the antioxidants in amino acids is to protect sperm against cold shock during cooling (Sangeeta et al., 2015).

Therefore, the present study aims to determine the biochemical changes in the cryopreserved seminal plasma and spermatozoa that were transported in a styrofoam box using dry ice as a preservative material. The levels of total protein and amino acids were assessed as the biochemical parameters. The study also aims to compare between the cryopreserved seminal plasma in cryotubes and in the straw. The purpose of using the two types of containers is to compare the results between them as they have different advantages. Cryotube is more robust compared to straw, while, straw is easy to be kept in a liquid nitrogen tank. Straw can be stored inside calister but cryotube needs special apparatus to be kept and store in a liquid nitrogen tank.

Materials and methods

The method used by Fan et al. (2014) was modified to suit the seminal plasma of our tropical fish species. Seminal plasma was obtained from the brooders by pressing the abdomen area toward the genital pore, and it was inserted into a 10 mL plastic tube. We make sure the semen are directly flow into the plastic vial without touching the body. Then, it was mixed with an extender at a 1:3 ratio and it was perfectly through vigorous agitation. Cryoprotectant and dimethyl sulfoxide (DMSO) were mixed at 12% of the total diluent. The diluent was inserted into a 2 mL cryovial (Sigma Aldrich, USA) and a 0.25 mL plastic straw.

The cryotube and the straw were placed 6 cm above the liquid nitrogen for 10 min and was placed on the surface of the liquid nitrogen until its temperature reached −80 °C. The temperature was measured using a thermocouple (Hanna, Portugal) by inserting the probe into the hole punched on top of the cryotube cap. The cryotube was immersed into liquid nitrogen contained in a 5 L cryotank, which was shipped to the laboratory. All the cryopreserved seminal plasma was held in the permanent storage tank until the time of the experiment. The thawing method used followed Fan et al. (2014) and the cryopreserved sperm was immersed in a water bath (37 °C) for 1.5 min.

Experimental design

The experiment was conducted by transporting the cryopreserved sperm in the straw and the cryotube inside a styrofoam box, loaded with dry ice as a refrigerant. The experiment control was post-thawed seminal plasma immersed into liquid nitrogen. The experimental design was explained in Table 1.

There are two parameters was observed in seminal plasma and spermatozoa which are total protein and fifteen amino acid level. For the total protein assay, the effect of added of extender and cryoprotechtant and cryopreservation procedure in the concentration of total protein was determined. The total protein and amino acid analysis described in detail below.

Total protein analysis

The total protein of the seminal plasma and spermatozoa of the giant grouper was determined using the Bradford's method (Bradford, 1976). The total protein reagents and standards used were brought from Sigma Aldrich USA. The proteins extracted from the spermatozoa were re-suspended in a lysis buffer before analysis. Fresh or frozen/thawed seminal plasma was diluted (1:250) with sea water and inserted into 2 mL plastic tubes. The samples were centrifuged at 1200 rpm for 30 min at 4 °C. The extracted proteins were re-suspended in a lysis buffer (50 mM Tris/HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Phenylmethane sulfonyl fluoride, 1 μg/μL leupeptin, 1 mM sodium orthovanadate [Na_3VO_4], 1 mM natrium fluoride [NaF]). The suspension was centrifuged at 18,000 rpm for 15 min at 4 °C. The supernatant containing the total soluble proteins was transferred into Eppendorf tubes. To determine the total protein in the seminal plasma, the seminal plasma was diluted three times with distilled water, and then 5 μL of the seminal plasma sample were added into a 96-well plate. In each well, 250 μL of Bradford Reagent were added and mixed on a shaker for ~30 s. The samples and standards were incubated for 45 min at room temperature and measured by a microplate reader (Multiscan™ FC, Thermo Fisher Scientific Inc.) with a wavelength absorbance of 595 nm. A standard curve was developed from a dilution series with known concentration that allow comparison with the samples. The concentration of the total protein of the sample was obtained through comparing the values of the sample against the standard curve.

Table 1
Independent variables (control and treatments).

Control (C)	Post-thawing of cryopreserved seminal plasma from liquid nitrogen
Treatment 1 (T1)	Cryopreserved seminal plasma transferred from liquid nitrogen to dry ice (-79 °C) for 24 h
Treatment 2 (T2)	Exposure of cryopreserved seminal plasma to dry ice for 24 h and immersed back into liquid nitrogen
Treatment 3 (T3)	Cryopreserved seminal plasma transferred from liquid nitrogen to dry ice (-79 °C) for 48 h
Treatment 4 (T4)	Exposure of cryopreserved seminal plasma to dry ice for 48 h and immersed back into liquid nitrogen
Treatment 5 (T5)	Cryopreserved seminal plasma transferred from liquid nitrogen to dry ice (-79 °C) for 72 h

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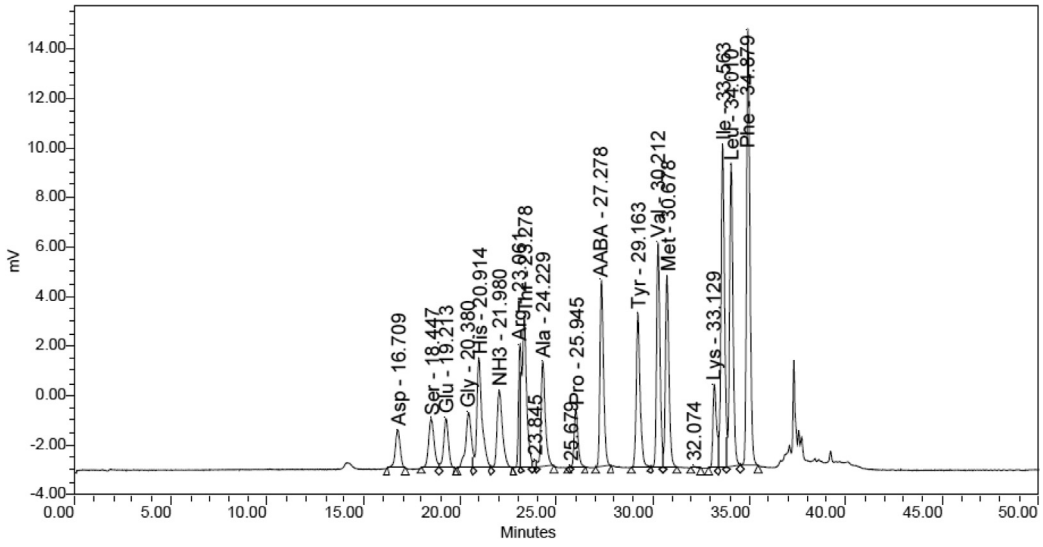

Fig. 1. The chromatogram of fifteen amino acid compounds and internal standards (AABA).

Amino acid analysis

Preparation of amino acid standards

For standards, 40 μL of amino acid internal standard and 40 μL of amino acid H standard (2.5 μmole/mL of amino acids & 1.25 μmole/mL of Cystine) were added to 920 μL of water. Then, 10 μL of the standard were added to 70 μL of borate buffer and 20 μL of AccQ flour reagent. Finally, 5 μL of the standard were injected into the High-Performance Liquid Chromatography HPLC (Waters 1525, Australia). Fig. 1 shows the chromatogram of amino acid standard. The peaks in the chromatogram indicated the amino acid compounds and the internal standards which were added to the amino acid standards.

Sample preparation and addition of internal standards

Dried samples in an amount of 0. 1–0. 2 g were added to 5 mL of 6 N HCl and were heated to 110 °C for 24 h; then, 4 mL of AABA stock was added to the sample which was filled with HPLC grade water to 100 mL. The sample solution was filtered through filter paper and then through a syringe filter. Then, 10 μL of the sample solution were added to 70 μL of borate buffer and 20 μL of AccQ reagent, and the resulting mixture was vortexed. The contents were transferred into a limited volume insert, placed in an injection vial and capped. The vial was heated to 55 °C for 10 min. Five μL of unknown and standard were injected into HPLC (Waters 1525, Australia).

Chromatographic conditions

The column used was AccQ tag (3.9 × 150 mm). The mobile phase featured a concentrated AccQ tag eluent A and an AccQ tag eluent B or 60% acetonitrile. The derivatization used AccQ Fluor Reagents. The standards were amino acid standard, hydro lysate (Standards H, "pierce"). Flow rate = 1 mL/minutes, detection = Fl uorescence Detector (Waters 2475), Eλ = 250 nm, E_m = 395 nm, gain = 1, filter = 1.5 s, injection volume = 5 μL.

Statistical analysis

The data on result was presented as mean ± SD. Significant differences in the data was analyzed using one way ANOVA followed by Tukey's post hoc test with 95 percent of confident level ($P < 0.05$). Statistical analysis was performed using SPSS 21, Chicago, IL, USA).

Results

Changes of the levels of total protein in the seminal plasma

Table 2 shows the level of total protein in the seminal plasma of four treatments before and after the addition of the extender and Cryoprotectant (DMSO). The cryoprotectant lowered the level of

Table 2
Levels of total protein in the seminal plasma in four treatments. T1: Fresh seminal plasma before the addition of the extender and cryoprotectant; T2: Fresh seminal plasma after the addition of the extender and cryoprotectant; T3: Post-thaw from liquid nitrogen (loaded into straw); T4: Post-thaw from liquid nitrogen (loaded into cryotube). Super indices letters indicate significant differences ($P > 0.05$) (n = 5).

Treatment	Levels of total protein (mean ± SD) (mg/mL)
T1	6.86 ± 1.35^{a}
T2	2.57 ± 1.06^{b}
T3	2.73 ± 0.66^{b}
T4	2.49 ± 1.13^{b}

Table 3
Comparison of total protein levels in the seminal plasma in straw and cryotube containers. C: Post-thaw from liquid nitrogen; T1: 24-h exposure to dry ice; T2: 24-h exposure to dry ice and immersion in liquid nitrogen; T3: 48-h exposure to dry ice; T4: 48-h exposure to dry ice and immersion in dry ice; T5: 72-h in dry ice. Super indices letters indicate significant differences ($P > 0.05$) (n = 5).

Treatment	Levels of total protein (mean ± SD) (mg/mL)	
	Straw	Cryotube
C	2.26 ± 0.47^{ab}	2.16 ± 0.59^{ab}
T1	2.5 ± 0.46^{ab}	2.36 ± 0.16^{ab}
T2	2.27 ± 0.29^{ab}	1.17 ± 0.96^{a}
T3	2.45 ± 0.34^{b}	2.62 ± 0.30^{b}
T4	2.33 ± 0.38^{ab}	2.61 ± 0.17^{b}
T5	1.88 ± 0.47^{a}	2.25 ± 0.24^{ab}

Table 4
Level of total protein in spermatozoa before and after addition of extender and cryoprotectant. Super indices letters indicate significant differences ($P > 0.05$) (n = 5).

Treatment	Levels of total protein (mg/mL)
Before the addition of the extender and the cryoprotectant	1.95 ± 0.54^{a}
After the addition of the extender and the cryoprotectant	1.94 ± 0.32^{a}
Post-thaw from liquid nitrogen (loaded into straw)	2.26 ± 0.47^{a}
Post-thaw from liquid nitrogen (loaded in cryotube)	2.16 ± 0.53^{a}

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total protein in the seminal plasma from 6.86 ± 1.31 mg/mL to 2. 57 ± 1.02 mg/mL. Meanwhile, Table 3 shows the level of total protein in seminal plasma which loaded into straw and cryotube. The comparison has been made between the control and five treatments. In straw, the result shows there are no significant difference between the control and all treatments except between T3 and T5. In cryotube, T3 and T4 shows significant difference than the others.

Changes of the levels of total protein in the spermatozoa

Table 4 shows that there were no significant differences ($P > 0.05$) in the levels of total protein in the spermatozoa before and after the addition of the extender and cryoprotectant. The results also show that there were no significant differences ($P > 0.05$) in the levels of protein in the spermatozoa before and after cryopreservation whether in cryotube or straw. Table 5 reveals a comparison of the levels of total protein among the treatments and the control for each container. For the cryopreserved sperm in straw, there were no significant differences ($P > 0.05$) among the treatment and the control groups with the exception of those preserved for 48 h in dry ice (Treatment 3) and 72 h in dry ice (Treatment 5). Likewise, for the cryopreserved sperm in a cryotube, there were no significant differences ($P > 0.05$) between the treatments and the control groups except for Treatments 2 (24 h in dry ice and immersed in liquid nitrogen) and 3 (48 h in dry ice) and between Treatments 2 and 4.

Table 5
Comparison of total protein levels in the spermatozoa of the control and the five treatments transferred into two different container types (straw and cryotube). C: Post-thawed from liquid nitrogen; T1: 24-h exposure to dry ice; T2: 24-h exposure to dry ice and immersion back into liquid nitrogen; T3: 48-h exposure to dry ice; T4: 48-h exposure to dry ice and immersion back into dry ice; T5: 72 h in dry ice. Super indices letters indicate significant differences ($P > 0.05$).

Treatment	Levels of total protein (mean ± SD) (mg/mL)	
	loaded into straw	loaded into cryotube
C	2.26 ± 0.47^{ab}	2.16 ± 0.59^{ab}
T1	2.5 ± 0.46^{ab}	2.36 ± 0.16^{ab}
T2	2.27 ± 0.29^{ab}	1.17 ± 0.96^{a}
T3	2.45 ± 0. 34^{b}	2.62 ± 0. 30^{b}
T4	2.33 ± 0.38^{ab}	2.61 ± 0. 17^{b}
T5	1.88 ± 0.47^{a}	2.25 ± 0.24^{ab}

Table 6
Comparison of the levels (g/100 g) of the amino acid compounds in seminal plasma loaded into straw. C: Post-thaw from liquid nitrogen; T1: 24-h exposure to dry ice; T2: 24-h exposure to dry ice and immersion in liquid nitrogen; T3: 48-h exposure to dry ice; T4: 48-h exposure to dry ice and immersion in dry ice; T5: 72 h in dry ice. Super indices letters indicate significant differences. ($P > 0.05$) ($n = 3$).

Treatment	His	Arg	Thr	Tyr	Val	Met	Lys
C	1.95 ± 0.07^{a}	2.15 ± 0.25^{a}	1.26 ± 0.07^{a}	1.69 ± 0.08^{a}	1.06 ± 0.05^{a}	1.20 ± 0.35^{a}	1.04 ± 0.01^{a}
T1	0.95 ± 0. 01^{b}	1.40 ± 0. 15^{b}	0.48 ± 0. 02^{b}	1.21 ± 0. 06^{b}	0.59 ± 0. 02^{b}	1.02 ± 0.02^{a}	0.54 ± 0. 03^{b}
T2	0.0041 ± 0. 0031^{c}	0.0660 ± 0. 0440^{c}	0.02 ± 0. 011^{c}	0.0019 ± 0. 0009^{c}	0.0087 ± 0. 00039^{c}	0.00059 ± 0. 00015^{b}	0.00765 ± 0. 00056^{d}
T3	0.002499 ± 0. 00046^{c}	0.0230 ± 0. 0010^{c}	0.0527 ± 0. 0011^{c}	0.0038 ± 0. 000153^{c}	0.0122 ± 0. 00023^{c}	0.0017 ± 0. 00088^{b}	0.0104 ± 0. 00046^{d}
T4	0.0033 ± 0. 00026^{c}	0.017 ± 0. 01186^{c}	0.0450 ± 0. 00031^{c}	0.0026 ± 0. 00040^{c}	0.0102 ± 0. 00015^{c}	0.00076 ± 0. 00015^{b}	0.0085 ± 0. 00023^{d}
T5	0.0050 ± 0. 0003^{c}	0.3136 ± 0. 0014^{c}	0.0253 ± 0. 00040^{c}	0.00035 ± 0. 00023^{c}	0.00304 ± 0. 00152^{c}	0.0020 ± 0. 00071^{b}	0.0523 ± 0. 00728^{c}

Treatment	Ile	Leu	Phe	Asp	Ser	Glu	Gly	Ala
C	1.10 ± 0.14^{a}	1.15 ± 0.18^{a}	1.43± 0.21^{a}	0.94 ± 0.05^{a}	0. 87± 0.18^{a}	1.22 ± 0.30^{a}	0.59 ± 0.22^{a}	0.71 ± 0.08^{a}
T1	0.85 ± 0.23^{a}	0.77 ± 0. 01^{b}	1.13 ± 0. 01^{b}	0.75 ± 0. 22^{b}	0.85 ± 0. 32^{b}	0.99 ± 0. 04^{b}	1.03 ± 0. 47^{b}	0. 01± 0. 0002^{b}
T2	0.0078 ± 0. 00015^{b}	0.00829 ± 0. 00065^{c}	0.0049 ± 0. 00023^{c}	0.010 ± 0. 00018^{c}	0.0097 ± 0. 042^{c}	0.0448 ± 0. 001^{c}	0.010 ± 0. 00036^{b}	0.0096 ± 0. 00025^{b}
T3	0.0068 ± 0. 00023^{b}	0.0138 ± 0. 00061^{c}	0.0071 ± 0. 00023^{c}	0.0150 ± 0. 00071^{c}	0. 011± 0. 00130^{c}	0.0187 ± 0. 0014^{c}	0.0137 ± 0. 00049^{b}	0.0126 ± 0. 00092^{b}
T4	0.0057 ± 0. 00023^{b}	0.0110 ± 0. 00015^{c}	0.00596 ± 0. 00015^{c}	0.0116 ± 0. 00045^{c}	0.01065 ± 0. 00038^{c}	0.0171 ± 0.00045^{c}	0.0113 ± 0. 00019^{b}	0.0109 ± 0. 00012^{b}
T5	0.0377 ± 0. 00023^{b}	0.0228 ± 0. 00031^{c}	0.0172 ± 0. 00031^{c}	0.0492 ± 0. 0026^{c}	0.0446 ± 0. 00039^{c}	0.0677 ± 0.00099^{c}	0.0415 ± 0. 00039^{b}	0.0379 ± 0. 00018^{b}

Table 7
Comparison of the levels (g/100 g) of the amino acid compounds in seminal plasma loaded into cryotube. C: Post-thaw from liquid nitrogen; T1: 24-h exposure to dry ice; T2: 24-h exposure to dry ice and immersion in liquid nitrogen; T3: 48-h exposure to dry ice; T4: 48-h exposure to dry ice and immersion in dry ice; T5: 72-h in dry ice. Super indices letters indicate significant differences ($P > 0.05$) ($n = 3$).

Treatment	His	Arg	Thr	Tyr	Val	Met	Lys
C	3.59 ± 0.43^{a}	7.14 ± 1.48^{a}	4.17 ± 0.6^{a}	3.32 ± 0.3^{a}	2. 77 ± 0. 032^{a}	2.99 ± 0.007^{a}	2.24 ± 0.02^{a}
T1	1.25 ± 0.01^{b}	2.25 ± 0.01^{b}	0.72 ± 0.001^{b}	0.98 ± 0.082^{b}	0.56 ± 0.009^{b}	0.35 ± 0.02^{b}	0
T2	0.036 ± 0.00070^{c}	1.0351 ± 0.10^{c}	0.2371 ± 0.147^{b}	0.0246 ± 0.00053^{c}	0.0999 ± 0.00045^{c}	0.00994 ± 0.00115^{c}	0.0610 ± 0.00407^{b}
T3	0.0061 ± 0.0013^{c}	0.036 ± 0.0011^{d}	0.067 ± 0.0264^{b}	0.0055 ± 0.00085^{c}	0.0206 ± 0.00065^{d}	0.0051 ± 0.00042^{c}	0.0094 ± 0.00476^{c}
T4	0.0100 ± 0.0004^{c}	0.2709 ± 0.0227^{d}	0.0209 ± 0.00024^{b}	0.0069 ± 0.0015^{c}	0.0245 ± 0.00819^{d}	0.0099 ± 0.0026^{c}	0.0453 ± 0.00229^{bc}
T5	0.0049 ± 0.0019^{c}	0.1927 ± 0.0088^{d}	0.0166 ± 0.00072^{b}	0.0060 ± 0.00308^{c}	0.0191 ± 0.00055^{d}	0.00096 ± 0.00055^{c}	0.0344 ± 0.00130^{c}

Treatment	Ile	Leu	Phe	Asp	Ser	Glu	Gly	Ala
C	2.89 ± 0.04^{a}	3.22 ± 0.01^{a}	3.62 ±0.03^{a}	4.33± 0.50^{a}	2.73 ± 0.22^{a}	3.96 ± 0.49 ^{a}	3.15 ± 0.54^{a}	2.55 ± 0.009^{a}
T1	0.97 ± 0.01^{b}	0.94 ± 0.01^{b}	1.059 ± 0.01^{b}	0.11± 0.04^{b}	0.88 ± 0.02^{b}	0.24 ± 0.01^{b}	0	0
T2	0.0575 ± 0.000701^{c}	0.1086 ± 0.0007^{c}	0.0619 ± 0.0020^{c}	0.1285 ± 0.00337^{c}	0.1121 ± 0.0018^{c}	0.18375 ± 0.00151^{b}	0.1188 ± 0.00037^{b}	0.1031 ± 0.0423^{b}
T3	0.0121 0.00065^{c}	0.022 ± 0.00089^{d}	0.0132 ± 0.00042^{d}	0.0847 ± 0.0823^{c}	0.083 ± 0.0031^{c}	0.0369 ± 0.0029^{b}	0.0268 ± 0.00053^{b}	0.0214 ± 0.00040^{c}
T4	0.0150 ± 0.00066^{c}	0.0323 ± 0.00066^{d}	0.013 ± 0.00086^{d}	0.0301 ± 0.00147^{c}	0.0323 ± 0.00194^{c}	0.045 ± 0.0016^{b}	0.0330 ± 0.00093^{b}	0.0287 ± 0.00093^{bc}
T5	0.0193 ± 0.00075^{c}	0.0121 ± 0.00075^{d}	0.0083 ± 0.00096^{d}	0.0199 ± 0.0013^{c}	0.0233 ± 0.00045^{c}	0.0336 ± 0.00123^{b}	0.0208 ± 0.00078^{b}	0.0210 ± 0.00145^{c}

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Changes in amino acid levels in the seminal plasma

In Tables 6 and 7, a comparison is given of the levels of amino acid compounds in the cryopreserved seminal plasma for the control and the treatment groups. The results of the statistical analysis showed significant differences ($P < 0.05$) in every amino acid level in the cryopreserved seminal plasma in different containers for post-thaw (control) and Treatments 2 (24 h in dry ice and immersion into liquid nitrogen), 3 (48 h in dry ice), and 4 (48 h in dry ice and immersed in liquid nitrogen). However, there were no significant differences between ($P > 0.05$) treatments 1 (24 h in dry ice) and 5 (72 h in dry ice). In addition, the statistical analysis showed a similar pattern for the levels of each amino acid compound; significant differences were found among the control and Treatments 1 and 2. However, no significant differences were found among Treatments 2, 3, 4, and 5.

Discussion

A similar study performed by Fatihah *et al.* (2016) determined the levels of total protein in spiny lobster seminal plasma relative to the duration of storage in the liquid nitrogen. The study showed significant differences in the levels of total protein in relation to the storage duration. While the dependent variables were similar, namely, the total protein of cryopreserved marine animal seminal plasma, the independent variables were not. Fatihah *et al.* (2016) used the duration of storage of the seminal plasma in liquid nitrogen as an independent variable, while here, it was exposure of cryopreserved seminal plasma (-169 °C) in dry ice (-79 °C) for different durations. This difference robs the comparison of meaning.

The biochemical compositions such as fatty acids, amino acids and lipids play an important role in performing physiological functions of certain aquatic species (El-Kassas, 2013; El-Karim et al. 2016; Dridi et al. 2017; El-Ghafour et al. 2018). Unlike total protein, amino acid levels in the seminal plasma of the treated samples were significantly decreased. Therefore, the transportation of the cryopreserved seminal plasma in a styrofoam box with dry ice as refrigerant affected the levels of each compound of amino acid tested. While no significant differences were found in the levels of total protein in the seminal plasma stored in cryotube and straw, by the contrary was found in amino acid levels. Significant differences appeared for every amino acid compound for every treatment between straw and cryotube. This shows that the thickness of the container helped protect the amino acids in cryopreserved seminal plasma after exposure to −79 °C.

The current results (data in Table 5) indicated that the exposure of cryopreserved seminal plasma to dry ice inside a styrofoam box during transportation for a certain period does not affect the total protein levels, although it negatively affects the amino acid levels. Total protein levels in the seminal plasma had significantly decreased after the addition of the extender and the cryoprotectant but they did not in spermatozoa. Furthermore, no significant difference appeared in the total protein levels in the cryopreserved seminal plasma after exposure to dry ice for a certain period either in cryotube or straw. However, the amino acid levels did exhibit a significant difference between the two containers for all types.

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