Research Article

Morphological alterations in cryopreserved spermatozoa of scallop
Argopecten purpuratus

Carlos Espinoza[1], Martha Valdivia[2] & Enrique Dupré[3]
[1]Laboratorio de Biología Experimental, Instituto del Mar del Perú
Esquina Gamarra y General Valle s/n, Chucuito Callao, Perú
[2]Laboratorio de Fisiología de la Reproducción Animal, Universidad Nacional Mayor de San Marcos, Perú
[3]Laboratorio de Criopreservación, Universidad Católica del Norte, Coquimbo, Chile

ABSTRACT. The present work identifies and quantifies the morphological alterations of scallop *Argopecten purpuratus* spermatozoa caused by long-term cryopreservation. Percentages of motility, fertilization and injured spermatozoa were quantified by optic microscopy and scanned electron microscopy. These parameters were evaluated in sperm without treatment (CTR), spermatozoa incubated in cryoprotective solution but not freezed (ICS) and freezed-thawed spermatozoa (FTS). Spermatozoa of ICS treatment remained motile longer than those of CTR, whereas those of FTS treatment were lowest. Morphology of the spermatozoa was affected in several ways by the freeze-thawing treatment; some had their head deformed or swollen, others had their cell membrane folded or broken; acrosome reaction; anomalous positions or absence of mitochondria as well as broken, stiff or loss of lineal structure of tail. CTR and ICS treatments had higher percentages of undamaged sperm (87.7% and 79.0% respectively), while FTS samples had 14.2% of undamaged sperm. The tail was the spermatic structure most commonly injured in FTS (77.0%), the percentage of sperm with head injury was 55.1% and with acrosome reaction was 28.7%, whereas middle piece was affected in 23.9% of sperm. Percentages of fertilization were 68.3%, 67.9% and 58.2% for CTR, ICS and FTS respectively, which were not significantly different. There was a higher correlation between injuries and motility than between injuries and fertilization success. Correlation between motility and fertilization was low (0.605 and 0.668 with motility at 5 and 30 min, respectively).

Keywords: cryopreservation, *Argopecten purpuratus*, scallop, sperm, Chile.

Alteraciones morfológicas en espermatozoides criopreservados de concha de abanico
Argopecten purpuratus

RESUMEN. El presente trabajo identifica y cuantifica las alteraciones morfológicas en espermatozoides de concha de abanico *A. purpuratus* causadas por la criopreservación en nitrógeno líquido. Porcentajes de motilidad, fecundación de ovocitos frescos y espermatozoides lesionados (en cabeza, acrosoma, pieza media y flagelo) fueron determinados bajo microscopía óptica y electrónica de barrido. Estos parámetros fueron evaluados en un control sin tratamiento (CTR), espermatozoides incubados en solución crioprotectora pero sin congelamiento (ICS) y espermatozoides congelados-descongelados (FTS). Los espermatozoides del tratamiento ICS mostraron mayor motilidad que de CTR, mientras que la motilidad de los espermatozoides del tratamiento FTS fue la más baja. La morfología externa de los espermatozoides fue afectada de varias formas por el congelamiento-descongelamiento; cabeza deformada o hinchada, membrana celular plegada o rota, reacción acrosómica, anormal posición o ausencia de las mitocondrias, ruptura, rigidez o pérdida de la estructura lineal del flagelo. El CTR e ICS presentó los mayores porcentajes de espermatozoides ilesos (87,7% y 79,0% respectivamente), mientras que las muestras de FTS tuvieron 14,2% de espermatozoides ilesos. El flagelo fue la estructura más afectada por el tratamiento FTS (77,0%), el porcentaje de espermatozoides con lesiones en la cabeza fue 55,1% y con reacción acrosómica 28,7%, mientras que los afectados en la pieza media fue de 23,9%. Los porcentajes de fecundación fueron 68,3%, 67,9% y 58,2% para CTR, ICS y FTS respectivamente, los cuales no mostraron diferencias significativas entre ellos. Se obtuvo mayor correlación

entre lesión y motilidad que entre lesión y fecundación. La correlación entre motilidad y fecundación fue baja (0,605 y 0,668 con motilidad a 5 y 30 min respectivamente).

Palabras clave: criopreservación, *Argopecten purpuratus*, concha de abanico, ostión, espermatozoides, Chile.

_____

Corresponding author: Carlos Espinoza (cespinoza@imarpe.gob.pe)

INTRODUCTION

One of the critical points for the development of aquaculture of native species is the reproduction in captivity, which needs a suitable management of breeding condition and incorporation of assisted reproductive techniques such as hormonal induction of spawning (Patiño, 1997; Zohar & Mylonas, 2001) and gametes cryopreservation (Caffey & Tiersch, 2000; Espinoza & Dupré, 2001). One of the reproductive problems of fish in captivity is asynchrony of gametes release, which can leads to failure of the effort to obtain brood, since ovulation does not happen at the expulsion sperm moment, ovulated eggs become "overripe" and cannot be fertilized (Zohar & Mylonas, 2001). One alternative to solve this problem is the use of induction methods for spawning, thermal shock for shellfish (Robert & Gérard, 1999) and hormonal induction for fish (Zohay & Mylonas, 2001), complemented with preservation at low temperatures techniques, since sperm freezed in liquid nitrogen could be used anytime and anywhere.

However, to optimize cryopreservation techniques, it is important to know and understand the effects that freezing-thawing has on sperm structure to, thus, design suitable freezing methodologies that allow us to minimize the percentage of sperm injured. It is known that during freezing and thawing, cells suffer osmotic stress because constant chemical potential changes between intra and extracellular medium caused by ice formation (Mazur, 1984). Damages in sperm might be different according to the structure affected, since sperm plasmatic membrane possesses three different domains, one in front region head, other in back region head and other in tail; which have different both lipids and membrane proteins (Alberts *et al.*, 2000), doing that freezing phenomena cause different deficiencies in motility and fertilizing capacity.

In previous investigation it has been reported different alterations in sperm as a result of freezing and thawing; being the most common, axonem structure alteration, folds formation in head plasmatic membrane (Billard, 1983), lysed cells and mitochondrias (Mediavilla *et al.*, 1995), damage in plasmatic membrane (Curry & Watson, 1992), acrosome eversion and broken tail (Bury & Olive, 1993). However, these alterations have not been quantified to correlate neither with motility nor fertilizing capacity of sperm.

The present work identified and quantified morphologic alterations caused on *Argopecten purpuratus* sperm by cryopreservation process with a system of mechanical freezer, designed by Dupré & Espinoza (2004). The goal is to recognize the injuries caused by freezing-thawing in sperm of this species to optimize an easy handling and lower cost methodology that could be used for preservation of other shellfish and fish in the field.

MATERIAL AND METHODS

Ripe scallops were obtained from cultures of La Herradura Bay in Coquimbo, Chile, transported to hatchery at the Universidad Católica del Norte and placed in 500 L tanks with constant aeration and water flow for conditioning. Then, gonads were dissected and the male portion was cut into pieces and placed into 10 mL Petri dishes containing filtered and sterilized sea water (SSW) for 10 min to obtain a spermatic suspension.

Spermatic suspension was mixed with cryoprotective solution (1:3) into cryotubes to be equilibrated for 5 min at 5ºC. The composition of the cryoprotective solution used is shown in Table 1. Freezing was performed in a portable mechanical freezer, cooling rate used was -8ºC min^{-1} (to -100ºC) and finally, the cryotubes were plunged into liquid nitrogen according to the methodology designed by Dupré & Espinoza (2004). After 24 h, thawing was performed by immersing cryotubes in a 50ºC water bath by 20 s and other in a room-temperature water bath until the last ice crystal had melted. Thawed samples were transferred into Petri dishes to evaluate spermatic motility and morphology (at 5, 30, 60, 110 and 150 min) and fertilization trials.

Sperm motility was observed under optic microscope (250x) and recorded as percentage of motile spermatozoa in a determinate area of hema-

Table 1. Composition of cryoprotective solution used to freeze sperm of *A. purpuratus.*

Tabla 1. Composición de la solución crioprotectora utilizada para congelar espermatozoides de *A. purpuratus.*

	Cryoprotective solution	Sterilized sea water
ME_2SO	10% (v/v)	
Sucrose	125 mM	
Egg hens yolk	10% (v/v)	
Distilled water	80% (v/v)	
Osmolarity ($mOsm\ kg^{-1}$)	2003	1387

tocytometer. Thus, motility was evaluated in sperm without treatment (CTR), spermatozoa incubated in cryoprotective solution but not freezed (ICS) and freezed-thawed spermatozoa (FTS).

To analyze spermatic extern morphology, samples were fixed in glutaraldehyde 3% (diluted in SSW), dehydrated through a series of ethanol baths and dried in a critical point drying apparatus (Sandri-780 Tousimis) using liquid CO_2. Subsequently, samples were placed on bronze porta-samples and sputter-coated with gold using fine-coat Ion Sputter JFC-1100, JEOL. Samples were examined in a scanning electron microscope JEOL-T300. They were tested 100 spermatozoa by sample and there were identified spermatozoa that had malformations in acrosome, head, middle piece and tail.

Oocytes obtained by artificially induced spawning and frozen-thawed sperm were placed in 60 mL Petri dishes and gently mixed together. Were used 1200 oocytes mL^{-1} and 15 spermatozoa per oocyte. After being washed and hydrated, eggs were transferred to a 250 mL bowl. Fertilization rates were determined by percentage of mobile trochophore larvae, 24 h after fertilization. The control was fertilized with fresh spermatozoa and a control of self-fertilization was also tested.

Data were presented in mean ± SE. Data normality was tested using Lilliefors test and variances homogeneity with Cochran test. When it was necessary, data were converted using arc-sine transformation. To determine significant differences in motility percentage, repeated measurement ANOVA and Tukey test were used for comparisons among time in the same treatment, and t-Student test with Bonferroni adjustment to compare among treatments of the same period. To determine correlation between *i*) injuries-fertilization, *ii*) injuries-motility and *iii*)

fertilization-motility, Pearson was used. We employed Systat 8.0 or SPSS 8.0 for Windows.

RESULTS

Sperm motility

Frozen-thawed sperm (FTS) showed significant decrease in motility compared with control (CTR) and spermatozoa incubated in cryoprotective solution (ICS) ($p < 0.001$). No significant differences were found among CTR (81.1 ± 2.3%) and ICS (76.6 ± 4.2%) ($p > 0.05$) to 30 min. Motility duration in FTS also was considerably lower than ICS and CTR. Motility in ICS stayed high without significant changes during 110 min (72.3 ± 5.5%) ($p > 0.05$), whereas in CTR this time was 30 min (81.1 ± 2.3%) and in FTS was 60 min (45.2 ± 4.9%) (Fig. 1).

Alterations in the external morphology of sperm

Typical sperm of *A. purpuratus* is differentiated in an acrosome in the anterior part of the head, an oval
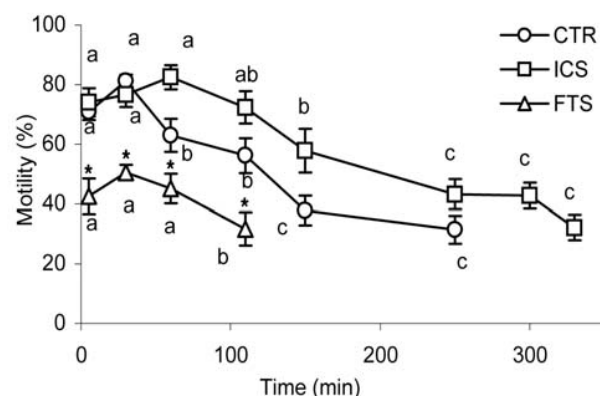

Figure 1. Total motility of frozen-thawed spermatozoa (FTS), spermatozoa incubated in cryoprotective solution but not freezed (ICS) and fresh sperm of control (CTR) in *A. purpuratus*. Values represent means and error bars represent standard deviation. Different letters indicate statistically significant differences in the time ($p < 0.05$). Asterisks indicate statistically significant differences between FTS and both CTR and ICS.

Figura 1. Motilidad total de espermatozoides congelados-descongelados (FTS), espermatozoides incubados en solución crioprotectora pero no congelados (ICS) y espermatozoides frescos del control (CTR) en *A. purpuratus*. Valores representan medias y barras de error la desviación estándar. Diferentes letras indican diferencias estadísticamente significativas en el tiempo ($p < 0.05$). Asteriscos indican diferencias estadísticamente significativas entre FTS y los otros dos tratamientos.

head, the middle piece conformed by four mito-chondrias (being evident as convex small projections) and a posterior tail (Fig. 2a). Considering these characteristics, morphological alterations observed in each of the spermatic structures were: head (HE): plasmatic membrane folded or broken, deformed, swollen or lysed (smooth), (Fig. 2b); acrosome (AC): acrosomic reaction (Fig. 2c); middle piece (MP): mitochondrias out of its normal position or absent (Figs. 2d-2e); tail (TA): it was the most affected structure, broked, rigid or loss of linear structure (Figs. 2f, 2g, 2h).

In control group, $87.7 \pm 3.4\%$ of sperm were undamaged, being significantly different of injured sperm percentages ($p < 0.001$). The $79.0 \pm 3.0\%$ of ICS were undamaged ($p < 0.05$), percentages of damage in tail ($13.8 \pm 2.0\%$) and in head ($12.4 \pm 1.3\%$) were significantly more than those observed in the middle piece ($2.8 \pm 1.4\%$) and acrosome ($2.1 \pm 1.2\%$) ($p < 0.05$). Only $14.2 \pm 2.8\%$ of FTS were undamaged, and structures more injured were the tail ($77.0 \pm 3.0\%$) and head ($55.1 \pm 7.4\%$) with regard to acrosome ($28.7 \pm 3.3\%$) and middle piece ($23.9 \pm 4.1\%$) ($p < 0.05$) (Fig. 3).

Fertilization rate

Fertility trials showed that frozen-thawed sperm could fertilize oocytes. Fertilization rates were $68.3 \pm 6.6\%$, $67.9 \pm 4.2\%$ and $58.2 \pm 7.3\%$ with fresh sperm (CTR), ICS and FTS respectively; no significant difference was observed among the three values ($p > 0.05$) (Fig. 4).

Correlation injure *vs* motility and injure *vs* fertilization

Correlation indexes between injured percentages and motility were highly significant ($p < 0.05$), being -0,739 between Mot5 and HE; -0,888 between Mot5 and AC; -0,859 between Mot5 and MP; -0,882 between Mot5 and TA (Tabla 2). Nevertheless, there was no significant correlation between injures percentages and fertilization rates ($p > 0.05$). Significant correlation was observed between fertilization rates and spermatic motility evaluated to 30 min (0,668; $p < 0.05$), but not between fertilization rates and spermatic motility to 5 min (0,650; $p > 0.05$) (Table 2).

DISCUSSION

Motility observed in ICS was higher than in CTR. It suggests few toxicity of cryoprotective solution during equilibration time. Even more, it has a positive
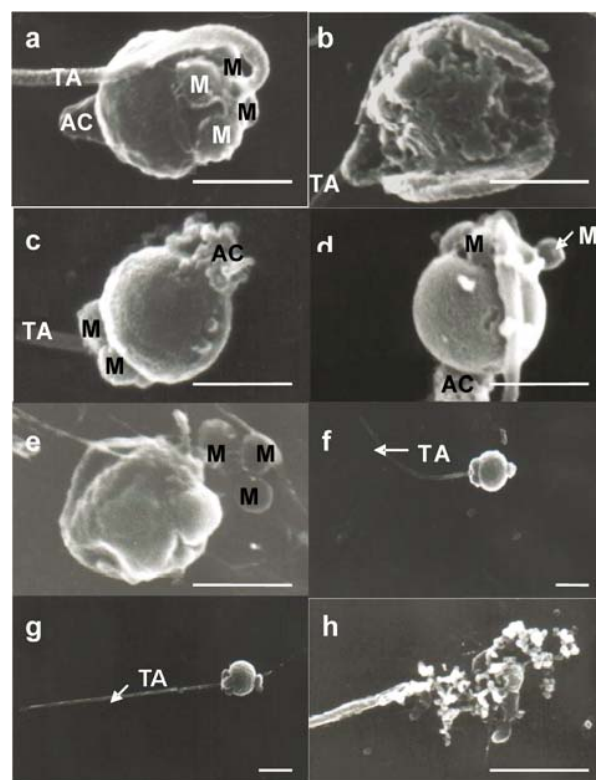

Figure 2. Morphologic alterations in frozen-thawed sperm of *A. purpuratus*. a) undamaged sperm, b) head lysed, c) acrosome eversion, d) sperm with a mitochondria stay away of its normal position, e) sperm with the four mitochondrias stay away of its normal position, f) break tail, g) rigid tail, h) rupture of membrane and loss of the linear structure of tail. AC: acrosome, TA: tail, M: mitochondria. Bar = 1 μm.

Figura 2. Alteraciones morfológicas en espermatozoides congelados-descongelados de *A. purpuratus*. a) esperma-tozoide ileso, b) cabeza lisada, c) eversión del acrosoma, d) espermatozoide con una mitocondria fuera de su posi-ción normal, e) espermatozoides con las cuatro mitocon-drias fuera de su posición normal, f) flagelo roto, g) fla-gelo rígido, h) ruptura de membrana y pérdida de la es-tructura lineal del flagelo. AC: acrosoma, TA: flagelo, M: mitocondria. Barra = 1 μm.

effect on the spermatic motility, since major tıme of activity was observed in ICS (Fig. 1). Osmolarity of cryoprotective solution was higher than SSW (Table 1); such factor would have been influencing motility and time of activity of *A. purpuratus* sperm like it was observed in other species (Morisawa & Suzuki, 1980; Chambeyron & Zohar, 1990; Ohta *et al.*, 1997). Minimal or no toxicity of the cryoprotective solution on *A. purpuratus* sperm could have been favored by inclusion of cryoadditives (sucrose and egg hen yolk),
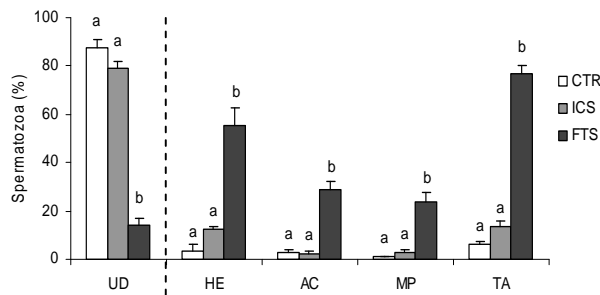
Figure 3. Percentages of injured *A. purpuratus* sperm. Undamaged sperm (UD) and sperm injured in head (HE), in acrosome (AC), in middle piece (MP) and in tail (TA). CTR: control of fresh sperm, ICS: sperm incubated in cryoprotective solution but not freezed and FTS: frozen-thawed sperm. Values represent means and error bars represent standard deviation. Letter on the bars indicate statistically similar groups in each estructure injured.

Figura 3. Porcentajes de espermatozoides lesionados de *A. purpuratus*. Ilesos (UD) y lesionados en cabeza (CA), en acrosoma (AC), en pieza media (MP) y en flagelo (TA). CTR: control de espermatozoides frescos ICS: espermatozoides incubados en solución crioprotectora pero no congelados y FTS: espermatozoides congelados-descongelados. Valores representan medias y las barras de error indican el desviación estándar. Letras sobre las barras indican grupos estadísticamente similares en cada estructura lesionada.

Figure 4. Fertilization rates of fresh oocytes with fresh sperm (CTR), sperm incubated in cryoprotective solution but not freezed (ICS) and frozen-thawed sperm (FTS) in *A. purpuratus*. CSF: control of self-fertilization. Values represent means and error bars represent standard deviation.

Figura 4. Porcentajes de fecundación de ovocitos frescos con espermatozoides frescos (CTR), espermatozoides incubados en solución crioprotectora pero no congelados (ICS) y espermatozoides congelados-descongelados (FTS) en *A. purpuratus*. CSF: control de autotecundación. Valores representan medias y barras de error indican desviación estándar.

since spermatic motility in tests performed with only ME$_2$SO, was always less than fresh sperm (Dupré *et al*., 1999).

Previous works have also reported better results in sperm cryopreservation of other species using cryoaditives as sucrose (Babiak *et al.*, 1998; Usuki *et al.*, 1997), egg hen yolk (Wheeler & Thorgaard, 1991) and lyophilized milk (Leung, 1987; Sztein, *et al.*, 2001) than those that only use cryoprotective. Cryoaditives would have fulfilled different functions of protection during cryopreservation process, complementing the action of permeable cryoprotective, such as decreasing the melting point (Rana, 1995), to serve like osmotic buffers during freezing and thawing (McWilliams *et al.*, 1995), or possibly to strengthen the cellular membrane. However, the effect of cryoaditive could be species-specific, since there are cases in which they do not fulfill the desired cryoprotective function; for example, good results were obtained with egg hen yolk in cryopreservation of brown trout (Piironen, 1993) and rainbow trout (Wheeler & Thorgaard, 1991) sperm, but not in cyprinid *Aspius aspius* sperm (Babiak *et al.*, 1998).

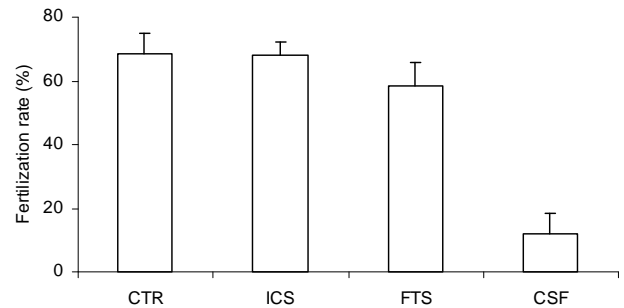The significant decrease of spermatic motility in treatment FTS and not in ICS indicates that damages

that affected spermatic motility was occasioned by ice formation during freezing; more than by the osmotic processes and toxicity during equilibrate time.

Although motility of frozen-thawed sperm diminished considerably with regard to CTR, fertilization rates obtained with these sperm did not change in the same proportion (Fig. 4). It was observed low correlation between motility and fertilization percentages of freezed-thawed sperm of *A. purpuratus*. Similar results were brought for *Pleuronectes ferrugineus* (Richardson *et al.*, 1999), they observed motility percentages of 80.0% and 43.3% with fresh and cryopreserved sperm respectively, and fertilization rates of 61.5% and 56.1% respectively. In the same way, Ritar (1999) observed lower percentages of motility in cryopreserved sperm than in fresh sperm in *Latris lineate*, but fertilization rates were not statistically different (82.7% and 71.2% respectively). To note such differences, probably, it is necessary to decrease sperm/oocytes ratio.

In this experiment, we observed that tail was the most vulnerable structure, owed to its fragility and thin shape, and it seems also that cellular membrane in tail is more vulnerable than in head or acrosomic region. In spite of microscope observations and video analysis, it was observed that sperm with broken tail had motility and it fertilized oocytes without much difficulty. It seems that energy offered to mitochondrias would play an important role in motility and fertilization capacity.

Table 2. Pearson correlation indexes of injured sperm structures whit motility or fertilization rates in *Argopecten purpuratus*. Percentages of sperm injured in every structure was correlated whit motility evaluated to 5 min because it was when samples took for morphologic analysis. Asterisks indicate significant correlation. Mot 5: motility quantified to 5 min post-dilution in sea water; Mot 30: motility quantified to 30 min post-dilution in sea water; Fer: fertilization percentage. Injured sperm in head (HE), acrosome (AC), middle piece (MP) or tail (TA).

Tabla 2. Correlación de Pearson entre estructuras lesionadas en espermatozoides con la motilidad o con las tasas de fecundante en *Argopecten purpuratus*. Los porcentajes de espermatozoides lesionados en cada estructura fueron correlacionados con la motilidad a 5 min debido a que fue el momento en que las muestras fueron colectadas para el análisis morfológico. Asteriscos indican correlación significativas. Mot 5: motilidad cuantificada a 5 min post-dilución en agua de mar; Mot 30: motilidad cuantificada a 30 min post-dilución en agua de mar; Fer: porcentaje de fecundación. Espermatozoides lesionados en cabeza (HE), acrosoma (AC), pieza media (MP) o flajelo (TA).

	Pearson index	Significance
Mot 5 *vs* HE	– 0.739	0.023*
Mot 5 *vs* AC	– 0.888	0.000*
Mot 5 *vs* MP	– 0.859	0.003*
Mot 5 *vs* TA	– 0.882	0.002*
Fer *vs* HE	– 0.432	0.245
Fer *vs* AC	– 0.556	0.120
Fer *vs* MP	– 0.651	0.058
Fer *vs* TA	– 0.653	0.057
Mot 5 *vs* Fer	0.650	0.058
Mot 30 *vs* Fer	0.668	0.049*

Sperm with rigid tail had slow motility; this rigidity could be owned to the loose of capacity of slide between adjacent doublets of microtubules of the axoneme; which makes us suppose that molecules of dineine are affected. Something similar would be happening with the sperm that presented circular motility in samples FTS. Possibly, they have had some injury in dineine arms that prevents the correct slide of microtubules of the axoneme towards one of the sides. However, this injury would be slightly frequent due to the scarce quantity of sperm with this characteristic.

Deformed heads of the sperm could be caused by swollen or injuries by crystal growth of ice. Of the sperm with head injuries, only 4.5% was lysed, the rest (51.7%) were swollen (possibly a state previous to lysis) owned to osmotic processes caused by ice crystal formations during freezing and thawing (Mazur, 1984). In contrast, when Mediavilla *et al.* (1995) cryopreserved sperm of common trout, it was observed that biggest alteration of frozen-thawed sperm was the cellular lysis (between 60 and 80%). Higher percentages of sperm with reacted acrosome in FTS samples compared to ICS and CTR were observed; this because stress during freezing and thawing produces changes in the superficial polarity of the cell (Fernández *et al.*, 2000), it start the acrosomic reaction (Longo, 1987).

In general, injuries in whatever spermatic structures are related to the cellular membrane, in this way, lysis of the head is due to the rupture of membrane, acrosomic reaction also involves alterations in membrane as well as expulsion of mitochondrias and discontinuity of the tail. This fact is because the membrane is a barrier between intra and extracellular matrix (which is highly changeable) and because it regulates osmotic processes caused by changes of chemical potential intra and extracellular during frozen of the matrix. These changes of concentration generate water flow and solutes towards both sides of the membrane, which change cell volume constantly. In this sense, the life of the cell depends on the structure of the membrane and, basically, of the fluidity and permeability that its components give.

Our results indicate that sperm incubation in cryoprotective solution used does not affect significantly the spermatic morphology, being freezing-thawing the cause of injuries in cryopreservation process.

Although major interrelation was observed between structures injured with motility than structure injured with fertilization, our results show that injury in any spermatic structure has equal incidence on its viability. Possibly, the main cause of the decrease of fertilizing capacity of sperm would be ultrastructural alterations than external morphology. In this sense, it would be necessary to continue investigating the effects of cryopreservation of sperm cells at ultrastructural and genetic level. It would be also more interesting to continue growing larvae obtained with cryopreserved sperm, since finding malformations and high mortalities during the larval development might indicate some damage in the spermatic-ADN.

REFERENCES

Babiak, I., J. Glogowski, R. Kujawa, D. Kucharczyk & A. Mamcarz. 1998. Cryopreservation of sperm from asp *Aspius aspius*. Progr. Fish-Cult., 60: 146-148.

Caffey, R.H. & T.R. Tiersch. 2000. Cost analysis for integrating cryopreservation into an existing fish hatchery. J. World Aquacult. Soc., 31: 51-58.

Chambeyron, F. & Y. Zohar. 1990. A diluent for sperm cryopreservation of gilthead seabream, *Sparus aurata*. Aquaculture, 90: 345-352.

Dupré, E., C. Arqueros, J. Barraza & J. Maureira. 1999. Toxicidad de diferentes crioprotectantes sobre espermatozoides y larvas de ostra *Crassostrea gigas* y del ostión *Argopecten purpuratus*. Informe Final: Proyecto Dirección General de Investigación y Cooperación Técnica (DGI-98), 32 pp.

Dupré, E. & C. Espinoza. 2004. Congelamiento de espermatozoides del ostión del norte *Argopecten purpuratus* mediante un congelador mecánico. Invest. Mar. Valparaíso, 32(2): 3-9.

Espinoza, C. & E. Dupré. 2001. Criopreservación: aplicaciones en acuicultura. Rev. Per. Acuic., 1(2): 50-54.

Fernández, M., G. Font de Valdez & A. Disalvo. 2000. Changes in the surface potencial of *Lactobacillus acidophilus* under freeze–thawing stress. Cryobiology, 41: 10-16.

Leung, L. 1987. Cryopreservation of spermatozoa of the Barrumundi, *Lates calcarifer* (Teleostei: Centropomidae). Aquaculture, 64: 243-247.

Longo, F. 1987. Fertilization. Chapman & Hall, London, 183 pp.

Mazur, P. 1984. Freezing of living cells: mechanisms and implications. Am. J. Physiol., 247 (Cell Physiol. 16): C125-C142.

McWilliams, R., W. Gibbons & S. Leibo. 1995. Osmotic and physiological responses of mouse zygotes and human oocytes to mono- and di-saccharides. Hum. Reprod., 10(5): 1163-1171.

Mediavilla, M., R. Álvarez & P. Herráez. 1995. Estudio de la viabilidad espermática en semen congelado de trucha común (*Salmo trutta*). Proceedings V National Congress on Aquaculture, pp. 392-397.

Morisawa, M. & K. Suzuki. 1980. Osmolality and potassium ion: their roles in initiation of sperm motility in teleosts. Science, 210(5): 1145-1146.

Ohta, H., K. Ikeda & T. Izawa. 1997. Increases of concentrations of potassium and bicarbonate ions promote acquisition of motility *in vitro* by Japanese eel spermatozoa. J. Exp. Zool., 277: 171-180.

Patiño, R. 1997. Manipulation of the reproductive system of fishes by means of exogenous chemicals. Prog. Fish Cult., 59: 118-128.

Piironen, J. 1993. Cryopreservation of sperm from brown trout (*Salmo trutta m. lacustris* L.) and Arctic charr (*Salvelinus alpinus* L.). Aquaculture, 116: 275-285.

Rana, K. 1995. Preservation of gametes. In: N.R Bromage & R.J. Roberts (eds). Broodstock management and egg and larval quality. Blackwell, Oxford, pp. 53-75.

Richardson, G., C. Wilson, L. Crim & Z. Yao. 1999. Cryopreservation of yellowtail flounder (*Pleuronectes ferrugineus*) semen in large straws. Aquaculture, 174: 89-94.

Ritar, A. 1999. Artificial insemination with cryopreserved semen from striped trumpeter (*Latris lineata*). Aquaculture, 180: 177-187.

Sztein, J., K. Nobel, J. Farley & L. Mobraaten. 2001. Comparison of permeating and nonpermeating cryoprotectants for mouse sperm cryopreservation. Cryobiology, 41: 28-39.

Usuki, H., H. Masami & I. Hiroko. 1997. Long-term cryopreservation of Pacific oyster, *Crassostrea gigas*, sperm. Bull. Nansei Natl. Fish. Res. Inst. Nanseisuikenho, 30: 115-123.

Wheeler, P. & G. Thorgaard. 1991. Cryopreservation of rainbow trout semen in large straws. Aquaculture, 93: 95-100.

Zohar, Y. & C. Mylonas. 2001. Endocrine manipulation of spawning in culture fish: from hormones to genes. Aquaculture, 197: 99-136.