FAS http://e-fas.org
Fisheries and Aquatic Sciences

Note
Fish Aquat Sci 18(4), 417-420, 2015

Potential Association between Insulin-like Growth Factor-1 Receptor Activity and Surf Clam *Spisula sachalinensis* Larvae Survival

Youn Hee Choi and Taek Jeong Nam*

Institute of Fisheries Sciences, Pukyong National University, Busan 46041, Korea

Abstract

We investigated the relationship between viability and IGF-1 receptor (IGF-1R) activity in D-shaped and umbo larvae of the surf clam *Spisula sachalinensis* after treatment with vitrification solution (VS) or freezing. In a toxicity assay, VS1, containing 5 M dimethyl sulfoxide (DMSO), was very harmful to D-shaped and umbo larvae. However, VS2, containing 5 M ethylene glycol (EG), was not harmful to either larval stage. Although VS2 had a promising toxicity test outcome, none of the larvae survived vitrification. After immersion into VSs and freezing, IGF-1R β-subunits were detected in all larvae; however, tyrosine phosphorylation of intracellular β-subunits was detected only in the control and live groups. These results suggest that activation of IGF-1R may influence surf clam larvae viability.

Key words: *Spisula sachalinensis*, Surf clam, D-shaped larvae, Umbo larvae, Vitrification, IGF-1R

Introduction

A number of attempts to develop a cryopreservation process in mammals and aquatic organisms have been made. Although vitrification is appealing because of its simplicity, it faces the challenges of intra/extracellular ice formation and the toxicity of high concentrations of cryoprotective agents. Recently, methods for vitrification of fish embryos, including zebrafish (*Brachydanio rerio*) (Zhang and Rawson, 1996), sea perch (*Lateolabrax japonicas*) (Tian et al., 2003), and flounder (*Paralichthys olivaceus*) (Chen and Tian, 2005), were established. However, despite several studies, neither vitrification nor traditional cryopreservation techniques have been successful in shellfish due to problems with the survival and growth of frozen-thawed embryos/larvae (Gwo, 1995; Chao et al., 1997; Paredes et al., 2013).

With regard to the survival of embryos/larvae, we investigated the relationship between insulin-like growth factor (IGF) and the viability of vitrified surf clam larvae. The IGF system plays an important role in controlling development and growth in vertebrates (Duan, 1997). The IGF-1 receptor (IGF-1R) has a heterotetrameric structure with a tyrosine kinase domain in the cytoplasmic portion of the β-subunit (Czech, 1989). Each receptor binds to its respective ligand, resulting in autophosphorylation and activation of multiple signal transduction cascades (Codina et al., 2008). Through these mechanisms, IGF regulates cell survival, proliferation, and protein synthesis.

In the current study, we investigated the toxicity of vitrification solutions (VSs) (modified formulations based on Robles et al., 2004) in D-shaped and umbo larvae of the surf clam, *Spisula sachalinensis*, using immunoblot analysis to assess the potential relationship between larval viability and IGF-1R activity after VS treatment or freezing.

Received 21 September 2015; **Revised** 27 October 2015
Accepted 8 November 2015

***Corresponding Author**
E-mail: namtj@pknu.ac.kr

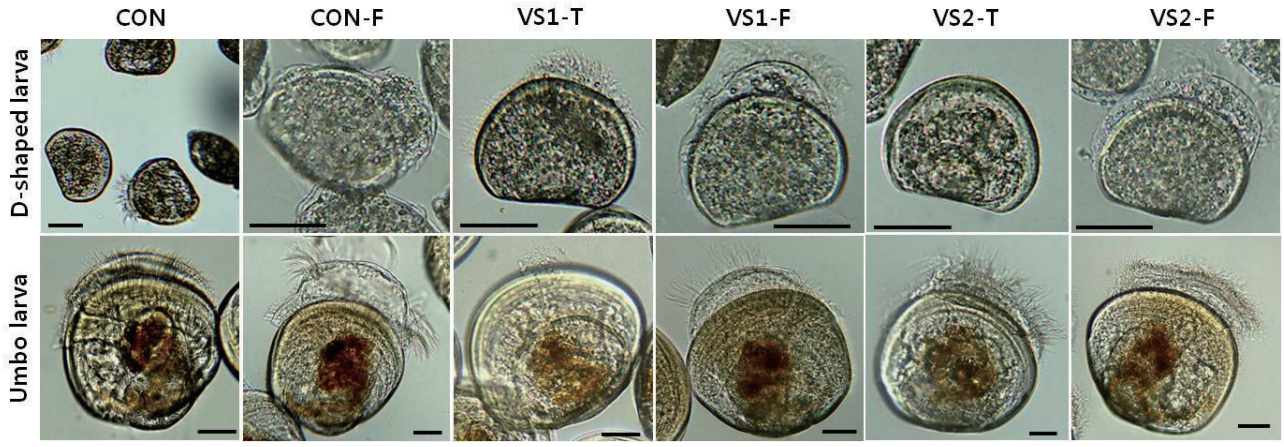

Fig. 1. Surf clam *Spisula sachalinensis* D-shaped and umbo larvae after treatment with vitrification solutions (VSs) and after thawing following liquid nitrogen (LN_2) vitrification. CON, without VS treatment or vitrification; CON-F, directly immersed in LN_2 without VS; VS1-T, larvae treated with VS1; VS1-F, vitrified larvae with VS1; VS2-T, larvae treated with VS2; VS2-F, vitrified larvae with VS2. Bar = 50 μm.

Materials and Methods

Preparation of larvae

D-shaped and umbo larvae were obtained 3 and 14 days after fertilization, respectively, from Gangwon Province Deep Ocean Water & Fishery Resource Center, Gangwon-do, Korea, in May and June, 2014. The larvae were transported to the laboratory for VS toxicity and vitrification analyses. All pre-frozen and freeze-thawed larvae were fed a mixture of *Isochrysis galbana*, *Chaetoceros calcitrans*, and *Pavlova lutheri*. The shell length (SL) and shell height (SH) of the larvae were calculated as 102.4 ± 3.9 μm and 80.8 ± 3.8 μm, respectively, in D-shaped larvae, and 213.1 ± 7.5 μm and 200.2 ± 8.8 μm, respectively, in umbo larvae using image analysis software (Motic Images Plus, China Group Co., Ltd., Xiamen, China).

VS toxicity and vitrification

First, the toxicity of two VS formulations on the larvae was evaluated: VS1 (5 M DMSO, 2 M EG, 1 M methanol, and 0.2 M sucrose) and VS2 (5 M EG, 2 M DMSO, 1 M methanol, and 0.2 M sucrose). All chemicals were reagent-grade and the VSs were prepared with 0.22 μm-filtered (Millipore, Express™ Plus, Billerica, MA, USA) seawater. The larvae were immersed in VS for 5 min at room temperature (RT; 20°C) and washed with fresh seawater. To assess the effect on larval vitrification, the larvae were loaded into 0.5 mL straws (FHK, Hokkaido, Japan) after immersion in VS at RT, and the straws were plunged directly into liquid nitrogen (LN_2). After 1 h of storage in LN_2, the straws were transferred to a 37°C water bath for several seconds. After thawing, the larvae were rinsed several times with seawater to remove the VS. The survival rates of larvae were determined by counting the number of larvae with a heartbeat and activated cilia using light microscopy (BX-41; Olympus, Tokyo, Japan).

Immunoblot analysis

The larvae were homogenized in RIPA lysis buffer (50 mM Tris, 1 mM EGTA, 150 mM NaCl, 1% NP-40, and 0.25% Na-deoxycholate) containing protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM Na_3VO_4, 1 mM NaF, and 1 mM PMSF). The samples were centrifuged at 12,000 rpm for 10 min and the supernatants collected. Total protein concentration was measured using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Proteins (35-50 μg) were separated by 8-10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to polyvinyl difluoride membranes (Millipore). Membranes were blocked with Tris-buffered saline containing Tween 20 (TBS-T) (10 mM Tris–HCl, 150 mM NaCl, pH 7.5, and 0.1% Tween 20) with 1% bovine serum albumin (BSA) and incubated with the indicated primary antibodies (1:1,000 in TBS-T) on a rocker overnight at 4°C. The primary antibodies used for immunoblotting included monoclonal mouse p-Tyr and polyclonal rabbit anti-IGF-1R β-subunit, purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After washing in TBS-T, the membranes were incubated for 1 h at RT with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) or HRP-conjugated goat anti-rabbit IgG (Sigma-Aldrich) diluted 1:10,000 in TBS-T. Monoclonal mouse anti-β-actin (C4) antibody (Santa Cruz Biotechnology) was used as a control. Membranes were developed using enhanced chemiluminescence detection reagents (Advansta, Menlo Park, CA, USA) and visualized using the GeneSys imaging system (SynGene Synoptics, Ltd., London, UK).

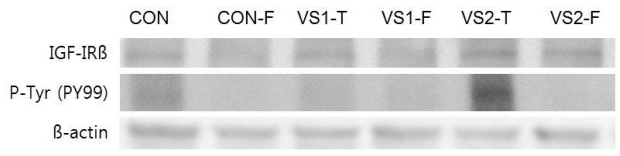

Fig. 2. Activation of IGF-1R β-subunits by tyrosine phosphorylation (PY99) after VS treatment and vitrification of surf clam *Spisula sachalinensis* umbo larvae. Cell lysates were resolved using SDS-PAGE and analyzed using Western blotting.

Statistical analysis

The survival rates of the larvae in each experiment are expressed as the mean ± standard deviation (SD) (n = 50). Significant differences among the means of the survival rates for each factor were evaluated using an analysis of variance (ANOVA) test. Differences with a probability value (P) of less than 0.05 were considered significant.

Results and Discussion

VS toxicity and vitrification

VS1, which contained 5 M DMSO, adversely affected D-shaped and umbo larvae after 5 min of immersion. None of the umbo larvae survived and only 2.8 ± 0.4% ($P < 0.05$) of D-shaped larvae survived. However, VS2, which contained 5 M EG, was not harmful to either larval stage, showing survival rates of 92.9 ± 2.5% and 100% for D-shaped and umbo larvae, respectively ($P < 0.05$). In this study, DMSO was more toxic than EG. However, the toxicity of a cryoprotective agent is species-specific and depends on its chemical properties (Robles et al., 2008). Although VS2 was associated with a high larval survival rate in our toxicity test, none of the vitrified larvae, including larvae frozen without VS (CON-F), survived. The larvae exhibited somewhat damaged intracellular inclusions, but cilia and shells remained intact (Fig. 1). Vitrification has been used mainly in mammals, owing to several advantages: it does not require expensive equipment such as a programmable freezer, it is simple and requires only seconds for freezing, and it can be used to preserve samples in the field (Cuevas-Uribe et al., 2011; Choi et al., 2013). Recently, attention has turned to vitrification for the preservation of fish sperm and embryos (Hagedorn and Kleinhans, 2011). However, successful vitrification methods have yet to be developed for many organisms because of the toxicity of VSs at high concentrations.

IGF-1R and tyrosine phosphorylation

IGFs play a role in cell proliferation, differentiation, migration, survival, metabolism, and contractility at the cellular level. They exert their biological actions by interacting with specific receptors localized to the cell membrane (Duan, 1997; Duan and Xu, 2005). IGF-1R has two α- and two β-subunits, with a tyrosine kinase domain in the cytoplasmic portion of the β-subunit (Czech, 1989). In this study, the IGF-1R β-subunit was detected in all umbo larvae after immersion into VSs and vitrification (Fig. 2). However, tyrosine phosphorylation of receptor β-subunits was associated with larval survival. The live umbo larvae treated without freezing and those surviving the VS toxicity test showed phosphorylation of the IGF-1R β-subunits (Fig. 2; CON and VS2-T). A similar result was observed in the D-shaped larvae (data not shown). Therefore, IGF-1R existed in the larvae, but was not activated in the larvae that did not survive. These results suggest that activation of IGF-1R may play a role in the survival of surf clam larvae. Generally, IGF-1 activation is regulated by triggering the kinase domain within the β-subunits, leading to receptor autophosphorylation and tyrosine phosphorylation of various substrates (Adams et al., 2000). Further studies regarding the association between the IGF-1 signaling pathway and cell survival after tyrosine phosphorylation are necessary.

Acknowledgments

The authors thank Gangwon Province Deep Ocean Water & Fishery Resource Center, Gangwon-do, Korea, for providing access to surf clam larvae. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2012R1A6A1028677).

References

Adams TE, Epa VC, Garrett TPJ and Ward CW. 2000. Structure and function of the type 1 insulin-like growth factor receptor. Cell Mol Life Sci 57, 1050-1093.

Chao NH, Lin TT, Chen YJ, Hsu HW and Liao IC. 1997. Cryopreservation of late embryos and early larvae in the oyster and hard clam. Aquaculture 155, 31-44.

Chen SL and Tian YS. 2005. Cryopreservation of flounder (*Paralichthys olivaceus*) embryos by vitrification. Theriogenology 63, 1207-1219.,

Choi YH, Nam TJ and Kuwano K. 2013. Cryopreservation of gametophytic thalli of *Porphyra yezoensis* (Rhodophyceae) by one-step fast cooling. J Appl Phycol 25, 531-535.

Codina M, de la serrana DG, Sanchez-Gurmaches J, Montserrat N, Chistyakova O, Navarro I and Gutierrez J. 2008. Metabolic and mitogenic effects of IGF-II in rainbow trout (*Oncorhynchus mykiss*) myocytes in culture and the role of IGF-II in the PI3K/Akt and MAPK signalling pathways. Gen Comp Endocrinol 157, 116-124.

Cuevas-Uribe R, Leibo SP, Daly J and Tiersch TR. 2011. Production of channel catfish with sperm cryopreserved by rapid non-equilibrium

cooling. Cryobiology 63, 186-197.

Czech MP. 1989. Signal transmission by the insulin-like growth factors. Cell 59, 235-238.

Duan C. 1997. The insulin-like growth factor system and its biological actions in fish. Ame Zool 37, 491-503.

Duan C and Xu Q. 2005. Roles of insuli-like growth factor (IGF) binding proteins in regulating IGF actions. Gen Comp Endocrinol 142, 44-52.

Gwo JC 1995. Cryopreservation of oyster (*Crassostrea gigas*) embryos. Theriogenology 43, 1163-1174.

Hagedorn M and Kleinhans FW. 2011. Problems and prospects in cryopreservation of fish embryos, in: T.R. Tiersch, C.C. Green (Eds.), Cryopreservation in Aquatic Species, second ed., World Aquaculture Society, Baton Rouge, Louisiana, USA, 483-502.

Paredes E, Bellas J and Adams S. 2013. Comparative cryopreservation study of trochophore larvae from two species of bivalves: Pacific oyster (*Crassostrea gigas*) and blue mussel (*Mytilus galloprovincialis*). Cryobiology 67, 274-279.

Robles V, Cabrita E, de Paz P, Cuñado, Anel L and Herráez MP. 2004. Effect of a vitrification protocol on the lactate dehydrogenase and glucose-6-phosphate dehydrogenase activities and the hatching rates of zebrafish (*Danio rerio*) and turbot (*Scophthalmus maximus*) embryos. Theriogenology 61, 1367-1379.

Robles V, Cabrita E, Acker JP and Herráez P. 2008. Embryo cryopreservation: what we know until now. CRC Press, Taylor & Francis Group, London.

Tian YS, Chen SL, Yan AS, Ji XS and Yu GC. 2003. Studies on vitrification method of sea perch (*Lateolabrax japonicus*) embryos. Acta Zool Sinica 49, 843-850.

Zhang T and Rawson DM. 1996. Feasibility studies on vitrification of intact zebrafish (*Brachydanio rerio*). Cryobiology 33, 1-13.