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Effect of Fetal Bovine Serum on the Sperm Quality of Depik (*Rasbora tawarensis*) after Short-term Cryopreservation

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ABSTRACT

Background: Sperm cells are susceptible to oxidative stress during cryopreservation. Therefore, an antioxidant is necessary to protect them from damages. Fetal bovine serum (FBS) is one of potent antioxidants for fish sperm cryopreservation. Hence, the aims of this study are to examine the effect of FBS on sperm quality after a short period and to determine its optimum concentration on depik (*Rasbora tawarensis*).

Methods: Depik fish were obtained from the Fish hatchery of Lukup Badak, Aceh Tengah District, Indonesia. Sperms collected from the fish were diluted in Ringer extenders containing FBS concentration of 10% (P1), 20% (P2), 30% (P3), 40% (P4), 50% (P5) and 60% (P6), filled into 2 ml cryotubes and equilibrated prior immersed into liquid nitrogen for 15 days. The parameters observed were sperm motility, consistency, pH, fertilization and hatching rates and DNA fragmentation post-thawing.

Result: The ANOVA test indicates that the application of FBS in Ringer had a significant effect on sperm motility, fertilization and hatching rates ($P<0.05$). The highest motility (58.33%) was recorded at FBS 60% and significantly different from those at other concentrations. The laddering analysis showed that applying FBS protected the integrity of depik sperms DNA. It is concluded that the optimum concentration of FBS on depik sperm led to a short-term cryopreservation of 60%.

Key words: Cryopreservation, Depik fish, DNA integrity, Fetal bovine serum, Hatching rate.

INTRODUCTION

Depik (*Rasbora tawarensis*) is one of the endemic and predominant fish with high economic value in Lake Laut Tawar, Central Aceh District, Indonesia (Muchlisin and Azizah, 2009). Presently, its population has been decreasing due to overexploitation, unfriendly fishing practices and ecological perturbation (Muchlisin, 2011; Muchlisin *et al*., 2011). The International Union for Conservation of Nature (IUCN) categorizes depik as a threatened fish species (CSBG, 2003; Lumbantobing, 2019). Therefore, it is crucial to save its population by prohibiting uncontrolled fishing while encouraging the Government of Aceh Tengah to develop aquaculture programs. The main obstacles faced by the program are the difficulty in locating high-quality broodstock throughout the year due to unsynchronous gonad maturation between male and female species during the rainy season in April, September and December (Muchlisin *et al*., 2010; Muchlisin *et al*., 2011b). Therefore, introducing aquaculture reproductive technology through cryopreservation program could provide high-quality germ cells as an alternative solution.

According to Eriani *et al*. (2008), sperm cryopreservation is a significant effort used to save the population of vulnerable and endangered animals by providing sustainable male germ cells. The success of male germ cells, however, strongly depends on the quality of sperm (Eriani *et al*., 2008), therefore, the best time to gather it is during spawning season (Moczarski and Koldras, 1982; Fauvel *et al*., 1999; 2000; Momin and Memis, 2018). Seminal plasma compositions is very important for high grade spermatozoa quality (Abishag *et al*., 2020) and the information about semen characteristics is helpful in selecting good quality semen for artificial fertilization thus could contribute to fishery development (Charak *et al*., 2020). Generally, sperm quality decreases after cryopreservation owing to temperature shock and extenders toxicity (Fauvel *et al*., 1999; Kasimanickam *et al*., 2007; Muchlisin and Azizah, 2009b;

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Bansal and Bilaspri, 2010). Cryoprotectant and antioxidant are the choices to overcome these potential problems. Since antioxidant can maintain the motility, viability, acrosomal integrity and plasma membrane of frozen-thawed sperms (Eriani *et al*., 2018), the better results are expected to be achieved by using it for the preservation of the depik sperm. Fetal bovine serum (FBS) is one of the potential antioxidants for fish sperm,this serum is extracted from a bovine fetuses blood (Maurer, 1986; Brunner *et al*., 2010).

The successful application of FBS in semen cryopreservation has been reported in several animals such as cattle (Reyes-Moreno, 2000), rabbit (Sarıözkan *et al*., 2013) and alpacas (Bravo and Valdivia, 2018). It was also applied the sperm of Sakhalin taimen fish (Kusuda *et al*., 2005), European eel (Garzon *et al*., 2008) and grouper (Yusoff *et al*., 2018). However, the limitation of these previous studies is that FBS was used at higher concentrations reaching 85-90%. FBS is expensive, rarely available in the local market, costly at higher concentration and not practical. In this study, the effect of FBS at low concentration on depik sperms is examined to determine its optimum value in the Ringer extender of depik spermatozoa.

MATERIALS AND METHODS

Location and time

The research was conducted in June-August 2019 at Fish Hatchery of Lukup Badak, Aceh Tengah District, Indonesia.

Experimental design

The completely randomized design (CRD) consisting of six levels of treatments (FBS concentration of 10%, 20%, 30%, 40%, 50% and 60%) and three replications was used in this study.

Brood fish and sperm collection

Sixty male broodfish length ranged from 75.98-113.31 mm and body weight ranged from 2.98-7.32 g) were randomly sampled using a *dedeuseun* trap. The fish were kept in the plastic bag filled with oxygen then transported to the Lukup Badak Hatchery at Aceh Tengah District. The broodfish was acclimatized in a pond for 7 days before sperm collection. A total of 20 mature males fish were taken randomly from the pond, then the genital pore was wiped by the tissue to avoid contamination, after which its abdomen was gently pressured. The sperms were collected by a syringe and pooled in the beaker glass kept in an icebox (4°C), after which it was assessed for initial quality using macroscopic and microscopic evaluations. Only sperms with motility higher than 60% were used for cryopreservation.

Extender preparation

The ringer solution was mixed with six different concentrations of FBS (10%, 20%, 30%, 40%, 50% and 60%) in a total reaction volume of 10 ml. Depik sperms were diluted in the solutions at a ratio of 1:20 (Muchlisin *et al*., 2009a).

Cryopreservation process

Diluted sperms were filled in separate cryotubes (vol. 2 ml) and kept at 4°C for 10 minutes for equilibration. It was evaporated at a distance of 5 cm above the surface of liquid nitrogen (-60°C) for 5 minutes and immersed into the liquid nitrogen (-196°C) for 15 days.

Semen evaluation

Semen quality was measured twice. The first was on the fresh sperm including color, pH, consistency, mass movement, motility and concentration. The second was on the post-thawed semen including motility, fertility, hatching rate and DNA integrity.

Evaluation of sperm motility, fertility and hatching rates

Sperm motility was microscopically evaluated in accordance to (Muchlisin and Azizah, 2009b). Fresh or cryopreserved semen was diluted in equal volume of tap water and observed using a stereo microscope (AmScope 40X-2000-3WLED Trinocular) at 400x magnifications. The motility rate was calculated by randomly assessing at least 50 spermatozoa. The fertility and hatching rates were calculated by dividing the total numbers of fertilized and hatched eggs with total numbers of incubated sperm, respectively (Muchlisin *et al.* 2015; Mutmainnah *et al.* 2018).

DNA fragmentation analysis

Quality of spermatozoa DNA was determined by the laddering method (Zilli *et al*., 2003). Genomic DNA was extracted from both fresh and frozen semen using the Genomic DNA Purification Kit (Promega) and subjected to electrophoresis in 1.5% agarose gel at 135 V for 30 minutes. The DNA fragmentation and laddering were characterized by using a Uvi-Doc Machine (Yusoff *et al*., 2018).

Data analysis

The data of motility, fertility and hatching rates were analyzed by one-way ANOVA and followed Duncan's multiple range tests using SPPS version 20 forsa Windows. The data of sperm viability, pH and DNA fragmentation were analyzed descriptively.

RESULTS AND DISCUSSION

Evaluation of fresh sperm quality indicates that depik sperm was condensed and had a white-milky color. The pH ranged from 7.4-8.0, spermatozoa density was 23.58×10^9 cells ml^{-1}, the initial motility and viability were 71.83% and 75.00%, respectively. Sperm abnormality was 11.00% (Table 1).

ANOVA test showed that FBS gave a significant effect on the sperm motility and fertility as well as egg hatching rate of the depik ($P<0.05$). The highest motility was found in 60% FBS (58.33% motility) and significantly different from those in other concentrations. The highest fertility and hatching rate were also recorded at 60% FBS, but the values were not significantly different with those at 50% FBS (69% vs 66.33% and 47% vs 45.67%, respectively) (Table 2).

The DNA electrophoresis results showed the presence of smears in both fresh and post-thawed sperm indicates the occurrence of DNA fragmentation. However, the application of 40% FBS showed thick and long smears, thereby, indicating a high degree of DNA fragmentation whereas the bright and short smears detected at 50% and 60% FBS applications showed low degree DNA fragmentation (Fig 1).

The results showed that the addition of FBS had a positive effect on the sperms motility and fertility and egg hatching rate of depik fish. This is presumably that FBS can protect sperm during freezing due to its protein, hemoglobin, glucose, insulin, cortisol, parathyroid hormone and prostaglandin E compositions (Hayman *et al*., 1985; Chen *et al*.,1992; Garzon *et al*., 2008).

According to Reyes-Morino *et al.* (2000), protein and glucose play an essential role in protecting sperms from excessive damage during cooling, freezing and thawing. The protecting effect of glucose presence in extender for better semen quality after freezing and thawing has been proved by better post-thawing and post-freezing motilty of Turkey sperms diluted in glucose 5% containing dimethyl sulphoxide over those diluted in other extenders (tris-glucose, lactated Ringer and lactated Ringer's glucose) (Kuzlu and Taskin, 2016).

In general, serum has multifunctional effects on sperms due to its high macromolecules and antioxidants contents (Alcay *et al*., 2019). It can act as an extracellular cryoprotectant for frozen spermatozoa by protecting the plasma membrane from crystallization, recrystallization or ice melting during different phases of freezing and thawing processes (Watson,1995). Besides, serum can induce capacitation (Xia and Ren, 2009) and acrosomal reactions (Hossain *et al*., 2007). This similar positive effect was reported by Kusuda *et al*. (2005) in Sakhalin taimen fish and by Yusoff *et al*. (2018) in groupers. However, these authors used a higher concentration of FBS (90% and 85%, respectively). Meanwhile, 50-60% was found optimal for short preservation of depik sperms without suppressing the results.

The study showed that there was a correlation between sperms motility and sperm fertility and egg hatching rate of depik as fertility and hatching rates increase proportionally to sperm motility rate. Similar results were reported by Ohta *et al*. (1995) in post-thawed sperm of masou salmon and turbot fish (Dreanno *et al*., 1998), indicating motility is an important indicator of sperm quality. Contrarily, Mutmainnah *et al*. (2018) reported no correlation between motility and fertility in cryopreserved sperms of seurukan fish when using glutathione as an antioxidant. The unclear relationship was also found in the sperms of brook and rainbow trout fish (Lahnsteiner *et al*., 2011), African catfish (Muchlisin *et al*., 2015) and muskellunge (Ciereszko *et al*., 1999). According to Ohta *et al*. (1995), several factors might reduce fertility including low post-thawing motility and egg stimulation. The ability of inactive sperms to fertilize egg is assumably related to hormones contained in it (Gilkey, 1981). The teleosts egg produces pheromones for example to attract sperm (Stehr and Hawkes, 1983). Gimnogamon hormone produced by egg can activate and draw spermatozoa (Yanagimachi *et al*., 2017). In addition, glycoprotein contained in the herring egg can activate spermatozoa (Griffin *et al*., 1996; Cherr *et al*., 2008).

The analysis of sperm DNA fragmentation after cryopreservation revealed the presence of smears in both fresh (column 2 in Fig 1) and preserved sperm (column 3, 4 and 5 in Fig 1 for 40%, 50% and 60% FBS, respectively). These results were in agreement with Zilli *et al*. (2003) who found DNA damage in both fresh and frozen sperms of sea bass. The long smear of DNA bands after electophoresis show fragmentation whereas clear DNA bands without smears indicate purity or integrity (Karp *et al*., 2012).

The results of this study showed the addition of concentrated FBS (line 3 and 4) could maintain DNA integrity of frozen depik sperms which is similar to that of fresh sperm (line 1). This might be responsible for the high fertilization and hatching rates of the sperm. The similar findings were reported by Yusoff *et al*. (2018) when analyzing DNA of groupers sperm cryopreserved in a combination of polyethylene glycol 15% and FBS 85% that a slight DNA smear did not affect fertilization and hatching rates.

Table 1: Quality of fresh semen of depik fish (*Rasbora tawarensis*).

Parameters	Result
Color	Milky white
pH	8
Consistency	Condensed
Mass movement	+++
Motility (%)	71.83%±0.18
Concentration (cells/ml)	23.58×10^{9}
Abnormality (%)	11 00±1.00
Live spermatozoa (%)	75.00±1.75

Table 2: Average post-thawed motility and fertility of frozen depik semen and the resulted egg hatching rate.

FBS concentration	Sperm motility (%)	Sperm fertility (%)	Egg hatching rate (%)
Fresh sperm	71.33 ± 2.08^{d}	65.25 ± 4.20^{c}	59.61 ± 12.91^{e}
10%	26.00 ± 1.73^{a}	33.00 ± 2.65^{a}	19.67 ± 1.52^{a}
20%	30.00 ± 3.60^{a}	35.00 ± 4.36^{ab}	22.67 ± 1.52^{ab}
30%	31.67 ± 2.51^{a}	37.33 ± 2.52^{ab}	25.33 ± 1.52^{c}
40%	40.67 ± 1.15^{b}	43.67 ± 4.04^{b}	30.33 ± 3.05^{cb}
50%	46.67 ± 3.05^{b}	66.33 ± 3.21^{c}	45.67 ± 0.57^{d}
60%	58.33 ± 2.88^{c}	69.00 ± 5.29^{c}	47.00 ± 2.00^{d}

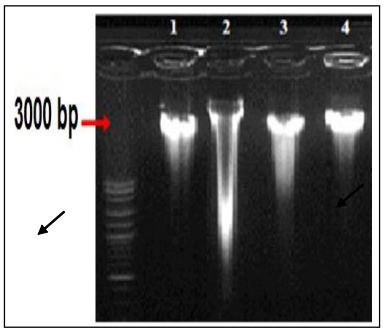

Fig 1: The DNA electrophoresis results of the depik (*R. tawarensis*) sperm. Line 1: Fresh semen, Line 2: Semen treated with FBS 40%, Line 3: Semen treated with FBS 50%, Line 4: Semen treated with FBS 60%.

CONCLUSION

FBS gave a positive effect on the sperm quality of depik (*Rasbora tawarensis*) as shown by increased sperm motility, fertility and hatching rates as FBS concentration intensifies, with the optimum concentration at 60%.

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REFERENCES

Abishag, M.M., Betsy, J.C., Kumar, S.S.J. (2020). Comparative study on spermatological parameters and seminal plasma compositions of *Lobeo rohita* strains from Tamil Nadu, India. Indian Journal of Animal Research. 54: 1229-1234.

Alcay, S., Toker, M.B., Gökçe, E., Önder, N.T., Üstüner, B., Nur, Z. (2019). Long term incubation resilience of post-thaw ram semen diluted with lecithin-based extender supplemented with bovine serum albumin. Kafkas Universitesi Veteriner Fakultesi Dergisi. 25: 291-297.

Bansal, A.K. and Bilaspuri, G., (2010). Impacts of oxidative stress and antioxidants on semen functions. Veterinary Medicine International. 7: 686137. DOI: 10.4061/2011/686137.

Bravo, Z. and Valdivia, M. (2018). Effect of foetal bovine serum on sperm motility, acrosome reaction and spermatic interaction to zona pellucida in alpacas (*Vicugna pacos*). Reproduction in Domestic Animals. 53: 695-699.

Brunner, D., Frank, J., Appl, H., Schöffl, H., Pfaller, W., Gstraunthaler, G. (2010). The serum-free media interactive online database. ALTEX-Alternatives to Animal Experimentation. 27: 53-62.

CBSG, (2003). Management Plan for Sumatran Threatened Species. Apple Valley, MN USA: IUCN-SSC Conservation Breeding Specialist Group. 5-9.

Charak, R.S., Chaudhary, K.D., Agarwal, K.N. (2020). A study on the physical and biochemical characteristics of semen of semen of *Tor putitora* - An endangered fish species in Himalayan water. Indian Journal of Animal Research. 55: 744-750.

Chen, L., Mao, S., Larsen, W.J., (1992). Identification of a factor in fetal bovine serum that stabilizes the cumulus extracellular matrix. A role for a member of the inter-alpha-trypsin inhibitor family. Journal of Biological Chemistry. 267: 12380-12386.

Cherr, G.N., Morisawa, M., Vines, C.A., Yoshida, Y., Smith, E.H., Matsubara, T., Pillai, M., Griffin, F.J., Yanagimachi, R. (2008). Two egg-derived molecules in sperm motility initiation and fertilization in the Pacific herring (*Clupea pallasi*). International Journal of Development Biology. 52: 743-752.

Ciereszko, A., Dabrowski, K., Lin, F., Christ, S., Toth, G. (1999). Effects of extenders and time of storage before freezing on motility and fertilization of cryopreserved Muskellunge spermatozoa. Transactions of the American Fisheries Society. 128: 542-548.

Dreanno, C., Suquet, M., Desbruyeres, E., Cosson, J., Le Delliou, H., Billard, R. (1998). Effect of urine on semen quality in turbot (*Psetta maxima*). Aquaculture. 169: 247-262.

Eriani, K., Boediono, A., Djuwita, I. Sumarsono, S.H., Azhar, A. (2008). Development of domestic cat embryo produced by preserved sperms. HAYATI Journal of Biosciences. 15: 155-160.

Eriani, K., Azhar, A., Ihdina, M., Rosadi, B., Rizal, M., Boediono, A. (2018). Quality enhancement of aceh swamp buffalo (*Bubalus bubalis*) frozen semen by supplementing β-carotene. Tropical Animal Science Journal. 41(Suppl. 1): 1-7.

Fauvel, C., Savoye, O., Dreanno, C., Cosson, J., Suquet, M. (1999). Characteristics of sperm of captive seabass in relation to its fertilization potential. Journal of Fish Biology. 54: 356-369.

Garzón, D., Peñaranda, D., Pérez, L., Marco Jiménez, F., Espert, X., Müller, T., Jover, M., Asturiano, J. (2008). Effects of pH, sodium bicarbonate, cryoprotectants and foetal bovine serum on the cryopreservation of European eel sperm. Reproduction in Domestic Animals. 43: 99-105.

Gilkey, J.C. (1981). Mechanisms of fertilization in fishes. American Zoologist. 21: 359-375.

Griffin, F.J., Vines, C.A., Pillai, M.C., Yanagimachi, R., Cherr, G.N. (1996). Sperm motility initiation factor is a minor component of the Pacific herring egg chorion. Development, Growth and Differentiation. 38: 193-202.

Hayman, E.G., Pierschbacher, M.D., Suzuki, S., Ruoslahti, E. (1985). Vitronectin - A major cell attachment-promoting protein in fetal bovine serum. Experimental Cell Research. 160: 245-258.

Hossain, M.S., Hyeong, L.J., Miah, A.G., Tsujii, H. (2007). Effect of fatty acids bound to bovine serum albumin V on acrosome reaction and utilization of glucose in boar spermatozoa. Reproductive Medicine and Biology. 6: 109-115.

Karp, A., Ingram, D.S., Isaac, P.G. (2012). Molecular Tools for Screening Biodiversity: Plants and Animals. Springer Science and Business Media, New York.

Kasimanickam, R., Kasimanickam, V., Pelzer, K.D., Dascanio, J.J. (2007). Effect of breed and sperm concentration on the changes in structural, functional and motility parameters of ram-lamb spermatozoa during storage at 4°C. Animal Reproduction Science. 101: 60-73.

Kusuda, S., Koide, N., Kawamula, H., Teranishi, T., Nakajima, J.-I., Yamaha, E., Arai, K., Ohta, H. (2005). Cryopreservation diluents for spermatozoa of Sakhalin taimen Hucho perryi. Fish Science. 71: 293-298.

Kuzlu, M. and Taskin, A. (2016). The effect of differentd externders on the sperm motiity and viability fof frozen Turkeu semen. Indian Journal of Animal Research. 51: 235-241.

Lahnsteiner, F., Mansour, N., Kunz, F.A. (2011). The effect of antioxidants on the quality of cryopreserved semen in two salmonid fish, the brook trout (*Salvelinus fontinalis*) and the rainbow trout (*Oncorhynchus mykiss*). Theriogenology. 76: 882-890.

Lumbantobing, D. (2019). *Rasbora tawarensis*. The IUCN Redlist of Threatened Species 2019: e.T19316A2204120.

Maurer, H. (1986). Towards chemically-defined, serum-free media for mammalian cell culture. Animal Cell Culture. 13-31.

Moczarski, M. and Koldras, M. (1982). Properties of tench (*Tinca tinca* L.) sperm and experiments with freezing it at -196 degrees celsius. Acta Ichthyologica et Piscatoria. 12: 41-49.

Momin, M. and Memiş, D. (2018). Sperm quality analysis of normal season (NG) and out-season by photoperiod manipulation (PG) of male rainbow trout broodstock (*Oncorhynchus mykiss*). Fish Physiology and Biochemistry. 44: 1551-1560.

Muchlisin, Z. (2011). Analisis kebijakan introduksi spesies ikan asing di perairan umum daratan Provinsi Aceh. Jurnal Kebijakan Sosial Ekonomi Kelautan Perikanan. 1: 79-89.

Muchlisin, Z. and Azizah, M.S. (2009a). Diversity and distribution of freshwater fishes in Aceh waters, northern Sumatra Indonesia. International Journal Zoological Research. 5: 62-79.

Muchlisin, Z. and Azizah, M.S. (2009b). Influence of cryoprotectants on abnormality and motility of baung (*Mystus nemurus*) spermatozoa after long-term cryopreservation. Cryobiology. 58: 166-169.

Muchlisin, Z., Musman, M., Azizah, M.S. (2010). Spawning seasons of *Rasbora tawarensis* (Pisces: Cyprinidae) in Lake Laut Tawar, Aceh Province, Indonesia. Reproduction Biology Endocrinology. 8: 1-8.

Muchlisin, Z., Nadiah, W. and Siti Azizah, M.N., (2015). Exploration of natural cryoprotectants for cryopreservation of African catfish, *Clarias gariepinus*, Burchell 1822 (Pisces: Clariidae) spermatozoa. Chezch Journal of Animal Sciences. 60: 10-15.

Muchlisin, Z.A., Fadli, N., Rudi, E., Mendo, T. and Siti-Azizah, M., (2011a). Estimation of production trend of the depik, *Rasbora tawarensis* (Teleostei, Cyprinidae), in Lake Laut Tawar, Indonesia. AACL Bioflux. 4: 590-597.

Muchlisin, Z.A., Musman, M., Fadli, N., Siti-Azizah, M.N. (2011b). Fecundity and spawning frequency of *Rasbora tawarensis* (Pisces: Cyprinidae) an endemic species from Lake Laut Tawar, Aceh, Indonesia. AACL Bioflux. 4: 273-279.

Muthmainnah, C., Eriani, K., Hasri, I., Irham, M., Batubara, A., Muchlisin, Z. (2018). Effect of glutathione on sperm quality after short-term cryopreservation in seurukan fish *Osteochilus vittatus* (Cyprinidae). Theriogenology. 122: 30-34.

Ohta, H., Shimma, H., Hirose, K. (1995). Relationship between fertility and motility of cryopreserved spermatozoa of the amago salmon Oncorhynchus masou ishikawae. Fish Science. 61: 886-887.

Reyes Moreno, C., Gagnon, A., Sullivan, R., Sirard, M.A. (2000). Addition of specific metabolites to bovine epididymal cell culture medium enhances survival and motility of cryopreserved sperm. Journal of Andrology. 21: 876-886.

Sarıözkan, S., Türk, G., Cantürk, F., Yay, A., Eken, A., Akçay, A. (2013). The effect of bovine serum albumin and fetal calf serum on sperm quality, DNA fragmentation and lipid peroxidation of the liquid stored rabbit semen. Cryobiology. 67: 1-6.

Stehr, C.M. and Hawkes, J.W. (1983). The development of the hexagonally structured egg envelope of the C O sole (*Pleuronichthys coenosus*). Journal of Morphology. 178: 267-284.

Watson, P. (1995). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reproduction Fertility Development. 7: 871-891.

Xia, J. and Ren, D. (2009). The BSA-induced Ca (2+) influx during sperm capacitation is CATSPER channel-dependent. Reproduction Biology Endocrinology. 7: 1-9.

Yanagimachi, R., Harumi, T., Matsubara, H., Yan, W., Yuan, S., Hirohashi, N., Iida, T., Yamaha, E., Arai, K., Matsubara, T. (2017). Chemical and physical guidance of fish spermatozoa into the egg through the micropyle. Biology of Reproduction. 96: 780-799.

Yusoff, M., Hassan, B.N., Ikhwanuddin, M., Sheriff, S.M., Hashim, F., Mustafa, S., Koh, I.C.C. (2018). Successful sperm cryopreservation of the brown-marbled grouper, *Epinephelus fuscoguttatus* using propylene glycol as cryoprotectant. Cryobiology. 81: 168-173.

Zilli, L., Schiavone, R., Zonno, V., Storelli, C., Vilella, S. (2003). Evaluation of DNA damage in *Dicentrarchus labrax* sperm following cryopreservation. Cryobiology. 47: 227-235.