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A REVIEW ON FACTORS INFLUENCING SUCCESS OF GROUPER SPERM CRYOPRESERVATION

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Abstract: This review was conducted to support information gathering on development of sperm cryopreservation techniques and protocols including factors such as type of cryoprotectants, extenders, cooling rates and dilution ratio and the impacts of those factors towards sperm cryopreservation of 10 different grouper species. Groupers are high-value marine species that contribute significantly to the income of Southeast Asian countries which are important as aquaculture producers. Thus, sperm cryopreservation is essential to support the development of grouper seed production and culture. Giant grouper is the most studied grouper species due to the fast-growing ability which enables to produce high-value interspecies hybrids for commercialization purposes. The most used cryoprotectant for sperm cryopreservation among various grouper species is dimethyl sulphoxide (DMSO). Among the extenders, sodium chloride (NaCl) is reported to be the most commonly used due to easy preparation, relative effectiveness and availability of the solution which enables local farmers to easily extend storage of the grouper sperm. Most grouper species seem to have a wide range of optimal cooling rates. However, some species exhibit the narrow range of optimal cooling rates. Optimal cooling rates were also possibly affected by the type and concentration of cryoprotectant. Dilution ratios used to cryopreserve grouper sperm seemed to vary. Sperm of brown-marbled grouper and seven band grouper to report dilution ratios of 1:49 while other grouper species sperm worked better at dilution ratios of 1:1, 1:2, 1:4 and 1:9. Grouper sperm cryopreservation protocol in general is species-specific and the optimization has to be done species by species to increase overall productivity. Grouper sperm cryopreservation is applicable and can be used to enhance seed production.

Keywords*: Cooling rates, cryoprotectant, extender, groupers, sperm cryopreservation*

Introduction

Grouper species play a significant role as a high-value commodity in the international markets of various countries including Hong Kong and China (Lee & Sadovy,1998; Sadovy *et al.,* 2003). Sadovy *et al,* 2003 also mentioned that countries such as Indonesia, Malaysia, Philippines and Australia are the primary exporters of reef fishes, particularly grouper fishes. However, grouper seed production is declining as to the grouper seeds are rarely available in the wild due to their unpredictable hermaphroditic life cycle (Yashiro, 2008), large size, high maintenance cost spent on broodstocks and unsynchronized spermiation of groupers (Kiriyakit *et al.,* 2011a). Grouper species are hermaphrodite, whereby the fish primarily matures first as females and then eventually changes sex to males. Bhandari *et al.* (2003) reported that factors which activate sex conversion from female to functional male remain unknown. Therefore, the development of sperm cryopreservation is necessary to assist with the production of hybrid groupers, avoid space consumption of both male and female broodstocks, reduce the cost of male grouper broodstock management and maintain constant production of other commercial groupers.

Cryopreservation is a technique that is used to preserve the sperm or other cells by cooling the sample to the cryogenic temperatures (Kaufman, 1976). Currently, cryopreserved sperm is regularly used in various interspecies hybrid productions to explore a wide variety of high offspring culture characteristics (Kiriyakit

et al., 2011b). This review was conducted to evaluate the factors affecting grouper sperm cryopreservation and to identify the impacts of those factors towards the aquaculture industry. In addition, this study provides recommendations to improve the overall cryopreservation success rate by controlling the factors that affects grouper sperm cryopreservation.

Materials and Method

Data collection

This review mainly targets the factors affecting the success of sperm cryopreservation protocols of various groupers. Manuscripts, journals, articles and research papers regarding sperm cryopreservation of groupers were gathered through online sources. The Sub-family Epinephelinae, particularly fishes from three specific genera - *Ephinephelus*, *Mycteroperca* and *Cromileptes* - were primarily targeted during data collection.

Gathered data were primarily related to the factors affecting grouper sperm cryopreservation such as species, cryoprotectant, extender, cooling rate and extender, sperm cryopreservation success rate analytic parameters such as post-thaw motility rate, fertilization rate, hatching rate and sperm-to-egg ratio, the author of relevant manuscripts, year of publication and other supporting elements from variety of journals. The gathered data was tabulated and sorted according to the species in an alphabetical order.

Results and Discussion

Species

A variety of different reasons and protocols was observed in all past studies on grouper sperm cryopreservation. The first record of sperm cryopreservation of grouper was that of *E. malabaricus* which was developed by Gwo *et al.* (1993) due to the limited number of Malabar grouper males in Taiwan (Gwo *et al.,* 1993). Since then, protocols for more than 10 species of grouper sperm cryopreservation has been developed for many other Epinephelus species. The most common reason for the studies was to support the dwindling capture of groupers in the wild by acquiring germplasm resources from the cryopreservation technique and also the increase in demand of hybrid groupers (Che-Zulkifli et al., 2020). Hence, cryopreservation technique was practised in most of grouper cultured-places to support the sustainable grouper production by preserving the germplasms.

The *E. lanceolatus* is the most studied grouper species for sperm cryopreservation from 2014 to 2019. The giant grouper is popular in the aquaculture industry due to the fast growing ability of the respective fish (Fan et al., 2014). Apart from that, moderate sized giant groupers are considered favourite food fish among Chinese community in countries like China, Taiwan, Hong Kong and Malaysia (Vatanakul *et al*., 1999). However, recently, the focus on *E. lanceolatus* is primarily due to use of its sperm for producing various fast-growing combinations of hybrids, especially with the rise in popularity of the *E. lanceolatus*♂♀ × *E. fuscoguttatus*♀ hybrid (Che-Zulkifli et al., 2020).

Cryoprotectant

Cryoprotectants are used to avoid damage of cells during freezing and thawing. Cryoprotectants are often toxic to cells (Best, 2015; Bhattacharya & Prajapati, 2016; Bhattacharya, 2018). Hence, the choice of cryoprotectant which balances between protection and toxicity is crucial (Tiersch *et al.,* 2000). Both permeating and non-permeating cryoprotectants were used in studies for grouper sperm cryopreservation. Permeating cryoprotectants function by entering into the cell and lessening the freezing point of the solution, minimizing the osmotic shock by removing the cell's water content and eliminating intracellular ice formation (Leung & Jamieson, 1991). Non-permeating cryoprotectants on the other hand such as sugars and polymers are unable to enter the cell and help to stabilize the membrane during cryopreservation (Meryman, 1971).

Dimethyl sulphoxide (DMSO) proved to be the most commonly used permeating cryoprotectant; it was used for 6 different grouper species at various concentrations which are 5% DMSO for *C. altivelis,* 10% DMSO for *E. akaara,* 12% and 15% DMSO for *E. lanceolatus,* 20% DMSO for *E. malabaricus,* 10% DMSO for *E. marginatus* and 5% and 10% DMSO for *E. septemfasciatus.* DMSO is a commonly used cryoprotectant at concentrations of 5%–25% for various marine species apart from groupers (Yusoff et al., 2018). DMSO treatment had 85% fertilization rate and 70% hatching rate of sea perch, *Lateolabrax japonicas* (Ji et al., 2004) likewise, sperm of red snapper, *Lutjanus argentimaculatus,* cryopreserved with 10% DMSO had higher post-thaw motility compared to other cryoprotectant treatment including DMA, glycerol and formamide (Vuthiphandchai et al., 2009). Fabbrocini et al., (2000) mentioned that DMSO offers superior protection to the cell prior to vitrification. However, DMSO was reported toxic at higher concentration in several studies (Sean In *et al.,* 2009).

Trehalose is a non-permeating cryoprotectant that is reported to be used for grouper sperm cryopreservation. Trehalose worked well in combination with NaCl as an extender and showed maximum sperm post-thaw motility of 100% for sperm cryopreservation of *E. coioides* (Peatpisut & Bart, 2010). Trehalose was also successfully used for sperm cryopreservation of *E. moara* without the presence of any extender due to the its ability of the trehalose solution to maintain the sperm osmolality (Miyaki *et al.,* (2005). However, the trehalose solution is much more expensive than DMSO. Besides DMSO and trehalose, propylene glycol, glycerol and soybean milk were also reported to be successful as cryoprotectants for grouper sperm cryopreservation. Soybean milk in particular, was introduced as a new potential cryoprotectant for *E. lanceolatus* sperm which was tested in Indonesia.

Extender

An extender is beneficial by diluting sperm and supplying a higher volume of diluted sperm for artificial breeding purposes (Muchlisin, 2004b). In cryopreservation, the extender also serves to reduce the toxicity of the cryoprotectant while maintaining osmolality of the solution. Nine different solutions which are sodium chloride (NaCl), fetal bovine serum (FBS), glucose, TS19, MPRS, Marine Fish Ringer, LG-ASP2, ELRS3 and ES1-3 were used as extenders for grouper sperm cryopreservation. All those extenders can be classified into 4 groups such as sodium chloride-based extenders (NaCl), sugar-based extenders (glucose), artificial seminal plasma-based extenders (TS19, MPRS, Marine Fish Ringer, LG-ASP2, ELRS3 and ES1-3) and bovine plasma-based extender (FBS). The NaCl solution was most commonly used extender due to the easy preparation, relative effectiveness and availability of the solution. It was used to cryopreserve sperm from 4 types of groupers, with 0.9% NaCl for *C. altivelis*, 150mM for both *E. coioides and E. malabaricus* and 1% NaCl for *E. marginatus* and yielded post thaw motility rate with the range of 36.80 ± 10.20% to 100%.

Cooling rate

The cooling rate is primarily influenced by cryoprotectant type and concentration, species, cell type, equilibration time and final temperature before plunging in liquid nitrogen and associated interactions (Viveiros *et al.,* 2000; Hu *et al.,* 2011). Similarly, Muchlisin, (2005) also agreed that optimal cooling rates often rely on the nature and concentration of the cryoprotectant that is used. Optimal cooling rate should be rapid enough to minimize the duration of exposure to prevent the occurrence of concentrated solute and slow enough to allow water osmosis to prevent intracellular ice crystal formation (Watson, 2000). Cooling rates for grouper sperm cryopreservation varied between species, with 2 groups being observed, one with low or moderate cooling rates such as with 10°C/min for *C. altivelis*, 5°C/min for *E. akaara*, 17.6 °C /min for *E. fuscoguttatus*,

20°C for *E. malabaricus*, 54.2 ± 0.9 °C /min for *E. septemfasciatus,* and high cooling rates of 154°C/min for *E. malabaricus*, 180°C /min for *E. lanceolatus* and 243°C ± 64°C/min for *E. moara*. There was an absence of optimal cooling rate that is applicable for all grouper species.

The differing cooling rates used was due to the different methods used for the cooling and freezing processes. High cooling rates were recorded on *E. moara* sperm cryopreservation at -243°C ± 64°C/min because a one-step freezing method was used for the experiment. The one-step freezing method is a method of plunging cryo-straws/cryovials or other containers containing sperm samples directly into liquid nitrogen at -196°C. Thus, the sperm samples experience high cooling rate in a short time period. The two-step cooling method for freezing protocol was used for the low, moderate cooling rates, with straws being chilled atop liquid nitrogen vapour before immersion into liquid nitrogen itself. Hence, the cooling rate obtained is relatively lower. The decision to use either a one-step or two-step cooling method is usually dependent on the type of cryoprotectant and concentration used. If a high concentration of toxic type cryoprotectant is used, slow methods (two-step cooling method) cannot be used as the sperm will die due to the cryoprotectant toxicity. Hence, a high cooling rate is recommended for the one-step cooling method, so the sperm samples quickly freeze before the toxicity effect takes place. However, the one-step cooling method is not necessarily suitable for all species is not suitable for all species, as some may not be so tolerant to high concentrations of cryoprotectants. Therefore, most studies tend to use and most prefers two-step cooling method.

Some grouper species seem to tolerate a wide range of cooling rates, while others expressed a narrow range of optimal cooling rate. Both *E. septemfasciatus* and *E. malabaricus* had a wide range of effective cooling rates (Gwo *et al.,* 1993, Koh *et al.,* 2013), while sperm cryopreservation of *E. lanceolatus* by Yoshiki et al., (2014) reported that only high cooling rate of 180°C/min was successful. Hence, optimal cooling rate is referred to be species-specific for grouper sperm cryopreservation. However, this is also partly due to the difference in type and concentration of cryoprotectants that were used. When a higher concentration of toxic cryoprotectant is used, higher cooling rates are required as sperm may not withstand the exposure to toxic conditions, and faster cooling rates reduces the exposure time of the sperm to the cryoprotectant.

Dilution ratio

Generally, the dilution ratios used for groupers were low and ranges from 1:1-1:9. Only 2 studies observed high dilution ratios which were 1:49 from Koh *et al.* (2010) and Yusoff *et al.* (2018) which was probably due to the low volume of sperm obtained. Asturiano et al., (2004) argued that lower dilution ratios have better cryopreservation effects for fish sperm, while Imaizumi et al., (2005) stressed that dilution ratios as high as 1:50 – 1:200 have better cryopreservation effects. Lahnsteiner, (2000) mentioned low dilution ratios caused the spermatozoa to become compressed and damaged during cryopreservation. However, Lahnsteiner *et al.,* (2003) routine technique, much more detailed information is required. Therefore, the present study was conducted to investigate various fertilization techniques and media, straw volumes as well as optimal semen volume for cryopreservation. The bleak (Chalcalburnus chalcalburnus also warned that high dilution ratios reduce the sperm concentration in the fertilization medium which, in turn, negatively affects the fertilization rate. Hence, optimal dilution ratio for grouper sperm cryopreservation seemed to be variable.

Post-thaw motility, fertilization and hatching

The success of the developed sperm cryopreservation protocols were evaluated using post thaw sperm motility, fertilization and hatching. Out of the 13 studies reviewed, 11 reported on the post thaw motility, 9 on fertilization rate and only 5 on the hatchability. High post thaw motility (63.68 ± 4.16% to 100%) was observed for all studies except for *E. marginatus* (Cabrita *et al.,* 2009) which reported low motility rates (36.8 ± 10.2%). Fertilization ranged from 56% to 94.6% while hatching rate ranged from 40.1 ± 0.4% to 76.83±18.31%.

Evaluation of success using sperm motility is easily done and straightforward and rapid, which is why it is almost always used in sperm studies. High sperm motility is also usually directly correlated to high fertilization and high hatching rates. Due to the difficulty of fertilization and hatching test involving artificial maturation of females groupers, the fertilization test and hatching trials were not done in all grouper sperm cryopreservation study. In those studies, only the post-thaw motility was assessed. The studies by Fan *et al.,* (2014) for *E. lanceolatus*, Gwo *et al.,* (1993) for *E. malabaricus*, Cabrita *et al.,* (2009) for *E. marginatus*, Miyaki *et al.,* (2005) for *E. moara* showed no records of hatching rate even though the fertilization assessments were done. We assume that the reason behind this is that the assessment of the hatching ability of cryopreserved sperm is not the part of objective for respective studies.

The variable results may also be due to the differences in sperm-to-egg ratio that were used during the fertilization and hatching trials in each study. Results of most studies showed that the sperm-to-egg ratio has a proportional relationship towards fertilization rate as high sperm-to-egg ratio results in high fertilization rate. Therefore, even the protocols which reported low post thaw motility might be able to produce high fertilization and hatching rates if higher sperm amount is used. This suggests that optimizations for the fertilization protocols might still be required until a high and promising hatching rate is recorded. From this review, we can summarise that all the grouper sperm cryopreservation protocols are viable and effective and have the potential for usage in commercialization purposes.

Table 1: The protocol of cryopreservation of grouper semen studied by past researchers

Species	Author	Cryoprotectant	Extender	Cooling Rate	Post-thaw motility rate, %	Fertilization rate, %	Hatching rate, %	Dilution ratio	Sperm-to-egg ratio
Cromileptes altivelis	Sean In et al., 2009	5% DMSO	0.9% NaCl	10 °C/min	80.00 ± 0.00	-	-	1:1 or 1:4 or 1: 9	-
Epinephelus akaara	Ahn et al., 2018	10% DMSO	300mM glucose	5 °C/min	85.00 ± 2.90	81.4 ± 4.30	40.10 ± 0.40	1.1	-
Epinephelus bruneus	Lim & Le, 2013	10% Glycerol	LG-ASP2	-	66.30 ± 2.00	-	-	-	-
Epinephelus coioides	Peatpisut& Bart, 2010	20% Trehalose	150 mM NaCl	-	100	82.10 ± 1.10	47.30 ± 4.80	1:1	2.8 x 10^4:1
Epinephelus fuscoguttatus	Yusoff et al., 2018	15% Propylene Glycol	85% FBS	17.6 °C /min	76.70 ± 8.80	90.90 ± 0.50	64.50 ± 4.10	1:49	3 x 10^5:1
Epinephelus lanceolatus	Fan et al., 2014	12% DMSO	MPRS	-	90.09 ± 3.40	92.27 ± 2.43	-	1:1 or 1:2	8.6–9.2 x 10^5: 1
			TS-19	-	90.85 ± 3.80				
	Yoshiki et al., 2014	15% DMSO + 10% FBS	ELRS3	180 °C /min	63.68 ± 4.16 to 74.75 ± 12.71	94.56	75.56	1:1	200:1 (v/v)
	Afni et al., 2019	15 % of Soybean Milk	Marine Fish Ringer	-	81.17 ± 1.92	-	-	-	-
Epinephelus malabaricus	Gwo et al., 1993	20% DMSO	150 mM NaCl	20°C to -154°C/min	-	56.00	-	-	-
Epinephelus marginatus	Cabrita et al., 2009	10% DMSO	1% NaCl	-	36.80 ± 10.20	69.50 ±17.70	-	1:9	3.7×10^3:1
Epinephelus moara	Miyaki et al., 2005	15% Trehalose		243°C ± 64°C/min	-	94.60	-	1:4	-
Epinephelus septemfasciatus	Koh et al., 2010	5% DMSO	95% FBS	54.20 ± 0.90 °C /min	77.60 ± 8.50	-	-	1:49	-
	Tian et al., 2013	10% DMSO	ES1-3	-	76.67 ± 0.00	68.08 ± 22.46	76.83±18.31	1:1	1x10^4:1
		10% Propylene Glycol		-	75.00 ± 5.00				

Conclusion

In conclusion, this review on factors influencing the success of grouper sperm cryopreservation highlights the factors affecting grouper sperm cryopreservation and the methods for identifying the success of the protocol. The factors affecting grouper sperm cryopreservation that have been identified include species, cryoprotectant, extender, cooling rate, dilution ratio and sperm-to-egg ratio. This study revealed that species, cryoprotectant, extender and cooling rate are the primary factors that affects grouper sperm cryopreservation. Post-thaw motility seems to be a simple and reliable method to evaluate cryopreservation success, while fertilization and hatching trial will be needed for the optimization of the fertilization protocols using cryopreserved sperm. In short, the review also serves to support additional references regarding the mechanism, protocol, advantages, and recommendation on grouper sperm cryopreservation to Malaysian local grouper breeders to add considerable value to the marine finfish aquaculture industry. However, proper understanding of grouper sperm cryobiology is essential in order to maximise the output.

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