Approaches for Improving Viability of Blue Catfish, *Ictalurus furcatus*, Stem Cells and Embryo Production of Channel Catfish, *I. punctatus*, Female × Blue Catfish Male Hybrids through Xenogenesis

by

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Abstract

The hybrid channel catfish, *Ictalurus punctatus*, female × blue catfish, *Ictalurus furcatus*, male possesses superior characteristics such as faster growth rate, higher survival rate, better disease resistance and low oxygen tolerance, and higher carcass and fillet yield is the best catfish for pond culture in the United States, accounting for approximately 75% of all catfish cultured. Artificial fertilization is the current technology for commercial production of the hybrid catfish embryos, but this method has the disadvantages of intensive labor, time, and sacrifice of valuable blue catfish males. Xenogenesis has been studied as an appropriate alternative for producing the hybrids by mating normal channel catfish female with xenogenic channel catfish male that produces blue catfish sperm, but this technology also needs improved efficiency. One-hundred % of the channel catfish fry and white catfish, *Ameiurus catus*, embryos that had been pressurized at 7,000 psi 5 min after fertilization were triploid while 85.7 and 82.9% of white catfish and channel catfish, respectively, were triploid when pressurized at 6,500 psi. To achieve 100% triploidy, hatch rate was about 20% of control diploid eggs. The triploid channel catfish in this experiment were transplanted with 4,000 type A spermatogonial stem cells (SSCs) and had a 0-16.7% xenogenesis rate. The triploid white catfish were injected with 9,000-10,000 SSCs, and had 3-4X higher xenogenesis rate. In the white catfish experiment, the donor cells were also treated with Rho protein kinase inhibitor (ROCK I). Injection of 8-14 primordial germ cells per

fry resulted in almost zero transplantation, and injection of blastulae resulted in almost zero hatch rate.

Experiments were conducted to determine survival rates of spermatogonial stem cells (SSCs) or oogonial stem cells (OSCs) cultured *in vitro* for 6 days including SSCs/OSCs dissociated from gonad tissues without enzymatic digestion (unsorted cells without enzymatic digestion), SSCS/OSCs dissociated with trypsin-EDTA 0.25% (unsorted cells with trypsin), and SSCs/OSCs dissociated with trypsin-EDTA 0.25% and enriched with discontinuous gradient density Percoll (sorted cells) and supplemented with ROCK I at 0, 10, and 50 µM. Methods to dissociate the SSCs/OSCs did not affect survival rates of the SSCs/OSCs during the 6-day culture period. Application of ROCK I at 10 or 50 µM yielded higher viability of SSCs compared to suspension without using ROCK I. Similarly, the survival rates were highest for the OSCs incubated with ROCK I 50 µM. The SSCs or OSCs had the highest viability in the first day right after being dissociated from the gonads.

Polymerase chain reaction (PCR) results from progeny of putative xenogenic channel catfish indicated that only 1 channel catfish male pressurized at 7,000 psi and transplanted with 4,000 SSCs was xenogenic. The fertilization and hatch rates of eggs fertilized by this xenogenic male were 7.3% and 12.3%, respectively. Although the results from the spawning of this xenogenic catfish were low, this is the first report on the success of producing 100% hybrids by mating a xenogenic channel catfish male with a normal channel catfish female via aquaria spawning. Future research should address introduction of larger numbers of donor cells, fine tuning of the best time to inject these cells and examining ROCK I to increase cell viability and colonization rate.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

SSCs	Spermatogonial stem cells type A
OSCs	Oogonial stem cells
PGCs	Primordial germ cells
ESC	Embryonic stem cells
EDTA	Ethylenediamine Tetraacetic Acid
MS- 222	Tricaine Methanesulfonate (MS-222)
HBSS	Hanks' Balanced Salt solution
L-15	Leibovitz-15 medium
GFP	Green Fluorescence Protein
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
PSI	Pound-force per square inch

CHAPTER ONE

Introduction

Catfish Industry in the United States

Production of channel catfish, *Ictalurus punctatus*, and the channel catfish female × blue catfish, *I. furcatus*, male hybrid has played an important role in the aquaculture industry in the United States. From the first commercial catfish farming established in Kansas in the 1930s and Alabama, Arkansas and Mississippi in the late 1960s, the farm-raised catfish production rapidly developed and become the leading aquaculture industry. The catfish production reached the high mark in 2002 when nearly 80,000 hectares were used to raise catfish. In 2003, around 300 million kg of catfish were processed (Hanson, 2006; Hanson and Sites, 2015). However, the amount of processed catfish and the total hectares for catfish production in 2014 declined by more than 50% and 65%, respectively, compared to the peak in the period 2002-2003. There were several factors contributing to the decrease in the US catfish industry, including increased fuel, feed, labor costs, the recession, competition of imported products from Asia, and control of fish diseases (FAO, 2011; Hanson and Sites, 2015). Recently, annual commercial production of catfish sales in the US reached $386 million dollars in 2016, which created 8,000 jobs and generated labor income of $211 million to the US economy. Alabama, Arkansas, Mississippi, and Texas were the top catfish-producing states, accounting for around 96% of the total

commercial catfish sales in 2016 (Posadas, 2017). In 2018, water surface area used for catfish production in three main producing states (Alabama, Arkansas, and Mississippi) reached 22.7 thousand hectares, slightly increasing to 4% compared to the catfish area from a year ago (NASS, 2018).

Hybrid catfish production

Among 42 interspecific hybrid catfish combinations from the seven major North American catfish species, the channel catfish female × blue catfish male hybrids are the most commercially valuable because of their superior performances (Dunham and Masser, 2012) and are the only hybrid with commercial potential. The hybrid catfish has showed overdominant traits compared to channel catfish such as 20-100% faster growth (Argue et al., 2003; Brown et al., 2011; Dunham et al., 1990; Dunham and Brummet,1999), 15-20% better feed conversion at high densities (Li et al., 2004; Yant et al., 1975), 50-100% better tolerance of low oxygen (Dunham and Smitherman, 1984), increased resistance to many bacterial diseases (Arias et al., 2012; Wolters et al., 1996), tolerance of crowded growth conditions in ponds, uniformity in size and shape (Dunham and Masser, 2012), higher dress-out percentage (Bosworth, 2012; Yant et al., 1975), increased harvestability by seining (Dunham and Argue, 1998), and increased vulnerability to angling (Dunham et al., 1986; Tave et al., 1981).

Although the potential of the channel catfish female × blue catfish male hybrid has been known for 53 years (Giudice, 1966), it was not applied on a large scale commericially for 39 years because of reproductive isolating mechanisms between the parent species. Hybridization between channel catfish females and blue catfish males is low, which limits the amount of hybrid fry produced (Goudie, et al., 1993; Kristanto et al., 2009; Silverstein et al., 1999). Spawning of

the hybrids in pens is also limited, ranging from 0 to 20% (Tave and Smitherman, 1982; Tieman, 1995) and averaging 15% (Dunham and Masser, 2012; Dunham et al., 2000).

From the beginning of the catfish industry until about 2012 channel catfish was the primary catfish species cultured in the aquaculture industry (Chapman, 1992; Hanson, 2006). Then, Auburn University developed an artificial spawning and fertilization technology based on carp pituitary extract injection for induced spawning (Dunham, 1993; Dunham et al., 1998; Dunham et al., 2011; Dunham et al., 2016; Lambert et al., 1999; Kim et al., 2016). This technology was adopted on a small scale by GoldKist (later purchased by HarvestSelect) in Inverness, Mississippi beginning in 1997. Hybrid embryo production ranged from 1-5 million from 1997-2004. Auburn University improved the hybrid embryo production technology with the key being the use of luteinizing hormone-releasing hormone analogue (LHRHa) as the hormone to induce ovulation (Perara, 2012; Phelps et al., 2011; Su et al., 2013). This allowed the doubling and tripling of the embryo production efficiency, and this technology was commercialized by Eagle Aquaculture, Auburn, Alabama in 2005, and then spread to several major catfish hatcheries. Embryo production immediately rose to 15.2 million in 2005 and was 275 million in 2018 (Fig. 1). It is now estimated that 65-75% of catfish production in the US comes from the culture of hybrids (Jimmy Avery, Mississippi State University, personal communication).

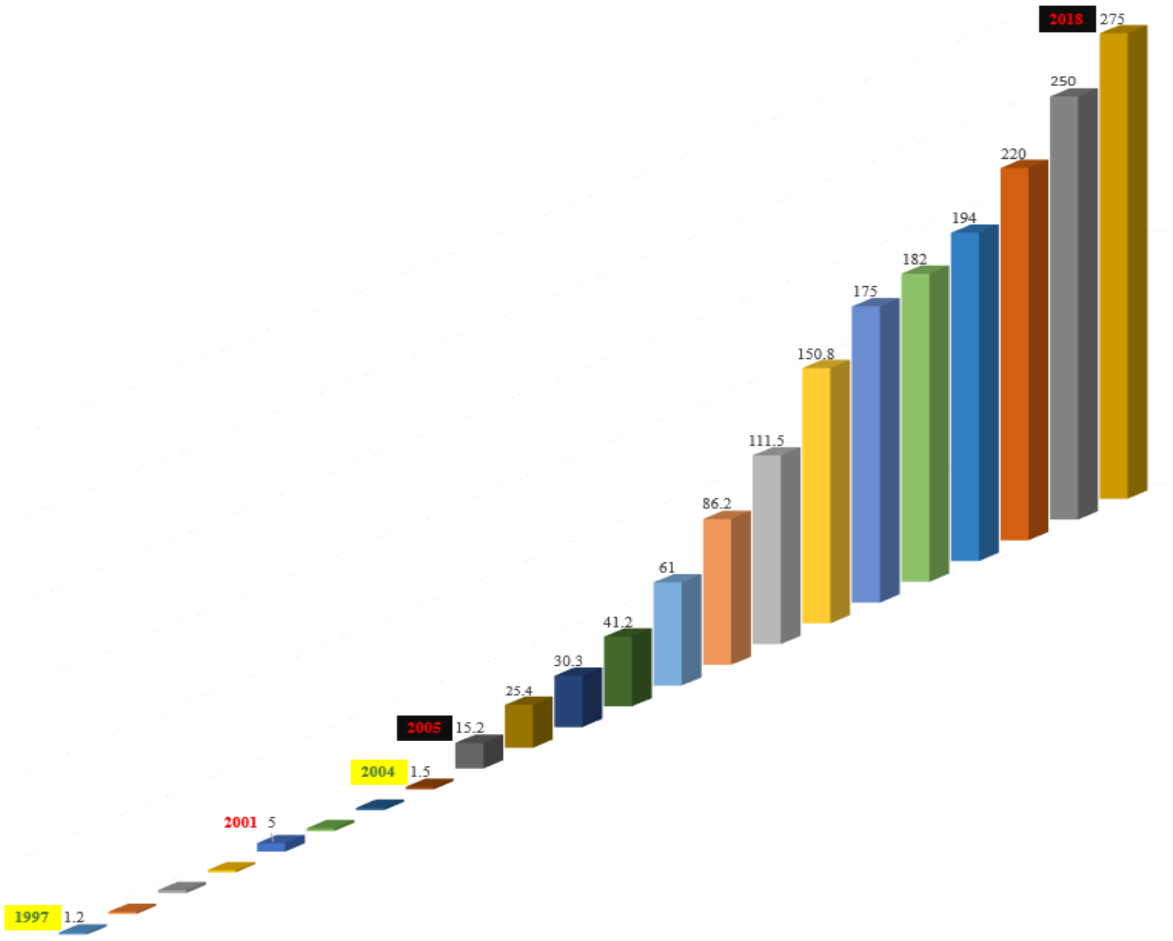

Fig. 1. Channel catfish, *Ictalurus punctatus*, female × blue catfish, *I. furcatus*, male hybrid embryos were commercially produced from 1997- 2018 in the United States. In 1997, there were 1.2 million hybrid embryos produced but the production of hybrid embryos remarkably increased 230 times more than the production in 1997, reaching 275 million embryos in 2018.

Hybrid embryo production still faces obstacles and inefficiencies. Artificial fertilization has been considered as an appropriate technology for commercial production of hybrid catfish (Dunham and Masser, 2012), but blue catfish males must be sacrificed for sperm collection to artificially fertilize channel eggs. In addition, artificial fertilization techniques are labor intensive and time-consuming. Advanced reproduction technologies such as xenogenesis have potential to simplify and make hybrid embryo production even more efficient and cost effective.

Xenogenesis, advanced technology for hybrid catfish production

Xenogenesis is a method of reproduction in which successive generations differ from each other (Dunham, 2011). This process can be accomplished by transplanting diploid germline stem cells, primordial germ cells (PGCs), type A spermatogonial stem cells (SSCs) or oogonial stem cells (OSCs), derived from donor diploid fish into sterile recipients so that the recipients have the capability of producing donor-derived gametes. The germline stem cells including PGCs, SSCs or OSCs are precursors of gametes that possess the ability to establish or recover gametogenesis (Wong et al., 2011; Wylie, 1999; Yoshizaki et al., 2010). Studies on germ cell transplantation of PGCs were reported for zebrafish, *Danio rerio* (Ciruna et al., 2002; Lin et al., 1992), medaka, *Oryzias latipes* (Shimada et al., 2008; Wakamatsu et al., 1993), rainbow trout, *Oncorhynchus mykiss* (Saito et al., 2010; Takeuchi et al., 2001), and the loach, *Misgurnus anguillicaudatus* (Nakagawa et al., 2002). Transplantation of SSCs was conducted for masu salmon, *Oncorhynchus masou* (Okutsu et al., 2006, 2007), Nile tilapia, *Oreochromis niloticus* (Lacerda et al., 2006) and channel catfish, *Ictalurus punctatus* (Perera et al., 2017). In terms of OSCs, manipulation with OSCs is important in the case of a ZW sex-determination system, where OSCs possess genetic information determination of both sexes. Procedures for transplantation of OSCs are similar to SSCs, and were successfully applied in trout (Yoshizaki et

al., 2010), zebrafish (Wong et al., 2011), and Siberian sturgeon, *Acipenser baerii* (Psenicka et al., 2015). Some of this early research was impeded and made less than efficient because of issues such a cell viability during harvest and handling. Compounds like Rho protein kinase (ROCK) inhibitor may help to solve these obstacles.

ROCK I, compound for enhancing cell viability

ROCK inhibitor is a compound that might enhance cell viability during harvest and handling, and is a general term for inhibitor of Rho protein kinase, which was discovered as a downstream target of the small guanosine -5'-triphosphate (GTP)-binding protein Rho (Leung et al., 1995; Matsui et al., 1996). ROCK I has been used to regulate a broad range of cellular responses such as cellular growth, adhesion, migration, metabolism, and apoptosis through control of assembly actin cytoskeleton and cell contractibility (Riento and Ridley, 2003). As a result, ROCK inhibitor mediates membrane blebbing, enhances actin-myosin contraction, and activates caspase signaling cascades and cellular apoptosis (Kurosawa, 2012).

ROCK I enhanced the *in vitro* survival of mouse embryonic stem cells derived neural precursors, mouse intestinal stem cells, and human keratinocytes (Chapman et al., 2010; Koyanagi et al., 2008). This compound was demonstrated to improve cell recovery of human embryonic stem cells after fluorescence-activated cell sorting (Emre et al., 2010). To apply to fluorescence-activated cell sorting system, the cells must be dissociated into single cells, therefore the cell recovery after sorting will be low due to dissociation-induced apoptosis. When ROCK I was added into the plating medium, the viability of post-sorted cells was improved. Until now, there have not been studies of using ROCK I to improve the viability of catfish stem cells.

Germ cell transplantation in white catfish

Saito et al. (2010) conducted an experiment to transplant germ cells between distantly related species, not only different genera (goldfish, *Carassius auratus*,to zebrafish) but also from different families (loach to zebrafish). For this approach, they successfully obtained germline chimeras and donor-derived gametes from all combinations. Furthermore, Saito et al. (2011; 2014) applied this technique to produce germline chimeras between more distant species from different orders (Japanese eel, *Anguilla japonica*, to zebrafish, or sturgeon, *Acipenser ruthenus*, to goldfish). They stated that donor germ cells localized at the genital ridges of the hosts, but they did not obtain donor-derived gametes from the combinations. White catfish, *Ameiurus catus*, have advantages of short maturation time and small handling size, making them potentially amenable for genetics research. According to Dunham and Smitherman (1981) and Fobes (2013), white catfish can become mature in 1-2 years compared to 2-4 and 4-6 years of age for channel catfish and blue catfish, respectively.

Objectives

A series of experiments were conducted to evaluate the success rate for germ cell transplantation by transplanting SSCs or PGCs of blue catfish into the sterile channel fry when newly hatched. ROCK I was evaluated to enhance cell viability by *in vitro* culturing of blue catfish sorted and unsorted SSCs and OSCs supplemented with ROCK I at 0, 10, and 50 µM. The last experiment was designed to produce xenogenic white catfish that can produce channel catfish gametes by transplanting unsorted and sorted channel catfish SSCs into triploid white catfish host at the newly hatched stage. Overall, the major objective of those experiments was to produce hybrid catfish by mating xenogenic channel catfish males with normal channel catfish

females or mating between xenogenic white catfish females transplanted with channel catfish stem cells with xenogenic white catfish male transplanted with blue catfish stem cells.

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CHAPTER TWO

Production of channel catfish, *Ictalurus punctatus*, female × blue catfish, *I. furcatus*, hybrids by mating xenogenic channel males with normal channel females in tanks

Abstract

Although channel catfish, *Ictalurus punctatus*, female × blue catfish, *I. furcatus*, male hybrid is one of the most important catfish cultured in the United States, production of the hybrid catfish embryo still has many challenges and needs improvements. Xenogenesis has been studied as the potential alternative for producing hybrid catfish via mating xenogenic channel males with normal channel females. In this study, primordial germ cells (PGCs) collected from blue catfish embryos or spermatogonial stem cells type A (SSCs) collected from young fingerling blue catfish were transplanted into the peritoneal cavity of the triploid channel catfish when newly hatched. The triploid fry were produced using hydrostatic pressure shock for 5 min at 6,500 psi and 7,000 psi five min after fertilization. Each triploid fry received 8-14 PGCs or 7,200 sorted cells (4,000 SSCs). After transplantation, the triploid fry were kept in indoor systems, branded and pit tagged before stocking them in ponds. Blood from transplanted 6-month-old channel catfish fingerlings was collected and analyzed with a Coulter counter for triploid confirmation. Two years later, the transplanted catfish were seined and selected for spawning in aquaria. One-hundred percent of the catfish fry that had been treated with 7,000 psi was triploid while the triploid percentage of catfish pressurized at 6,500 psi was 82.9%. The hatch rates of eggs

pressurized to induce triploidy ranged from 8.3-12.6%, which was significantly lower than the rate of control eggs, which was 43.8% ($P < 0.0001$). In terms of spawning, 61 transplanted fish including females and males were induced to spawn by mating with transplanted or normal channel catfish. The results from the spawning showed that only 19.7% of transplanted fish induced with hormones spawned at 2 years old. Polymerase chain reaction (PCR) results from progeny of those spawned fish indicated that only 1 channel male pressurized at 7,000 psi and transplanted with 4,000 SSCs was xenogenic. The fertilization and hatching rates of eggs fertilized by this xenogenic male were 7.3% and 12.3%, respectively. Although the results from the spawning of this xenogenic catfish were low, this was the first success for producing hybrid catfish by mating xenogenic channel male with normal channel female via aquaria spawning.

Keywords: Triploidy, hydrostatic pressure, xenogenesis, primordial germ cells, spermatogonial stem cells, channel catfish, *Ictalurus punctatus* × blue catfish, *I. furcatus*, male hybrids.

Introduction

Hybrids, superior catfish for aquaculture production

Channel catfish female, *Ictalurus punctatus*, × blue catfish, *I. furcatus*, male hybrid catfish possess favorable production traits compared to channel catfish, and culture of hybrid catfish is more economical than that of channel catfish (Ligeon et al., 2004). The average cost of hybrid catfish fingerling production was 15-22.5% lower than the cost of channel catfish production. Hybrid catfish exhibit superior growth compared to channel catfish as Guidice (1966) found that hybrid catfish had 41% higher body weight than channel catfish. The hybrids had more favorable growth rates than channel catfish, growing 35% more rapidly when raised under communal pond conditions (Dunham and Brummett, 1999). Dunham et al. (1990) observed that growth of hybrid catfish fry was more rapid than channel catfish when they were stocked at high density in ponds. Besides better growth, survival rates of the hybrids are also greater than channel catfish as Li et al. (2004) found that the survival rates of hybrid and channel catfish were 93.8% and 85.4%, respectively when grown in ponds. Dunham et al. (1990) also found that hybrid fry survival was greater than the survival of channel catfish when they were cultured separately in ponds (100% and 29.5%, respectively). Another advantage of hybrid catfish is that hybrids are more tolerant to diseases as compared to channel catfish. Wolters et al. (1996) reported that hybrid catfish and channel catfish had survival rates of 73.8% and 62.0%, respectively when subjected to an immersion bath with *Edwardsiella ictaluri*. Truong (2011) found that hybrid catfish were less susceptible to the parasitic infection, compared to channel catfish. Additionally, the hybrids exhibit increased tolerance to low dissolved oxygen. Dunham

et al. (1983) found that hybrids exhibited enhanced survival over channel catfish when subjected to low dissolved oxygen levels in concrete tanks, cages, and ponds. In oxygen-deprived ponds, survival rates were about 49.5% for the channel catfish and 92.5% for the hybrid catfish. In cages, about 87.5% of channel catfish and 51.0% of hybrids perished. The mortality rate in the concrete tanks was 100% for the channel catfish and 33% for the hybrids. In terms of feed conversion ratio (FCR), Chappell (1979) reported the mean FCR of the channel catfish female × blue catfish male hybrids was better than the mean FCR of its maternal and paternal species in earthen ponds. Yant et al. (1975) determined that the average FCR for hybrids and channel catfish was 1.35 and 1.56, respectively. Hybrid catfish are also known to have a better dress-out percentage than channel catfish. Argue et al. (2003) found that the channel × blue hybrid has a dress-out percentage of 61.1% while the channel catfish had a dress-out percentage of 57.5%. The hybrids have been reported to differ in their ease of seining in comparison to channel catfish. The amount of 75.0% hybrids catfish could be harvested by taking only one seine in the ponds while the same amount of channel catfish harvested from the ponds needed at least two seines (Yant et al., 1975). Chappell (1979) found that channel × blue hybrids were less difficult to seine than the channel catfish as 64.6% of the hybrid population was captured while the percentages of channel catfish captured per seine haul during the summer ranged from 18.5% to 24.3%. Susceptibility to angling is another important feature of the hybrids, which may be quite beneficial to a fee fishing enterprise. Tave et al. (1981) stated that hybrid catfish were more vulnerable to capture by angling than channel catfish when stocked together in communal ponds. In this study, the hybrids represented 29.5% of the fish in the pond by number and 37.4% in terms of weight, but they made up 57.3% of fish captured by number and 63.8% of the fish in terms of weight. Dunham et al. (1986) also found that the hybrids were more easily caught by

hook and line when compared to variety of genetic lines of channel catfish. Finally, the hybrids display growth that is more uniform than channel catfish at 7.400 fish/ha. Yant et al. (1975) reported that when fish were harvested the channel catfish were less uniform in total length than the hybrids. Three principle size classes were identified: 35.6 cm (14 inches), 38.1 cm (15 inches), and 40.6 cm (16 inches). Seventy-six percent of the channels could be categorized into three size classes as opposed to 87% of the hybrids.

Several methods for producing the hybrid catfish including open-pond spawning, pen spawning, and induced spawning with artificial fertilization have been studied and reported (Dunham and Masser, 2012; Dunham and Smitherman, 1987; Dunham et al. 2000; Tave and Smitherman, 1982). The open-pond spawning does not consistently produce hybrids because of behavioral incompatibility in the natural environment (Dunham and Smitherman, 1987). Confinement of channel females and blue males in pens without hormone injection rarely results in successful spawning. Tave and Smitherman (1982) obtained no spawns from nine channel female × blue male pairings when placed in outdoor pens. However, hybridization of channel females and blue males can happen in pen condition if the fish are induced with hormones. Tave and Smitherman (1982) reported the spawning success of channel female × blue male held in pens was 40% but success rates in later studies ranged from 0 to 20%, averaging 15% (Dunham et al. 2000; Tieman, 1995). Artificial fertilization technology by injecting hormones in channel females to stimulate ovulation had stripping eggs, and fertilizing with blue catfish sperm is considered to be efficient method for producing hybrid catfish. Phelps et al. (2007) injected the channel catfish females with luteinizing hormone-releasing hormone analogue (LHRHa) at 20 µg/kg body weight followed 12 h later with 100 µg/kg and placed them in aquaria at different temperatures (24-28 ^{o}C). Results from the study showed that 52.9-95.5% channel catfish females

ovulated after 24-64 h, and 59.1-70.0% eggs fertilized when the channel catfish females artificially fertilized with blue catfish males. Kim et al. (2016) found that channel catfish females injected with carp pituitary extract (CPE) at 2 mg/kg body weight for the first dose and 6 mg/kg for the second dose resulted in 100% of the females ovulated. A mean of 78.1% channel catfish eggs were fertilized with blue catfish sperm when the females received 2 mg/kg for the first dose and 7 mg/kg for the second dose. Dunham et al. (2016) found that 90.3% females yielded eggs when they first hand stripped and 71.5% eggs from the first stripping attempt were able to be artificially fertilized with blue catfish sperm to produce hybrids. However, the artificial fertilization method is labor intensive and time-consuming (Dunham and Masser, 2012).

Spawning of channel catfish

Channel catfish, a native species in the eastern United States, usually reach sexual maturity two to three years earlier than blue catfish (Tucker and Robinson, 1990). The channel catfish spawn in the natural environment from February to August when the water temperature ranges 21-30 ^{o}C (Lenz, 1947; Wolters, 1993). For natural spawning, containers are placed in ponds as spawning nests and checked periodically every few days for the presence of eggs (Busch, 1983; Steeby, 1987; Tucker and Robinson, 1990). Spawning rates of channel catfish brooders used the open pond method was around 30-50% (Bondari, 1983; Brauhn, 1971; Wolters, 1993).

Spawning in pens or aquariums was an alternative, but this method requires more control and effort to obtain egg masses. For the pen method, individual fish are selected and confined in pens placed next to pond bank. Females may be allowed to spawn naturally or induced with hormones (Graham, 1999). Spawning success rates of channel catfish induced with HCG in pens

was 88.0% (Tave and Smitherman, 1982) or 67.0% with Ovaprim injection (Goedie et al., 1992). Spawning in aquaria requires careful selection and use of hormones (Graham, 1999). The successful rate for spawning of channel catfish stocked as pairs or groups in rectangular 120 L fiberglass tanks ranged 22-58% (Bates and Tiersch, 1998). Dunham et al. (unpublished) routinely obtained 75-90% spawning in aquaria. Channel females were observed to have only spawn once a year while males were observed to spawn several times under hatchery condition (Legendre et al., 1996). Eggs of channel catfish were released in a glycoprotein matrix that could be dissolved in 1.5% sodium sulfite solution (Issac and Fries, 1991; Lenz, 1947). Eggs hatch 5-10 days after spawning (Wolters, 1993), depending on water temperature.

Fish germ cell transplantation

To overcome obstacles of the hybrid catfish embryo production, xenogenesis could be an alternative. Primordial germ cells (PGCs) or type A spermatogonial stem cells (SSCs) or oogonial stem cells (OSCs) are usually used for transplantation because they possess the ability to establish or recover gametogenesis through processes of proliferation, differentiation, and recombination of genetic information (Okutsu et al., 2006; Takeuchi et al., 2003; Wong et al., 2011;Yoshizaki et al, 2010). Lin et al. (1992) used needles to transplant PGCs of donor embryos into the host embryos that were at the same developmental stage, producing germline chimeras in zebrafish, *Danio rerio*. This was the first report of PGC transplantation in fish. However, the limitation of this method was that germ cells were transplanted together with somatic cells. Later, the same approach was applied in medaka, *Oryzias latipes* (Wakamatsu et al., 1993), rainbow trout, *Oncohunchus mykiss* (Takeuchi et al., 2001) and loach, *Misgurnus anguillicaudatus* (Nakagawa et al., 2002). Efficiencies of the germline chimera production were not high, ranging from 17.9% in zebrafish to 31.6% in trout. Takeuchi et al. (2003, 2004)

isolated PGCs from the genital ridge of newly hatched trout embryos and transplanted into the peritoneal cavity of the salmon embryos at the same stage as the donor. The results showed that 13.5% of females and 17.0% of male hosts produced donor-derived gametes. In 2010, Saito et al. (2010) identified PGCs of loach by injecting green fluorescent protein (GFP)-*nos*1 3'UTRmRNA into the eggs, dissociated the cells with enzyme digestion, and isolated single germ cells before transplanting the single cells into sterilized host embryos. A single stem cell was sufficient for a gonad to produce gametes. Then, this technique was applied to produce germline chimeras between species that belong to different orders, Japanese eel, *Anguilla japonica*, to zebrafish (Saito et al., 2011), sturgeon, *Acipenser* genus to goldfish, *Carrasius auratus* (Saito et al., 2014).

Okutsu et al. (2006, 2007) proved that spermatogonial stem cells were functionally similar to PGCs and could be used as the donor cell source for transplantation. They performed isogeneic spermatogonial stem cell transplantation in rainbow trout and then xenogeneic transplantation from rainbow trout to masu salmon, *Oncorhynchus masou*. The testicular cells containing SSCs were isolated from adult trout using trypsin 0.5% with some supplements, marked with GFP driven by the *vasa* gene, and sorted by flow cytometry. The SSCs were then transplanted into the peritoneal cavity of newly hatched embryos of both sexes, differentiated into spermatozoa in male recipients and functional eggs in female recipients. In addition, donor-derived spermatozoa and eggs from the recipients produce normal offspring. Also, Lacerda et al. (2006) carried out the study by sorting SSCs of tilapia, *Oreochromis niloticus*, using discontinuous gradient density Percoll and transplanted into mature fish through urogenital papilla. The results from this experiment showed the presence of PKH26 labeled germ cells cysts in the seminiferous tubules of the recipient tilapia testes. In terms of oogonia, a similar procedure

was applied to transplant the oogonial stem cells in rainbow trout (Yoshizaki et al., 2010), zebrafish (Wong et al., 2011), and Siberian sturgeon, *Acipenser baerii* (Psenicka et al., 2015). Trout and zebrafish oogonia also showed sexual plasticity after transplantation and differentiation into both male and female gametes. In catfish, Perera et al. (2017) reported that putative spermatogonia type A from blue catfish were transplanted into the gonads of sub-adult triploid channel catfish via catheterization or surgical insertion. Ten months after transplantation, nearly 90% of the recipients were confirmed as xenogenic fish. Two years after transplantation, sperm from one of those xenogenic males were able to fertilize eggs from a normal channel catfish female.

Triploid fish production and its performances

Polyploidy, a state in which individuals possess extra sets of chromosomes, has been studied in fish and shellfish. Triploidy refers to the 3N state. Triploidy can be induced by allowing normal fertilization and then force retention of the second polar body (Chrout, 1984; Lou and Purdom, 1984) by applying temperature, hydrostatic pressure, or chemical shocks after fertilization (Cassani and Caton, 1986; Chrout, 1984; Johnstone et al., 1989; Wolters et al., 1981). Hydrostatic pressure produces more consistent results, survival of treated eggs, and percent triploidy than other treatments. This technique is applied to induce triploidy in rainbow trout, grass carp, *Ctenopharygodon idella*, common carp, *Cyprinus carpio*, bighead carp *Hypophthalmichthys nobilis*, loach, *Misgurnus fossilis*, zebrafish, channel catfish, and yellowtail flounder, *Pleuronectes ferrugineus*.

One of the advantages of triploid fish is that they are sterile (Chrout et al., 1984; Wolters et al., 1981) because of lack of gonadal development (Casani and Caton, 1986). Therefore,

triploidy can be used to control natural spawning in tilapia and prevent the establishment of exotic species. Sterility in triploid has been observed in loach (Suzuki et al., 1985), common carp (Cherfas et al., 1994), and Atlantic salmon, *Salmo salar* (Refstie, 1984). Triploid channel × blue hybrid catfish had gonadsomatic index (GSI) equivalent to those of diploids, but diploids had normal gonadal development and histology while triploids had abnormal gonadal development (Lilyestrom et al., 1999). Other advantages of triploid fish can, but does not always include increased growth rate (Chrout et al., 1986), carcass yield, survival, and flesh quality (Bye and Lincoln, 1986; Dunham, 1996; Hussain et al., 1995).

Flow cytometry and the Coulter counter have been used to determine the polyploid state of fish. The flow cytometry was used to identify triploid and diploid coho salmon, *Oncorhynchus kisutch*, and chinook salmon, *Oncorhynchus tshawytscha* (Johnson an Wright, 1984), rainbow trout, *Oncorhynchus mykiss*, and brown trout, *Salmo trutta*, Amazon molly, *Poecilia formasa* (Lamatsch et al., 2000). Wattendrof (1986) used a Coulter counter to evaluate triploidy in grass carp. Generally, the Coulter counter was an effective technique and less expensive as compared to flow cytometry method.

The first objective of this experiment was to produce triploid channel catfish by applying hydrostatic pressure shock on fertilized eggs at 6,500 and 7,000 psi. Then, the PGCs from blue catfish embryos and SSCs from young blue catfish fingerlings were isolated and transplanted into the triploid channel catfish fry at the newly hatched stage to produce the xenogenic channel catfish. The overall objective was to produce channel catfish female × blue catfish male hybrids by mating the xenogenic channel catfish males with normal channel catfish females.

Materials and Methods

Production of triploid channel catfish fry

The general procedure for xenogenesis is outlined in Fig. 2. Mature channel catfish females and males were carefully selected for spawning. To induce ovulation, gravid channel females were intramuscularly implanted with luteinizing hormone-releasing hormone analogue (LHRHa) at 90 µg per kg body weight, following the procedures of Dunham et al. (2000); Hutson (2006); Kristanto et al. (2009); and Lambert et al. (1999). Then, the implanted channel females were placed in spawning bags submerged in flow-through spawning tanks. Channel males with excellent secondarily sexual characteristics were sacrificed for the sperm collection. When the females began to ovulate, several eggs could be seen outside the spawning bags. The ovulated channel females were selected and anesthetized in 100 mg/L tricaine methanesulfonate (MS-222, Ferndale, WA) until their opercula stopped moving. The ovulated females were hand stripped into spawning pans (25 g of eggs/pan) and these eggs fertilized with channel males sperm before adding Fullers' earth solution into the pans to activate the fertilization process. Fullers' earth solution was prepared by adding 6g of Fullers' earth powder (MP Biomedicals, Santa Ana, CA) into 1 L of pond water maintained at 27 ^{o}C. At 3 min post-fertilization, the fertilized eggs were transferred into round chamber (34 cm height, 7 cm diameter) placed on the hydrostatic pressure machine. At 5 min after fertilization, the eggs were pressure shocked for 5 min. Two levels of pressure, 6,500 and 7,000 psi were applied to induce triploidy. After completing pressure shock, the eggs were removed from the chamber and placed in the tanks supplemented with calcium chloride ($CaCl_2$) for 1 h before incubating them in flow through

hatching troughs aerated with paddle wheels. Those triploid eggs were hatched after 5-7 days at 25.5-27.4 ^{o}C. After 6 months, the triploidy of the channel catfish was verified by using a Coulter counter to measure erythrocyte nucleic volume (Beck and Biggers, 1983).

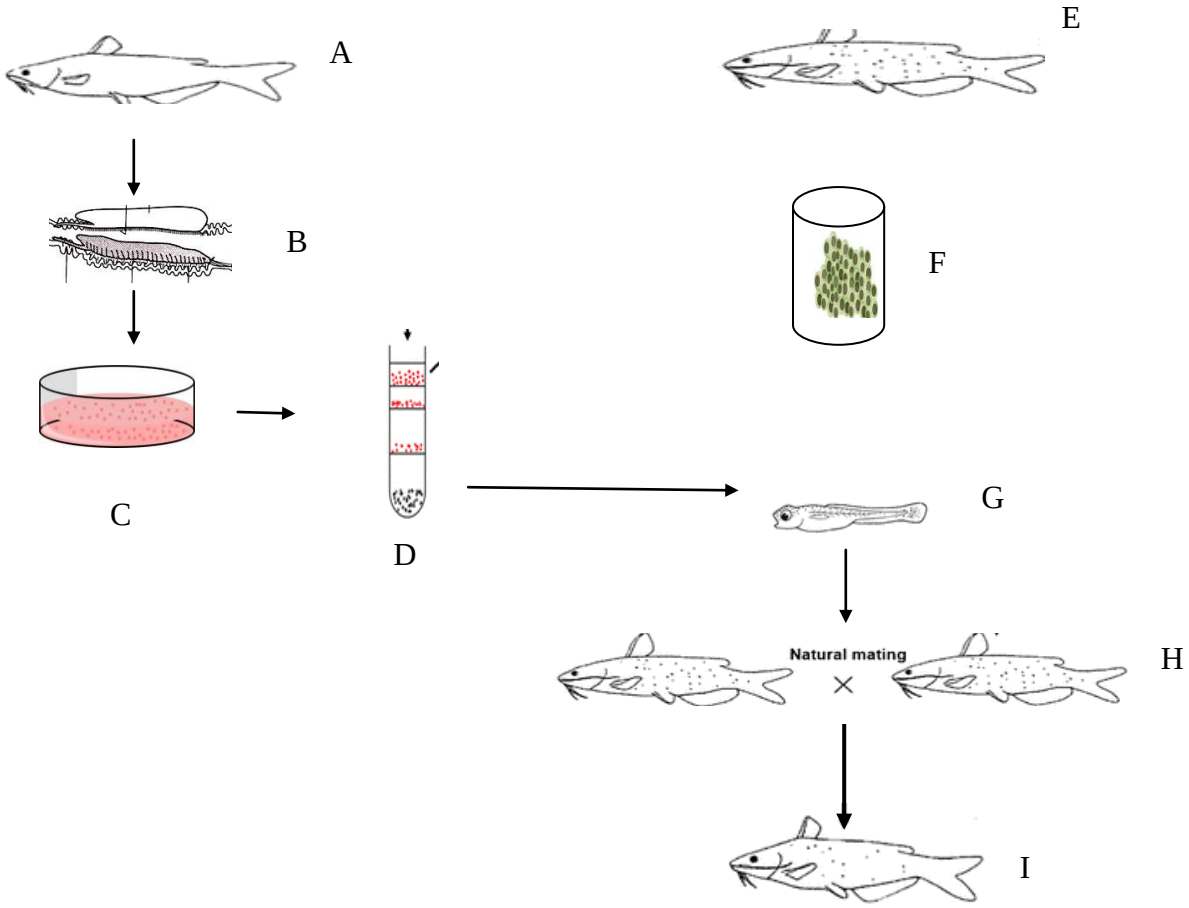

Fig 2. The general procedure of producing channel catfish, *Ictalurus punctatus*, female × blue catfish, *Ictalurus furcatus* male hybrids by mating xenogenic channel catfish male with normal channel catfish female. A: 2-year-old blue catfish males were used for isolating type A spermatogonial stem cells (SSCs); B: Testes from blue catfish males; C: Isolation SSCs with trypsin ethylenediamine tetraacetic acid (EDTA) 0.25%; D: Enrichment of SSCs with discontinuous gradient density centrifugation (Percoll); E: Diploid channel catfish females used to produce diploid eggs; F: Induction of triploidy of channel eggs with hydrostatic pressure; G: triploid channel catfish fry used as recipient donor-derived stem cells from blue catfish 12-36 h after hatch; H: Mating between normal channel catfish female with transplanted channel catfish male; I: hybrid catfish produced by mating xenogenic channel catfish male with normal channel female.

Isolation of donor stem cells form blue catfish

Two types of stem cells isolated from blue catfish were used for this experiment including type A spermatogonial stem cells (SSCs) and primordial germ cells (PGCs). For the collection of SSCs, sexually immature, male blue catfish (mean body weight 538.8±274.1g; mean testis weight 0.33±0.42g) were selected and euthanized with 300 mg/L MS-222 for 10 min until opercular movement ceased. Then, they were washed with tap water and placed on ice before being dissected. The external surface of the donor fish was sterilized with 70% ethyl alcohol (Pharmco-Aaper, Shelbyville, KY). Individual fish were weighed before the abdomen was opened for gonadal collection. Testes were carefully removed to avoid contamination of connective tissues, weighed and washed in 0.5% bleach solution for 1 to 2 min before placing them in a petri dish containing 5 mL of anti-agent medium (Hank's Balanced Salt Solution (Corning Cellgro, Manassas, VA) with 1µg/mL $NaHCO_3$ (Sigma, St. Louis, MO), and 100 unit/mL penicillin and 100 µg/mL streptomycin (Gibco, Grand Island, NY)). Under a biosafety cabinet (The Baker Company, Sanford, Maine), connective tissues and blood vessels were separated and discarded. The testes were washed three times with phosphate-buffered saline (PBS) 10X (Lonza, Walkersville, MD) and three times with anti-agent medium before being minced with two sterilized blades. Minced tissues were transferred to autoclaved flasks containing a magnetic bar and 0.25% trypsin-EDTA (Gibco). The flasks were incubated on ice for 30 min followed by 60 min at room temperature on a stirrer (Corning, Manassas, VA). The suspension was filtered with a 40-µm strainer (Cat. no. 10999-654, VWR, Radnor, PA) and centrifuged at 500 g for 10 min (Sorvall RT6000). After centrifugation, supernatant was discarded and the pellets were re-suspended with 2mL cell culture medium (L-15 Leibovitz (Lonza, Walkersville, MD) supplemented with 25mM HEPES (Sigma, St. Louis, MO), 100

unit/mL penicillin, 100 µg/mL streptomycin (Gibco), 1.0 µg/mL $NaHCO_3$ (Sigma), 0.3 µg/mL L-glutamine (Gibco), 20% ES Cell Fetal Bovine Serum (Gibco) and 1ng/mL bFGF (Gibco). The number of SSCs or total cells were determined under a microscope 40X (Olympus, Center Valley, PA) using a hemocytometer (Reichert, Buffalo, NY).

For the purpose of sorting cells, discontinuous density gradient Percoll (GE Healthcare, Uppsala, Sweden) was used to separate the cells. Volumes of 2 mL each of Percoll 35, 45, and 70% were transferred into a 15 mL centrifuge tube. A volume of 2 mL of testicular cells was added on top of Percoll 35% and centrifuged at 800 g for 40 min. The first layer, which contained the highest percentage of spermatogonial type A stem cells was collected (Shang et al., 2015). The sorted cells were centrifuged at 500 g for 10 min and re-suspended with cell culture medium before transplanting into the triploid fry.

Besides the SSCs, primordial germ cells (PGCs) from blue catfish embryos were also collected and used for this experiment. Procedure for collecting blue catfish PGCs has followed the same procedure for collecting the SSCs. Before hatching, midsections including genital ridges from the blue catfish embryos were dissected. Those midsections were processed in the same way described for collecting the SSCs.

Transplantation of sorted SSCs or PGCs into the triploid recipients

Approximately 9-10 h post-fertilization, channel catfish embryos pressurized at 6,500 and 7,000 psi that developed to blastula stage were transplanted with PGCs or SSCs. Each egg was microscopically injected with 1 µL of cell suspension containing 4,000 SSCs or 8-14 PGCs via a gastight syringe attached to a repeater (Hamilton, Reno, NV). After injection, the transplanted eggs were incubated in baskets placing in flow through hatching troughs.

The newly hatched triploid channel fry (8.9±0.5 mm) were anesthetized by placing them in a 10 mg/L MS-222 buffered with 10 mg/L sodium bicarbonate solution. Anesthetized fry were placed in the petri dish and observed microscopically at 1.5X (Amscope, Irvine, CA). Each fry was injected with 1 µL sorted stem cell solution containing 7,200 cells (4,000 SSCs) or 8-14 PGCs via a 33 gauge needle and a gastight syringe attached to a repeater. Triploid channel catfish fry were laid on the side and the needle was gently inserted in the cavity between anal fin and yolk sac where the genital ridge was expected to be formed (Fig 3). After injection, the fry were placed in recovery troughs aerated for 6 months before tagging and stocking them in the ponds.

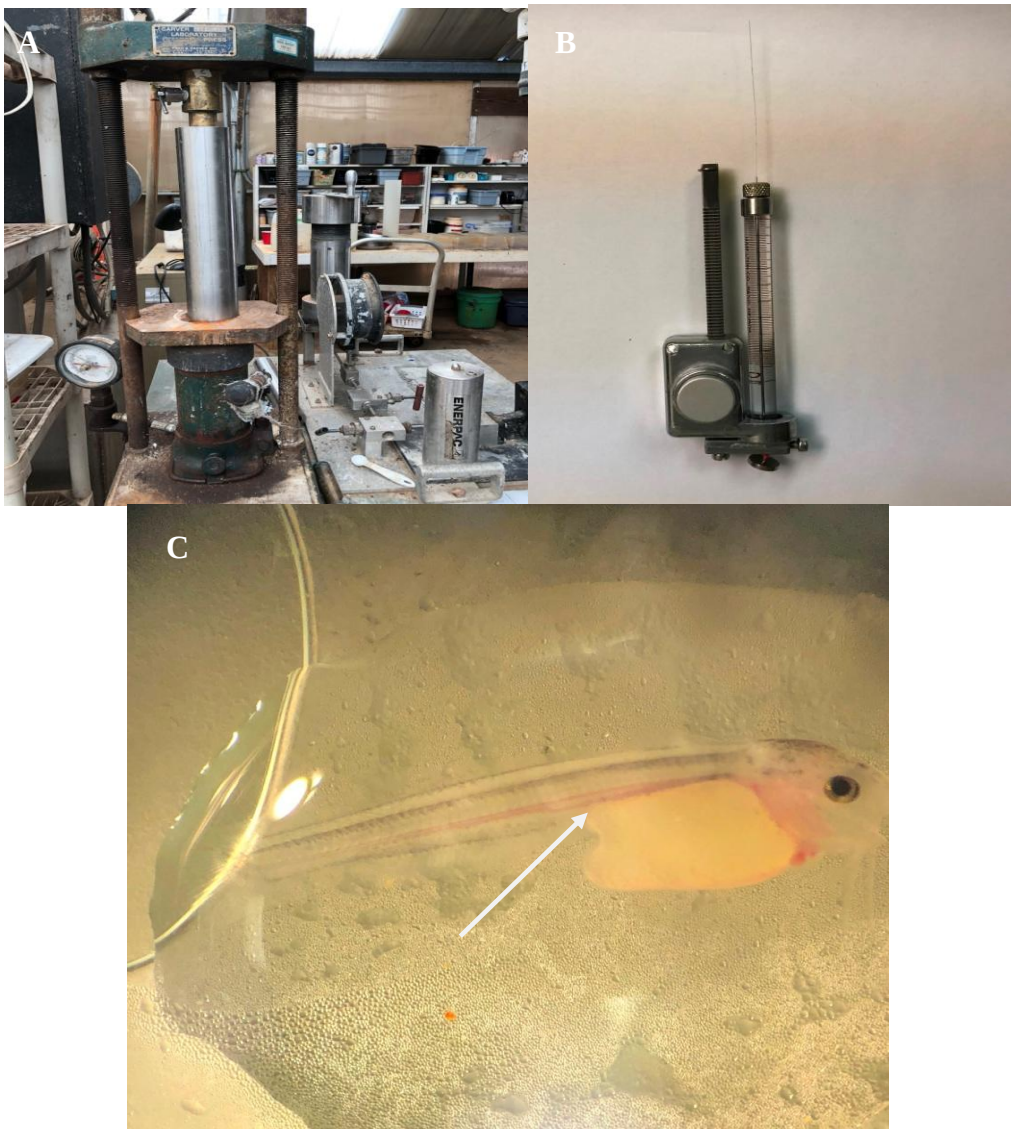

Fig 3. Carver press with hydrostatic pressure chamber, manual injector, and triploid hatched channel catfish, *Ictalurus punctatus*, fry used for transplantion of blue catfish, *Ictalurus furcatus*, type A spermatogonial stem cells (SSCs) and primordial germ cells (PGCs). A: Carver press with hydrostatic pressure chamber to make triploid eggs. Five min post-fertilization, eggs were loaded into the steel chamber and the pressurized at 6,500-7,000 psi for 5 min. B: A gastight syringe attached to a repeater was used to transplant the SSCs or PGCs into the peritoneal cavity of triploid newly hatched fry. C: Channel catfish fry at 12-36 h post-hatch pressurized at 6,500 and 7,000 psi using hydrostatic pressure shock and transplanted with

germline stem cells (PGCs or SSCs) with a gastight syringe attached to a repeater. A pair of genital ridges are located on the wall of the peritoneal cavity. The white arrow shows the site where the needle should be inserted into the cavity. The total length of newly hatched fry was approximately 9 mm.

Spawning of transplanted fish

In 2018, the two-year-old transplanted fish were seined from the ponds and selected for spawning. Those fish with excellent secondary sexual characteristics were carefully selected and moved to indoor facilities. Normal females and males used to mate with transplanted channel catfish were also selected. The transplanted males and females were separated and induced to spawn with LHRHa implantation at 90 µg/kg body weight. The same dose of LHRHa was also applied for normal channel males and females that were used to pair with transplanted fish. A male was individually placed with a normal or transplanted female in a spawning aquarium (91cm x 32 cm x 61cm) and vice versa. The water flow rate was maintained at 4 L/min, water temperature 26.4-27.8 ^{o}C, and dissolved oxygen 4.8-5.7 mg/L. After spawning, the egg masses from spawned pairs were collected and sampled for deoxyribonucleic acid (DNA) analysis. Then, the egg masses were transferred into baskets and suspended in hatching troughs aerated with air diffusers and water flow. Formalin or chelated copper 100 mg/L were applied to prevent fungus and bacteria growth every 8 h. The treatments were given statically for 15-30 min, 3 times a day. Newly hatched fry were sampled and transferred to the rearing tanks fed with 50% protein feed.

DNA extraction from eggs and newly hatched fry samples

Eggs and newly hatched fry were sampled and placed into 1.5 mL microfuge tubes on ice then held in a -80 °C freezer. DNA was extracted using proteinase K digestion following protocols for protein and ethanol precipitation described by Liu et al. (1998) and modified by Waldbieser and Bosworth (2008). DNA from diploid channel catfish, blue catfish, and hybrid catfish were also extracted and used as controls. Briefly, 300 µL cell lysis buffer containing 100

mM NaCl, 10 mM Tris-HCl, pH 8, 25 mM/ EDTA, and 0.5% sodium dodecyl sulfate was added into tube that contained 20-30 mg of tissue. Then, 1.5µL of Proteinase K (Sigma) was added into the tube before incubating 2-4 h at 55 ^{o}C. During incubation, the tube was checked and vortexed to ensure the tissue was completely dissolved. Once, the tissue was completely dissolved, the tube was dried and vortexed for 15 s. Then, 170 µL protein precipitation solution was added and vortexed for 20 s before being placed in a -20 ^{o}C freezer for 10 min. The tube was centrifuged at the highest speed (13.2 x 10^3 revolution per minute, RPM) for 7 min before the supernatant was transferred into a new tube containing 600 µL 100% ethyl ethanol. After centrifuging the new tube at the maximum speed at 13.2 x 10^3 RPM, liquid was removed by pipetting and white pellet retained. Then 600 µL of 75% ethyl ethanol was added in the new tube, vortexed several s, and centrifuged at 13.2 x 10^3 RPM for 3 min to precipitate the DNA. Then, the ethyl ethanol was removed by pipetting and white pellet was eluted by adding 10-100 µL RNA/DNA free water. DNA concentration was quantified with an ND-1000 spectrophotometer (NanoDrop Technologies). DNA from control samples from diploid channel catfish, blue catfish, and hybrid catfish were also extracted by the same procedure.

Polymerase chain reaction (PCR) detection

PCR was used to differentiate channel catfish, blue catfish, and hybrid catfish following the protocol from Waldbieser and Bosworth (2008). Primers used for differential PCR-amplification of channel catfish and blue catfish genes are listed in Table 1. Marker genes were follistatin (*Fst*), and hepcidin antimicrobial protein (*Hamp*) for channel catfish and blue catfish. PCR reactions were prepared in 10.0 µL volume containing 20-250 ng genomic DNA in 1× buffer (1.0 µl 10 mM Tris-HCl [pH: 8.0], 0.4 µL 50 mM MgCl2, 0.8 µL 2.5 mM of each dNTP, 0.6 µL 10 µM each *Fst* primer, 0.3 µL 10 µM each *Hamp*, 0.1µl 5U/µL platinum Taq

polymerase, 3.9 µL water. The PCR procedures were performed using the following thermoprofile: after an initial desaturation at 95 °C for 3 min; the first PCR amplification was carried out at 95 °C for 1 minute, 65 °C for 1 minute, and 70 °C for 1 minute for 35 cycles; followed by a second PCR amplification at 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 1 minute for 35 cycles; and the final extension at 72 °C for 10 min. The *Fst* and *Hamp* amplification products were analyzed on an ethidium bromide stained 2.0% agarose gel. Amplicon size was measured by Tracklt™ 100 bp™ 100bp DNA Ladder (Invitrogen, Carlsbad, CA).

Mitochondrial DNA (mtDNA)

The DNA sequence of the mitochondrial cytochrome oxidase 1 (CO1) gene in progeny of hybrid catfish produced by xenogenic channel catfish male was determined. DNA from muscle tissue was isolated and the COI gene amplified using the polymerase chain reaction (PCR) and sequenced by capillary electrophoresis. The subsequent DNA sequence was compared with that of control samples.

Genomic DNA was extracted using the same procedures described in the previous section followed by sequencing of the cytochrome oxidase 1 (CO1) gene recovered from the experimental preparations. A partial sequence of CO1 gene was amplified using the primers listed in Table 1. Four samples were collected for testing and two samples from channel catfish and blue catfish were used as controls. PCR was performed in a 10 µL reaction volume in a 0.5-mL micro-centrifuge tube containing 10 µl PCR grade water; 1X PCR buffer; 200 µM of dATP, dCTP, dGTP and dTTP each; 2 mM MgCl2; 1 µM of blue catfish (BC) COI and channel catfish (CC) COI each; 0.5 unit of Platinum® Taq DNA polymerase (Invitrogen) and 20 ng DNA. PCR cycling conditions were as follows: initial denaturation at 94°C for 2 min; 35 cycles of

denaturation at 94°C for 30 s, annealing at 52°C for 40 s, extension at 72°C for 1 min; and a final extension at 72°C for 10 min.

Table 1. Primers used for *Fst* (*follistatin*), *Hamp* (*hepcidin* antimicrobial protein), *Mtcol* (*Mitochondrial cytochrome oxidase* 1) genes to differentiate channel catfish, *Ictalurus punctatus,* and blue catfish, *I. furcatus*. Primers were previously described by Waldbieser and Bosworth (2008).

			Amplicon (bp)	
Gene	Forward Primer	Reverse Primer	Channel Catfish	Blue Catfish
Fst	ATAGATGTAGAGGAGCATTTGAG	GTAACACTGCTGTACGGTTGAG	348	399
Hamp	ATACACCGAGGTGGAAAAGG	AAACAGAAATGGAGGCTGGAC	222	262
Mtcol-CC	TATTCACTGTAGGGATAGACGTG	CCAACAGTAAAGAGGAAGATGAAA	178	None
Mtcol-BC	TGTTTACTGTAGGAATGGACGTA	CCAACGGTAAATAGGAAAATGAAG	None	178

Results

Triploid percentages

Two levels of pressure, 6,500 and 7,000 psi, were applied to channel catfish eggs to produce triploid fish. Six months later, blood samples from 41 fish pressurized at 6,500 psi (11.9±0.6 g), 42 fish pressurized at 7,000 psi (12.3±0.6 g), and 5 diploid channel catfish (14.3±0.4 g) as controls were measured for ploidy level by using a Coulter Counter (Beckman Coulter). At 6,500 psi, the triploid percentage was 82.9% while more, 100%, of the fish pressurized at 7,000 psi were triploid ($P = 0.005$) (Fig. 4). Hatching rates of those eggs pressurized at 6,500 and 7,000 psi were also different ($P = 0.006$), averaging 16.2% and 8.3%, respectively. These hatching rates were much lower than the rate of eggs without pressurization, 43.8% ($P < 0.0001$) (Fig. 5).

Survival rates of channel catfish embryos shocked with hydrostatic pressure and injected with cell suspension at blastula stage ranged from 0-4.9% while the survival rates of channel catfish embryos when newly hatched and transplanted with cell suspension were higher, ranging from 6.9-37.3%.

Rate of Xenogenesis

Eighty 9-month-old channel catfish fingerlings (mean body weight 48.7±19.2 g) that had been injected with the blue catfish stem cells died from *Ichthyophthirius multifiliis*. DNA

analysis of their gonads revealed. Rate of xenogenesis was very low 16.7, 6.7 and 0.0% for the treatments 7,000 psi injected with SSCs, 7,000 psi injected with PGCs and 6,500 psi injected with PGCs, respectively (Table 2 and Fig. 6).

Spawning results

Approximately, 20% of 250 fish that were injected as fry appeared to be sexually mature at 2 years of age (Table 3). The transplanted channel catfish were seined and selected for spawning after 2 years grown in the ponds. Sixty-one transplanted fish including 17 channel catfish (11 males and 6 females) fish pressurized at 6,500 psi and transplanted with PGCs, 15 channel catfish (9 males and 6 females) pressurized at 7,000 psi and transplanted with PGCs, 12 channel catfish (8 males and 4 females) pressurized at 6,500 psi and transplanted with SSCs, and 17 channel catfish (12 males and 5 females) pressurized at 7,000 psi and transplanted with SSCs were paired together or with normal channel catfish in aquaria. There were 21 transplanted channel catfish males were paired with 21 transplanted channel catfish females in aquaria. Two pairs among these combinations spawned, but only 1 pair fertilized eggs. Nineteen transplanted males were paired with 19 normal channel females in aquaria and induced to spawn with hormone. Nine pairs spawned, and 4 males were able to fertilize egg masses. Overall, most of the 2-year-old transplanted fish did not spawn, only 19.7% of the transplanted fish spawned in which one channel female pressurized at 6,500 psi and transplanted with PGCs spawned and was fertilized by male pressurized with same level and transplanted with same cell type; three males pressurized at 6,500 psi and transplanted with PGCs or SSCs and one male pressurized at 7,000 psi and transplanted with SSCs can fertilized the egg masses of control channel catfish females (Table 4). Among transplanted fish that spawned and fertilized the eggs, only one male

pressurized at 7,000 psi and transplanted with SSCs was confirmed xenogenic with PCR analysis on its progeny's samples (Fig. 7 and Fig. 8). The fertilization and hatching rates from this xenogenic male spawning were 7.3% and 12.3%, respectively. Although these rates were not high, and the successful rate of stem cell transplantation was low, this was the first report of successful spawning of stem cell transplanted channel catfish spawned in tank conditions.

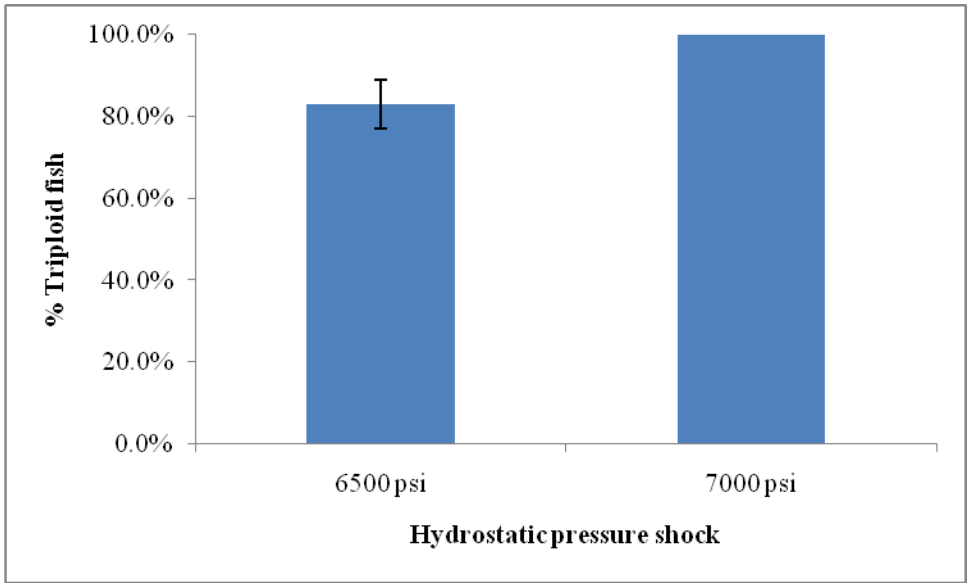

Fig 4. Triploid percentages of channel catfish, *Ictalurus punctatus*, embryos induced by hydrostatic pressure shock for 5 min at 6,500 and 7,000 psi 5 min after fertilization at 27 ^{o}C. Blood samples from 41 fish pressurized at 6,500 psi (11.9±0.6 g), 42 fish pressurized at 7,000psi (12.3±0.6g), and 5 diploid channel catfish (14.3±0.4 g) as controls were measured for ploidy level by using a Coulter counter. Triploid percentages of channel catfish pressurized at 6,500 psi and 7,000 psi were 82.9% and 100%, respectively. Values analyzed with t-test were significantly different at $P < 0.05$.

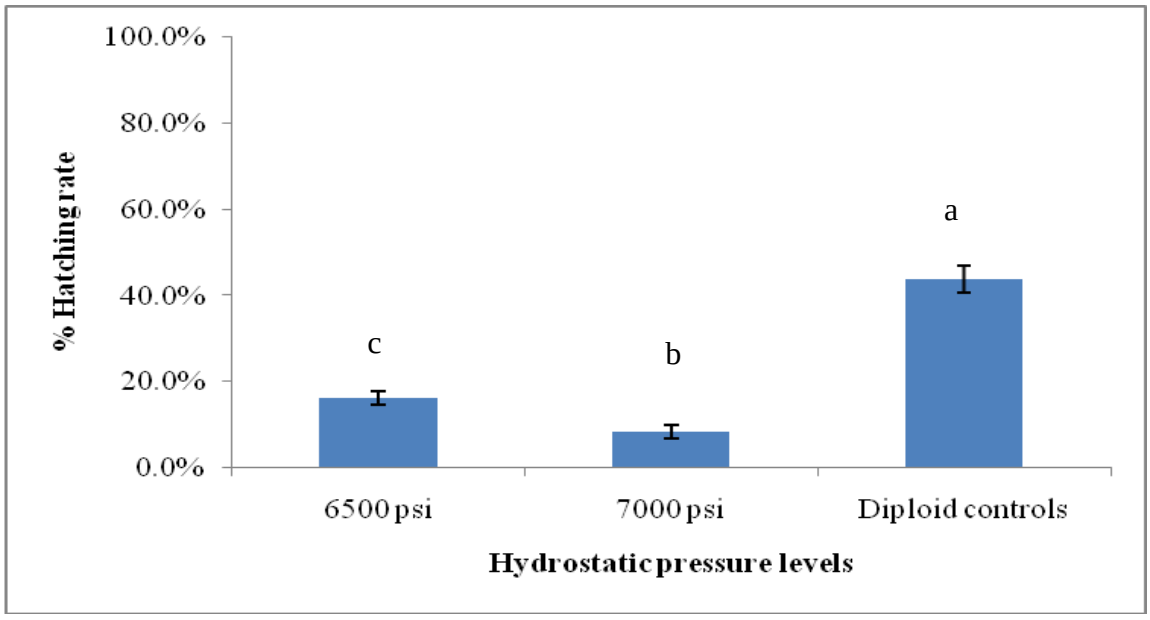

Fig 5. Hatching percentages of triploid channel catfish, *Ictalurus punctatus*, embryos induced by hydrostatic pressure shock at 6,500 and 7,000 psi and the diploid control without pressurization. Hatching percentages of channel catfish pressurized at 6,500 psi and 7,000 psi were 12.6 % and 8.3 %, respectively, which were significantly lower than the percentage of the controls, 48.3% ($P < 0.0001$). Hatching percentages were shown as mean ± SE. Values analyzed with Analysis of variance (ANOVA) and Tukey-Kramer tests with different letters were significantly different at $P < 0.05$.

Table 2. The xenogenic numbers of channel catfish, *Ictalurus punctatus,* pressurized at 6,500 and 7,000 psi and transplanted with blue catfish, *Ictalurus furcatus*, primordial germ cells (PGCs) or type A spermatogonial stem cells (SSCs) 9 months after transplantation. Deoxyribonucleic acid (DNA) sampled from their gonads revealed that xenogenic percentages of fish pressurized at 7,000 psi and transplanted with SSCs and PGCs were 16.7 and 6.7 %, respectively, while the percentage of channel catfish pressurized at 6,500 psi and transplanted with PGCs was 0.0%.

Pressure (PSI)	Type of cells	N	N Transplanted fish possessing blue catfish DNA
7000	SSCs	12	2
6500	PGCs	16	0
7000	PGCs	30	2

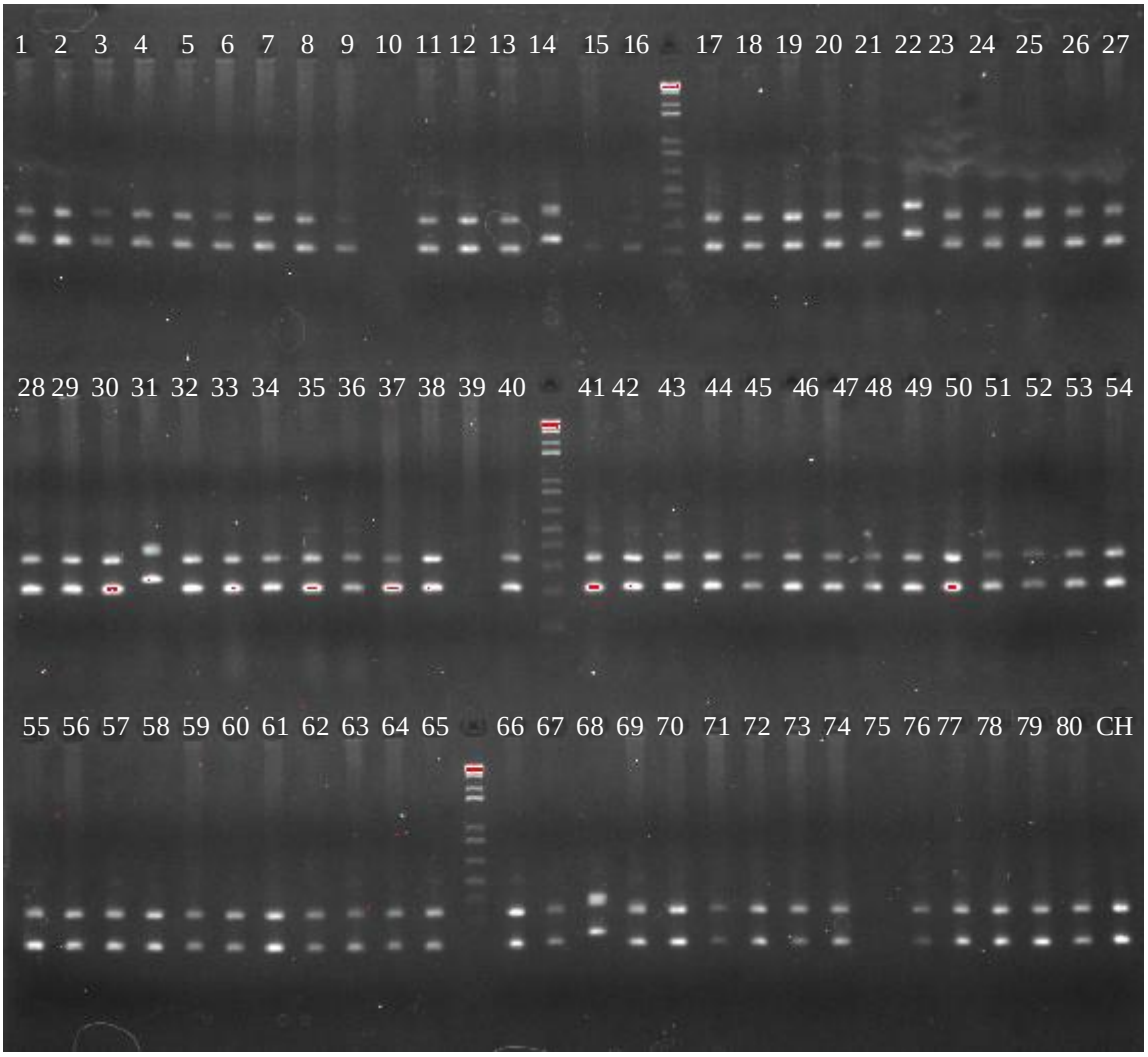

Fig 6. Polymerase chain reaction (PCR) results for detecting blue catfish, *Ictalurus furcatus,* DNA in gonadal tissues (testes or ovaries) of triploid channel catfish, *Ictalurus punctatus*, 9 months after transplantation. Channel catfish embryos were pressurized and 6,500 and 7,000 psi and transplanted with 8-14 primoridal germ cells (PGCs) or 4,000 type A spermatogonial stem cells (SSCs) from blue catfish. Channel catfish and blue catfish DNA were differentiated with PCR using follistatin, *Fst* and hepcidin antimicrobial protein, *Hamp* genes as markers. CH is channel catfish control. Eighty samples from all treatments (channel catfish embryos pressurized at 6,500 psi and transplanted with blue catfish PGCs; channel catfish

embryos pressurized at 7,000 psi and transplanted with blue catfish PGCs; channel catfish embryos pressurized at 6,500 psi and transplanted with blue catfish SSCs; channel catfish embryos pressurized at 7,000 psi and transplanted with blue catfish SSCs) were analyzed. There were four samples (10, 15, 39, and 75) that had low deoxyribonucleic acid (DNA) concentration and were not able to be analyzed. Among 76 analysed samples, four samples (14, 22, 31, and 68) contained blue catfish DNA. They were channel catfish embryos pressurized at 7,000 psi and transplanted with blue catfish PGCs (sample 22 and 68) and channel catfish embryos pressurized at 7,000 psi and transplanted with blue catfish SSCs (sample 14 and 31).

Table 3. Total numbers, mature numbers, and maturity rates of channel catfish, *Ictalurus punctatus*, pressurized at 6,500 and 7,000 psi and transplanted with blue catfish, *Ictalurus furcatus*, primordial germ cells (PGCs) or type A spermatogonial stem cells (SSCs) were checked ans selected 2 years post-transplantation. Overall, there were around 61 of 250 fish from all treatments appeared to be sexually mature at 2 years of age.

Pressure-Types of cells	N	Selected fish	Maturity rate
6500-PGCs	60	17	28.3%
7000-PGCs	50	12	24.0%
6500-SSCs	80	15	18.8%
7000-SSCs	60	17	28.3%

Table 4. Total pairs, spawned pairs, unfertilized pairs, and fertilized pairs, and fertilization percentages for pairings of channel catfish, *Ictalurus punctatus*, males pressurized at 6,500 psi and 7,000 psi transplanted with primordial germ cells (PGCs) or spermatogonial stem cell type A (SSCs) of blue catfish, *Ictalurus furcatus*, with channel catfish females pressurized at 6,500 and 7,000 psi transplanted with either PGCs or SSCs or control (diploid) channel catfish females. One channel catfish male pressurized at 7,000 psi and transplanted with SSCs fertilized a control female produced hybrid progeny. The fertilization and hatching rate of eggs fertilized with this xenogenic male were 7.3 and 12.3%, respectively.

Male		Female		Total	Spawned	Unfertilized	Fertilized	%
Pressure	Cell type	Pressure	Cell type	pairs	pair(s)	pair(s)	pair(s)	Fertilization
6500	PGC	6500	PGC	2	1		1	60.8
6500	PGC	7000	PGC	3	0			
6500	PGC	Control		6	3	2	1	75.9
6500	SSC	6500	SSC	1	1	1		
6500	SSC	6500	PGC	1	0			
6500	SSC	7000	PGC	1	0			
6500	SSC	7000	SSC	1	0			
6500	SSC	Control		4	2	0	2	69.4

7000	PGC	6500	PGC	1	0			
7000	PGC	6500	SSC	2	0			
7000	PGC	7000	PGC	1	0			
7000	PGC	Control		5	2	2		
7000	SSC	6500	PGC	2	0			
7000	SSC	6500	SSC	1	0			
7000	SSC	7000	PGC	1	0			
7000	SSC	7000	SSC	4	0			
7000	SSC	Control		4	2	1	1	7.3*

* The offspring of male pressurized at 7,000 psi and transplanted with SSCs that mated with normal channel female were confirmed all hybrids.

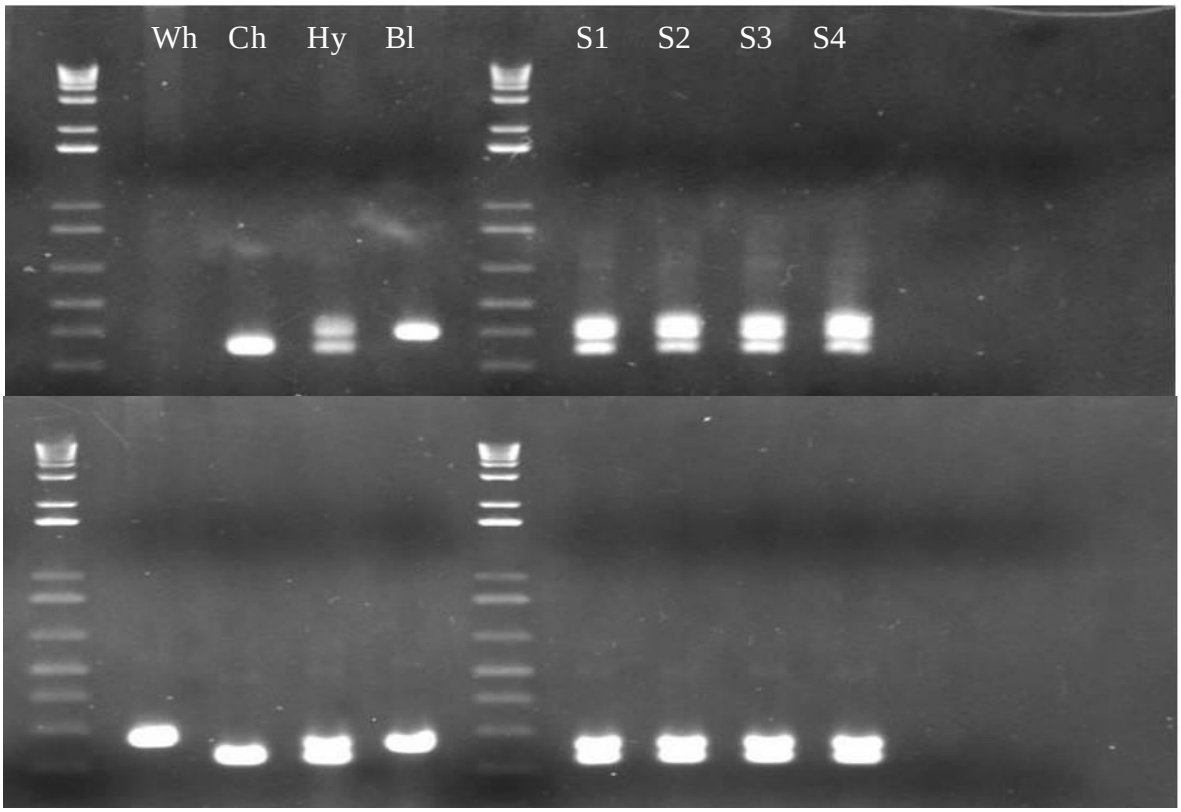

Fig 7. Polymerase chain reaction (PCR) results for progeny of a triploid channel catfish, *Ictalurus punctatus*, male pressurized at 7,000 psi and transplanted with 7,200 sorted cells from blue catfish, *Ictalurus furcatus*, males mated with normal channel catfish female. Blue catfish and channel catfish were differentiated using follistatin, *Fst*, and hepcidin antimicrobial protein, *Hamp* genes as markers. Wh = White catfish control; Ch = channel catfish control; Bl = blue catfish control; Hy = channel catfish female × blue catfish male hybrid control. Four fry (S1 – S4) from the spawn of the transplanted male and normal channel catfish females were analyzed. All four fry showed the genotype of channel catfish × blue catfish hybrids.

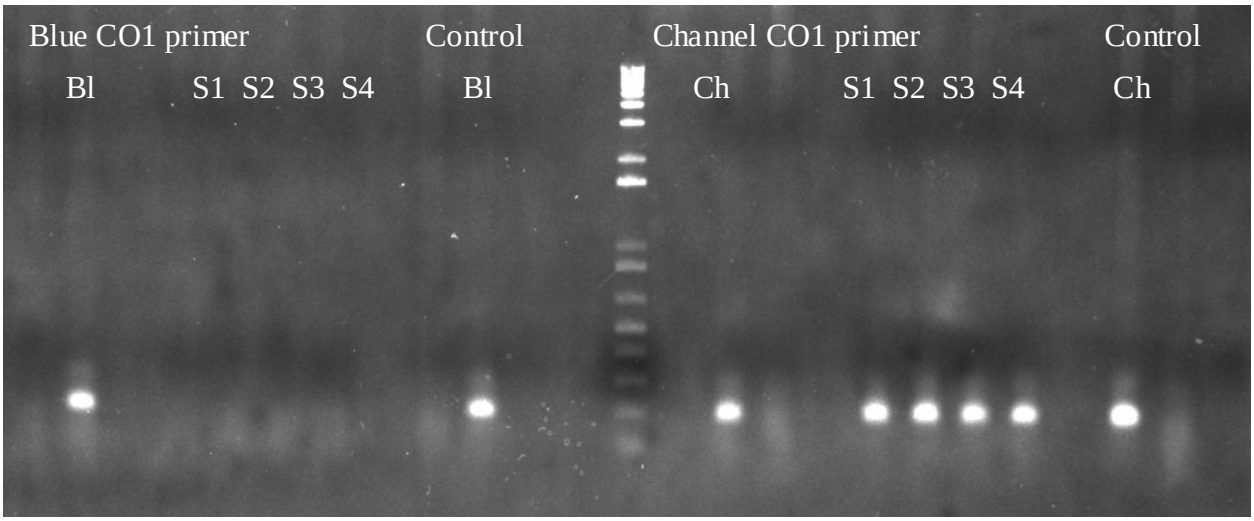

Fig 8. The sequence of cytochrome oxidase 1 (CO1) genes were amplified using *Mtcol BC* and *Mtcol CC* primers of blue catfish, *Ictalurus furcatus*, (left) and channel catfish, *Ictalurus punctatus*, (right). Four samples were collected for testing and two samples from channel, blue catfish were used as controls. With the blue catfish CO1 primer, all samples were negative, indicating these hybrids did not inherit mtDNA of blue catfish female. The result showed that four hybrid catfish inherited mtDNA from channel catfish female which indicated that the dam of these hybrids was a channel catfish.

Discussion

A hydrostatic pressure of 7,000 psi was required to produce 100% triploid channel catfish hosts. This resulted in low hatching rates of 8% compared to 48% for diploid controls. Colonization rates were quite low, 16%, for injected SSCs, and 0-7% for PGCs, indicating the injected cell numbers were too low, timing of injection was wrong or Percoll purification of the cells damaged colonization ability. Correlated to this, spawning and apparent xenogenesis rate in two-year-old putative xenogens was also very low. However, one male triploid xenogenic channel catfish produced all hybrid progeny when mated with a normal channel catfish female. Fertilization rate was low and hatch rate was low, but this is the first report of a xenogenic channel catfish male being able to produce hybrid progeny through mating and ejaculation after induce spawning.

Unsterile fish that received donor-derived germ cells produces a considerable number of their own gametes together with donor-derived gametes. Therefore, a method to produce surrogate recipients capable of producing only donor-derived gametes was developed. Since the sterile fish were incapable of producing their own gametes, triploid fish were used as germ cell recipients (Yoshizaki and Lee, 2018). Triploid fish have been used as hosts for germ cell transplantation in several studies (Okutsu et al., 2007; Perera et al., 2017; Yoshizaki et al., 2012). Although triploid fish are sterile, they do have a normal ability to nurse diploid germ cells once they were incorporated into the recipient genital ridges (Yoshizaki and Lee, 2018). Yoshizaki et

al. (2016) also found that germ cell transplantation using the triploid or germ cell-free recipients resulted in effective colonization compared with recipients carrying endogenous germ cells.

In comparison to other methods (thermal and chemical shock), hydrostatic pressure shock used to induce triploidy is straightforward to apply and produces more consistent results and higher survival rate for treated eggs (Bury, 1989; Dogankaya and Bekcan, 2014). Triploid rates induced by heat or cold shocks were variable for different species, depending on temperatures, initiation time after fertilization and treatment duration time. For example, Reddy et al. (1990) reported that heat shocks at 42 ^{o}C in 1-2 min induced only 12% triploidy in rohu (*Labeo rohita*). Utter et al. (1983) found the triploid rates of coho salmon, and chinook salmon shocked at 28-30 ^{o}C in 10 min averaged 58-84%. The results from our experiment showed that 82.9-100% of channel catfish eggs pressurized at 6,500-7,000 psi were triploid. These results were similar to the results found by Lilyestrom (1989) for channel catfish eggs as hydrostatic pressures initiated 5 min after fertilization induced 67.0-100% triploidy, depending on the levels of pressure. Hydrostatic pressure at 7,000 psi is a suitable level for producing triploid catfish. At this level, 100% of rainbow trout or 98.0% of grass carp pressurized were triploid (Aldridge et al., 1990; Chrout, 1984).

The hatching rates of these triploid eggs from this experiment were low, ranging from 8.3-16.2%. The lower pressure shock (6,500 psi) resulted in a significant higher hatching rate, nearly doubled the hatching rate of eggs pressurized at higher pressure (7,000 psi). The hatching rates of those control eggs in the experiment was also low (43.8%) which indicated that egg quality, fertilization procedure, water quality, and bacterial infection could be limiting factors for the hatching rate of channel catfish (Hatch et al., 1990; Kjorsvik et al., 1990; Sapkale et al., 2011). These hatching rates for triploid eggs were quite low when compared to hatching rates

reported by Lilyestrom (1989) for the same species and techniques. As he reported that around 50 - 100% of triploid induced eggs hatched. However, Lilyestrom (1989) also observed that the hatching rate of eggs at a low-pressure level had almost double the hatch rate of eggs at high pressure.

To select the most suitable developmental stage for germ cell transplantation, catfish recipients were injected at three different stages: blastula, newly hatched embryo, and sub-adult. Overall, when the triploid blastula used as recipients, the survival rate of manipulated embryos was very low, ranging from 0-4.9% while the survival rate of manipulated embryos at the newly hatched stage was much higher and ranged from 6.9-37.3%. This result was also similar to the result found by Takeuchi et al. (2003) when survival rate of transplanted recipients at blastula was 26% at 30 days posttransplantation while the survival rate of hatched embryos used as recipients was 94% at the same period after transplantation. Transplantation in subadult resulted in a survival rate of nearly 100%, but this methodology could not be used in large scale treatments (Perera et al., 2017), and the proliferation after transplantation appeared to be too low at this developmental stage. A high mortality of microinjected blastulae limits their potential large-scale application. The data to date indicate that fry at the newly hatch are the best life stage for germ cell transplantation. In addition, at the newly hatched stage the immune system of the recipients was found previously to also not be fully functional (Manning and Nakanishi, 1996), thus, they would not have the ability to reject donor-derived foreign cells.

The Percoll density gradient centrifugation method is the best approach for separating the stem cells. The density gradient is longitudinally layered in a test tube and the cell sample is layered on the top. The cells move along the density gradient and accumulate at a position where the density of the medium matches that of the cells under centrifugal forces (Brakke, 1951). In

our study, three different concentrations of Percoll, 35, 45, and 70% were used, resulting in distinct cell bands after centrifugation. The results of SSCs separation from this experiment were also similar to the results reported by Shang et al. (2015) when the first band above the 35% Percoll fraction contained around 50% of type A spermatogonia. In this study, the percentage of type A spermatogonial stem cells after being sorted with Percoll averaged 55.5%. Percoll density ingredient has also been used to separate germ cells in loach (Yoshikawa et al., 2009), tilapia (Lacerda et al. 2006), and sturgeons (Psenicka et al., 2015). In loach, SSCs type A and B were also found in the 30-36% Percoll layer. In sturgeons, approximately 80% of the SSCs were located in 10-30% Percoll layer. Overall, Percoll density gradient centrifugation was a good method for SSC enrichment, although the percentage of spermatogonia type A was lower than that from flow cytometry sorting (Takeuchi et al., 2003; Yoshizaki et al., 2000). However, flow cytometry sorting required more effort to make transgenic individuals or transgenic lines, which requires high technology and time-consuming efforts as well as use of transgenesis, which for practical application would cause government regulatory impediments.

Both PGCs and SSCs were transplanted into triploid channel catfish fry at the newly hatched stage in this experiment. However, PGCs accounted for a very small percentage (less than 1%) of the total cells retrieved by proteolytic digest from blue catfish embryo's genital ridges. Yoshizaki and Lee (2018) also reported only 10-20 PGCs were seen among 3,000 cells digested from the genital ridges. PGCs are accessible only during a short period of time before gonadal sex differentiation (Braat et al., 1999). In addition, manipulation of small blue catfish fry (9-10 mm) to extract the PGCs was more difficult as compared to the SSCs collected from the young catfish (2 years old). Spermatogonia have been identified as an alternative cell type to overcome these difficulties. A larger number of spermatogonia can be found in the testes at all

developmental stages. Furthermore, spermatogonia have the ability of self-renewal and can give rise to specialized cell types, which has proved that these cells are a suitable choice for germ cell transplantation. Indeed, the SSCs have also been reported to transplant successfully in trout, salmon, tilapia, and catfish (Lacerda et al., 2006; Okutsu et al., 2006; Perera et al. 2017). Thus, SSCs are recommended for germ cell transplantation. From our experiment, the triploid channel host transplanted with SSCs also successfully produced donor-derived gametes (blue catfish), but the ability to fertilize eggs was low as 7.3% eggs were fertilized and 12.6% hatched. Perera et al. (2017) reported that only 1% of eggs hatched that were fertilized by a channel male transplanted with blue catfish SSCs at the sub-adult stage mated with a normal female, however, his sperm quantity was low and the hybrid progeny were produced by sacrifice of the male and artificial fertilization. There are obstacles in spermatogenesis of xenogenic catfish that needs to be further investigated.

In this experiment, the spawning rates of triploid channel catfish transplanted with germline stem cells were around 19.7%, and only one spawn was a result of successful transplantation. Four channel catfish diploid spawns were produced that hatched as a result of the triploidy not being 100%. Seven egg masses did not pass, and these fish were likely triploids with unsuccessful transplantation. Thus, one of eight is a 12.5% transplantation rate, which matches the xenogenesis rate measured in the fish at 9 months of age, 6.7-16.7%. Channel catfish can start to spawn at 2 years old, but are more likely to be sexually mature at 3 years old or older, thus better results might be obtained when these fish are older. Additionally, it is not yet known how the donor cells (from a late maturing species) might affect sexual maturation rates in the host. The triploid channel catfish in this experiment were transplanted with 4,000 SSCs. The triploid white catfish in chapter 4 were injected with 9,000-10,000 SSCs, and had 3-4X higher

xenogenesis rate. In the white catfish experiment, the donor cells were also treated with ROCKi. Future research should address introduction of larger numbers of donor cells, fine tuning of the best time to inject these cells and examining ROCKi to increase cell viability and colonization rate.

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CHAPTER THREE

Survival rates of sorted and unsorted spermatogonial type A stem cells of blue catfish (*Ictalurus furcatus*) incubated with Rho protein kinase inhibitor (ROCK I)

Abstract

A 3 × 3 × 6 repeated factorial experiment was designed to evaluate survival rates of gonadal stem cells of blue catfish (*Ictalurus furcatus*) isolated by three different methods (i. dissociation without enzymatic digestion; ii. dissociation with trypsin ethylenethiamine tetraacetic acid (EDTA) 0.25%; iii. dissociation with trypsin-EDTA 0.25% then enrichment with discontinuous density gradient centrifugation (Percoll)) and incubated with three different concentrations of ROCK I (i. 0; ii.10; iii. 50 µM) from the 1st to 6th day (repeated factor) after isolation. Use of trypsin-EDTA 0.25% yielded the highest number of spermatogonial A stem cells (SSCs), 2.2×10^{7}/g of testes, and oogonial stem cells (OSCs), 2.1×10^{6}/g of ovary while dissociation without enzymatic digestion resulted in 34.8% and 38.5% fewer OSCs and SSCs, respectively. Use of Percoll to separate the OSCs and SSCs resulted in 30.3% and 37.5% cell loss compared to the gonadal stem cells without being separated by Percoll. Both spermatogonial type A stem cells (SSCs) from blue male catfish and oogonial stem cells from female blue catfish were cultured *in vitro* with cell culture medium at 27 ^{o}C in the incubator. Overall, mean survival rates of unsorted SSCs without trypsin, unsorted SSCs with trypsin, and sorted SSCs were 71.1, 73.9 and 76.3%, respectively, after 6 days. The sorted SSCs that separated with Percoll had the

highest observed survival percentage ($P < 0.05$). Additionally, SSCs reached the highest survival rates on the first day (90.0%) with a remarkable reduction by approximately 30.0% from the first day to 6th day ($P < 0.0001$). Using ROCK I at 10 or 50 µM had higher survival rates than stem cell suspensions without using ROCK I ($P < 0.0001$). Results for OSC culture were similar with no differences in survival rates of unsorted OSCs with and without trypsin digestion and sorted OSCs, averaging 68.5 - 69.7%. Use of ROCK I at 10 or 50 µM to culture OSCs gave around 4.0 -10.0% higher mean survival rates than those cultured without ROCK I ($P < 0.0001$). The OSCs should be used at the first day after dissociation from the ovaries to yield the highest survival rate (84.0%), compared to lower survival percentage of OSCs at 6th day (59.5%) ($P < 0.001$). Therefore, the SSCs or OSCs should be transplanted at the first day after dissociation from the gonads with supplement of 50 µM ROCK I in cell culture medium to yield the greatest viable numbers of stem cells for transplantation in catfish. The use of trypsin for dissociation followed immediately with application of ROCK I will yield the highest number of stem cells with best viability.

Keywords: Blue catfish, *Ictalurus furcatus*, spermatogonial type A stem cells, oogonia, enzymatic digestion (trypsin-EDTA 0.25%), ROCK I, *in vitro* cell culture, survival rates.

Introduction

Fish stem cell culture

Cells have been cultured *in vitro* since the 1950s and have provided important models for biological and physiological research to produce enzymes, vaccines, growth factors, and monoclonal antibodies and hormones (Evans et al., 2006). Stem cells can be defined as precursor cells that can divide to produce daughter cells which are identical to each other and to their mother cells, as well as having the ability to give rise to differentiated cell types (Till and McCulloch, 1980). These make the stem cells different from other cells. The first stem cell cultures were derived from early developing mouse embryos in 1981 by Evans and Kaufman (1981). Fish stem cell culture began in 1992 as Collodi et al. (1992) used a feeder layer technique to culture cells from blastula stage diploid and haploid zebrafish, *Branchydanio rerio*, embryos. Two years later, Wakamatsu et al. (1994) established the pluripotent cell line, OLES1, from the blastula of medaka, *Oryzias latipes*. The feeder cells consist of a layer of cells unable to divide which provides extracellular secretions to help other cells to proliferate (Liames et al., 2015), including zebrafish embryonic fibroblasts or rainbow trout, *Oncorhynchus mykiss*, spleen cell lines (Collodi et al., 1992; Ma et al., 2001). Ma et al. (2001) also demonstrated that germline chimera could be produced using short-term zebrafish embryo cell culture. Hong et al. (1996) developed a feeder-free layer technique for fish embryonic stem cells (ESCs) derived from medaka which led to the establishment of three ESC lines, MES1, MES2, and MES3. Zebrafish and medaka are among the most primitive vertebrates, so success with medaka ES cell line culture provided direct evidence for possibilities to derive ESCs from other vertebrate species. In 1998, human ESCs were obtained from the inner cell mass of the blastocyst of an early staged embryo (Thomson et al., 1998). According to Hong et al. (1996), an essential component of ESC

conducive medium in the feeder-free culture system is the fish embryo extract from medaka. This component was combined with basic fibroblast growth factor and fish serum enabling self-renewal of dissociated midblastula embryo cells in a dish coated with gelatin. The result showed that a zebrafish cell line was successfully obtained from the blastula stage embryos. Therefore, both feeder and feeder-free conditions seem to work in zebrafish ESC culture. Applications of feeder-free culture system has also been used to establish ESC lines in several marine fish species, including the gilthead sea bream, *Sparus aurata* (Bejar et al., 2002), red sea bream, *Pargus major* (Chen et al., 2003), sea perch, *Lateolabrax japonicas* (Chen et al., 2007), Asian sea bass, *Lates calcarifer* (Parameswaran et al., 2007), and Atlantic cod, *Gadus morhua* (Holen et al., 2010).

Germ cells including primordial germ cells (PGCs) and gonadal stem cells (type A spermatogonial stem cells (SSCs) in the testes and oogonial stem cells (OSCs) in the ovaries) attracted interest for the development of germline culture. Spermatogonial stem cell culture is an important tool to understand the mechanisms that control stem cell survival, self-renewal, proliferation, and differentiation. The first spermatogonial stem cell culture was reported for mice (Nagano et al., 1998). Then, Shinohara et al. (2000) developed laminin selection technique to enrich spermatogonial stem cells and improve the efficacy of spermatogonia culture. In addition, the discovery of a Glial cell line derived neurotrophic factor (GDNF) and basic fibroblast growth factor (FGF2) were crucial to the development of these cultures (Meng et al., 2000; Kanatshu-Shinohara et al., 2003). Kanatshu-Shinohara et al. (2005) developed a method to improve their cell culture conditions by eliminating cell feeder or serum. This reduced introduction of unknown factors into the culture environment. To achieve this, laminin or fetuin were added to the cell culture system.

In fish, gonadal cells cultured *in vitro* under experimental conditions remain viable and maintain essential cellular functions such as proliferation and differentiation (Song and Gutzeit, 2003). *In vitro* transformation from spermatogonia to spermatozoa was reported in different species such as Japanese eel, *Anguilla japonica* (Miura et al., 1991), zebrafish, *Danio rerio* (Sakai, 2002), medaka, *Oryzias latipes* (Saiki et al., 1997). Hong et al. (2004) reported the first success for establishing a cell line from spermatogonial stem cells generated from a mature medaka testis. Spermatogonial stem cells underwent meiosis and spermatogenesis to generate motile sperm in the test tube under appropriate culture conditions. The success of this study has stimulated an increasing interest in other species.

Shikina et al. (2008) reported SSCs isolated from a trout transgenic line maintained the survival and mitotic activity in L-15 medium supplemented with 10% FBS at 10 ^{o}C. Shikina and Yoshizaki (2010) studied spermatogonial stem cells from transgenic rainbow trout, *Oncorhynchus mykiss*, and reported that overgrowth of testicular somatic cells could be suppressed by adjusting fetal bovine serum to 1%. Spermatogonial stem cell survival, mitotic activity, and transplantation capacity can be enhanced by adding soluble factors such as bovine serum albumin, adenosine, and salmonid serum. Panda et al. (2011) cultured purified populations of spermatogonia *in vitro* for more than 2 months in L-15 media containing 10% FBS, 1% carp serum, and other nutrients. Lacerda et al. (2013) established the cell culture conditions for Nile tilapia spermatogonia, containing DMEM/F12 medium supplemented with some other ingredients.

Some years ago, the absence of spermatogonial stem cells markers limited their identification. Spermatogonial stem cells have been mainly identified by morphological characteristics (Lacerda et al., 2014; Miura et al., 2003; Nobrega et al., 2010; Schulz and Miura,

2002). The enrichment of spermatogonia population has been based on cell size and granularity, using density gradient media (Lacerda et al., 2006; Shang et al., 2015; Wong et al., 2013; Yoshikawa et al., 2009) or flow cytometry (Kise et al., 2012). Recently, spermatogonia specific antigen 1 (SGSA-1), lymphocyte antigen 75 (Ly75 or CD205), *gfra1*and *nanos2* proteins, *oct 4* were found to detect spermatogonia A in medaka, loach, *Misgurnus anguillicadatus*, Nile tilapia, *Oreochromis niloticus*, and rainbow trout (Schulz et al., 2010; Nagasawa et al., 2010; Yoshikawa et al., 2009; Lacerda et al., 2013). For ictalurid catfish, Shang et al. (2015) reported that *plzf* and *integrin6* have high expression in spermatogonial stem cells of the channel and blue catfish and could be used as SSCs specific markers.

Primordial germ cells (PGCs) are precursors of sperm and eggs (Magnusdottir and Surani, 1987). The PGCs have been studied in a number of fishes using morphological criteria. Teleost PGCs can be distinguished from somatic cells by their distinct morphology, having a bigger size (10-20 µm) and a larger nucleus (6-10 µm). The number of PGCs varies from 10 to 30 during pregonial development in most fish (Braat et al., 1999). For the identification of PGCs, zebrafish *vasa* provided significant improvement for fish germ line evaluation from a molecular aspect and was considered as the first marker in the germline of zebrafish (Braat et al., 2000; Krovel and Olsen, 2002). Besides *vasa*, other molecular markers in fish germ cells have been also discovered such as *nanos, dnd1, dalz, and sdf1a/cxcr4b* (Knaut et al., 2003; Koprunner et al., 2001; Kosaka et al., 2007; Liu and Collodi, 2010). *Vasa* and or *nanos* promoters have been successfully used for the generation of transgenic lines in several fish species, including medaka (Liu et al., 2009), zebrafish (Fan et al., 2008), and trout (Yoshizaki et al., 2000). These transgenic fishes expressed green or red fluorescent protein in germ cells (PGCs) that allowed for visualization of PGCs in developing embryos. In addition, those molecular markers have been

studied in other commercially valuable species such as Atlantic salmon, *Salmo salar*, Atlantic cod, *Gadus morhua*, and turbot, *Scophthalmus maximus* (Lin et al., 2013; Nagasawa et al., 2013; Skugor et al., 2014).

Embryonic germ cells were first isolated from mouse and cultured in a supplemented medium including stem cell factor (SCF), leukemia inhibitory factor (LIF), and basic fibroblast growth factor (bFGF) (Matsui et al., 1992). Later, PGCs from chicken blood were also cultured using feeder supplemented with LIF, SCF, bFGF and insulin-like growth factor (IGF-1) (Van de Lavoir et al., 2006). In fish, zebrafish PGCs were isolated, marked, and cultured by Fan et al. (2008). For this study, the transgenic embryos that expressed the red fluorescent protein (RFP) under PGC-specific vasa promoter were used which led to the isolation of pure populations of PGCs by fluorescence-activated cell sorting (FACS) and to optimize the culture conditions by counting the number of PGC colonies in different culture media. In addition, these authors also studied the effects of growth factors (Kitlga and Kitlgb) and stromal-cell derived factor 1a and 1b on PGC proliferation. Then, Wong et al. (2013) established zebrafish female germline stem cell (FGSC) cultures from a transgenic line of fish that expresses Neo and DsRed under control of germ cell-specific promoter, *ziwi*. The cell line was selected in G418 and engineered to expressed zebrafish leukemia inhibitory factor (Lif), basic fibroblast growth factor (Fgf2), and glial cell line-derived neurotrophic factor (Gdnf). These components enhanced FGSC growth and survival in culture condition.

ROCK Inhibitor

ROCKs are protein kinases that share 45 to 50% homology to other actin cytoskeletal kinases such as myotonic dystrophy kinase (DMPK), myotonic dystrophy related cdc42-binding

kinase (MRCK) and citron kinase (Riento and Ridley, 2003). ROCK inhibitor is a general term for inhibitors of Rho protein kinase which was discovered as a downstream target of the small guanosin -5'-triphosphate (GTP)-binding protein Rho (Leung et al,. 1995; Matsui et al., 1996). ROCK I has been used to regulate a broad range of responses such as cellular growth, adhesion, migration, metabolism, and apoptosis through control of assembly actin cytoskeleton and cell contractibility (Riento and Ridley, 2003). As a result, ROCK inhibitor-mediated membrane blebbing enhances actin-myosin contraction and activates caspase signaling cascades and cellular apoptosis (Kurosawa, 2012).

A pyridine derivative, Y-27632 known as ROCK I which has been used widely in many biological systems such as cultured cells, isolated tissues, and animal models. This compound is soluble in distilled water and stable at room temperature for at least 4 weeks. When Y-27632 is added into the culture medium, it was taken up by the cells in a time and temperature dependent and saturable manner (Ishizaki et al., 2000). Therefore, this compound is quite suitable for *in vitro* applications of isolating tissues and culturing stem cells.

Frisch and Screaton (2001) experimentally demonstrated that ROCK I (Y-27632) was able to prevent apoptosis of dissociated human pluripotent stem cells (hPSCs) and increase their survival rate and plating efficiency. Dissociation induced apoptosis in hPSCs called anoikis, a type of apoptosis induced by inappropriate cell-cell or cell-extracellular matrix interactions. According to Nichols and Smith (2009), pluripotent stem cells could be sorted into two types, the inner cell mass (naïve state of pluripotency) and epiblast (primed state of pluripotency). Mouse embryonic stem cells were naïve pluripotent stem cells with no apicobasal polarity and were not vulnerable to dissociation. Human embryonic stem cells were primed pluripotent stem cells with apical-basal polarity that underwent dissociation-induced apoptosis, which has been a technical

problem for many cellular manipulations (Ohgushi and Sasai, 2011). According to Krawet et al. (2009), Y-27632 allowed hPSCs to escape anoikis by increasing cell-cell interaction and adhesion. Ohguishi et al. (2010) proposed a molecular pathway of dissociation-induced apoptosis of human embryonic stem cells. They demonstrated that the apoptosis of stem cells was caused by the ROCK-dependent hyperactivation of myosin. In dissociated human embryonic stem cells the loss of E-cadherin mediated cell-cell interaction immediately activated the Rho-ROCK/myosin signaling cascade causing myosin hyperactivation and finally leading to apoptosis.

Y-27632 inhibits the activation of ROCK and consequently blocks the Rho/ROCK/myosin cascade. Chen et al. (2010) suggested that actin-myosin contraction is a major mechanism promoting the death of single dissociated hESCs. Cell survival could be increased by disrupting contraction of the actin-myosin motor. ROCK I reduced phosphorylation of myosin light chain which activated the actin-myosin contraction lead to an increase in the survival of human ESCs.

ROCK I applications for culturing stem cells

ROCK I enhanced the *in vitro* survival of mouse embryonic stem cells derived neural precursors, mouse intestinal stem cells, and human keratinocytes (Chapman et al., 2010; Koyanagi et al., 2008). ROCK I has been applied to improve cell recovery of human embryonic stem cells after fluorescence-activated cell sorting. To apply to fluorescence-activated cell sorting system, the cells must be dissociated into single cells. Under such conditions, therefore the cell recovery after sorting will be low due to dissociation-induced apoptosis. When Y-27632 was added into the plating medium, the survival rate of post-sorted cells was improved. Cells

undergo morphological changes including contraction, dynamic membrane blebbing, and nuclear disintegration during apoptosis, which is driven by ROCK-mediated actin-myosin contractile force generation (Coleman and Olson, 2002). When a cell loses survival signals derived from contact with other cells, they may undergo anoikis, a kind of apoptosis induced by lack of correct cells and extracellular matrix (ECM) attachment. ROCK I has been reported to prevent anoikis in the ethanol-induced death of primary rat astrocytes (Minambres et al., 2006). It has been proven that culturing human embryonic stem cells *in vitro* and isolated individual cell clones following gene transfer or during differentiation procedures in suspension culture conditions was a technical challenge because of the high rate of anoikis-induced cell death (Koyanagi et al., 2008).

ROCK I was reported to protect against anoikis in dissociated human ESC and mouse ESC derived neural precursors (Koyanagi et al., 2008; Watanabe et al., 2007). In addition, survival of ESC derived neural precursor cells was increased by 20-30% when cells were pretreated with Y-27632 before dissociation and injected into the striatum of mice (Koyanagi et al., 2008). Pacary et al. (2006) found that ROCK I could be effective in combination with hypoxia mimetics in promoting mesenchymal stem cell differentiation into neuron-like cells. In fish, ROCK inhibitor/feeder layer supplementation is found to offer the advatages of selecting for epithelial like cell type and decreasing time to immortalization of Mozambique tilapia, *Oreochromis mossambicus* (Gardell et al., 2014).

Trypan blue and hemocytometer for cell counting

Trypan blue, an intraoperative stain, that is used to facilitate anterior segment surgery (Melles et al., 1999), improved the visualization of peripheral epiretinal membrane in patients

with proliferative vitreoretinopathy (Feron et al., 2002). Previous studies also demonstrated the safety of intravitreal trypan blue in rabbit eyes and in retinal pigment epithelium cell culture (Stalmans et al., 2003; Veckerneer et al., 2001). Stalmans et al. (2003) also reported that a concentration of up to 3 mg/mL trypan blue applied for 5 min was nontoxic on cultured cells. Narayanan et al. (2005) studied that human retinal pigment epithelial cells and rat neurosensory retinal cells grown in tissue culture and treated with four different concentrations of trypan blue (0.1, 0.05, 0.025, and 0.0125%) for 2 min and then exposed to halogen light source, showing that cell morphology was not altered in both types of cells after being treated. Katsares et al. (2009) also used 0.4% trypan blue to evaluate the survival of umbilical cord blood stem cells and compared trypan blue extension method with Cytotox - Glo kit. The results showed that both assays gave similar viability percentages.

Therefore, trypan blue dye can be used to determine the number of viable cells present in the cell suspension by selectively coloring the dead cells or tissues (Tran et al., 2010). The mechanism of this staining is based on it being negatively charged and not interacted with cells unless the membrane of cells is damaged. In this case, live cells possess intact cell membranes that excluded the certain dyes where dead cells with damaged membrane are stained in a distinctive blue color (Tran et al., 2011). Avelar-Freitas et al. (2014) found that trypan blue at 0.002% was defined as the optimum concentration for distinguishing unstained living cells from fluorescent dead cells and fluorescence emission was stable for 30 min after cell treatment. Trypan blue has been used to discriminate the living and dead stem cells in this experiment because of its popularity and ease of use.

The aim of this research was to evaluate factors that can lead to increased yield and viability of catfish stem cells for germ cell transplantation by 1) determining the effects on

cultured stem cell survival from different methods of dissociating stem cells, including dissociation with or without enzymatic digestion (trypsin-EDTA 0.25%) and dissociation with trypsin then enrichment with discontinuous gradient density seperation (Percoll); 2) determining the best concentrations of ROCK I add to the cell culture medium to enhance the survival of dissociation -derived stem cells that are *in vitro* cultured; and 3) determining the length of time that the stem cells can be cultured prior to cell transplantation.

Materials and Methods

Experimental blue catfish

Twelve 2-year-old blue catfish males (mean body weight: 478.9±114.7 g, mean testis weight: 0.08±0.03 g) and ten blue catfish females (mean body weight 362.1±73.8 g, mean ovary weight: 0.65±0.34 g) were euthanized with 300 ppm tricaine methanesulfonate MS-222 for gonadal collection. All the fish were kept on ice and the weight of each fish was collected before sterilizing the external surface of those fish with 70% ethyl alcohol. Then, an incision was made on the abdomen of the fish and testis or ovary of each fish was gently collected. Connective tissues were discarded before weighing the gonad with a digital scale. Testes or ovaries were washed in 0.5% bleach solution for 1 minute before transferring them to separate dishes containing anti-agent medium Hank's Balanced Salt Solution (HBSS) with 1µg/mL $NaHCO_3$, and 100 unit/mL penicillin and 100 µg/mL streptomycin. The gonads were washed three times with phosphate -buffered saline (PBS) 10X and three times with anti-agent medium before being dissociated.

Cell culture medium and ROCK I preparation

Cell culture medium used in this experiment was L-15 Leibovitz (Lonza, Walkersville, MD) supplied with 25mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 100 unit/mL penicillin, 100 µg/mL streptomycin, 1.0 µg/mL $NaHCO_3$, 0.3 µg/mL L-glutamine, 20% ES Cell Fetal Bovine Serum (FBS) and 1ng/mL bFGF (basic fibroflast growth factor). After

mixing all components together, the cell culture medium was filtered and sterilized with a 0.22 µm filter (Millipore).

ROCK I was prepared following the protocol from the producer. A volume of 1.5 mL of sterile water was added into the 5 mg vial of ROCK I and mixed thoroughly to produce a 10 mM stock solution. The aliquot was stored at -20 to -80 ^{o}C into the working volume to avoid repeated freeze-thaw cycles. The stock solution was diluted into cell culture medium immediately before use. The concentration of the final solution (0, 10, 50 µM) was prepared from the stock solution, followed the formula below.

Concentration of final solution = concentration of stock solution x volume of stock / volume of final solution.

Treatments of stem cells and ROCK I concentrations

There were three treatments of SSCs dissociated from blue catfish males using in this experiment. The first treatment was unsorted SSCs dispersed freshly from testes of experimental blue catfish. The second treatment was unsorted SSCs isolated from the testes of blue catfish and trypsinized with trypsin-EDTA 0.25%. The third treatment was sorted SSCs dissociated from blue catfish testes with trypsin-EDTA 0.25% and enriched with discontinuous gradient density centrifugation (Percoll).

General procedures for peparing three treatments for SSCs were described as follows: Connective tissues or blood vessels were carefully removed from the testes. The testes were then washed with PBS 10X and anti-agent medium before being minced with a pair of sterilized blades. The minced testes were equally divided into three sterile Petri dishes later used for three

different treatments of stem cells before transferring into three autoclaved glass flasks which contained stir bars. A volume of cell culture medium (a ratio of 1 volume gonadal tissue: 15 volumes of cell culture medium) was added into the first flask for the first treatment while a volume of trypsin-EDTA 0.25% (1 volume gonadal tissue: 15 volumes of trypsin-EDTA 0.25%) was respectively added into the second and third flask for the second and third treatment before incubating the flasks on ice for 30 min. Then, the flasks were placed on the hot plate stirrer at 22 ^{o}C for 60 min to achieve higher dissociation efficiency. During the incubation process, the suspension from each flask was sampled and checked under the microscope to ensure that all cells were individually separated. Cell suspensions from the flasks were filtered with a 40 µm cell strainer (nylon mesh, Falcon) and centrifuged at 1.5×10^{3} revolutions per minute (rpm) for 90 s using an Eppendorf centrifuge (Model 5415D, Hauppauge, NY). The supernatants included unwanted cell types were discarded.

For the first and second treatment, the pellets at the bottom were kept and resuspended with cell culture medium. The cells in the suspension were enumerated and divided into six-well plates coated with an attachment factor of 0.1% Gelatin (Gibco). Each well contained 0.4 mL cell suspension and 1.6 mL cell culture medium supplemented with ROCK I at 0, 10 or 50 µM. The numbers of SSCs from blue catfish males in each well of the first treatment averaged 4.5-4.7×10^{4} while each well for the second treatment contained 5.2-5.6 $\times$ 10^{4} SSCs. Three replicates for each treatment of stem cells incubated with each concentration of ROCK I were used.

For the third treatment, the pellet was resuspended in 2mL Hanks' Balanced Salt Solution (HBSS). Discontinuous density gradient centrifugation (Percoll) was conducted by using 70%, 45%, and 35% Percoll to enrich the SSCs following the protocol described by Shang et al. (2015). The volume of each 70%, 45%, and 35% Percoll was gradually and carefully transferred

into the 15 mL tube. Then, 2 mL of the suspension was carefully transferred on the top of 35% Percoll layer. The tube was centrifuged at 800 g by using a Sorvall machine at 4 ^{o}C for 40 min. The top band, which contained the highest number of SSCs, was collected and transferred into a centrifuge tube before centrifugation at 500 g in 10 min with a Sorvall centrifuge. The supernatant was removed while the pellet was collected and resuspended with cell culture medium. The cells in the suspension were determined and divided into 6-well plates that contained cell culture medium supplemented with ROCK I at three different concentrations. Each well contained 4.5-4.8×10^{4} SSCs. Three replicates for this treatment of stem cells incubated with each concentration of ROCK I were also utilized.

All the plates from all treatments were incubated in the incubator (Queue, model 02710) at 27 ^{o}C air temperature. Every day during the culture, the cells were sampled, stained with trypan blue, and observed under the microscope to determine stem cell viability.

The oogonial stem cells (OSCs) extracted from ovaries of ten blue catfish females were also studied. The same procedures to make three treatments of OSCs (unsorted OSCs without trypsin-EDTA 0.25%, unsorted OSCs with trypsin-EDTA 0.25%, and sorted OSCs) incubated with three concentrations of ROCK I (0, 10 and 50 µM) were tested. Three replicates for each treatment and each concentration of ROCK I were applied. The numbers of unsorted OSCs without trypsin-EDTA 0.25%, unsorted OSCs with trypsin-EDTA 0.25%, sorted OSCs cultured in each well were and 2.7-3.1×10^{4}, 3.4-3.5 ×10^{4}, and 3.0-3.2×10^{4}, respectively.

Trypan blue extension and hemocytometer

Trypan blue 0.4% dye was used to determine the number of viable cells present in the cell suspension. A viable cell had a clear cytoplasm, whereas a dead cell had blue cytoplasm

upon staining. The dye solution was made by diluting 1 volume of trypan blue with 1 volume of cell suspension and mixing gently with pipets before loading on a hemocytometer.

To prepare the hemocytometer, the mirror-like polished surface was carefully cleaned with Kimwipes. A coverslip was also cleaned. The coverslip was placed over the counting surface prior to adding cell suspension. The hemocytometer was divided into 9 major squares 1 mm x 1 mm each. The height of the chamber formed with the cover glass was 0.1 mm, so the hemocytometer had a volume of 0.1 mm^3 or 10^{-4} mL. The number of alive and dead SSCs/OSCs in all four outer squares were counted then divided by 4 to give the mean number of SSCs/OSCs. Percentage of live cells was calculated by dividing the number of viable SSCs/OSCs by the total number of SSCs/OSCs (alive and dead cells) and multiplying by 100.

Statistical analysis

Statistical procedures were anlayzed using SAS® version 9.4 (SAS Institute, Inc., Cary, NC, USA). A repeated measures factorial measures ANOVA was used to analyze the interactions of three factors including treatments of gonadal stem cells (unsorted stem cells without trypsin, unsorted stem cells with trypsin, and sorted stem cells), concentrations of ROCK I supplemented in the culture media (0, 10, 50 µM), and incubation times (repeated factor; day 1 - day 6) for both SSC and OSC survival. If interaction terms were non-significant the models were rerun with these non-significant interactions removed. Alpha was set at 0.05 for main effects and interactions. Tukey-Kramer tests were applied to determine significant differences of all possible pairwise comparisons.

Results

The numbers of oogonial stem cells (OSCs) dissociated from the ovaries of blue catfish, *Ictalurus furcatus,* females in three treatments (unsorted OSCs without trypsin, unsorted OSCs with trypsin, and sorted OSCs) were 2.6×10^6, 4.0×10^6, and 2.8×10^6, respectively (Table 5). Overall, use of trypsin-EDTA 0.25% yielded 34.8% higher numbers of OSCs compared to the treatment without using trypsin for digestion. Additionally, the results indicated that 30.3 % of OSCs were lost when the stem cells were separated with discontinuous gradient density centrifugation.

The survival rates of blue catfish OSCs dissociated by three different methods were not different during and after 6 days of culture ($P = 0.68$), averaging 68.5 to 69.6% (Fig. 9). However, the OSCs in the cell culture medium supplemented with ROCK I at 10 or 50 µM had significantly higher survival percentages (68.7% and 74.0%) compared to those oogonial stem cells cultured in the medium without ROCK I, in which OSCs supplemented with ROCK I at 50 µM yielded the highest survival rate ($P < 0.0001$) (Fig. 10). Additionally, the mean survival rate of OSCs at 1st day after being dissociated from the ovaries peaked at 84.0%, which was significantly higher than the mean survival rates from the 2nd to 6th experimental day ($P < .0001$). The viability of oogonial stem cells were reduced by around 24% from the 1st to 6th day (Fig. 11). These results indicated that OSCs should be used 24 h after being dissociated from gonadal tissues for maximum yield and ROCK I at 50 µM should be added in the cell culture medium to yield the highest viable rate for OSCs.

Similar results were found for blue catfish spermatogonial type A stem cells (SSCs) *in vitro* culture for 6 days. The numbers of SSCs dissociated from the testes of blue catfish males in

three treatments (unsorted SSCs without trypsin, unsorted SSCs with trypsin, and sorted SSCs) were 4.1×10^6, 6.7×10^6, and 4.2×10^6, respectively (Table 5). Use of trypsin-EDTA 0.25% yielded 38.5% more SSCs compared to the treatment without using trypsin for digestion. In addition, use of Percoll to enrich the SSCs resulted in 37.5% of SSCs being lost during process of separation.

Mean survival rates of unsorted SSCs without trypsin, unsorted SSCs with trypsin, and sorted SSCs were different ($P = 0.038$), in which mean survival rate of unsorted SSCs without enzymatic digestion was around 5.0% lower than the survival percentage of sorted SSCs (Fig. 12). In addition, the supplement of ROCK I at 10 or 50 µM in cell culture medium yielded the higher survival percentages (74.7% and 78.6%, respectively) compared to the control treatment without using ROCK I (68.0%) after 6 days of culture ($P < 0.0001$, Fig.13). The survival rate of SSCs was highest at day one (90.0 %) immediately after being dissociated from the testes and gradually decreased from the first day to 6^{th} day ($P < 0.0001$, Fig. 14). After 6 days, approximately 60% of SSCs were alive.

Table 5 . The numbers of spermatogonial type A stem cells (SSCs) and oogonial stem cells (OSCs) yielded from the testes and ovaries of 2-year-old blue catfish, *Ictalurus furcatus*, males and females by three different methods including unsorted SSCs and OSCs dissociated without trypsin ethylenediamine tetraacetic acid (EDTA) 0.25%, unsorted SSCs and OSCs dissociated with trypsin-EDTA 0.25%, and sorted SSCs and OSCs dissociated with trypsin-EDTA 0.25% and enriched with discontinuous gradient density centrifugation (Percoll). Uses of trypsin-EDTA 0.25% yielded the higher number of SSCs and OSCs from the gonadal tissues while dissociation without enzymatic digestion resulted in 34.8 and 38.5% less number of OSCs and SSCs, respectively. Uses of Percoll to separate the OSCs and SSCs resulted in 30.3 and 37.5% cell loss compared to the gonadal stem cells without being separated by Percoll.

Treatment	Testes weight (g)	Total cells	Ovaries weight (g)	Total cells
Unsorted cells without trypsin	0.3050	4.1×10^6	1.8765	2.6×10^6
Unsorted cells with trypsin	0.3010	6.7×10^6	1.8767	4.0×10^6
Sorted cells	0.2990	4.2×10^6	1.8780	2.8×10^6

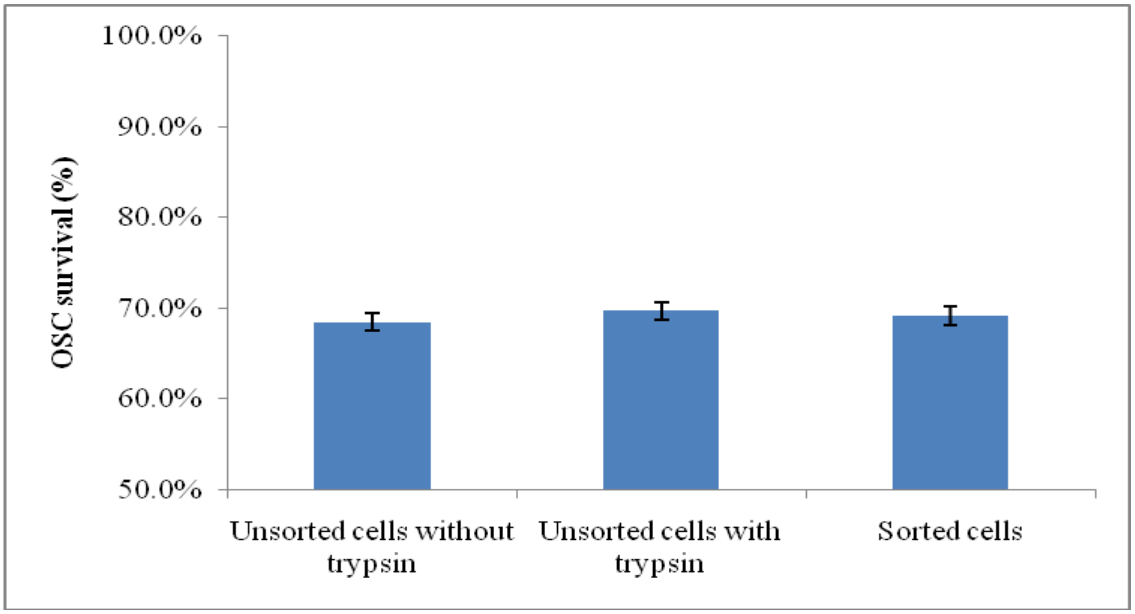

Fig. 9. Mean survival rates of oogonial stem cells (OSCs) dissociated from ovaries of blue catfish, *Ictalurus furcatus*, females without trypsin Ethylenediamine tetraacetic acid (EDTA) 0.25% (unsorted cells without trypsin); with trypsin-EDTA 0.25% (unsorted stem cells with Trypsin); with trypsin-EDTA 0.25% and enrichment with discontinuous gradient density separation (sorted cells) cultured *in vitro* for 6 days. The average number of OSCs per well *in vitro* cultured was 2.7-3.1×10^4 for treatment of unsorted cells without trypsin, 3.4-3.5×10^4 for treatment of unsorted cells with trypsin, and 3.0-3.2×10^5 for treatment of sorted cells. The mean survival rates of unsorted OSCs without trypsin, unsorted OSCs with trypsin treatment, and sorted OSCs was 68.5, 69.7, and 69.2%, respectively. The survival rates were shown as mean ± SE. There were no differences in survival rates of three treatments of stem cells ($P > 0.05$) using a repeated measures analysis of varinace (ANOVA). Tukey-Kramer tests were applied to determine significant differences of all possible pairwise comparisons.

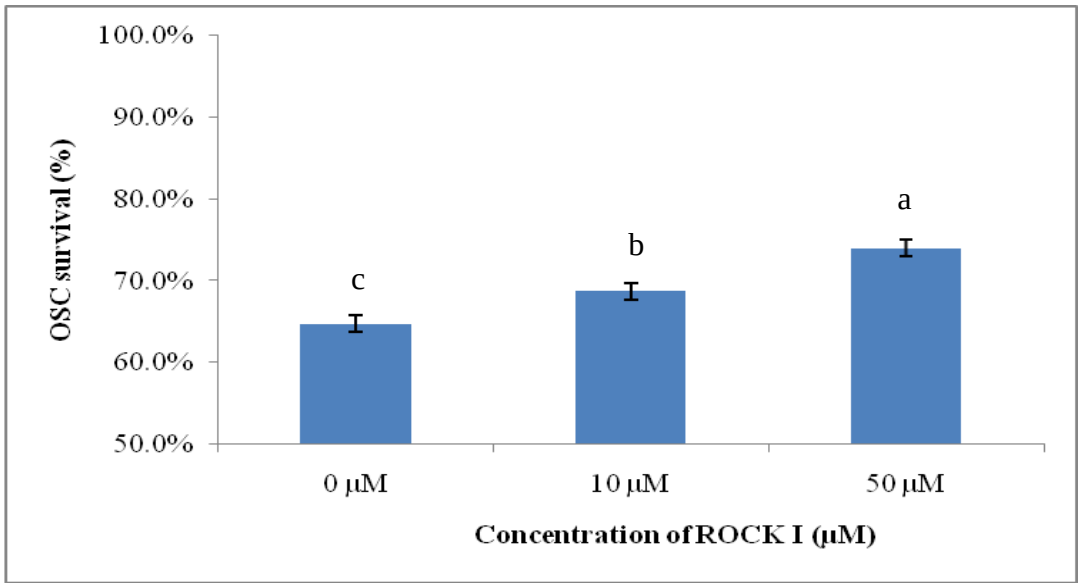

Fig. 10. Mean survival rates of oogonial stem cells (OSCs) dissociated from ovaries of blue catfish, *Ictalurus furcatus*, females cultured *in vitro* in 6-well plates and supplemented with Rho protein kinase inhibitor (ROCK I) at 0, 10 and 50 µM for 6 days. The mean survival rate of OSCs during the 6-day culture period supplemented with ROCK I at 0, 10, and 50 µM was 64.7, 68.6, and 73.9 %, respectively. Overall, the supplement of ROCK I at 10 or 50 µM in the cell culture medium gave the significantly higher survival rates compared to the survival rates of OSCs cultured without ROCK I supplement. The survival rates were shown as mean ± SE. All the values were significantly different ($P < 0.05$), analyzed with repeated measures Analysis of Variance (ANOVA). Tukey-Kramer tests were applied to determine significant differences of all possible pairwise comparisons.

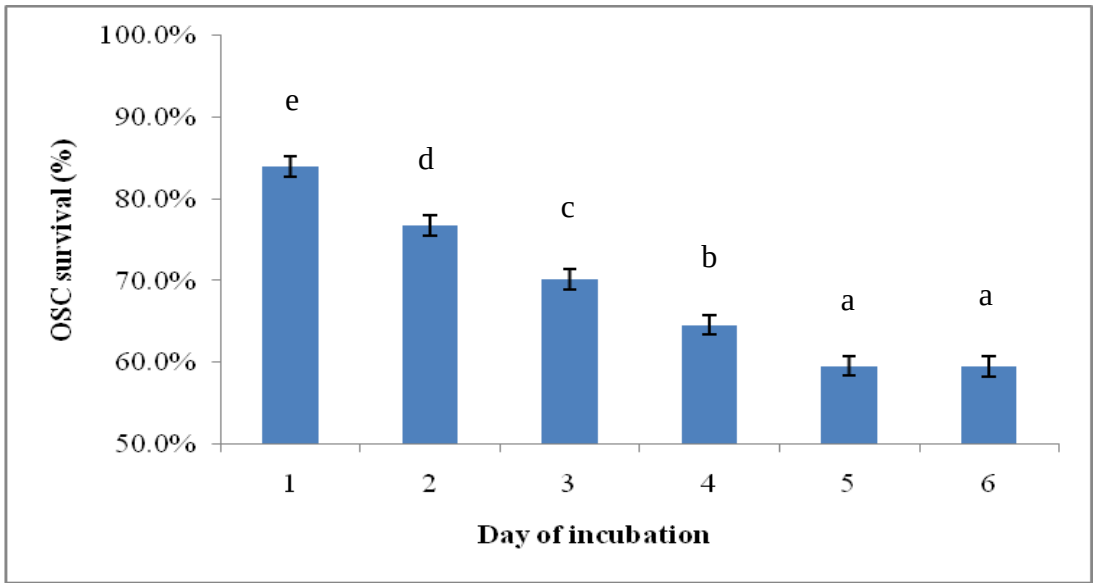

Fig. 11. Cumulative survival rates of oogonial stem cells (OSCs) dissociated from ovaries of blue catfish, *Ictalurus furcatus*, females cultured *in vitro* in 6-well plates for 6 days. The OSCs had the significantly highest survival rate on the 1st day after being dissociated from gonadal tissue (84.0%). There was a decrease in the survival rates of OSCs after the 1st day ($P < 0.0001$). The survival rate was shown as mean ± SE. Values followed by different letters were significantly different ($P < 0.05$) by using repeated measures analysis of variance (ANOVA). Tukey-Kramer tests were applied to determine significant differences of all possible pairwise comparisons.

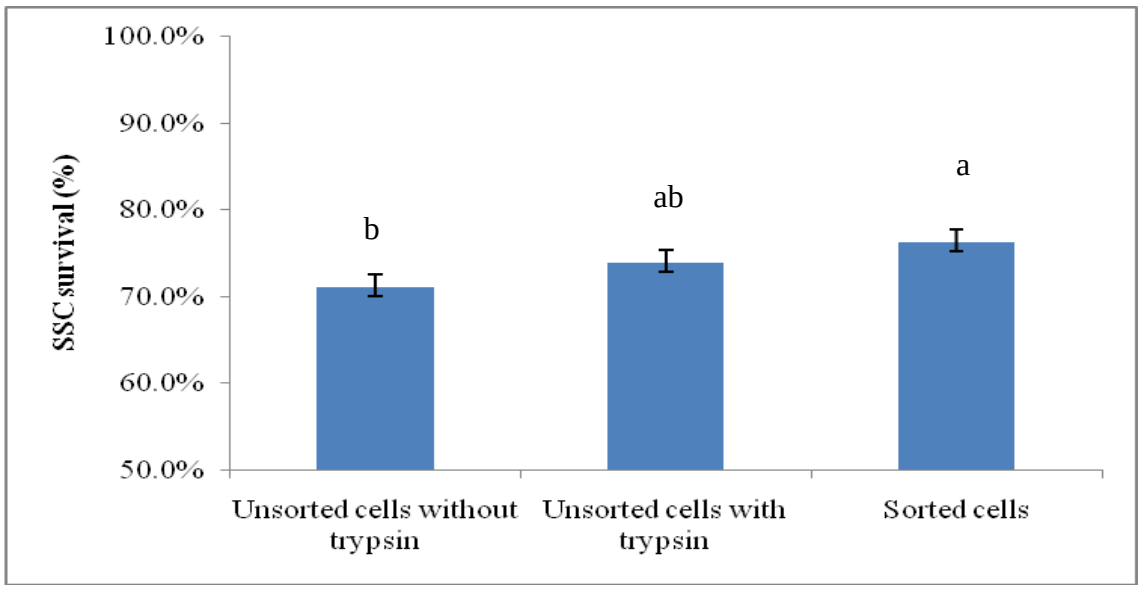

Fig. 12. Mean survival rates of spermatogonial type A stem cells (SSCs) dissociated from testes of blue catfish, *Ictalurus furcatus*, males without trypsin Ethylenediamine tetraacetic acid (EDTA) 0.25% (unsorted cells without trypsin); with trypsin-EDTA 0.25% (unsorted stem cells with Trypsin); with trypsin-EDTA 0.25% and enrichment with discontinuous gradient density separation (sorted cells) cultured *in vitro* for 6 days. The average number of SSCs per well *in vitro* cultured was 4.5-4.7×10^4 for treatment of unsorted cells without trypsin, 5.2-5.6×10^4 for treatment of unsorted cells with trypsin, and 4.5-4.8×10^4 for treatment of sorted cells. The mean survival rates of unsorted SSCs without trypsin, unsorted OSCs with trypsin treatment, and sorted OSCs was 71.1%, 73.9%, and 76.3%, respectively, in which the survival rate of sorted SSCs was significantly higher than the rate of unsorted SSCs without trypsin. The survival rates were shown as mean ± SE.The differences in survival rates of three treatments of stem cells ($P < 0.05$) were analyzed with a repeated measures analysis of varinace (ANOVA). Tukey-Kramer tests were also applied to determine differences of all possible pairwise comparisons.

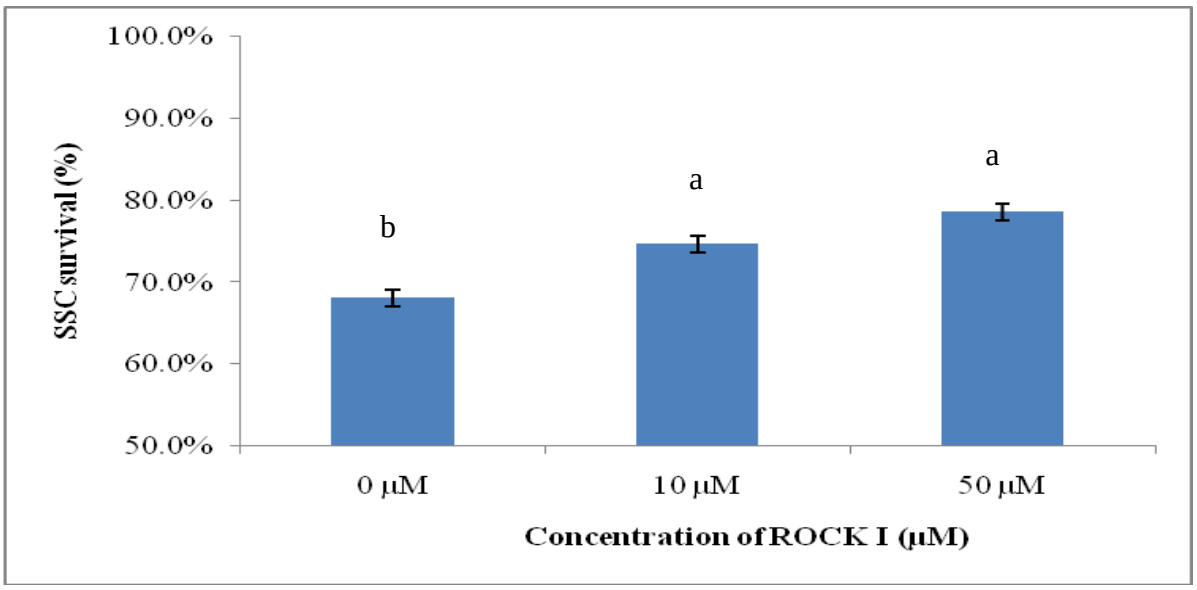

Fig. 13. Mean survival rates of spermatogonial type A stem cells (SSCs) dissociated from testes of blue catfish, *Ictalurus furcatus*, males cultured *in vitro* in 6-well plates and supplemented with Rho protein kinase inhibitor (ROCK I) at 0, 10 and 50 µM for 6 days. The mean survival rate of SSCs during the 6-day culture period supplemented with ROCK I at 0, 10, and 50 µM was 68.0, 74.6, and 78.6%, respectively. Overall, the survival rates of SSCs supplement with ROCK I 50 µM in the cell culture medium were significantly greater than the survival rates of SSCs cultured without ROCK I ($P < 0.0001$). The survival rates were shown as mean ± SE. The differences in survival rates of SSCs supplemented with ROCK I ($P < 0.05$) were analyzed with a repeated measures analysis of varinace (ANOVA). Tukey-Kramer tests were also applied to determine differences of all possible pairwise comparisons.

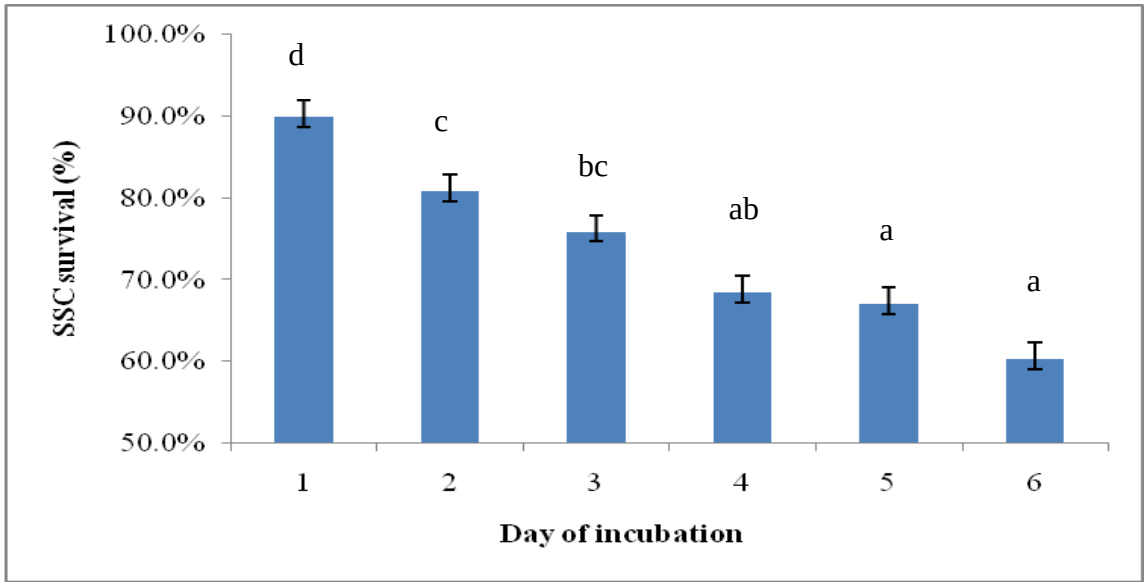

Fig.14. Cumulative survival rates of spermatogonial type A stem cells (SSCs) dissociated from testes of blue catfish, *Ictalurus furcatus*, males cultured *in vitro* in 6-well plates for 6 days. The SSCs had the significantly highest survival rate on the 1st day after being dissociated from gonadal tissue (90.0%). There was a decrease in the survival rates of SSCs after the 1st day ($P<0.0001$). The survival rates were shown as mean ± SE. Values followed by different letters were significantly different ($P < 0.05$) analyzed with repeated measures analysis of variance (ANOVA). Tukey-Kramer tests were applied to determine significant differences of all possible pairwise comparisons.

Discussion

Spermatogonial type A stem cells (SSCs) and oogonial stem cells (OSCs) from blue catfish, *Ictalurus furcatus*, were successfully cultured *in vitro* for transplantation. Approximately 60% of SSCs or OSCs were alive after 6 days of culture. The highest percentages (90.0 and 84.0%) of viable SSCs and OSCs were seen in the first day after being dissociated from the gonadal tissues. Then, there was a decrease in the survival rates of the SSCs and OSCs after the first day. Supplement of ROCK I at 10 or 50 µM in the culture medium of SSCs and OSCs yielded the significantly higher percentages of viable cells compared to those cells cultured without ROCK I. The numbers of SSCs and OSCs dissocated with Trypsin-EDTA 0.25% were higher than the numbers dissociated without trypsin-EDTA 0.25%. Use of discontinuous gradient density to sort the SSCs and OSCs resulted in 30.3-37.5% less cells compared to the unsorted SSCs or OSCs digested with trypsin-EDTA 0.25%.

In our experiments, we derived the SSCs and OSCs from the 2 year-old blue catfish males and females. According to Shang (2013), testes from young blue catfish males (3 months old) only contained undifferentiated spermatogonia and Sertoli cells. At 2 years of age, the testes have villiform lobed structures containing mostly spermatogonia type A and B stem cells and spermatocytes. In adult males, spermatids, spermatozoa and a very small percentage of spermatogonial stem cells were found in the basement membrane of spermatogenic cysts. Lacerda et al. (2014) also found similar results in the testes of adult tilapia. In terms of blue catfish females, ovaries of 3-month females contain oogonia and early-stage primary oocytes. In

2-year-old blue catfish females, the oogonial nests were surrounded by a monolayer of granulose cells filled with lumina. The number of oogonial stem cells was low as compared to other germ cells such as primary and secondary oocytes. A very low number of oogonia were found while most immature eggs were seen in mature blue catfish females. Although the percentages of SSCs and OSCs in juvenile blue catfish are smaller than the percentage in very young blue catfish, the gonads are much larger and yield a much higher number of the desired SSCs or OSCs. Obviously, adult blue catfish had the largest testes or ovaries, but the densities of oogonial and spermatogonial stem cells are really low and extraction SSCs/OSCs from gonadal tissues of adult catfish require more effort to complete. Thus, juvenile blue catfish are the best choice for stem cell isolation and culture.

In fish, the SSCs have been cultured *in vitro* and used as a valuable tool in both basic and applied biology. The testicular stem cells of medaka were studied *in vitro* under experimental conditions, remaining viable and exhibiting cell type specific properties such as cell proliferation and differentiation (Song and Gutzeit, 2003). Under *in vitro* culture, the transformation from spermatogonia to spermatozoa have been reported in eel, zebrafish, tilapia, medaka, and rainbow trout (Miura et al., 1991; Sakai, 2002; Saiki et al., 1997; Shikina et al., 2008; Tokalov and Gutzeit, 2005). In our study, SSCs were seen to be alive until the 6th day, but the number of alive SSCs declined during the experimental period. Shang (2013) also reported that live dissociated testicular germ cells of blue catfish could be seen after 16 days of in vitro culture, but they did not display proliferation and attachment. In addition to SSCs, OSCs have been also used for germ cell transplantation in rainbow trout (Yoshizaki et al., 2010), zebrafish (Wong et al., 2011), and Siberian sturgeon, *Acipenser baerii*, (Psenicka et al. 2015). Trout and zebrafish oogonia had sexual plasticity after transplantation and differentiation into both male and female

gametes. The OSCs cultured *in vitro* have been reported in mice (Pacchiarotti et al., 2010; Lu et al., 2016) and human (White et al., 2012; Dunlop et al., 2014), but there have been no studies of *in vitro* culture reported in fish. In this study, blue catfish oogonial stem cells isolated and cultured *in vitro* in cell culture medium supplemented with ROCK I for a period of 6 days The result showed that around 60% of OSCs were ailve after 6 days. Cell death could be a result of several factors. Necrosis, passive cell death, is the result of sudden and severe environmental stress, causing cell swelling, and eventually cell lysis. Apoptosis, programmed cell death can be triggered by external and internal factors, leading to cell shrinkage, increased cytosolic concentration of calcium cation, and plasma membrane blebbing. Anoikis, a type of apoptosis induced by inappropriate cell-cell or cell-extracellular matrix interactions as the cells were isolated individually for gene transfer or during differentiation procedures in suspension culture conditions (Frisch and Screaton, 2001; Koyanagi et al., 2008). Shikina et al. (2008) suggested that the over-growth of somatic cells in cell culture inhibited the survival and proliferation of spermatogonia. Therefore, purified spermatogonia culture enhanced survival and proliferation of SSCs in rainbow trout. This same result was found in the current experiment when enriched SSCs or OSCs tended to have higher survivals than the unsorted stem cell solutions. In addition, stem cells could not be maintained *in vitro* for extended time periods because they rapidly differentiated or died (Glettig and Kaplan, 2013). This may be the reason leads to the decrease of SSCs and OSCs in the cultured populations.

Enzymatic digestion is commonly used for tissue dissociation. Trypsin, a digestive serine protease, is extensively used in protein sequencing and proteomic applications. Most of cells tolerate short exposure to trypsin solution. Other enzymes such as collagenase, dispase and actinase E also can be used for germ cell isolation. Higaki et al. (2010) used 0.1% trypsin, 0.1%

actinase E, and 0.4% urea to digest zebrafish embryos. Lacerda et al. (2013) used 0.03% DNase and trypsin 0.25% to isolate spermatogonial stem cells from Nile tilapia testes. Panda et al. (2011) used collagenase 500 units/ml to digest the testicular tissue from carp, *Labeo rohita*. The type of enzyme and time of digestion vary among the studies and these differences could have an influence on cell properties (Bourin et al., 2013). To overcome this obstacle, a method to isolate stem cells without enzymatic digestion was proposed (Busser et al., 2014). The results from these authors showed that cells obtained from isolation without enzymatic digestion were equivalent to the cells isolated with enzyme, but the cells without enzymatic digestion had better long term hematopoietic support than those with enzymatic digestion. Moreover, enzymatic-free digestion method is easier, safer, faster, and less expensive compared to enzymatic digestion. Overall, enzymatic digestion can induce cell injury and alter cell functions (Dean et al., 2005).

In this experiment, blue catfish SSCs and OSCs isolated without trypsin had similar percentages of viable cells as compared to the percentages of viable cells isolated with trypsin. However, the number of OSCs and SSCs dissociated from gonadal tissues without enzymatic digestion was 34.8 and 38.5% less than the number of cells dissociated with enzyme (trypsin-EDTA 0.25%). Shang (2013) also conducted an experiment to evaluate the digestive efficiency of trypsin –EDTA at 0.25% and 0.05% on testicular tissue of blue catfish. The results from her experiment indicated that blue catfish testes digested with a higher concentration of trypsin-EDTA yielded higher germ cells but cell viability was lower, but this lower viability was counteracted by the much higher number of cells derived from trypsin at 0.25%.

ROCKs are important regulators of cellular growth, migration, metabolism, and apoptosis through control of the actin cytoskeletal assembly and cell contraction. During apoptosis, cells undergo significant morphological changes including contraction, dynamic membrane blebbing,

and nuclear disintegration. Culturing embryonic stem cells *in vitro* has proven to be technically challenging, especially for human ESC when attempting to isolate individual cell clones in suspension culture conditions because of the high rate of anoikis-induced cell death (Koyanagi et al., 2008). Recently, ROCK I was reported to be protective against anoikis in dissociated human ESC (Watanabe et al., 2007) and mouse ESC–derived neural precursors (Koyanagi et al., 2008). In our experiments, significantly more viable SSCs/OSCs were seen in the suspensions incubated with a high concentration of ROCK I. The results showed that ROCK I can help to protect fish stem cells from anoikis in dissociated suspension.

Previous research found that ROCK I could be effective in promoting mesenchymal stem cell differentiation (Pacary et al., 2006). ROCK I at a concentration of 10 µM was applied to improve cell recovery of human embryonic stem cells after fluorescence-activated cell sorting (Emre et al., 2010). The addition of ROCK I 10µM to both freezing medium and post-thawing medium exhibited an additive effect to enhance the efficiency of colony formation. Treatment with ROCK I during cryopreservation significantly increased the survival rate and cell adhesion of free-thawed dissociated hESCs (Classen, 2009).

In the current experiment, application of 10 µM of ROCK I resulted in the higher viability of blue catfish SSCs or OSCs as compared with those stem cells that were not supplemented with ROCK I. An increase to 50 µM ROCK I led to a higher survival percentage of OSCs, and survival rate of SSCs incubated with 50 µM trended toward a viability than stem cells supplemented with 10 µM.

In conclusion, survival rates of SSCs and OSCs dissociated from the testes and ovaries of blue catfish with trypsin-EDTA 0.25% were similar to the rates of SSCs or OSCs dissociated

without trypsin but the numbers of SSCs and OSCs dissociated with trypsin were higher, so trypsin-EDTA 0.25% should be used to dissociate gonadal stem cells (SSCs and OSCs) from the gonadal tissues of donor fish. The SSCs and OSCs separated with Percoll tended to yield the higher viable cells but the total number of cells decreased by 30.3-37.5% after centrifugation. ROCK I (Y-27632) at 10 or 50 µM added in the cell culture medium reduced mortality rates of stem cells, so ROCK I should be supplemented in the cell culture medium before culturing them or transplanting them into recipients. The SSCs or OSCs had the highest survival rates in the first day after being dissociated from testes or ovaries. Thus, they should be used to transplant into the recipients immediately after being dissocated from the gonadal tissues to achieve the highest rate of viable cells and enhance ability of stem cell colonization. In the future, higher concentrations of ROCK I need to be tested to see if viability of stem cells can be further improved. To achieve the maximum amount of stem cells after being sorted, other methods to separate stem cells need to be investigated and tested. The incubation time needs to be extended to see how long the stem cells can survive and when they can begin to proliferate in cultured conditions.

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CHAPTER FOUR

Production of xenogenic catfish by transplanting type A spermatogonial stem cells of channel catfish, *Ictalurus punctatus*, into newly hatched white catfish, *Ameiurus catus*, triploid fry

Abstract

A factorial experiment was designed to test the rate of xenogenesis in white catfish fry, *Ameiurus catus*. Newly fertilized eggs were pressurized at 6,500 or 7,000 psi to produce triploid embryos that were transplanted with two types of type A spermatogonial stem cells (SSCs), sorted and unsorted after hatching. The SSCs were isolated from testes of channel catfish, *Ictalurus punctatus*, with a mean body weight of 332.4±164.2 g and a mean testes weight of 0.67 ± 0.42 g. A mean of 14,639 and 15,680 sorted cells were transplanted into the abdominal cavity of newly hatched white catfish pressurized earlier at 6,500 or 7,000 psi, respectively, while the mean number of unsorted SSCs transplanted into the abdominal cavity of white catfish fry pressurized earlier at 6,500 or 7000 psi were 37,104 and 41,328 cells, respectively. Twenty-eight days after transplantation, gonadal regions from the white catfish fry were dissected using laser capture microdissection (LMD). The DNA analysis from the gonads showed that 41.2% of the white catfish fry from all treatments were xenogenic, possessing cells from channel catfish. The xenogenic rates of white catfish shocked with two levels of hydrostatic pressure (6,500 and 7,000

psi) were not different (P = 0.96). However, the xenogenic rate (47.7%) of triploid white catfish transplanted with unsorted SSCs was higher than the rate (34.9%) of triploid white catfish transplanted with sorted SSCs (P = 0.04). This is the first report of successful xenogenesis for white catfish transplanted with channel catfish stem cells, which demonstrates future potential for production of hybrid catfish and other applications.

Keywords: White catfish, *Ameiurus catus*, xenogenic catfish, laser microdissection (LMD), triploid, unsorted and sorted type A spermatogonial stem cells (SSCs).

Introduction

Advantages of hybrid catfish in aquaculture

The hybrid catfish from mating of female channel catfish, *Ictalurus punctatus*, and male blue catfish, *I. furcatus* is widely used by US catfish producers as it is the best genetic type of catfish for pond culture because of its desirable characteristics such as overdominant growth in earthen ponds (Argue et al., 2003; Brown et al., 2011; Dunham and Brummet, 1999; Goudie et al., 1995; and Li et al., 2004), higher survival rate (Chatakondi et al., 2000; Dunham et al., 1990), better disease resistance (Arias et al. 2012; Wolter et al. 1996), higher dress-out and fillet percentages (Argue et al., 2003), easier catch by seines, hooks and lines (Chappell, 1979; Dunham et al., 1986; Dunham and Argue, 1998; Dunham and Masser, 2012; Tave et al., 1981; Yant et al., 1976). However, the two species seldom mate in the natural environment because of spawning behavior incompatibility (Dunham and Masser, 2012). Production of hybrid catfish by inducing ovulation of channel females with hormones, hand stripping ovulated eggs and artificially fertilizing with blue sperm is the most productive and consistent technique for commercially making the hybrids. However, this technique is labor intensive and requires sacrifice of mature blue catfish males. Xenogenesis, a method of reproduction in which successive generations differ from each other, may be a potential tool to address these problems.

White catfish production

White catfish have advantages of short maturation time and small handling size, making them potentially amenable as a model for genetics research as well as practical applications.

According to Dunham and Smintherman (1981) and Fobes (2013), white catfish can become mature in 1-2 years compared to 2-4 and 4-6 years for channel catfish and blue catfish, respectively. White catfish begin to reach sexual maturity as early as 152 mm in fork length in northern California populations (Borgeson and Mc Cammon, 1967) and up to 230 mm total length in New Jersey populations (Keller, 2010). Spawning of white catfish could be observed in late May when the temperature of water ranges 19-21 ^{o}C (Wang, 2010). Few studies address artificial spawning of this species. Fobes (2013) reported that using luteinizing hormone-releasing hormone analog (LHRHa) implants at 90µg/kg in domestic white catfish resulted in 100% ovulation of the females after 72 h. Carp pituitary extraction (CPE) injections (primary dose of 2 mg/kg and resolving dose of 8 mg/kg) yielded 28.6% ovulation of wild white catfish females. Interruption of water flow might play an important role in the spawning of white catfish as 75% of domestic white catfish injected with CPE ovulated in static water, while no white catfish injected with LHRHa and constant water flow. White catfish grow rapidly as fingerlings, but their growth is slow when they begin sexually mature at 1 year old. The white catfish are less resistant to bacterial diseases, have poor dressing percentage, more difficult to catch with seines compared to channel catfish (Dunham and Smitherman, 1984).

Triploid induction

Triploids are sterile because of the irregular meiotic division of chromosomes, resulting in repressed gonadal development, and no production of viable gametes (Piferrer et al., 2009). Thus, triploid fish have been used as recipients for germ cell transplantation, producing only donor-derived offspring (Okutsu et al. 2007; Yoshizaki et al. 2016). Among methods for producing triploidy, hydrostatic pressure produces more consistent results, survival of treated eggs, and percent triploidy than thermal and chemical (Bury, 1989; Dogankaya and Bekcan,

2014; Rottmann et al., 1991). This technique is applied to make triploid rainbow trout, *Oncorhynchus mykiss*, grass carp, *Ctenopharygodon idella*, common carp, *Cyprinus carpio*, bighead carp, *Hypophthalmichthys nobilis*, loach, *Misgurnus fossilis*, zebrafish , *Danio rerio*, channel catfish, *Ictalurus punctatus*, and yellowtail flounder, *Pleuronectes ferrugineus*.

Germ cell transplantation

Xenogenesis is a potential alternative for making hybrids. The first steps in the process are very labor intensive, but long-term the need to sacrifice blue catfish is eliminated and theoretically, xenogenic brood stock should be easy to spawn and can be used for multiple years.

In fish, three types of cells have been isolated and introduced into the fish hosts including primordial germ cells (PGCs) (Lin et al., 1992; Saito et al., 2010; Takeuchi et al., 2003), spermatogonial stem cells (SSCs) (Lacerda et al., 2006; Okutsu et al., 2006; Perera et al., 2017), and oogonial stem cells (OSCs) (Wong et al., 2011; Yoshizaki et al., 2010). Xenogenic fish can be produced by transplanting germline stem cells into three different life stages of recipients. Transplantation of PGCs into blastula stage fish embryos *via* micromanipulation is the first approach to produce xenogenic fish (Ciruna et al., 2002; Giraldez, 2005). Saito et al. (2008, 2010) also carried out a study by using a single PGC from pearl danio, loach, or goldfish microinjected into the blastodisc of zebrafish to generate xenogenic males that produced 100% gametes of the donors.

The second technique to produce xenogenic fish is transplantation of isolated PGCs or SSCs from donors into recipient fry at the newly hatched stage. Since the newly hatched fry do not have a functional immune system, injection at this stage can avoid immune rejection of exogenous germ cells (Lacerda et al., 2012; Yoshizaki et al. 2005). Takeuchi et al. (2003)

injected PGCs into triploid newly hatched embryos and investigated migration and colonization of donor stem cells in the genital bridge of recipient embryos. The PGCs proliferated and matured into eggs and sperm in the gonads, which resulted in normal offspring with the donor-derived phenotype. Isolated SSCs from transgenic adult male rainbow trout expressing green fluorescent protein (GFP) driven by the *vasa* promoter were injected into peritoneal cavities of newly hatched fry. After the recipients reached maturation, donor SSCs differentiated into functional spermatozoa in males and eggs in females. Fertilization of these spermatozoa and eggs produced normal rainbow trout offspring, indicating SSCs possess a high level of sexual plasticity (Okutsu et al., 2006). Andriani (2012) studied transplantation of isolated SSCs held at 4 ^{o}C from giant gourami, *Osphronemus goramy,* into tilapia at 3-day post hatch. The results from this study showed that SSCs were able to colonize and develop inside the newly hatched recipients. The technique of injection at newly hatched fry was also applied to marine species such as nibe croaker, *Nibea mitsukurii* (Higuchi et al., 2011), yellowtail mackerel, *Trachurus novaezelandiae (*Morita et al. 2012), and grass puffer, *Takifugu niphobles* (Hamasaki et al., 2017).

The third technique to produce xenogenic fish has been accomplished by introducing immature SSCs in Nile tilapia, *Oreochromis niloticus*, through the urogenital papilla of adult fish. These adult fish were treated with busulfan at high temperature (35 ^{o}C) to deplete endogenous spermatogonial development. Donor stem cells were labeled with fluorescent lipophilic dye PKH26-GL, transplanted into the adult testes of the recipients, and checked at 14 h, 2 weeks, 4 weeks post-transplantation. The results showed that PKH26 labeled stem cells cysts were presented in the seminiferous tubules of the recipients. Majhi et al. (2009) transplanted SSCs from the pejerrey, *Odontesthes bonariensis*, into the gonads of sexually

mature Pantagonian pejerrey, *O. hacheri*, that had been partially depleted of endogenous SSCs with a combination of busulfan and high water temperature. Mating between transplanted *O. hacheri* males with normal *O. hacheri* females produced 12% hybrid fry. Nobrega et al. (2010) also examined the spermatogonial transplantation in both male and female adult recipients to assess the presence, biological activity, and plasticity of spermatogonial stem cells in zebrafish. Donor-derived spermatogenic cysts were found at different phases of spermatogenesis along the seminiferous epithelium of the recipients after 3 weeks of transplantation. Perera et al. (2017) transplanted spermatogonial stem cells type A isolated from blue catfish into gonads of triploid channel catfish via catheterization and surgery. After 2 years of transplantation, three of seven injected males were confirmed xenogenic. Sperms were collected from one of the three xenogenic channel catfish and used to fertilize normal channel catfish females resulting in 100% hybrids, although only one percent of fertilized eggs hatched and seven 6- month-old progeny survived.

Laser microdissection (LMD) and its applications

Laser capture microdissection (LMD) is a technology for isolating the pure cell population from histological specimens, living cells and cell cultures, plant material, formalin fixed paraffin embedded or fresh frozen and stained or unstained tissues, precisely targeting and capturing the cells of interest for a wide range of downstream assays (Liu, 2010). Optimal laser capture microdissection is achieved with tissue sections cut around 5-15 µm thick. Tissue sections thinner than 5 µm may not provide full cell thickness and sections greater than 15µm may not microdissect completely. Infrared and ultraviolet are two general classes of LMD system (Vandewoestyne et al., 2013). LMD is based on an inverted light microscope fitted with a laser device to facilitate the visualization and procurement of cells. The platform consists of an

inverted microscope, a solid state near-infrared laser diode, a laser control unit, a joystick controlled microscope stage, a camera and a color monitor (Fend and Raffeld, 2000). This system is based on the placement of a thin transparent thermoplastic film over a tissue section. Cells of interest are selectively adhered to the film with a fixed position, short duration, focused pulse from an infrared laser (Emmert-Buck et al., 1996). Adherence of cells to film exceeds adherence to the glass slide, allowing removal of cells of interest. Cells are removed by lifting of the film and transferring to a microcentrifuge tube containing buffer solution required for isolation of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) (Curran et al., 2000; Goldstein et al., 1998). Ultraviolet LMD uses tissues that have been mounted on a membrane and placed on a glass side on which the operator directs an ultraviolet (UV) laser beam under direct visualization (Gjerdrum et al., 2001). The narrow beam UV laser is used to draw around the cells, leaving the desired cell population intact while simultaneously removing unwanted tissue. This method can avoid any complicated operator and nonspecific adherence of tissue to the cap as well. LMD is very fast and does not destroy adjacent tissues (Bonner et al., 1997).

An issue encountered in LMD is failure to remove the selected region from the slide, resulting from lack of cell adherence to the membrane because of incomplete tissue dehydration or a low laser beam. Another problem of the LMD approach is the process of preparation of samples to obtain a balance between good tissue morphology and the integrity of biomolecules. The amount and degradation of biomolecules can affect the results, especially, in gene expression analysis. RNA is extremely susceptible to degradation by RNase from the tissue and the environment (Gallego-Romero et al., 2014; Opitz et al., 2010). Similarly, DNA has the same issue, although degradation is much less problematic (Bustin et al., 2009). Damaged DNA due to a long series of chemical, physical and enzymatic treatments can make it difficult or impossible

to interpret the results. In short, analyzing the genome and its expression through biomolecules such as DNA, and RNA from isolated cells requires optimization of LMD protocol for each tissue or cell type including sample preparation, staining, dehydration, and microdissection parameters. The procedure widely used to obtain the best morphology is formalin fixation followed by paraffin coating. The tissue fixed has good discriminating tissue morphology compatible with a wide range of staining and long-term preservation protocols. However, the ability to analyze nucleic acids and proteins with this method is limited due to nucleic acid fragmentation and amino acid changes (Masuda et al., 1999).

Some interesting applications of LMD were demonstrated by Simone et al. (2000) who utilized LMD to microdissect populations of normal prostate epithelial cells, cells from prostate intraepithelial neoplasia and invasive prostate adenocarcinoma cells. Zheng et al. (2003) reported a putative marker for prostate cancer using microdissected benign and malignant epithelial cells. Johann et al. (2009) used the LMD technique to examine solid tumor heterogeneity on a cellular basis using tissue proteomics.

The objectives of this experiment were to determine the best pressure levels to produce the triploid white catfish, the appropriate cell types to transplant into the triploid hosts, to develop a protocol using LMD to dissect the cells of interest from young fry and to produce channel catfish female x blue catfish male hybrid catfish by mating the xenogenic white catfish together. Ultimately, white catfish females transplanted with channel catfish SSCs will be paired with white catfish males that received blue catfish SSCs to produce the hybrids. If successful, this technology can help eliminate obstacles of current hybrid production as well as accelerate genetics research.

Materials and Methods

Triploid white catfish production

Gravid white catfish females were induced to ovulate by using carp pituitary extract (CPE, Stoller Fisheries USA, Spirit Lake, IA) or luteinizing hormone releasing hormone (LHRHa). CPE was used to induce ovulation of the white catfish females with two injections (the primary injection of 2 mg/kg and resolving injection of 5 mg/kg body weight). LHRHa was implanted in the females at 100 µg/kg body weight. The females were then placed in overhead aquaria with or without males. Thirty-six hours after receiving the resolving dose, the females received the resolving dose were checked for ovulation. When several eggs were seen on the bottom of the aquaria, the ovulating females were collected and placed in 100 ppm of tricaine methanesulfonate (MS-222, Ferndale, WA) for 5 min until movement of fish opercula was ceased. Anesthetized females were quickly rinsed in freshwater and carefully dried with clean towels before the eggs being hand stripped into a spawning pan. Each pan contained 25 g of eggs that were fertilized with 1 mL of white catfish sperm solution. Fuller's Earth solution (MP Biomedicals, Santa Ana, CA) was added to the spawning pan containing sperm and eggs to activate the fertilization process and to prevent adhesion of the eggs. Fuller's Earth solution was prepared by adding 6 g of powdered Fuller's Earth to 1 L of pond water at 27 ^{o}C. After 3min, the fertilized eggs were transferred into a steel round chamber (34 cm height, 7 cm diameter) and after an additional 2 min hydrostatic pressurize was applied at 6,500 to 7,000 psi for 5 min at 27 ^{o}C. After pressure shock, the eggs were removed from the chamber and incubated in baskets

placed in flow-through hatching troughs fitted with paddlewheel for 9-13 days at 22.4-25.3 ^{o}C until they hatched. Blood samples of triploid white catfish were verified with a Coulter counter to measure erythrocyte nuclei volume (Beck and Biggers, 1983).

Isolation of type A spermatogonial stem cells from donor channel catfish

Sexually immature, male channel catfish (mean body weight 332.4±164.2 g; mean testes weight 0.67±0.42 g) were collected and euthanized with 300 ppm MS-222 for 10 min until opercular movement ceased. Then, they were washed with tap water and placed on ice before dissection. The external surface of the donor fish was sterilized with 70% ethyl alcohol (Pharmco-Aaper, Shelbyville, KY). Total weight was recorded before an incision was made on the abdomen of the fish. Digestive organs were removed and testes were carefully collected to avoid connective tissues (peritoneum or blood vessels). The testes from individual fish were weighed and washed in 0.5% bleach solution for 1 to 2 min before being placed in a petri dish containing 5 mL of anti-agent medium (Hank's Balanced Salt Solution (Corning Cellgro, Manassas, VA) with 1µg/mL $NaHCO_3$ (Sigma, St. Louis, MO), and 100 unit/mL penicillin and 100 µg/mL streptomycin (Gibco, Grand Island, NY)). Under a biosafety cabinet (The Baker Company, Sanford, Maine), connective tissues and blood vessels were discarded. The testes were washed three times with phosphate-buffered saline (PBS) 10X (Lonza, Walkersville, MD) and three times with anti-agent medium before being minced with two sterilized blades. Minced tissues were transferred to autoclaved flasks containing a magnetic bar and 0.25% trypsin-EDTA (Gibco, Grand Island, NY) with a ratio of 1 unit volume of testes: 15 volumes of trypsin-EDTA 0.25%. The flasks were incubated on ice for 30 min followed by 40-60 min at room temperature on a stirrer (Corning, Manassas, VA). The suspension was filtered with a 40-µm strainer (Cat. no. 10199-654, VWR, Radnor, PA) and centrifuged at 500g for 10 min (Sorvall RT6000). After

centrifugation, the supernatant was discarded and the pellets were resuspended with 2mL cell culture medium (Dulbecco's Modified Eagle Media (Sigma, St. Louis, MO), with 10% ES Cell FBS (Gibco, Grand Island, NY), 1% glutamine (Gibco, Grand Island, NY), ND 1% 100 U penicillin/0.1mg/mL streptomycin (Gibco, Grand Island, NY) and 50 µM ROCK Inhibitor (Stem Cell Technology, Vancouver BC, Canada).

The number of spermatogonia type A or total cells was counted under a microscope 40X (Olympus, Center Valley, PA) using a hemocytometer (Reichert, Buffalo, NY). This suspension was used as freshly unsorted stem cells solution for transplantation. For the purpose of sorting cells, discontinuous density gradient Percoll (GE Healthcare, Uppsala, Sweden) was used to enrich type A spermatogonial stem cells. Volumes of 2mL each of Percoll 35, 45, and 70% were transferred into a 15 mL centrifuge tube. A volume of 2 mL of unsorted stem cell solution was added on top of Percoll 35% and centrifuged at 800 g for 40 min. The first layer, which contains the highest percentage of type A spermatogonial stem cells (Shang et al. 2015), was collected after centrifugation. The sorted cells were centrifuged at 500 g for 10 min and re-suspended with cell culture medium containing 50 µM rho protein kinase inhibitor (ROCK I).

Stem cell implantation into the triploid white catfish host

The newly hatched triploid fry (7.0±0.4 mm) were anesthetized by placing them in a 10 mg/L MS-222 buffered with 10 mg/L sodium bicarbonate solution. Anesthetized fry were placed in the petri dish and observed microscopically at 1.5X (Amscope, Irvine, CA). Each fry was injected with 1 µL sorted cell suspension containing 14,639-15,680 cells (9,032-9,063 SSCs) or unsorted cell suspension containing 37,104-41,328 cells (10,611 - 10,621 SSCs) through a 33-gauge needle with a gastight syringe attached to a repeater (Hamilton, Reno, NV). Fry were laid

on their side and the needle was gently inserted in the cavity between anal fin and yolk sac where the genital ridge is expected to be formed. After injection, the fry were placed in a recovery container that was aerated and then stocked back in the troughs.

Laser microdissection used to isolate gonadal tissues from transplanted white catfish

Twenty eight-day post injection white catfish fry were collected and freshly fixed in tissue fixative solution (Sakura, Torrance, CA) for 10 days before being further processed. After 10 days, heads and tails of those fry were removed while the midsection was placed back in the tissue fixative solution. Then, each midsection was separately transferred into an embedding ring and placed in 70% ethyl alcohol before moving the rings to an automated embedding machine (Autotechnicon). The embedding rings were processed by the machine for 16 h before the tissues were taken out of the rings and placed in molds. Samples were processed with 12 steps by moving them into different baths. The tissues were firstly dipped in 70% ethyl alcohol for 1.5 h. Then they were moved to 80% ethyl alcohol for 2 h. Next, the tissues were dipped in three different baths of 95% ethyl alcohol for 3 more h before being transferred to three different baths of 100% ethyl alcohol. Then tissues were twice washed with Hemo-De (Hemo-De) for 2 h. Finally, they were placed in warm Paraplast Plus (Mc Cormick) for 3.5 h. For general dehydration, standard graded series of alcohols (70, 80, 95, and 100%) were used to dehydrate tissue to prevent shrinkage or distortion of the cells. To ensure complete removal of water during dehydration, two changes of 100% ethyl alcohol of 1 h each were used. Hemo-D, a clearing agent, was used to clean the samples and served as a transition medium between two immiscible compounds, ethanol and paraffin. Then the samples were submerged in warm liquid paraffin for 3.5 h for infiltration.

The midsections of the fry were transferred to molds and filled with warm liquid Paraplast Plus. After the machine completed its processes, the paraffin-embedded tissues were sectioned with a microtome (American Optical) at 10 µm thickness and attached to MMI membrane slides (Molecular Machine and Industries). For purposes of DNA extraction, at least 25 sections of catfish tissue were needed. The slides were placed in the dryer for approximately 45-60 min at 42 ^{o}C. Then, they were deparaffinized and stained by placing them in the first container of Hemo-De (four min) before being moved to the second container of Hemo-De for three more min. These slides were also immersed several times to fast remove Paraplast Plus. Then, the slides were placed in 100% ethyl alcohol for 4 min, followed by 70% ethyl alcohol container for 2 min before being washed twice with RNase-free water until Hemo-De and ethyl alcohol disappeared. The slides were stained with MMI staining kit (hematoxilyn) for 1-2 min and placed the slides in 70% ethyl alcohol for 30-60 s until the stain began to clear. Then, they were moved to a container of 100% ethyl alcohol for 30 s. Finally, they were removed from 100% ethyl alcohol container and allowed to air dry for approximately 20 min before being dissected with the LMD machine.

Protocol for LMD process

Protocol for using laser microdissection to isolate the tissues were debcribed by Espina et al. (2006). First, the computer tower was turned on, followed by the computer screen and lamp. The computer was then allowed to boot up fully before turning on the laser and camera box. An orange and green light appeared when the key was turned followed by pressing a button on the blue laser and camera power box. Then, MMI software on the computer screen was started by double-clicking on the MMI software icon on the computer screen. Once the program was opened, the top left corner of the screen was checked to make sure the program was recognizing

the laser and camera. The MMI membrane slide was placed on a regular glass slide with a trough side up before securing both slides on the stage. Then, an MMI Isolation Cap tube was placed in the cap holder. The microscope and software objective was set to 4X and button in the right panel with a winding arrow was activated to map the slide. Next, the microscope and software were set to 20X, followed by moving the field of view to an empty area. Laser was fired 2-3 times by clicking the laser button in the right panel before locating the spot was where the laser fired. Under the UV Cut tab and laser tab, laser position set was clicked to calibrate the laser. To dissect tissues, region of interest was identified. A stylus was used to draw around the region of interest. When drawing was finished, the cap was lowered by hitting the arrow in the right panel. Finally, the cut button was clicked to fire the laser and excise the region of interest. When laser finished, the cap was raised by hitting the arrow button. The region of interest was now isolated on the MMI isolation cap. This process can be repeated as needed. The cap should be replaced when it is fully covered in tissues and when used to isolate different regions of interest.

Extraction of DNA from gonadal tissue samples

The excised gonad tissues were picked up with the MMI Cap tube and kept at -80°C until DNA was extracted. DNA from each tissue was extracted using proteinase K digestion followed by a standard protocol for protein precipitation and ethanol precipitation. The only difference was that with LMD samples, 5µL of the DNA extraction in the PCR tubes was used instead of the normal volume of 0.7 µL to get enough DNA. DNA from diploid channel catfish and white catfish were also extracted as controls.

Polymerase chain reaction (PCR) technique

Genes evaluated were follistatin (*Fst*), and hepcidin antimicrobial protein (*Hamp*) for channel catfish and white catfish. PCR reactions were prepared in 7.2 µL volume containing 10-300 ng genomic DNA in 6 µL of PCR Master Mix (proprietary reaction buffer [pH: 8.5], 400 µM dATP, 400 µM dGTP, 400 µM dTTP, and 3 mM $MgCl_2$), 0.4 µL Forward *Hamp* Primer, 0.4 µL Reverse *Hamp* Primer, 0.2 µL Forward *Fst* Primer, and 0.2 µL Reverse *Fst* Primer. The *Fst* and *Hamp* amplification products were analyzed on a Tris Borate EDTA 2.0% agarose gel. Amplicon size was measured by Tracklt™ 100 bp™ 100bp DNA Ladder (Invitrogen, Carlsbad, CA).

Statistical analysis

Statistical procedures were anlayzed using SAS® version 9.4 (SAS Institute, Inc., Cary, NC, USA). A two-way analysis of variance (ANOVA) was used to analyze the interaction of gonad stem cell treatments (unsorted and sorted SSCs) and pressure levels for inducing triploid channel catfish embryos (6,500 and 7,000 psi). Alpha was set at 0.05 for main effects and the interaction. Tukey-Kramer tests were applied to determine significant differences of all possible pairwise comparisons.

Results

The triploid percentages of white catfish, *Ameiurus catus*, pressurized at 6,500 and 7,000 psi were 85.7% and 100%, respectively. The hatching rates of white triploid eggs pressurized at 6,500 and 7,000 psi averaged 22.1±13.2% and 10.8±3.8%, respectively, which were lower than the rate of control white catfish (51.8±13.1%) ($P < 0.05$) (Table 6).

The survival rates of hatched embryos injected with SSCs at 1 day, and 20 days post-transplantation were 78.4%, and 53.4%, respectively. The survival rates of transplanted fry were not different compared to the survival rate of control treatment at same time intervals which were 83.2, and 60.4%, respectively (Table 6).

At 28 days post-transplantation, an average of 41.2% of the white catfish fry from all treatments were confirmed xenogenic fish, possessing channel catfish cells (Fig. 15, Table 7). Overall, there were no significant differences in rates of xenogenesis by pressure treatments (6,500 or 7000 psi, $P = 0.96$, Fig. 16). The xenogenic percentages of white catfish at 6,500 psi and 7,000 psi were 40.9% and 41.7%, respectively. The rate of xenogenesis was higher for the fry injected with the unsorted SSCs than for those injected with sorted SSCs (Fig. 17).

Table 6. Hatching rates of white catfish, *Ameiurus catus*, eggs pressurized at 6,500 psi, 7,000 psi with hydrostatis pressure and control embryos without pressurization incubated in flow through hatching troughs fitted with paddlewheels for 9-13 days at 22.4-25.3 ^{o}C, and survival rates of putative triploid white catfish fry that were transplanted with type A spermatogonial stem cells (SSCs) of channel catfish, *Ictalurus punctatus*, males 1 day and 20 days post-transplantation and triploid controls without transplantation. The hatching rate of control fry was significantly higher than the hatching rates of triploid white catfish eggs pressurized at 6,500 and 7,000 psi (P = 0.0002), one way ANOVA and Tukey-Kramer tests), but the survival rates of transplanted fry were not different from the survival rate of control triploid white catfish at 1 day (P = 0.341, ANOVA test) and 20 days (P = 0.292, ANOVA test) post-transplantation.

Treatment	White catfish embryos pressurized at 6,500 psi	White catfish embryos pressurized at 7,000 psi	Control white catfish embryos[1]
Hatching rate	22.1 ± 13.1[b]	10.8 ± 3.8[b]	51.8 ± 13.1[a]
Survival rate 1 day post-transplantation	78.8 ± 4.3	78.1 ± 6.2	83.2 ± 3.7
Survival rate 20 days post-transplantation	54.7 ± 8.3	52.2 ± 8.0	60.5 ± 4.4

[1]Control for hatch rate is diploid and control for survival is triploid.

Table 7. Pressure levels applied to produce triploid white catfish, *Ameiurus catus*, embryos, unsorted and sorted spermatogonial type A stem cells (SSCs) from channel catfish, *Ictalurus punctatus*, used to transplant into the triploid white catfish embryos, numbers of transplanted fish dissected by laser microdissection machine, numbers of total sorted and unsorted cells, percentages of SSCs in total cells, numbers of sorted and unsorted SSCs for transplantation, and percentages of xenogenic fish produced by using different pressure levels and transplanted with different treatments of stem cells were listed. There were no differences in xenogenic rates of white catfish fry by using two different hydrostatic pressure levels (6,500 and 7,000 psi) (P = 0.96) but the rate of xenogenesis was higher (P = 0.04) for the triploid fry injected with the unsorted stem cells than for those injected with sorted cells.

Treatment	Type of stem cells	Dissected fry	Cell numbers	% SSCs	SSCs numbers	% Xenogenic fish
6500psi	Sorted	11	14,639	61.7%	9,032	36.4
6500 psi	Unsorted	6	37,104	28.6%	10,611	45.5
7000 psi	Sorted	11	15,680	57.8%	9,063	33.3
7000 psi	Unsorted	6	41,328	25.7%	10,621	50.0

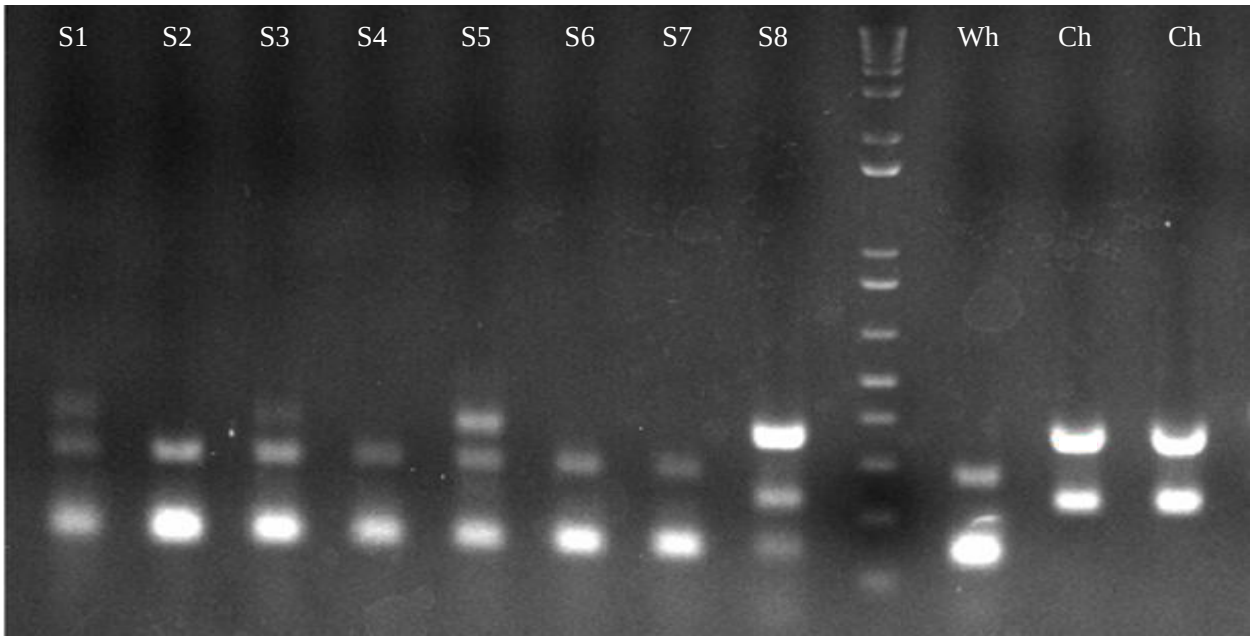

Fig.15. Polymerase chain reaction (PCR) results for detecting channel catfish, *Ictalurus punctatus*, cells in gonadal tissues of triploid white catfish, *Ameiurus catus*, of the first eight samples that were dissected with laser microdissection machine. Channel catfish and white catfish DNA were differentiated with PCR using follistatin, *Fst* and hepcidin antimicrobial protein, *Hamp* genes as markers. Wh was white catfish control while Ch was channel catfish control. The first three samples S1-S3 were triploid white catfish pressurized at 6,500psi and transplanted with unsorted stem cells; S4-S6 were triploid white catfish pressurized at 6,500 psi and transplanted with sorted stem cells; S7-S8 were triploid white catfish pressurized at 7,000 psi and transplanted with unsorted stem cells. The channel catfish cells were detected in four samples including S1, S3, S5, and S8, while the remaining samples contained no channel catfish cells.

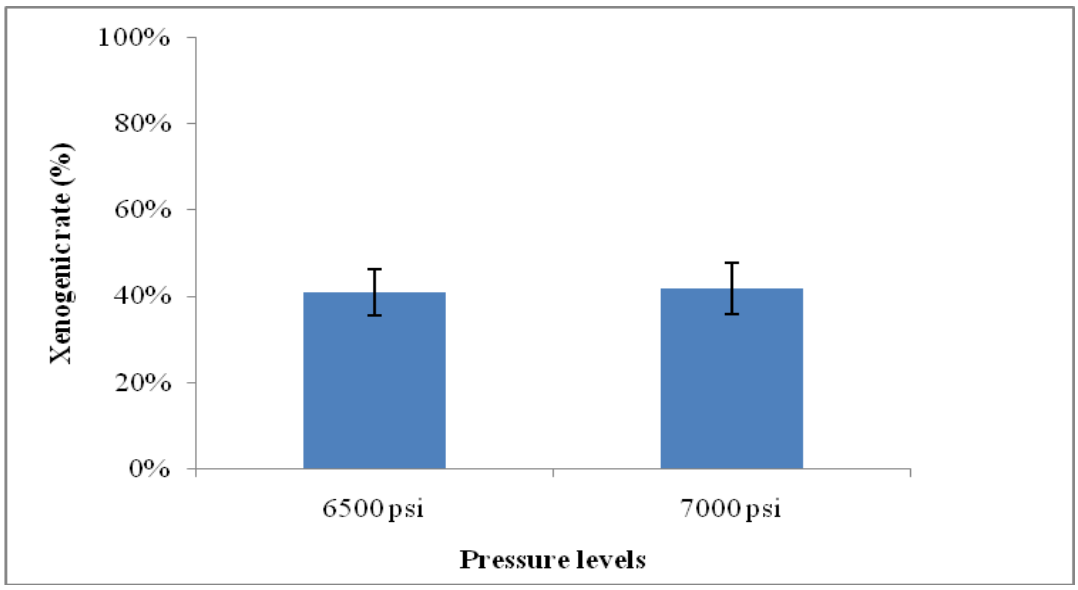

Fig. 16. Percentages of triploid white catfish, *Ameiurus catus*, embryos were pressurized at 6,500 psi and 7,000 psi that possessed channel catfish, *Ictalurus punctatus*, cells. The xenogenic percentages of white catfish at 6,500 psi and 7,000 psi were 40.9% and 41.7%, respectively. There were no differences (P = 0.96) in xenogenesis of triploid white catfish produced by two levels of hydrostatic pressure. The xenogenic percentages were shown as mean ± SE.

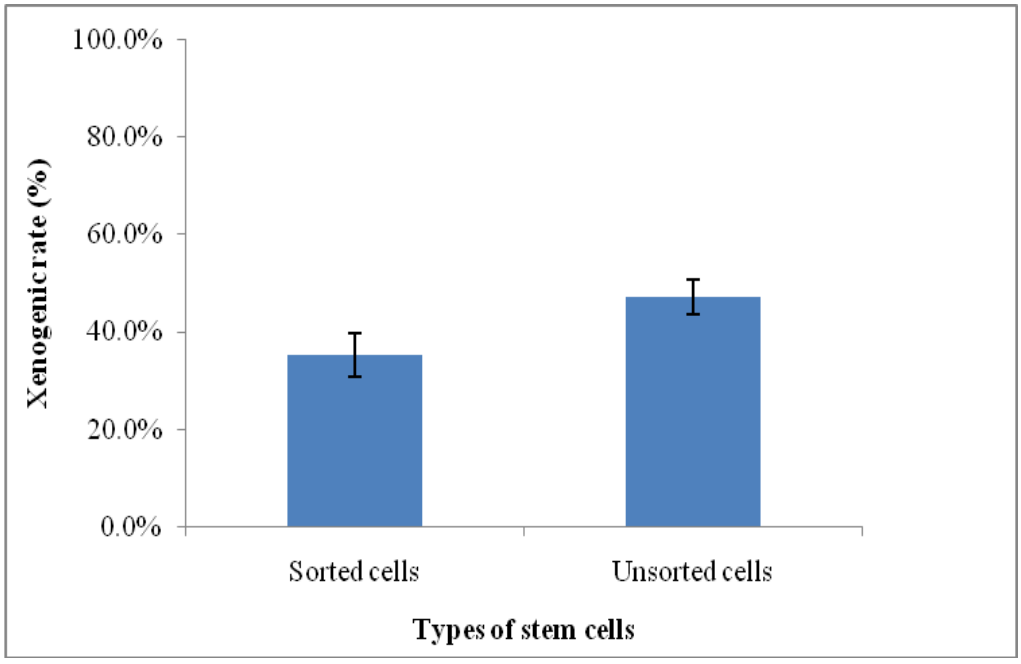

Fig. 17. The xenogenic percentage of triploid white catfish, *Ameiurus catus*, transplanted with sorted and unsorted channel catfish, *Ictalurus punctatus*, SSCs. The mean numbers of sorted and unsorted SSCs transplanted in the triploid white catfish fry were ranged 9,032- 9,063 and 10,611-10,621 cells, respectively. The xenogenic percentages of triploid white catfish fry transplanted with sorted and unsorted SSCs of channel catfish males were 34.9% and 47.7%, respectively. There was a significant difference in xenogenesis of triploid white catfish produced by two treatments of SSCs, sorted and unsorted ($P = 0.04$). The xenogenic percentages were shown as mean ± SE. All the values were significantly different at the level of $P < 0.05$.

Discussion

In this experiment, hydrostatic pressure shocks of 6,500 and 7,000 psi induced 85.7 - 100% triploid white catfish, *Ameiurus catus*, embryos. However, the hatching rates of the pressurized white catfish embryos were low, averaging 10.8-22.1%, which were much lower than the rate of control embryos (51.8%). Transplanted embryos had similar survival rates as compared to the embryos without transplantation after 20 days. Cumulative survival rates of transplated and control white catfish at 20th day post-transpantation were 53.4 and 60.4%, respectively. There was approximately 41.2% of triploid white catfish pressurized at 6,500 and 7,000 psi and transplanted with sorted and unsorted channel catfish, *Ictalurus punctatus,* type A spermatogonial stem cells that were xenogenic. Overall, xenogenic rates of white catfish embryos were not affected by levels of pressure shocks (6,500 or 7,000 psi), but treatments of stem cells (sorted and unsorted SSCs) yielded significantly different xenogenic rates.

There are several ways to produce sterile fish. The most widely used and easiest approach of fish sterilization is genome manipulation, especially triploidization (Piferrer et al., 2009). Induction of triploid involves the application of hydrostatic pressure, the thermal chemical shock to eggs after fertilization to disrupt the formation and expulsion of the second maternal meiotic polar body (Dunham, 2001). Triploid fish can also be produced by crossing between diploid and tetraploid fish (Nam and Kim, 2004). In addition, sterile fish can be produced through hybridization (Piferrer et al., 2009) or a knockout strategy to inhibit the functions of genes

involving in gonadal development (Li et al., 2017). The hydrostatic pressure shocking has been considered as an effective method for producing triploid fish compared to other methods.

Higher levels of pressure shock resulted in higher rates of triploidy, but lower hatching rates as the hatching rate of triploid white eggs pressurized at 6,500 psi (85.7% 3N) and 7,000 psi (100% 3N) averaged 22.1±13.1% and 10.8±3.8% hatch rate, respectively. These results are similar to results reported by Gima (2009) as flathead catfish, *Pylodictus olivaris*, embryos pressurized at 6,500 psi had 100% triploidy and 16.7% of pressurized embryos hatched. At the higher-pressure level, 7000 psi, the triploid rate of flathead catfish embryos was still 100%, but the hatching rate was decreased three fold as only 5.3% pressurized embyos hatched. Lilyestrom et al. (1989) found that the triploid rate and hatching rate of channel catfish embryos pressurized at 900 kg/cm^2 were 66.7 and 100%, respectively, while at higher pressure level, 1,500 kg/cm2, 100% triploid rate achieved but the survival rate of channel catfish embryos pressurized with high pressure was decreased by 50%. The hatching rates of white catfish embryos pressurized at 6,500 and 7,000 psi in this experiement were 2.3-4.8 times lower than the control treatment. Gima (2009) found that hatching rates of flathead catfish pressurized at 6,500 and 7,000 psi were approximately 1.3-4.1 times lower than the rates of control flathead catfish embryos.

Sterile triploid fish whose germ cells are unable to develop into fertile gametes (Arai, 2001) have been used as recipients for germ cell transplantation in salmon (Okutsu et al., 2007), medaka (Seki et al., 2017), Nibe croaker (Yoshikawa et al., 2017), grass puffer (Hamasaki et al., 2017). Yoshizaki et al. (2011) found that the fertility of triploid recipients was fully recovered by transplantation of diploid donor germ cells. Thus, triploid fish have the ability to nurse diploid germ cells once they are incorporated into the recipient genital ridges. Gonad somatic cells and their endocrine system functions normally until the donor-derived germ cells mature and produce

gametes. Yoshizaki et al. (2016) also stated that germ cell transplantation using triploid or germ cell-free recipients resulted in effective colonization compared with recipients carrying endogenous germ cells. Therefore, triploid fish should be used as recipients for germ cell transplantation. From this experiment, triploid white catfish can be induced by using hydrostatic pressure at 6,500 and 7,000 psi and these two levels of pressure did not give differences in success rates of xenogenesis. However, 7,000 psi gave 100% triploidy, which would simplify screening to ensure xenogenesis had been accomplished. However, this requires pressurizing twice as many eggs to meet brood stock quota goals. Alternatively, improving incubation protocols could result in better hatch rates of triploid embryos.

Unsorted and sorted spermatogonial stem cells were used to transplant into the newly hatch fry of white catfish in this experiment. The number of SSCs for the sorted and unsorted were similar. The unsorted cells resulted in a higher number of xenogenic fry. Either the Percoll centrifugation damages the SSCs, cells other than the SSCs somehow assist colonization or there are other factors in the cell suspension that promote colonization that are lost during the Percoll sorting. This is an unexpected result as survival rates of unsorted and sorted stem cells cultured *in vitro* were not different (Chapter 3).

There have been had several studies that either used sorted or unsorted SSCs to use for transplantation. Lacerda et al. (2006) used 2×10^6 sorted spermatogonial stem cells to transplant into adult of Nile tilapia and still detected the spermatogonial stem cells 4 weeks after transplantation. Perera et al. (2017), transplanted approximately 7×10^4-4.5×10^5 purified spermatogonial cells into the triploid sub-adult channel catfish and had xenogenic fish 2 years post-transplantation. Another study was conducted by Majhi et al. (2014) when approximately 3.75×10^6 sorted stem cells from young pejerrey, *Odontesthes bonariensis*, were transplanted into

adult fish Patagonian pejerrey, *O. hatcheri*. They confirmed the presence of donor-derived gametes by PCR in 17% and 5% of the surrogate, *O. hatcheri,* fathers and mothers, respectively, 7 months post-transplantation. Unsorted SSCs have been used in studies by Lujic et al. (2018) and Hamasaki et al. (2017). Approximately, 15,000 unsorted germ cells from the brown trout and the European grayling, *Thymallus thymallus,* were labeled with PKH 26 and injected into the abdominal cavity of each recipient larvae of rainbow trout three to five days post hatch. Detection of red fluorescent cells (PKH26) was used as evidence of successful colonization of donor stem cells after 60 days post transplantation. There were 23-28% of donor stem cells incorporated into the gonads of recipients (Lujic et al. 2018). Hamasaki et al. (2017), used a suspension containing 4,000-6,000 unsorted testicular cells to transplant into the peritoneal cavity of grass puffer larvae at 1 and 5 days post hatch (dph). Results from their experiment showed that 23.1-38.3% recipients could produce donor-derived gametes 2 years after transplantation. The results from these experiments indicated that both unsorted and sorted spermatogonial stem cells can be used for germ cell transplantation to make xenogenic fish, but none compared the efficacy of the two types.

The average percentage of xenogenic triploid white catfish transplanted with unsorted and sorted SSCs was 41.2%. This result is similar to the result reported by Okutsu et al. (2006) when they transplanted spermatogonial stem cells of rainbow trout into the newly hatched fry of allogenic rainbow trout, which resulted in 43% transplanted germ cells incorporated into the genital ridge of recipient fish after 20 days post-transplantation. Yazawa et al. (2013) transplanted spermatogonial type A stem cells collected from immature chub mackerel, *Scomber japonicas*, into larva of Pacific bluefin tuna, *Thunnus orientalis*. The result showed that the donor cells could be detected in only 5.4% of total observed fish after 21 days of transplantation.

Morita et al. (2012) studied allogeneic transplantation of spermatogonial in the yellowtail, *Seriola quinqueradiata*, by transplanting type A spermatogonia collected from immature (10 month old) males into peritoneal cavities of 8-day-old larvae. Florescence observation at 28 days post-transpantation indicated that 66.7% donor stem cells were incorperated.

Laser microdissection (LMD) is a technology that allows the rapid procurement of selected cell populations from a section of heterogeneous tissues in a manner conducive to the extraction of DNA, RNA, and proteins (Balestrini et al., 2009). LMD can be used to obtain single cells or homogenous population of cells for genomic, gene expression, and proteomic analysis (Nakamura et al., 2007). In this experiment, the gonadal tissues from the 28-day-old fry of triploid white catfish transplanted with spermatogonial type A stem cells are not visible to the naked eye while the region of gonadal tissues can be visualized under the microscope and harvested by using LMD for DNA extraction, PCR sequencing and species molecular identification. In addition, using LMD does not require labelling cells before transplanting into the recipients which resulted in damage or loss of desirable cell population. However, the LMD method is time consuming since the fry must be undergo different processes including fixation with tissue solution, embed with parrafin, and stain with hematoxilyn before being dissected. Additionally, LMD is quite expensive. The consumables, such as nuclease free membrane slides and collecting tubes, are more than 5 times more expensive than normal consumables. There is a considerable risk that the quality of microdissected tissues may not meet the standard quality for further analysis due to its long exposure to chemicals, fixatives and staining reagents. In this study, around 10% of white catfish samples dissected with LMD had low DNA quality that could not be used for genomic analysis.

This is the first time xenogenic catfish between two different genera of ictalurid catfish (white catfish and channel catfish) produced by transplanting spermatogonia stem cells type A from channel catfish into the newly hatched fry of white catfish. This is the first step for testing a system to produce channel catfish female × blue catfish male hybrids by mating xenogenic white catfish females transplanted with channel catfish SSCs and xenogenic white catfish males transplanted with blue catfish SSCs in traditional open ponds or spawning aquaria. Potentially, xenogenic white catfish will greatly reduce the time to reach sexual maturity of broodfish and reduce intensive labor and facility requirements compared to the current artificial fertilization technique. Even if commercial-scale application of xenogenesis for hybrid catfish production does not become reality, many potential applications of using xenogenic fish exist including conserving of endangered species, studying cellular and tissue communication, producing difficult to spawn species, and enhancing gene transfer.

In summary, xenogenic white catfish could be produced by transplanting sorted and unsorted type A spermatogonial stem cells (SSCs) of channel catfish males into triploid white catfish that were produced by hydrostatic pressure shocks at 6,500 and 7,000 psi. Approximately 9,000 and 10,000 sorted and unsorted SSCs, respectively, needed to be transplanted into the peritoneal cavity of newly hatched white catfish embryos to produce xenogenic fish. The transplantion of unsorted stem cells into triploid newly hatched embryos resulted in higher xenogenic percentage as compared to the percentage of triploid hatched fry transplanted with sorted stem cells. Further research needs to be focused on, (1) transplanting the higher number of unsorted SSCs of channel catfish males in the triploid white catfish, (2) transplanting unsorted SSCs from blue catfish to produce the xenogenic white catfish that are able to produce blue catfish gametes, and (3) mating the xenogenic white catfish females transplanted with channel

catfish stem cells with xenogenic white catfish males transplanted with blue catfish males to produce hybrids.

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