Assessment of the use of cell binding agents useful in the generation of viable offsprings – Biodiversity conservation strategy through production of androgenetic fish

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Abstract — Production of androgenetic clones is one of the important strategies in the conservation of endangered species. In conventional method of producing androgenetic clones, genome inactivation of eggs is done by using radiation, followed by subjecting to thermal /hydrostatic /pressure shock for diploidization after monospecific activation. Due to these two types of subsequent treatments, the eggs are noticed to get severe stress leading to embryonic aberrations and poor survival. Thus the present study was done to test the use of Poly Ethylene Glycol (PEG) and Calcium chloride to produce fused sperms which can activate the genome inactivated eggs thus using only one treatment. But success of production of putative diploid androgenetic clones depends on the availability of the number of viable fused sperms (dispermy). Hence influence of combination of Poly Ethylene Glycol (PEG) and High pH-Calcium chloride on the production of dispermy or the fusion efficiency, viability and motility of fused spermatozoa and rate of activation of genome inactivated eggs with the use of dispermy have been studied.

Key words — Androgenesis, dispermy, genome inactivated eggs.

1 INTRODUCTION

Conservation and management of aquatic resources is important for sustainable utilization of fisheries potential for the economic advancement of farmers and fishery workers. ***Ex situ*** strategies like cloning of fish gametes and embryos may prove globally important. But nuclear manipulation in fishes is found to be a difficult task due to the non- visibility of the egg nucleus (Pandian, 2003). Applications of androgenesis in genome banking programs address the needs of both the aquaculture industry to safeguard valuable strains and lines and natural resource conservation for ***in vitro*** protection of endangered species or populations. Due to radiation treatment and shock treatment used during the production of androgenotes the survival rates of putative androgenotes are presumed to be very low. Shock treatment was reported to cause more damage than radiation on survival (Bongers, 1995). One potential approach to avoiding loss of survival is artificial androgenesis through fertilization of egg with paired sperms (fused sperms).

Due to the absence of an acrosome in the fish sperm (Afzelius, 1978), entry of the sperm in to the egg is made possible through the micropyle (Ginsburg, 1972) during activation of egg. Absence of acrosome in spermatozoa of teleostean fishes and the presence of micropyle in egg facilitate not only heterogamy but also polyspermy (multiple sperm entry) (Clifton and Pandian, 2008). Membrane fusion techniques were introduced to experimental embryology in the early 1970s when they were used to fuse early embryonic blastomeres and to study the effects of the resulting tetraploidy on development (Jan Tesarik, 2000).

Chemical fusogens were used to fuse sperm cells in order to get dispermy. Gelatin, Dextran, Polyethylene Glycol (PEG), High p^H - high calcium, Poly Vinyl Alcohol, Sodium nitrate, Phytolectins, PEG plus DMSO, Pronase E, Seawater, Lysolethicin, Lectins such as concanavalin A (Con A), and other phytohemagglutins as well as immune antisera are the commonly used fusogens (Saunder J.A.1985). In order to achieve cell fusion, the plasma membrane of two or more cells must be physically appressed for some time interval. Earlier studies indicate that concentration of fusogen and cell treatment period may disrupt the cells and reduce viability. So, each step in the fusion process should be evaluated not only for yield of fusion products, but also for its overall effect on cell viability.

Hence the present study is carried out to test the use of different concentrations of Poly Ethyleneglycol and High pH (10.5) - high $CaCl_2$ on Zebrafish sperm binding (dispermy formation), viability, motility and fertility.

2 MATERIALS AND METHODS

2.1 Selection and maintenance of test species

Zebra fish ***Danio frankei*** were obtained from Southern Aqua Farms, Chennai, India. Fishes were reared separately in 4 large rectangular cement tanks (45cm x 30cm x15cm) containing well aereated water with Hydrilla and Pistia plants at a stocking density of 5 fish/L. Prior to use, the water was aerated over night. Fishes were fed on live tubifex worms and basal diet 3 times a day (photo period of 14h:10h Light: Dark regimen). 1/4th volume of water is exchanged with fresh water on weekly basis and pH is maintained at 8.0±0.2.

Figure 1

Female Danio frankei (dotted) *Male Danio frankei (albino)*

Collection of Sperms

Milt of male ***D. frankei*** *(albino)* was collected through stripping. Before collection, the fish were anesthetized in 0.01% Tricaine methanesulphonate for 1-2min until they stop swimming. The anesthetized fish were rinsed in fresh water, blotted dry on a paper towel and were placed on a holder with the belly facing up. Gentle pressure was applied from each side of the belly in the direction of the anal opening for the release of sperms. Sperms were collected into eppendorf tube containing 20µl Hank's solution (Westerfield, 1995) using a micropipette.

Sperm count

Sperm count of the milt was made using Neubauer haemocytometer (Belsely *et al.*, 1980). 10 µl aliquot was taken from milt sample and placed on the haemocytometer chamber. Different dilutions of sperm assemblage were prepared using Hank's solution (HIMEDIA) in order to obtain single layer of sperms on the slide for precise counting. The number of sperms in the appropriate squares of the haemocytometer was counted using Phase Contrast Microscope (Leica, Germany) at 400 times magnification. Sperm count/concentration refers to the number of spermatozoa per ml.

$$\text{Sperm Count} = \frac{\text{No. of sperms x dilution factor x depth factor}}{\text{No. of areas counted}} = \text{Millions / ml}$$

Sperm Viability

The ratio of live Vs dead sperms was determined using 1% trypan blue (Bjorndahl et al., 2003). Sperm sample (0.2 ml) used for counting was incubated with 0.2 ml of 1% trypan blue stain for 10 min at 37°C. A drop of the suspension was placed in Neubauer chamber under a cover slip, allowed to settle for 1 min and observed under Phase Contrast Microscope. The number of stained and unstained spermatozoa was scored in 10 to 20 separate fields. The sperms which were not stained with trypan blue were considered as viable. The number of unstained cells was determined and the percent viability was calculated as follows.

$$\text{Sperm Viability (\%)} = \frac{\text{No. of unstained sperms}}{\text{Total number of sperms}} \text{x100}$$

Sperm Motility

Based on the information of sperm count obtained, milt was diluted using Hank's solution. 10µl aliquot of each sperm sample was taken and placed on haemocytometer chamber and 10µl deionized water was added to activate the sperm just before the assessment starts. Sperm motility was estimated by visually examining the sperms at 400X magnification using dark-phase microscopy (Phase Contrast Microscope, Leica, Germany) and was expressed as the percentage of sperms that moved actively showing zig-zag movement within 30 seconds after activation at room temperature. Sperms vibrating at a single place were not considered as motile. The percentage of sperm motility was calculated by using the following formula.

$$\text{Sperm motility (\%)} = \frac{\text{No. of motile sperms}}{\text{Total number of sperms}} \times 100$$

Production of disperms using PEG & HIGH p^H(10.5)-high $CaCl_2$:

Milt was collected from male zebrafish ***D. frankei*** (*albino*) and suspended in 1ml Hank's solution followed by centrifugation at 1500 rpm for 5 min. Pellet was discarded and supernatant was centrifuged at 800 rpm for 5 min. Supernatant was discarded and to the pellet 0.5 ml of 2% PEG and 0.5ml of 1.5mM High p^H (10.5) High $CaCl_2$ solution were added to the pellet and incubated at room temperature for different time periods of 2, 4, 6, 8 and 10 min separately. Similar procedure was followed for the 1:1 of 2.5% PEG and 1.5mM High p^H (10.5) high $CaCl_2$ and 1:1 of 3% PEG and 1.5mM High p^H (10.5) High $CaCl_2$ treatments. Microscopic examination of dispermic condition was carried out.

Percentage of paired sperms formed against single sperms and clumps, motility and viability of disperms were assessed similar to the procedure followed for single sperms.

Testing fertility of disperms

Collection of eggs

Before collection of eggs female ***Danio.frankei***, were anesthetized using 0.01% Tricaine methanesulphonate for 1-2 min until they stop swimming. The anesthetized fish were rinsed in fresh water, blotted dry on a paper towel (since excess water may cause swelling of the eggs and prevent fertilization) and stripped as explained earlier. Eggs were collected into a petri dish containing synthetic ovarian fluid, in which the eggs remained competent for fertilization for at least 90 min and the fish were returned to the aquarium for recovery.

Fertilization with single sperms

Fertilization was induced by suspending eggs (330±20) of ***D. frankei*** (*dotted*) in a petri plate containing 2 ml of synthetic ovarian fluid (Bongers et al., 1995), and by adding100 µl single sperm suspension of fresh milt to the eggs. Approximately 0.5 ml water from the fish tank was immediately added and mixed using a soft quill. Few min later, the dish was filled with filtered water from fish tank. Fertilized eggs were kept at 28 ± 0.5^0 C for 30 min and observed for the formation of blastodisc. The embryos were transferred to a tray containing 500ml tap water with aeration and were maintained for further development. Observations were made regarding the onset of embryo development and assessed for percentage fertilization & hatchlings formed. This group serves as control for the group of androgenetic progeny produced in subsequent experiment.

$$\text{\% Fertilization} = \frac{\text{No. of fertilized eggs with blastodisc formation}}{\text{Total No. of eggs}} \text{x100}$$

Egg genome inactivation

For the production of androgenetic progeny, eggs were subjected to genome inactivation following a custom-built UV illumination chamber fabricated in collaboration with Labnet, Chennai, was used for egg genome inactivation. In the chamber, a UV lamp of 254 nm (1 mW at the surface of eggs) was used as source of irradiation. Eggs collected from female ***D. frankei*** *(dotted)* were suspended in a petri plate containing synthetic ovarian fluid. 5 ml water was added to the plate to provide slight buoyancy. UV lamp was switched on for at least 30 min prior to subjecting eggs for irradiation. Petri dish containing approximately 330±20 eggs was placed in the chamber on a rotator - shaker at 25 rpm, permitting eggs to roll in the fluid and thus ensuring the uniform exposure of eggs to the illumination. The distance between the light source and eggs was maintained at 26cm by adjusting the height of the shaker. Fertilizability of eggs irradiated for 2min was assessed.

Production of diploid androgenetic progeny using 1:1 PEG and High p^H - High $CaCl_2$ (10.5) fused sperms

Before fertilization, paired sperms were purified by subsequent washes with Hank's solution. After incubating the sperms in 1:1 PEG and High p^H (10.5) - High $CaCl_2$ for specific time periods the tubes were centrifuged at 800 rpm for 5min and the supernatant was discarded. The pellet was again washed with Hank's solution after which 1ml Hank's solution was added and the solution was mixed properly.

In order to produce diploid androgenetic clones, sperms treated with 1:1 ratio 2.5% PEG and 1.5mM High p^H (10.5) - High $CaCl_2$ which induced maximum dispermic formation was added to genome inactivated eggs of female ***D. frankei*** **(***dotted)* in a petri plate. Subsequently, 0.5 ml water was added to facilitate activation of sperms. Gentle swirling of the dish was done to ensure activation of embryonic development and then the plate was filled with filtered water from fish tank. Fertilized eggs were kept at 28 ± 0.5^0 C for 30 min; observed for the formation of blastodisc and the embryos were transferred to a tray containing tap water with aeration where they were maintained.

Statistical analysis

The experiments were carried out using 6 or 10 replicates based on the type of the parameter assessed. Two-way ANOVA and Duncan's multiple range test (DMRT) ($P<0.01$) were used to assess the effect of time, concentration and both time and concentration on percentage of paired sperms, motility and viability of disperms. Statistical analysis was performed using SPSS commercial statistical package (SPSS, version 16.0 for Windows, SPSS Inc., Chicago, USA).

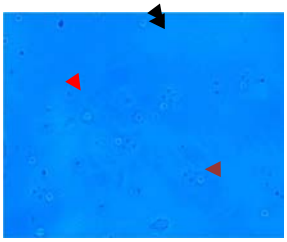
RESULTS AND DISCUSSION

Plasma membrane has a net negative surface charge and hence the adjacent cells, with similar charges tend to repel each other. Fusion efficiency of spermatozoa was found to be influenced by the fusing agent. In general, chemical fusogens were found to stimulate the fusion of cells in one of the two ways - i) they may induce cell agglutination or ii) alter the membrane in such a way as to make it susceptible to fusion(Constable and Kao,1978). These compounds override the repellant force of the membrane surface charges causing the cell to clump into tight clusters.

Table 1: Count of single sperms and fused sperms upon treatment with different ratios of PEG & High p^H (10.5) - High $CaCl_2$ for different time periods. (Values are Mean (%) ,±SD of 6 individual observations). Values in parenthesis represent the percentages.

1:1 of PEG & High p^H- High $CaCl_2$	Sperm Count (Percentage)	Duration of Incubation (Min)				
		2	4	6	8	10
0.0Mm (Control)	Total sperm count (Control)	40,44,240± 53,430 (98.4%)	37,93,530± 57,540 (92.3%)	33,82,530± 73,980 (83.2%)	31,89,360± 49,320 (77.6%)	29,79750± 78090 (72.1 %)
2% : 1.5mM	Single Sperms	3,320,880± 172,620 (80.8%)	30,049,620± 156,180 (74.2%)	2,708,490± 131,520 (65.9%)	2,379,690± 123,300 (57.9%)	2,149,530± 119,190 (52.3%)
	Double sperms	789,120± 65,760 (19.2%)	998,730± 78,090 (24.3%)	1,241,220± 106,860 (30.2%)	1,500,150± 115,080 (36.5%)	2,149,530± 123,300 (40.2%)
	Clumps	-	-	-	-	-
2.5%:1.5mM	Single Sperms	2,687,940± 139,740 (65.4%)	2,408,460 ± 127,410 (58.6%)	2,026,230± 115,080 (49.3%)	1,237,110± 82,200 (30.1%)	1,528,920± 98,640 (28.2%)
	Double sperms	1,130,250 ± 102,750 (27.5%)	1,364,520 ± 115,080 (33.2%)	1,635,780± 123,300 (39.8%)	2,285,160± 176,730 (55.6%)	1,775,520± 131,520 (43.2%)
	Clumps	291,810± 24,660 (7.1%)	337,020 ± 32,880 (8.2%)	447,990± 41,100 (10.9%)	587,730± 49,320 (14.3%)	805,560± 61,650 (29.6%)
3%:1.5mM	Single sperms	1,500,150± 90,420 (36.5%)	1,586,460± 106,860 (38.6%)	1,426,170± 78,090 (34.7%)	1,076,820± 69,870 (26.2%)	8,46,660± 57,540 (20.6%)
	Double sperms	1,565,910± 110,970 (38.1%)	1,401,510± 110,970 (34.1%)	1,241,220± 94,530 (30.2%)	1,175,460± 86,310 (28.6%)	982,290± 78,090 (23.9%)
	Clumps	1,043,940± 86,310 (25.4%)	1,204,230± 106,860 (29.3%)	1,442,210± 119,190 (35.1%)	1,870,050± 139,740 (45.2%)	2,285,160± 180,840 (55.6%)

Fig: 2: Single sperms (◄), disperms (◄) & clumps (◄)



D. frankie sperms treated with 2% PEG : 1.5mM $CaCl_2$ showed 19, 24, 30, 36 and 40% of sperms forming disperms while 81, 74, 66, 58 and 52% sperms remained free upon treatment for 2, 4, 6, 8 and 10 min while no clumps formed at this concentra-

tion (Table 1). Sperms treated with 2.5% PEG : 1. 5mM $CaCl_2$ showed 27, 33, 39 and 56 % disperms formation upon treatment for 2, 4, 6, 8 and 10 min respectively which later decreased to 43% after treating for 10 min. However 2.5% PEG : 1. 5mM $CaCl_2$ treatment was further found to induce 7, 8, 11, 14 and 29% sperms to form clumps while 65, 59, 49, 30 and 28% remain free upon treatment for 2, 4, 6, 8and 10 min respectively (Table 1). On the other hand, treatment with 3% PEG : 1.5mM $CaCl_2$ for 2min showed 38% disperm formation which decreased to 34, 30, 29, 24% upon treatment for 4, 6, 8 and 10 min respectively. With the same concentration 25, 30, 35, 45 and 56% sperms were found to form clumps while 36, 39, 35, 26 and 20 % sperms remained single upon 2, 4, 6, 8and 10 min treatment respectively (Fig 2)

Table 2:Viability and Motility of paired sperms formed upon treatment with different ratios of PEG & High p^H(10.5)-High $CaCl_2$ for different time periods. (Values are Mean (%) ±SD of 6 individual observations) Values in parenthesis represent the percentages.

1:1 of PEG & High p^H-high $CaCl_2$	Viability of Disperms (%)					
	0min	2min	4 min	6 min	8 min	10 min
2% : 1.5mM	42,20,000± 10,000 (97.8%)	30,13,080± 2,19,440 (71.4%)	29,20,240± 2,02,560 (69.2%)	27,55,660± 1,94,120 (65.3%)	26,75,480± 1,98,340 (63.4%)	24,94,020± 1,64,580 (59.1%)
2.5%: 1.5mM	42,20,000± 21,000 (98%)	29,46,956± 1,94,212 (69.8%)	27,82,298± 1,85,768 (65.9%)	26,68,304± 1,98,434 (63.2%)	25,41,644± 1,73,102 (60.2%)	22,96768± 1,51,992 (54.4%)
3%: 1.5mM	42,20,000± 22,000 (97.2%)	27,09,882± 1,77,282 (64.2%)	25,03,053± 1,64,619 (59.3%)	23,13,108± 1,47,735 (54.8%)	22,24,467± 1,43,514 (52.7%)	20,93,616± 1,35,072 (49.6%)
1:1 of PEG & High p^H-high $CaCl_2$	Motility of Disperms (%)					
	0min	2 min	4 min	6 min	8 min	10 min
2% : 1.5mM	42,20,000± 10,000 (97.8%)	29,45,560± 1,77,240 (69.8%)	28,31,620± 1,73,020 (67.1%)	27,34,560± 1,56,140 (64.8%)	26,41,720± 1,60,360 (62.6%)	24,26,500± 1,35,040 (57.5%)
2.5%: 1.5mM	42,20,000± 21,000 (98%)	28,66,738± 1,73,102 (67.9%)	27,18,968± 1,81,546 (64.4%)	25,83,864± 1,68,880 (61.2%)	25,20,534± 1,51,992 (59.7%)	22,46,104± 1,30,882 (53.2%)
3%: 1.5mM	42,20,000± 22,000 (97.2%)	26,80,335± 1,85,724 (63.5%)	24,86,169± 1,64,169 (58.9%)	23,04,666± 1,35,072 (54.6%)	21,61,152± 1,26,630 (51.2%)	20,42,964± 1,13,967 (48.4%)

PEG and Calcium Chloride are the two common effective binding agents which override the repellant force of the membrane surface charges causing the cells to clump into tight clusters (Sowers, 1987), were found to be highly useful in the formation of polysperms. In any case, the cell membrane must be considered a fluid, dynamic structure that can fuse with other membranes under appropriate conditions. Further the surface charge of the cell membrane may be modified by the p^H of the buffer, by polycations added to the media, or by the dehydrating effect of various chemical fusogens. PEG and calcium both which are used routinely for fusion of animal cells (Ahkong et al.,1980) are now found to need specific procedure and technique with appropriate modification from plant cells.

The fusion effect of combination of PEG and $CaCl_2$ studied for the first time in the current investigation showed that maximum of 55.6% disperms were formed after 8 min incubation treatment with 2.5% PEG:1.5mM $CaCl_2$. Out of 56% disperms formed more than half were found to show viability and motility which is basically required to ensure successful dispermic fertilization (Table 2). The present study showed that fish sperms are highly sensitive to proportions of PEG and $CaCl_2$ as well as duration of exposure to the chosen concentrations (Table 1 & 2).

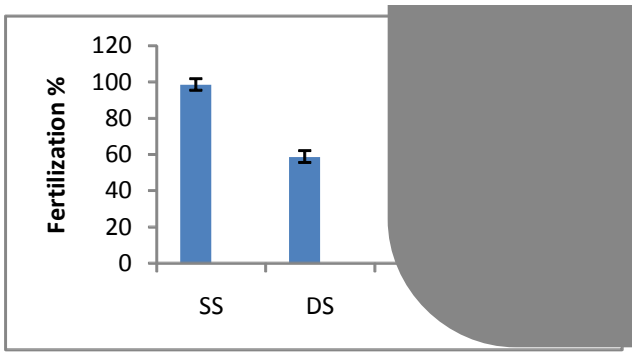


Figure 3: Percentage of viable fertilized eggs produced using single sperms (SS: control) and disperms (DS)

Fertilization between fresh eggs and fresh sperms showed 96.8% success. Fusion of genome inactivated eggs and disperms exhibited 58.8% fertilization success indicating the prospects of using disperms in the production of viable diploid androgenotes .(Fig 3)

Reduction in fertilization upon using disperms compared to that of single sperms can be attributed to the limitation encountered by fused sperms to make entry through the micropyle of the eggs. Reduction in the dispermic fertilization appears to be common to all the species as evidenced by earlier studies (Clifton and Pandian, 2008: Nagoya etal., 2010).

Conclusion:

In the context of existing problems associated with establishing conservation strategies for endangered species, the successful production of diploid androgenetic progeny using fused sperms with greater potential of survival provide scope for future advancements. Treatment of sperms with 2.5% PEG: 1.5mM $CaCl_2$ resulting in 56% sperms forming disperms with more than 50% viability and motility provide evidence for prospects of combinational use of fusing agents to achieve improved method of producing diploid androgenotes. Hence dispermic formation using a suitable agents such as 1:1 PEG and $CaCl_2$ in future studies would have better prospects to achieve successful androgenetic cloning to formulate conservation strategies of endangered fish species.

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