Original Article   **Open Access**

Assessment of survival and ploidy condition of Zebra fish progeny produced through androgenesis using Disperms

D Sai Vinathi[1], V Sridevi[1], DC Reddy[2], V Kalarani[1]*

[1]Department of Biotechnology, Sri Padmavati Mahila Visvavidyalayam, (Women's University), TIRUPATI-517 502, A.P., INDIA
[2]Former UGC Emeritus Scientist, S.V. University, Tirupati
*Corresponding author email: kala.dandala@gmail.com

ABSTRACT

Over exploitation, habitat modification, pollution load and introduction of exotic species affect fish genetic diversity directly or indirectly. Excessive mortality of fishes due to any of these factors lead to species reduction of population size or extinction. Hence conservation of aquatic genetic resources is essential to maintain ecological as well as socioeconomic equilibrium. *Ex situ* strategies like cloning of fish gametes and embryos are proven globally important. In conventional methods, production of androgenetic clones was done using X- rays or γ- rays followed by subjecting the eggs to thermal/ hydrostatic/ pressure shock for diploidization after monospermic activation. Due to two subsequent treatments of radiation and shock, the survival rates of putative androgenotes are presumed to be very low. The present study is carried out to assess the survival and ploidy condition of diploid androgenetic progeny of zebrafish, *Danio frankei*, produced through dispermic fertilization. Poor survival rates of hatchlings produced through fertilization of genome inactivated eggs with monosperms compared to the survival ability of hatchlings produced through fertilizing genome inactivated eggs with disperms was discussed. This study clearly showed that the use of combination of Poly Ethylene glycol (PEG) and Calcium chloride ($CaCl_2$) is more effective than any single agent to form viable disperms that can induce fertilization of genome- inactivated eggs similar to the effect shown by normal eggs.

Key words: Genome inactivated eggs, disperms, androgenetic clones, ploidy,

INTRODUCTION

Aquatic animals experience threats of habitat change, over exploitation, invasive alien species and pollution and the number of species added to the Red list is increasing gradually (www.iucn.org). Effects are being made to restore endangered species following conservation strategies such as *In situ* and *Ex situ* (Frankham *et al.,* 2002; Hiddink *et al.,* 2008).

While *in situ* is the conservation of species in their natural habitat, *ex situ* is the preservation of components of biological diversity outside their natural habitats. This involves conservation of genetic resources and draws on a diverse body techniques and facilities. Genetic manipulations are important tools to develop conservation strategies. Chromosomal manipulations namely gynogenesis and androgenesis have attained significance in developing protocols of restoration of endangered species through **ex situ** conservation. Androgenesis is considered one of the most promising and reliable techniques for recovering complete nuclear genome information (Babiak *et al.,* 2002). It allows obtaining individuals whose development proceeds under the control of male chromosomes without participation of maternal nuclear genome. But due to involvement of subsequent treatments of radiation and shock used during the process, putative androgenotes obtained were found to show poor survival (Corley Smith *et al.,* 1996). Shock treatment was reported to cause greater damage to survival than radiation (Bongers, 1995). Enhanced homozygosity suffered by the androgenotes due to suppression of the first mitotic division is also a common and decisive cause for the high embryonic mortality observed. Hence array of trials are under way to generate diploid progeny avoiding giving shock treatments.

As denoted by Clifton and Pandian (2008) absence of acrosome in spermatozoa of teleostean fish and the presence of micropyle in egg facilitate heterogamy or even polyspermy (multiple sperm entry). Hence one potential approach to avoid loss of genetic diversity and high mortality is suggested to be artificial androgensis where in two fused spermatozoa are used for fertilizing the egg (Nagoya *et al.*, 2010) which may also enhance heterozygosity. Accordingly several chemical fusogens were used to fuse sperm cells in order to get disperms. Gelatin (Kameya, 1973), Dextran (Kameya,1979), Polyethylene Glycol (PEG), High p^H - high calcium (Kao *et al.,* 1974), Polyvinyl alcohol (Nagata,1978), Sodium nitrate (Power *et al.*, 1970), Phytolectins (Chin and Scott, 1979), PEG plus DMSO (Haydu *et al.,*1977), Pronase E (Kameya, 1979), Seawater (Binding, 1974), Lysolethicin (Constabel and Kao, 1974), Lectins such as concanavalin A (Con A), and other phytohemagglutins (Saunder, 1985) as well as immune antisera are some of the fusogens used. Fusogens induce cell agglutination or alter the membrane in such a way so as to make it susceptible to fusion. In order to achieve cell fusion, the plasma membrane of two or more cells must be physically appressed for some time. PEG treatment has been proven useful for fusing plant protoplasts (Kao and Michayluk, 1974; Beránek *et al.*, 2007), mouse fibroblasts (Roos and Choppin, 1985) erythrocytes (Hui *et al.*, 1985; Herrmann *et al.*, 1993) and mammalian cells (Yang and Shen, 2006). Several reports attribute the mechanism of fusion of sperms to modifications in plasma membrane surface charges (Ueda *et al.*, 1985, Nagoya *et al.*, 2010). Hence membrane integrity plays a key role in deciding the ability of adhesion or fusion among the sperms.

Treatment of sperms with 2.5% PEG: 1.5mM $CaCl_2$ carried out in our laboratory showed 56% sperms forming into disperms with 50% viability and motility providing evidence for prospective use of combinational fusing agents to attain improved number of viable disperms (Sai Vinathi *et al.,* 2014). Thus the present study is carried out to assess the impact of the use of disperms formed through the treatment with PEG and High p^H – high $CaCl_2$ in producing the diploid androgenetic progeny of **Danio frankei** with good survival.

MATERIALS AND METHODS

Procurement and Maintenance of test species:
Male and female Zebra fish **D. frankei (albino)** obtained from Southern Aqua Farms, Kollathur located 20 Km South of Chennai, Tamil Nadu were reared separately in 4 rectangular cement tanks containing well aerated water (28 ± 1° C) with Hydrilla and Pistia plants under natural photoperiod with feeding 3 times a day *ad libitum* on dried/ live/ pelleted feed ensuring supply of 30% protein. Water was changed every alternate day by removing ¼th of water from the bottom of the tank using suction pump. Dissolved oxygen content was maintained at 5.5± 0.5 mg /L and p^H at 8.0±0.2.

Collection of Sperms:
Milt of male *D. frankei* was collected through stripping. Before collection, the fish were anaesthetized in 0.01% Tricaine methanesulphonate for 1-2 min until they stop swimming. The anaesthetized 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. The

tubes were kept at 4°C until further use. Fish were returned to the holding tanks for recovery.

Production of disperms using PEG & High p^H (10.5)-high CaCl$_2$:

Milt collected was suspended in 1ml Hank's solution and centrifuged at 1500 rpm for 5 min. The pellet was discarded and the supernatant was centrifuged at 1800 rpm for 5 min. The supernatant was discarded and mixture of 0.5 ml 2.5% PEG and mixture of 0.5ml 1.5mM High p^H (10.5)-high CaCl$_2$ solution was added to the pellet and incubated at room temperature for 8 minutes (Sai Vinathi *et al.,* 2015). Microscopic examination of dispermic condition was carried out using phase-contrast microscope (Leica, Germany).

Collection of eggs:

Eggs of female **D. frankei** were collected through stripping following the procedure as explained in case of sperm collection. Eggs were collected into a petri dish containing synthetic ovarian fluid, in which the eggs remained competent for fertilization for at least 90 min.

Egg genome inactivation:

For the production of androgenetic progeny, eggs were subjected to genome inactivation using a custom-built UV illumination chamber fabricated in collaboration with Labnet, Chennai. In the chamber, a UV lamp of 254 nm (1 mW at the surface of eggs) was used as source of irradiation. 5 ml water was added to the plate containing eggs 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 set up at 25 rpm, permitting eggs to roll in the fluid and thus ensuring uniform exposure to the illumination. A distance of 26cm was maintained between the light source and the eggs by adjusting the height of the shaker. Fertilizability and ploidy (Haploid / Aneuploid) condition were assessed following irradiation of eggs for 2.0min.

I. Fertilization of fresh eggs with single sperms:

Fertilization was induced by suspending eggs (330±20) of **D. frankei** (*dotted*) in a petri plate containing 2 ml 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. Further the live hatchlings were collected and ploidy condition was determined by making the metaphase spreads. This group serves as control for all the groups of androgenetic progeny produced in subsequent experiments.

$$\% \text{ Fertilization} = \frac{\text{No. of fertilized eggs with blastodisc formation}}{\text{Total No. of eggs}} \times 100$$

II. Production of diploid androgenetic progeny using monosperms:

a) Fertilization of genome inactivated eggs with monosperms.

UV-irradiated eggs of **D. frankei** were activated using freshly collected sperms of **D. frankei** and maintained at 28 ± 1° C on a rotator-shaker for 10 min. The dishes were then filled with filtered water from fish tanks and maintained for 15 min. The entire process was conducted in darkness. Fertilized eggs were subjected to heat shock for developing into diploid embryos.

b) Diploidization of fertilized genome inactivated eggs through heat shock:

In order to produce monospermic diploid progeny, the first mitotic division of genome inactivated fertilized eggs should be inhibited by giving heat shock. This is accomplished by exposing eggs for 10 min after fertilization, at 40.5 ± 0.5^0 C in a hot waterbath for 2.2 min. At the end of 2.2 min the eggs were returned to 28 ± 0.5^0 C in order to restore pre-shock condition (Corley-Smith et.al., 1996). Percentage fertilization and hatchlings formed were assessed. Further the ploidy condition of hatchlings was determined by making the metaphase spreads.

III. Production of diploid androgenetic progeny using disperms:

In order to produce diploid androgenetic progeny, disperms suspension was added to genome inactivated eggs in a petridish. The petridish was gently swirled to ensure activation of embryonic development followed by the addition of 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 then transferred to a tray containing aerated tap

water and maintained. Percentage fertilization and hatchlings formed were assessed. Further the ploidy condition of hatchlings was determined by making the metaphase spreads.

Parameters assessed:
Survival:

$$\% \text{ Survival of fertilized eggs} = \frac{\text{No. of fertilized eggs}}{\text{Total no. of eggs}} \times 100$$

$$\% \text{ Survival of hatchlings} = \frac{\text{No. of hatchlings}}{\text{Total no. of eggs}} \times 100$$

IV. Assessment of Ploidy condition of Hatchlings produced using genome inactivated eggs

24 hr after fertilization, subsamples of embryos were used for assessment of ploidy condition. The embryos were dechorionated; and suspended in 0.01 % freshly prepared colchicine solution; incubated at 28 ± 0.5^0 C for 90 min in the dark and transferred into a container with 1.1 % sodium citrate. Embryo yolk was punctured and the timer started. After 8 min, embryos were transferred onto ice, incubated for further 8 min; transferred to a Carnoy's fixative and incubated for 20 min. The fixative was changed and the embryos were incubated at 4^0 C overnight. Then they were blotted partially dry and suspended in 50% acetic acid for 1 min and aspirated using Pasteur pipette for cell dispersion. Dropping of cells onto a pre-warmed (45^0 C) glass slide was carried out following the method of Kligerman and Bloom (1977). Slides were stained in 4 % Giemsa for 30 min and the metaphase spreads were screened for assessing ploidy condition.

Statistical analysis:
Statistical analysis was performed using SPSS commercial statistical package (SPSS, version 16.0 for Windows, SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

Putative diploid androgenotes were produced by fertilizing genome inactivated eggs of ***D. frankie*** (dotted) with single sperms of ***D. frankie*** (albino) subjected to heat shock for chromosomal diplodization. Percentage of fertilization and hatching along with percent ploidy condition of putative diploid androgenotes were recorded and presented in Table 1.

Out of 330 genome inactivated eggs of ***D. frankei*** 258 were found to be fertilized by using fresh single sperms of ***D. frankei*** and 191 out of 335 after using PEG : CaCl$_2$ fused disperms (Table 1). Fertilization between genome inactivated eggs of ***D. frankei*** and single sperms of ***D. frankei*** diploidized through heat shock resulted in 78% fertilization, where as PEG & CaCl$_2$ associated disperms showed 57% fertilization success. These results indicate that the success of fertilization of genome-inactivated eggs appears to be better (Fig. 1) with monosperms than with disperms which could be explained on the basis of decreased probability of fertilization with disperms. In contrast 61.5% fertilization and 4.3% hatching rate was noticed in *Buenos* species with 2.5% PEG incubated disperms (Clifton and Pandian, 2008). Recently Nagoya *et al.*, (2010) observed 53.3% fertilization and 0.22% hatching rate in salmon species with 50% PEG incubated disperms.

Table 1: Number of unfertilized eggs, fertilized eggs and number of hatchlings obtained using Fresh Eggs and Fresh Sperms (FE x FS), Genome inactivated Eggs × Fresh sperms (GIE x FS) and Genome inactivated Egg disperms (GIE x DS) of zebra fish *D. frankei* (albino) along with proportions of variations in ploidy condition of putative androgenotes.

Crosses	No. of Eggs per fish	Fertilized eggs (Nos.)	Hatchlings (Nos.)	Ploidy condition			
				Haploid (n) (%)	Diploid (2n) (%)	Triploid (3n) (%)	Aneuploid (2n-1)
FE x FS	320±12	310±10	263 ± 8	-	100	-	-
GIE x FS	330±16	258±10	48 ± 4	2.2±0.2	81.3±3.7	2.8±0.8	14.2±1.2
GIE x PEG : CaCl₂ DS	335±18	191±8	92 ± 5	3.1±0.2	95.3±5.1	1.7±0.4	1.3±0.8

(Values are Mean (%) ±SD of 6 individual observations)
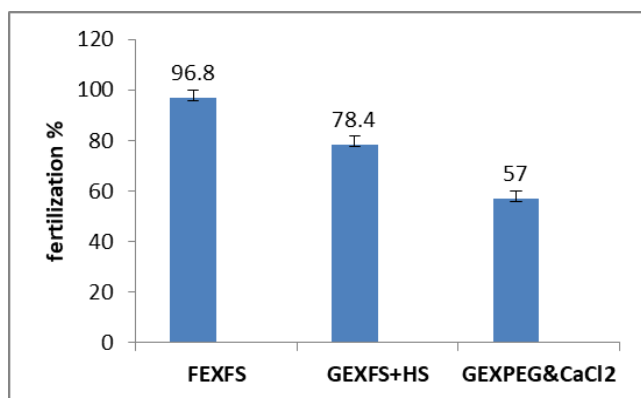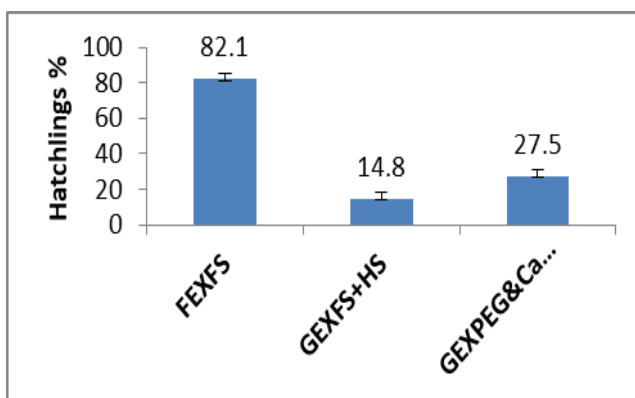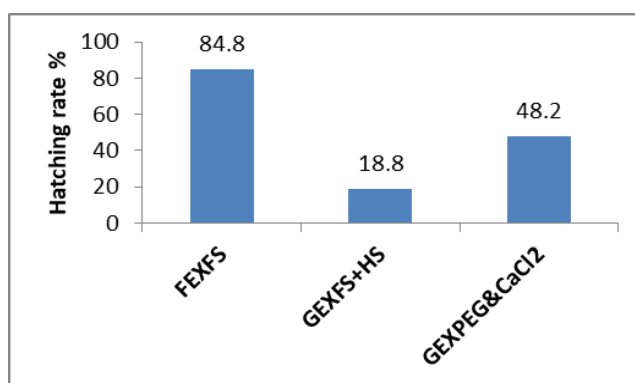
Figure 1

Figure 2

Figure 3

Figure 1: Percent survival of fertilized eggs produced through fertilization of Fresh Eggs and Fresh sperms (FEXFS), Genome inactivated Eggs and Fresh sperms subjected to Heat shock (GIE X FS+ HS) and Genome inactivated Eggs and Disperms produced using PEG & Cacl$_2$ (GIE X DS) of zebra fish *D. frankei* (albino).

Figure 2: Percentage of total number of eggs hatched through fertilization of Fresh Eggs and Fresh Sperms (FE x FS), Genome inactivated Eggs and Fresh sperms subjected to Heat shock (GIE x FS + HS) and Genome inactivated Eggs and Disperms produced using PEG & Cacl$_2$ (GIE x DS) of zebra fish *D. frankei* (albino).

Figure 3: Percentage of fertilized eggs hatched through fertilization of Fresh Eggs and Fresh Sperms (FE x FS), Genome inactivated Eggs and Fresh sperms subjected to Heat shock (GIE x FS + HS) and Genome inactivated Eggs and Disperms produced using PEG & Cacl$_2$ (GIE x DS) of zebra fish *D. frankei* (albino)
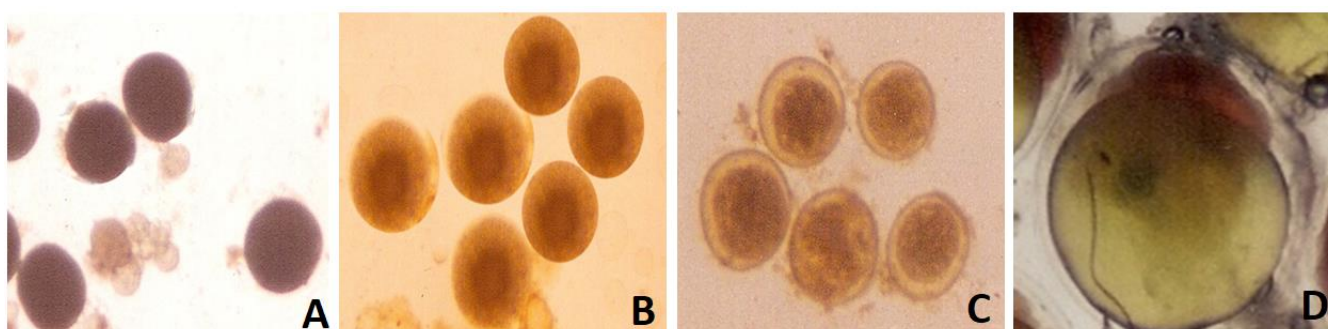

Figure-4: A)Unfertilized eggs B)Fertilized eggs produced (FExFS) using Fresh Eggs and Fresh Sperms C) Eggs 10 min after fertilization D) 8 cell stage fertilized egg
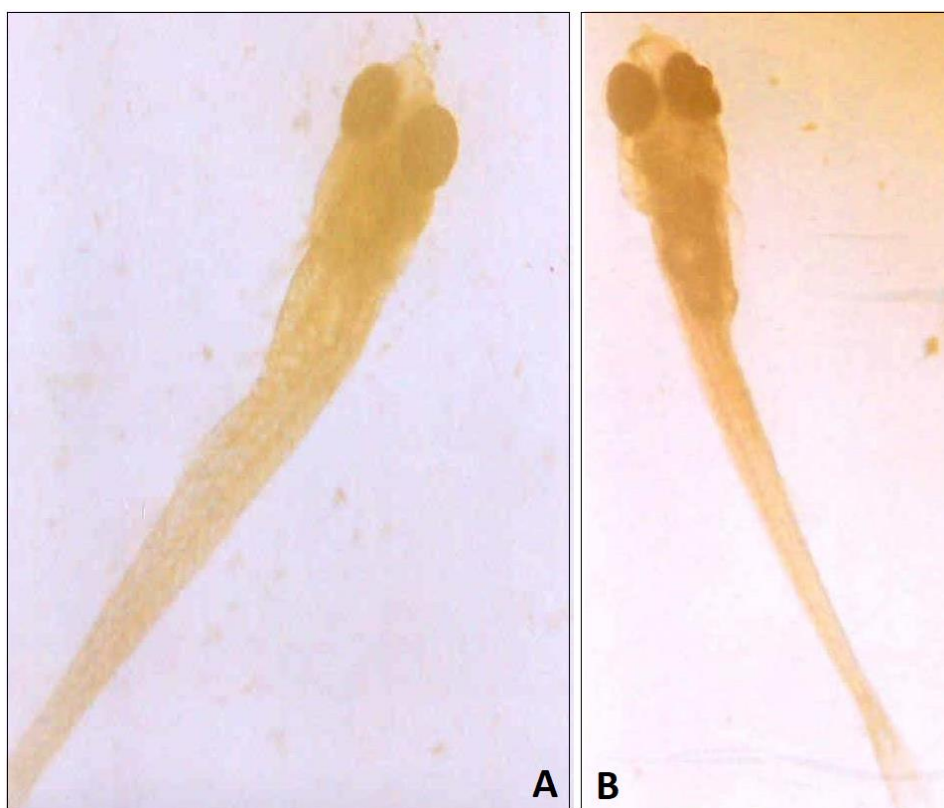
Fig 5: A) Fry produced through fertilization of fresh eggs with fresh sperms (FExFS) and **B)** Fry produced through fertilization of genome inactivated egg with disperm (FExFS) of *D. Frankei*

The percentage of eggs hatched out after fertilizing with 1:1 PEG and $CaCl_2$ treated disperms was highest (27.5%) followed by the genome-inactivated eggs fused with monosperms and heat shock (14.8%), (Fig.2).

Hatching success as a proportion of fertilized genome inactivated eggs further showed that highest proportion (48%) of the fertilized genome-inactivated eggs hatched after using 1:1 PEG & $CaCl_2$ fused sperms compared to heat shock treatment after monospermic fertilization (18%) (Fig.3 & 5). The hatching rate was calculated as the number of hatched larvae relative to the number of eggs that were successfully fertilized.

Fig. 4 shows the morphology of unfertilized fresh, fertilized eggs produced (FExFS) using fresh eggs and fresh sperms, eggs 10 min after fertilization and 8 cell stage fertilized egg. The rate of fertilization was lower with the sperm-fusion method than with the mitosis-inhibition method, but the reverse was true for the hatching rate. However, analysis of ploidy condition carried out by preparing metaphase spreads clearly showed the effect of expected genetic manipulations (Table 1). Larger number of diploids obtained by using fused sperms (Table 1) compared to the number of diploids obtained through heat shock, further showed that survival of androgenotes can be improved if heat shock treatment is avoided by using dispermic fertilization.

CONCLUSION

In summary, this study clearly showed that percent fertilization of genome-inactivated eggs with monosperms is highest. This could be due to the fact that diploidization through heat shock was found to cause greater stress, which results in decreased survival of hatchlings. 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. Since the main purpose of production of diploid androgenotes is to generate the progeny with survival of clones till maturation and their ability to produce subsequent generations studies in this direction are yet to be done considering different species/strains.

Conflicts of interest: The authors stated that no conflicts of interest.

REFERENCES

Babiak I, Dobosz S, Goryzko K, Kuzminski H, Brzuzan P, Cielielski S (2002) Androgenesis in rainbowtrout using cryopreserved spermatozoa the effect of processing and biological factors, *Theriogenology*, 57, 1229-1249.

Binding H (1974) Fusion experiments withisolated protoplasts of *Petunia hybrid* L, Z. *Pflanzenphysiol.* 72:422-426.

Bongers ABJ, Abraca JB, Doulabi Z, Eding EH, Komen J and Richter CJ (1995) Maternal influence on developmentof androgenetic clones of common carp, Cyprinus carpio L. *Aquaculture*, 137, 139–147.

Chin JC and Scott KJ (1979) Effect of phytolectins on isolated protoplasts from plants, *Ann.Bot.*, 43: 33-44.

Constabel F and Kao KN (1974) Agglutination and fusion of plant protoplasts by polyethylene glycol, *Can.J.Biol*. 24: 143-146

Constabel F (1984) Fusion of protoplasts by polyethylene glycol (PEG) in Cell culture and somatic Cell Genetics of plants, Vol I 414-422, Academic Press, New York.

Clifton JD, Pandian TJ (2008) Dispermic induction of interspecific androgenesis in the fish, Buenos Aires tetra using surrogate eggs of widow tetra. *Curr Sci.,* 95, 64–74

Corley-Smith GE, Lim CJ and Brandhorst BP (1996) Production of androgenetic zebrafish (Danio rerio). *Genetics*, 142, 1265–1276.

Haydu Z, Lazar G and Dudtis D (1977) Increased frequency of polyethylene glycol induced protoplast fusion by dimethylsulphoxide, *Plant Sci. Lett.,* 10: 357-360

Herrmann A, Clague MJ and Blumenthal R (1993) The role of the target membrane structure in fusion with influenza virus: effect of modulating erythrocyte transbilayer phospholipid structure Membrane, *Biochemistry* , 10, 3_ 15.

Hui SW, Isac T, Boni LT and Sen A (1985) Action of polyethylene glycol on the fusion of human erythrocyte membranes. *J . Membr. Biol*. 84, 137-146.

Kameya T (1973) The effects of gelatin on aggregation of protoplasts from higher plants, *Planta*, 115:77-82.

Kameya T (1979) Studies on plant cell fusion : Effects of dextran and pronase E on fusion, *Cytologia*, 44:449-456

Kao KN and Michayluk MR (1974) A method for high-frequency intergeneric fusion of plant protoplast, *Planta*. 115,355-367

Kligerman AD and Bloom SE (1977) Rapid chromosome preparations from solid tissues of fishes. *Journal of the Fisheries Research Board of Canada,* 34, 266–269.

Nagata T (1978) A novel cell fusion methods of polyvinyl alcohol, Naturwissenschaften 65:263-264.

Nagoya, Kouichi Kawanura and Hironiohta, (2010) Production of androgenetic amago salmon *oncorhynchus masou ishikawae* with disperrny fertilization. *Fisheries Sciences*, 305-313.

Roos DS, Choppin PW (1985) Biochemical studies on cell fusion. II. Control of fusion response by lipid alteration*. J . Cell Biol*. 101, 1591-1598.

Sai Vinathi DK, Rajyalakshmi M Leelavathi, Nirosha M and Kalarani V (2015) Assessment of the use of cell binding agents useful in the generation of viable offsprings – Biodiversity conservation strategy through production of androgenetic fish *International Journal of Scientific & Engineering Research*, Volume 6, Issue 2

Saunders JA (1985) Electrically induced fusion of cells and protoplasts, in ;*Fronteirs of Membrane Research in Agriculture,*vol.9

Ueda T, Kobayashi M and Sato R (1986) Triploid rainbow trouts induced by polyethylene glycol. *Proc. Jpn. Acad*., 62B,161–164.

Westerfield M (1995) The Zebrafish Book. University of Oregon Press, Eugene.

Yang J and Shen MH (2006) Polyethylene glycol-mediated cell fusion. *Methods Mol Bio*l. ;325:59-66.