Chromosomal Studies on Radiation-Induced Gynogenesis and Diploid Gynogenesis in the Fish Oryzias latipes

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INTRODUCTION

In the fish Oryzias latipes, it has been shown quantitatively that when the sperm were irradiated with ultraviolet light (UV) and were used to fertilize normal eggs, the so-called 'Hertwig effect' occurred, i.e. a high mortality of embryos at low doses but a better survival at high doses. This phenomenon induced by ultraviolet light (UV) or gamma-rays was previously studied quantitatively using the survival frequencies of embryos at various stages during their development. From the genetic analysis of both UV and gamma-ray effects, using the wild-type sperm of this species and then checking the appearance of melanophores on the yolk sacs of embryos, it was suggested that sperm chromosomes do not participate in embryonic development at high doses. The number of chromosomes in cells of the embryos which survive till stage 26 were counted, finding haploid embryos in the dose region of the Hertwig phenomenon. The analysis of chromosome number was mostly in agreement with the genetic studies, but there existed a few cases in which these two methods of analysis did show the opposite results. From these data, the validity of the genetic studies based on the appearance of melanophores on the yolk sac is discussed. Attempts to produce diploid gynogenesis through the cold temperature treatment are also reported.
the chromosome numbers in their embryonic cells. A preliminary attempt was made to produce diploid gynogenetic fish in this species by cold-temperature treatment.

MATERIALS AND METHODS

Sexually mature females of the orange-red variety of *Oryzias latipes* were sacrificed to obtain ripe eggs in an isotonic balanced salt solution. About 20—30 ripe eggs were collected from each female with usually 200—300 eggs being pooled, mixed, and then divided among 2—3 watchglasses. The attachment fibres of the eggs were carefully removed with forceps under a dissecting microscope.

**UV Irradiation**

The testes from 3 mature male fish of wild type (black) were isolated in the same manner, macerated in 2—3 ml of isotonic balanced salt solution, and a sperm suspension without testicular debris was prepared. A portion (c.a. 1 ml) of this suspension was put into a small watchglass and irradiated under a UV source that emitted nearly 95% of its UV energy at a wavelength of 253.7 nm. The dose rate reaching the surface of the sperm suspension was measured by a UV dosimeter (UVR-254, TOPCON). Two dose rates were used in the present experiment: 0.27 J·m⁻²·sec⁻¹ for the doses of 7.5, 15 and 30 J·m⁻², and 2.5 J·m⁻²·sec⁻¹ for the dose of 200 J·m⁻².

**Gamma-Ray Irradiation**

Irradiation of sperm with γ-rays was performed in vivo. Two or three mature male fish were put in a small plastic cylindrical vessel, 5 cm in diameter. The vessels were irradiated laterally with γ-rays from a source of about 4 kCi ¹³⁷Cs in an air-conditioned room at 25°C (Research Center for Nuclear Science and Engineering, University of Tokyo). The dose rates in air were 2.5 kR/min for the dose of 75 kR, and 0.25 kR/min for the dose of 2.5 kR. Immediately after the irradiation, each group of fish was transferred to a glass vessel with 1 liter of isotonic balanced salt solution. It has been already reported that with 75 kR fish survive at least a day. At two hours after the irradiation, the testes from the irradiated male fish were isolated in isotonic balanced salt solution, and the sperm were liberated by tearing these testes apart in a watchglass with 2—3 ml of isotonic balanced salt solution.

**Artificial Insemination and Embryonic Development**

Artificial insemination was carried out, by adding the sperm suspension to the eggs in the watchglasses and then agitating the mixture manually for several minutes; 10 min later, the samples were placed in glass vessels with isotonic
balanced salt solution. The embryos were incubated in the dark. All the UV irradiation procedures and examination of the embryos were done in a dark room at 25°C under a PR-safety light (National, FL 20Y-F). From 1 day after fertilization, the embryos were reared in tap water of which volatile chlorine had been removed.

Observations of the embryos were made every 12 hours for 0–3 days after fertilization and daily thereafter in order to check for surviving embryos and to stage their development according to Matui's normal table for this species.\textsuperscript{6,8} Dead embryos were removed every day after recording the stages at which they had been arrested. The use of color mutants permitted a check on whether or not the fusion of an egg nucleus and a sperm nucleus took place. The gene B (black) found in the wild type (BB) is dominant over the b of the orange-red variety (bb). This gene manifests its activity as an appearance of embryonic melanophores on the yolk sacs of embryos. The validity of this method will be checked and later discussed.

\textit{Chromosome Numbers in Embryonic Cells}

Chromosome numbers in cells of embryos at stage 26 were counted by the usual squash method.\textsuperscript{9} After tearing the chorion with sharp-pointed forceps, embryonic bodies without yolk were immersed in 1/100 M CaCl\textsubscript{2} solution for 20 min, placed in 45% aceto-orcein solution for 90 min, and squashed between a slide and a cover slip. Chromosome numbers in metaphase figures were counted using an oil immersion lens on the microscope.

\textit{Production of Diploid Gynogenetic Embryos (Cold-Temperature Treatment)}

It was reported previously that embryos with a haploid gynogenetic condition can develop no further than stage 27, that is they never hatch.\textsuperscript{4,5} Two types of administration of cold shocks were tested in this preliminary experiment to produce diploid gynogenetic embryos. The first trial was to give the eggs the cold shock after insemination. At various times after insemination with UV-irradiated wild-type sperm, eggs in the glass vessel with the isotonic balanced salt solution were placed in the cooling apparatus adjusted to 0 to +2°C. The duration of this cold treatment was 15 min, after it the embryos were again put in the incubator at 25°C.

In the second trial, cooling of the sperm and the unfertilized eggs was started about 6 hours before the artificial insemination, keeping the adult female and male fish in the cold room (4°C) for 6 hours. All the procedures of collection of eggs, preparation of sperm suspension, UV irradiation of sperm, and artificial insemination, were done in this cold room, under a PR-safety lamp. Inseminated eggs in the glass vessel filled with isotonic balanced salt solution were immediately stored in the cooling apparatus (0 to +2°C). After cold treatments of varying durations, eggs were placed in the incubator at 25°C.
Table 1. Survival frequencies for embryos at various stages after exposure of the sperm to different doses of UV or γ-rays

<table>
<thead>
<tr>
<th>Radiation and dose</th>
<th>γ-ray 2.5 kR (i)</th>
<th>γ-ray 75 kR (ii)</th>
<th>UV 7.5 J·m⁻² (iii)</th>
<th>UV 15 J·m⁻² (i)</th>
<th>UV 30 J·m⁻² (i)</th>
<th>UV 200 J·m⁻² (ii)</th>
<th>Control (i)</th>
<th>Control (ii)</th>
<th>Control (iii)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of eggs inseminated</td>
<td>116</td>
<td>75</td>
<td>62</td>
<td>71</td>
<td>122</td>
<td>68</td>
<td>62</td>
<td>77</td>
<td>52</td>
</tr>
</tbody>
</table>

Number of surviving embryos (Percentage to the number of inseminated eggs)

<table>
<thead>
<tr>
<th>Fertilized eggs</th>
<th>113(97)</th>
<th>73(97)</th>
<th>61(98)</th>
<th>70(99)</th>
<th>119(98)</th>
<th>65(96)</th>
<th>59(95)</th>
<th>74(96)</th>
<th>52(100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 14</td>
<td>102(88)</td>
<td>62(83)</td>
<td>59(95)</td>
<td>63(89)</td>
<td>97(80)</td>
<td>54(79)</td>
<td>53(85)</td>
<td>63(82)</td>
<td>52(100)</td>
</tr>
<tr>
<td>Stage 17</td>
<td>102(88)</td>
<td>47(63)</td>
<td>58(94)</td>
<td>48(68)</td>
<td>89(73)</td>
<td>43(63)</td>
<td>53(85)</td>
<td>57(74)</td>
<td>52(100)</td>
</tr>
<tr>
<td>Total</td>
<td>92(79)</td>
<td>46(61)</td>
<td>58(94)</td>
<td>26(37)</td>
<td>84(69)</td>
<td>39(57)</td>
<td>53(85)</td>
<td>57(74)</td>
<td>52(100)</td>
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<td>58</td>
<td>48</td>
<td>5</td>
<td>2</td>
<td>53</td>
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<td>45</td>
<td>0</td>
<td>0</td>
<td>84</td>
<td>41</td>
<td>0</td>
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<tr>
<td>Stage 19</td>
<td>92(79)</td>
<td>46(61)</td>
<td>58(94)</td>
<td>26(37)</td>
<td>84(69)</td>
<td>39(57)</td>
<td>53(85)</td>
<td>57(74)</td>
<td>52(100)</td>
</tr>
<tr>
<td>Total</td>
<td>56(48)</td>
<td>22(29)</td>
<td>51(82)</td>
<td>10(14)</td>
<td>36(30)</td>
<td>23(34)</td>
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<tr>
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<td>0</td>
<td>33</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

Sperm were taken from the male wild type (BB) and the eggs from the females of the orange-red type (bb). Parentheses express the percentage to the total number of eggs inseminated. The letters B and O denote the phenotypes judged by the appearance of melanophores on the yolk sac: B (black embryo, presumably Bb); O (orange-red, b).

The numbers (i), (ii) and (iii) indicate each set of experiments using the sperm suspension and pooled eggs prepared at the same time.
RESULTS

Survival Data and Genetic Studies
Table 1 summarizes the survival data obtained in the present experiments. Eggs of *Oryzias latipes* were inseminated with sperm irradiated at different doses of γ-rays or UV. Sperm were taken from the males of wild type (BB) and eggs from the females of the orange-red type (bb). Embryos in the control and low-dose groups (γ-rays of 2.5 kR; UV of 7.5 and 15 J·m⁻²) exhibited melanophores on yolk sac (named as 'black' embryos), whereas most embryos in the high-dose groups (γ-rays of 75 kR; UV of 30 and 200 J·m⁻²) lacked melanophores (Table 1 and Fig. 1). Studies on both the dose-survival relationship and the frequency of black embryos have already been reported in detail. The present results are in a good agreement with these earlier studies.⁴,⁵

Chromosome Number
Several embryos were picked from among the survivors at stage 26 from which squash preparations were made. Chromosome figures like those shown in Fig. 2 were observed under a light microscope.

Figure 3 illustrates examples of the results of counting the number of chromosomes in cells in each embryo. Each sample number written on the abscissa denotes one embryo, and each open circle expresses the chromosome number in one cell of that embryo. It has been established that the chromosomes of *Oryzias latipes* are invariably 48 in number.¹⁰,¹¹ Detailed karyotype analysis has recently been performed.¹² Most of the counts made in this report lay in a range less than 48 even in the embryos of the normal (control) group. This is obviously due to the limitations of the squash method, the technique employed in the
present experiments. The distribution of chromosome numbers in the irradiated 2N-type and in the control (non-irradiated) embryos showed no difference in the mean and standard deviation (see Fig. 3).

Table 2 summarizes the results obtained from the examination of the number of chromosomes in squashed embryos. In this table, embryos having a mean chromosome count of more than 30 were classified as 2N-type or diploid-type, and those having a mean less than or equal to 24 as N-type or haploid-type.

Fig. 3. Examples of the chromosome numbers of cells in embryos developed from sperm exposed to UV or γ-rays. Each sample number (1, 2, 3, . . . . . . .) on the abscissa denotes one embryo. Each open circle above it expresses a chromosome number scored on one cell in that particular embryo. The letter B or O just above the sample number denotes the result of genetic analysis: B denotes the appearance of melanophores on yolk sac (black embryo) and O indicates their absence (orange-red). Asterisk (*) expresses the sample which showed N-type chromosome numbers although melanophores could be detected on its yolk sac (B). The data of control embryos normally inseminated with intact sperm are shown at the top left of the figure.
As seen in Fig. 3, there was no difficulty in differentiating the embryos in these two types. Not all the embryos surviving at stage 26 were examined. A random selection was made from among the embryos of each group, in order to check the correlation between the genetic studies (appearance of melanophores on yolk sac) and the chromosome constitution of embryonic cells.

No clearly chimaeric embryos consisting of both 2N-type cells and N-type cells were observed on the squash preparations made at stage 26. In some embryos, only a few cells showed counts of 2N-type amongst predominant N-type (shown in Fig. 3, for example the sample number 1, 2, 5, 6 of 75 kR, γ-rays; 2,3 of 200 J·m\(^{-2}\), UV). Whether such 2N-type chromosome numbers represented true diploid cells or merely resulted from an overlapping of two adjacent cells was not discernible from the squash preparations.

**Production of Diploid Gynogenetic Embryos**

Embryos with a haploid gynogenetic condition can never develop further
than stage 27, and thus die before hatching. Attempts were made to produce diploid gynogenetic embryos, which then may be able to hatch.

The first trial: At different times (5, 10, 15 min) after insemination with UV-irradiated (200 J·m⁻²) sperm, embryos were kept cold (0 to +2°C) for 15 min, then returned to 25°C. Although the number of hatched fry was still very low, the cold treatment started 5 min after insemination seemed to improve hatching (about 1–2% of inseminated eggs).

The second trial: The experiment involving cooling the eggs and sperm before and after fertilization was next attempted. Prior to artificial insemination, adult female and male fish had been kept at 4°C for 6 hours. Sperm irradiation (200 J·m⁻²) and insemination were done also at this temperature, then the inseminated eggs were kept at 0 to +2°C. At different intervals after insemination embryos were returned to 25°C. Several hours of cold treatment after insemination were found to be detrimental to the embryonic development of this species: in the groups which received the cold treatment for 6 or 8 hours after insemination, most embryos died at stage 1–6, i.e. before blastula, and none survived until hatching. In the 1 hour- and 2 hour-treated groups, there was a slight improvement in the incidence of hatched fry (about 4–5% of inseminated eggs), and at one month after hatching these fry were still growing at the normal rate, with no differences in external appearance except that they showed the orange-red color (implying no participation of the sperm genomes).

DISCUSSION

Hertwig (1911) first observed that when frog sperm were exposed to radiation and were subsequently allowed to fertilize normal eggs, a situation emerged in which the embryos showed a high mortality at low doses but displayed a better survival at high doses. This phenomenon is now commonly referred to in the literature as the 'Hertwig effect'. Studies on the Hertwig effect have been reported in various species using the inactivation of sperm by ionizing radiations, ultraviolet light (UV) and several chemicals. However, the mechanism by which gynogenetic haploid embryos result at high doses is not yet completely understood.

It is a widely observed phenomenon that, like the irradiated embryonic nucleus, the irradiated sperm nucleus when fused with an egg nucleus results in chromosome aberrations and chromosome diminutions through bridges in the cells at early cleavage. In the blastula of Salmo gairdnerii developed from sperm irradiated with UV at a dose near that for the Hertwig effect, slightly different chromosome numbers were observed in several cells of the embryo. Similar observations were reported on loach (Misgurnus fossilis). The present experiment on O. latipes showed that, at later embryonic stages such as stage 26, each embryo contains cells of either 2N-type or N-type chromo-
some numbers and exhibits apparently a normal metaphase chromosome pattern. Embryos having both types (i.e. 2N- and N-types) of cells together were rare at such stages.

These data imply that most embryos exposed to low doses carrying severely damaged or abnormal chromosomes may have died in earlier stages, and those surviving till later stages must be the embryos which had a diploid set of genomes in normal looking chromosomes. It is also suggested that some haploid embryos found after low doses (such as 2.5 kR of \( \gamma \)-rays) may have originated from pronuclear syngamy, followed by a progressive loss of the paternal set of chromosomes. However, in most embryos after high doses the sperm nucleus should have failed to fuse with the female nucleus due to the far more severe damages inflicted to the sperm nucleus. After intermediate doses, embryos exhibiting the Hertwig effect may develop by the elimination of the damaged sperm chromosomes during early development. In order to clarify this statement, a time-course study of chromosome numbers from the time of syngamy to the later stages (such as stage 26) is necessary. Such experiments using appropriate doses of radiation are now underway.

In Table 2, out of 15 embryos (2.5 kR, \( \gamma \)-ray) which were judged by the author as black (i.e. melanophores on yolk sac), 2 embryos (with (*) in Table 2) showed an N-type chromosome constitution. Also, in the 75 kR group one embryo had an N-type chromosome number, being judged as a black embryo. The actual quantitative data for these embryos can be seen in Fig. 3. This disagreement may point out the limitations of the genetic techniques using the appearance of melanophores on the yolk sac, when applied to embryos which do not develop normally. The high frequency of black embryos after high doses of \( \gamma \)-rays (corresponding to those required for the Hertwig effect) previously reported,\(^5\) may be accounted for in this way, i.e. a limitation of the analysis when applied to malformed embryos. However, an alternative explanation is possible. The appearance of melanophores may be due to the expression of a part of sperm genomes retained as small fragments, although the cells have a nearly N-type chromosome number. This view has been discussed in detail in the previous paper.\(^5\) The tendency for a lower embryo survival formerly observed by the author after large doses of \( \gamma \)-rays, when compared with similar examples after UV, could not be demonstrated in the present experiment. Such may be ascribed to the condition of the eggs, which depends crucially on the months in which the experiments are performed.

Several experiments have been carried out on radiation-induced diploid gynogenesis in various species of fish.\(^{18,19,31-34}\) In the present experiment in *O. latipes*, the temperature shock (cold treatment) to the unfertilized or just fertilized eggs was employed. The effect of the cold shock on eggs is believed to suppress the metaphase of the second meiosis, thus producing a diploid from a haploid. The second trial performed here, namely the cold treatment both
before and after insemination was planned following the successful report of Romashov et al..\textsuperscript{19} This trial was encouraging. In order to increase the percentage of diploid gynogenesis, further experiments are necessary to find suitable conditions for the onset time of the cold treatment and its duration. For the production of homozygotes which are free from the possibility of the meiotic chiasma, replication of haploid maternal chromosomes was to be followed by a suppression of cell division at the one-cell stage. This was successfully done in zebra fish (\textit{Brachydanio rerio}).\textsuperscript{34} Attempts such as these using hydrostatic pressure, temperature shock or cytochalasin B could be usefully made in \textit{Oryzias latipes}.

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**REFERENCES**