Gamma-Ray Irradiation of the Sperm of the Fish

Oryzias latipes and Induction of Gynogenesis

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When sperm of the fish Oryzias latipes were irradiated with γ-rays (137Cs) and allowed to fertilize normal eggs, the so-called 'Hertwig effect' was observed with a dose-dependent decrease in survival rate at low doses (0-12.5 kR) but a better survival rate at higher dose range (50-150 kR). Compared with the UV-induced Hertwig effect previously reported, the result of γ-irradiation of sperm showed differences in survival rates and in percentage of embryos exhibiting paternal characteristics. It suggested the possibility of the difference in the mechanisms of Hertwig effect caused by UV and by γ-rays.

INTRODUCTION

In 1911, Hertwig found that, when frog sperm exposed to various doses of radiation (radium irradiation) are subsequently allowed to fertilize normal eggs, a paradoxical situation emerges in which the embryos show a high mortality at low doses but display better survival rates at higher doses. This phenomenon is now commonly referred to in the literature as the 'Hertwig effect.' The probable explanation of the Hertwig effect lies in the partial inactivation of the sperm chromatin at low doses with the result that the embryos develop with damaged chromosomal condition, which is detrimental to survival. At higher doses, however, the sperm chromatin is completely inactivated or destroyed, and only the maternal haploid chromosomes participate in further development. This gynogenetic haploid condition seems to be far less deleterious to development. The sperm essentially acts only to give an impetus to development. Studies have been reported in various species on the Hertwig effect through the inactivation of sperm by ionizing radiations, ultraviolet light (UV), and several chemicals.

Previously, in the fish Oryzias latipes, it was shown quantitatively that when sperm of the fish were irradiated with UV and fertilized to normal eggs, the Hertwig effect was observable. Illumination with visible light after fertilization showed the existence of photoreactivation (PR), demonstrating that pyrimidine dimers are a lesion in sperm DNA that is mainly responsible for the UV-caused Hertwig effect.
The present study concerns the induction of Hertwig effect by γ-irradiation of the sperm of *Oryzias latipes.*

**MATERIALS AND METHODS**

Sexually mature females of the orange-red variety of *Oryzias latipes* were killed to provide ripe eggs in an isotonic balanced salt solution of this species.  About 20-30 ripe eggs were obtained from each female, and a total of 200-300 eggs were pooled, mixed and divided into 2-3 watchglasses. Attachment fibers on the eggs were carefully removed with forceps under a dissecting microscope.

Irradiation of sperm was performed in vivo. Two or three mature male fish were put in a small plastic cylindrical vessel of 5 cm in diameter. Irradiation was performed laterally to the vessels with γ-ray source of about 4 kCi $^{137}$Cs in an air-conditioned room at 25°C. The dose-rate in air was 2.5 kR/min previously measured by Fricke dosimeter. Immediately after the irradiation, each lot of fish was transferred to a glass vessel with 1 liter of the balanced salt solution. As previously reported, some fish died during or immediately after the irradiation over the dose of 100 kR. At two hours after the irradiation, the testes from the irradiated male fish were isolated in the isotonic balanced salt solution in order to perform artificial insemination. Sperm were liberated by tearing these testes in a watchglass which contained ripe eggs previously prepared. Samples were agitated manually for several minutes, then ten minutes later they were placed in glass vessels with the balanced salt solution.

From one day after fertilization, embryos were reared in aged tap water. Observations of the embryos were made every 12 hours for 0-3 days after fertilization and once every day afterwards up to hatching, in order to check the surviving embryos and to plot their development according to Matui's normal table of this species. The developmental stages mentioned in this article are: fertilization; stage 17 (blastopore nearing closure; elongated embryonic form); stage 19 (optic bud formation); stage 27 (increased pigmentation of eyes; well-defined pectoral fins; embryo encircles 3/4 of yolk sac); stage 29 (enlargement of optic capsules; movement of pectoral fins; embryo encircles yolk sac entirely); and hatching. Dead embryos were removed every day after recording of the stages at which they had died.

Color mutants of this species were used to check whether or not paternal genomes participated during the development of embryos inseminated with irradiated sperm. The gene $B$ (black) found in the wild type (BB) is dominant over $b$ of the orange-red variety (bb), and can serve as an excellent marker in the developmental genetics of this species. This gene manifests itself in the appearance of embryonic melanophores on the yolk sacs of embryos within 48 hours after fertilization at 25°C. The male fish of the wild type were irradiated with γ-rays and then sperm were inseminated to the untreated eggs of orange-red females. The appearance of embryos carrying melanophores ($Bb$, named 'black embryos') was examined. The absence of black embryos can be interpreted as a sign that the embryos are developing without the participation of paternal
RESULTS

Figure 1 shows the rate of surviving embryos with respect to γ-ray dose, examined at several developmental stages from fertilization to hatching. Irradiated sperm were prepared from the males of the orange-red variety and inseminated to the eggs of the females of the orange-red variety. As can be seen, fertilization took place normally with irradiated sperm in all the dose ranges from 0 to 150 kR. As development proceeded, the survival rates decreased with increasing γ-ray dose. The figure shows the survival rates at different stages of development, including fertilization, stage 17, stage 19, stage 27, stage 29, and hatching. Open circles (○) indicate the survival rates of embryos developed from eggs inseminated with non-irradiated (control) sperm. The open circle at the dose of zero is taken as the average value of all these control data. The two survival rates (○ and ●) at a given dose were obtained using the same collection of eggs once pooled and mixed. Each point represents the survival rate for an initial of 50-150 eggs.

Fig. 1. Survival rates of *Oryzias latipes* embryos (orange-red variety) with respect to γ-ray dose, examined at several developmental stages. Eggs were inseminated with sperm irradiated by γ-rays ($^{137}$Cs) and were incubated at 25°C (●——●). Open circles (○) indicate the survival rates of embryos developed from eggs inseminated with non-irradiated (control) sperm. The open circle at the dose of zero is taken as the average value of all these control data. The two survival rates (○ and ●) at a given dose were obtained using the same collection of eggs once pooled and mixed. Each point represents the survival rate for an initial of 50-150 eggs.
advanced, taking stage 19 (optic bud formation) as a typical example, we saw a dose-dependent decrease in survival rate at low doses (0-12.5 kR). However, as the γ-ray dose increased beyond the value of 50 kR, better survival rates were obtained.

At stage 17 and 19, the characteristic pattern of the Hertwig effect—namely, the dose-dependent decrease in survival rate at low doses followed by a rise in survival capability at higher doses—was seen. Though the survival rates were very low, the similar pattern was also observable at stage 27. After this stage, the response pattern changed: all the embryos at high dose regions died or ceased development at stage 27.

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\text{Survival Rate vs. } \gamma\text{-Ray Dose (kR)}
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Fig. 2. Survival rates of \textit{Oryzias latipes} embryos with respect to γ-ray dose (\textsuperscript{137}Cs), examined at several developmental stages. Sperm of the wild-type fish were irradiated at different γ-ray doses and inseminated to the eggs of orange-red females (○—●). Embryos were incubated at 25°C. Open circles (○) indicate the survival rates of embryos developed from eggs inseminated with non-irradiated (control) sperm. The open circle at the dose of zero is taken as the average value of all these control data. The two survival rates (○ and ●) at a given dose were obtained using the same collection of eggs once pooled and mixed. Each point represents the survival rate for an initial of 50-150 eggs.
Sperm of the wild-type fish were irradiated at different γ-ray doses and inseminated to the eggs of orange-red females. The dose-survival relationships examined at several developmental stages are shown in Fig. 2, exhibiting the similar characteristics as those in Fig. 1. The rates of appearance of 'black embryos' (embryos carrying melanophores on yolk sac) are shown in Table 1. At the doses of 0-12.5 kR, almost all the embryos exhibited melanophores, showing that sperm chromosomes were participating in their development. However, at high doses of 50-125 kR, the rates of embryos carrying melanophores were low.

| Stages examined | 0 (control) | 6.25 | 12.5 | 18.75 | 25 | 50 | 75 | 100 | 125 |
|-----------------|-------------|------|------|-------|----|----|----|-----|-----|
| Stage 17        | 100%        | 58/61b | 37/43 | 11/22 | 7/15 | 4/11 | 4/19 | 6/22 | 2/18 |
| Stage 19        | 100%        | 50/53c | 23/25 | 2/2   | —   | 0/4 | 4/15 | 6/22 | 2/13 |

*a Survival rates are shown in Fig. 2.
*b (Number of black embryos at stage 17)/(Number of total embryos at stage 17).
*c (Number of black embryos at stage 19)/(Number of total embryos at stage 19).

DISCUSSION

The first investigation on the Hertwig phenomenon in fish was made by Oppermann in 1913 using radium irradiation on Salmo trutta sperm.8) Rugh and Clugston23) observed that X-irradiation of the sperm of Fundulus heteroclitus to the dose of 10 and 20 kR did not have so severe effect upon the subsequent development, although lower doses (5 kR) had caused high mortality and abnormality.8) In the loach Misgurnus fossilis, it was also reported that X-irradiation of the sperm showed the Hertwig phenomenon. Percentage of mortality, malformation and disturbed mitoses in embryos increased as the radiation dose increased only within certain limits (2-6 kR). Beyond this limit the damage was lessened and despite an increase in the X-ray dose a gradual return to normal was observed in terms of mortality and percentage of chromosome breaks. Apparently normal individuals could be found again at the doses of radiation starting from 20 kR.10)

As previously reviewed,5) the studies on radiation-induced diploid gynogenesis in fish open up interesting possibilities both in the theoretical and in the practical sense. Some of the doses successfully employed by the investigators in this field were 20 kR (loach),10) 75 kR (goldfish)34) and 100 kR (sturgeon)25) of X-rays. Gamma-rays of 100 kR (60Co) were also utilized (plaice).5)

The egg-activating properties of the sperm did not seem to be altered even by an exposure of sperm to 150 kR of γ-rays, so that fertilization was achieved in the same percentage as that of control (around 90%). Fertilization took place normally also with UV-irradiated sperm in all the dose ranges from 0 to 1000 J·m⁻².13,15)
In the study of UV-induced Hertwig effect in *Oryzias latipes*\(^{13,111}\), the genetic analysis showed a clear-cut result. At low doses of 8.0 and 13.3 J·m\(^{-2}\), almost all the embryos exhibited melanophores, and at high doses of 100-500 J·m\(^{-2}\), which correspond to the region of Hertwig phenomenon characterized with high survival rates of embryos, no embryos (or in some cases only one out of 30 embryos) were carrying melanophores. At the intermediate dose of 21.2 J·m\(^{-2}\), both types of embryos (*b* and *Bb*) emerged. PR enzymes have already been existing in unfertilized and fertilized eggs."\(^{11}\) The effective period for visible-light treatment for repairing UV damage of sperm nucleus was determined to be the early phase (up to around 20-30 min after insemination) of the single-cell stage. This critical time for PR efficiency coincides with the time of syngamy of sperm and egg nuclei in the normal development of this species.

The fate of UV-irradiated sperm after insemination into eggs has recently been observed in amphibians. It was reported that, in eggs inseminated with sperm irradiated at 540 J·m\(^{-2}\), both the sperm pronucleus and the egg pronucleus behaved normally until they moved in close apposition to each other. Thereafter, an apparent coagulation of nucleoplasm was observed in the center of the irradiated sperm nucleus."\(^{17}\)

These studies have led us to conclude that the primary damages to sperm chromatin responsible for the phenomenon are pyrimidine dimers formed in sperm DNA. Molecular damages of such type may lead to the coagulation of nucleoplasm, thus rendering the sperm nuclei incapable of syngamy and incapable of participating in embryonic development thereafter.

In the γ-ray-induced Hertwig effect reported here, the survival rates were lower compared with those of embryos in UV-induced Hertwig region. With the UV doses of 50-500 J·m\(^{-2}\), survival rates at stage 19 took the value around 0.7, whereas in the γ-irradiated case the survival rates at the same stage were about 0.2 (Figs. 1 and 2). The other difference was in the result of genetic analysis. Contrary to the clear-cut data of UV-irradiation, some embryos still exhibited the paternal characteristics even in the dose range of 75-125 kR, where relatively high survival rates were observed (Fig. 2 and Table 1). Such significant number of black embryos indicated that the X-irradiation had not prevented male pronucleus formation or syngamy, and that rather the loss of paternal chromatin occurred in subsequent development. This idea should be investigated by the cytological method, but the present data at least suggest the possibility of the difference in the mechanism of Hertwig effect caused by UV and γ-rays. Further studies remain to be performed on this problem.

Edwards\(^{6,12}\) studied the possibility of induction of gynogenesis in the mouse by UV and X-rays. In the mouse no Hertwig effect was observable because many potential gynogenetic haploids degenerated in very early stages. In comparing the effect of UV with that of X-rays, he stated that in the 3.5-day-old embryos inseminated with UV-irradiated sperm, haploid mitotic figures which possessed exactly 20 chromosomes (2n=40, in this species) were seen. This was quite different from the result of X-rays, which produced the near-haploid mitoses, which contained chromosome fragments (presumably, of paternal chromosomes) in addition to 20 normal...
chromosomes. This result also suggests the difference in the behavior between the UV-irradiated sperm nucleus and X-irradiated one.

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