Effect of γ -radiation on fowl sperm function *in vitro* and *in vivo*

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Summary. Doses of up to 300 Gy of ionizing radiation had little effect on fowl sperm morphology, ATP content and motility when measured *in vitro*. Fertility of eggs from hens inseminated with spermatozoa receiving 50 Gy, in terms of post-oviducal development, was less than 4%. However, 35% appeared 'fertile' by macroscopic examination of the germinal disc of unincubated eggs. These contained few embryonic cells, although the vitelline membrane contained many trapped spermatozoa. After doses of 100 Gy or more, inseminated spermatozoa were not found in the vitelline membrane and no fertile or apparently fertile eggs were produced; nor did such spermatozoa enter the utero-vaginal sperm-host glands.

Genetic transformation using fowl spermatozoa irradiated with doses in excess of 100 Gy appears to be an unlikely prospect.

Introduction

Pandey & Patchell (1982) reported that genetic transformation of egg and feather colour was achieved in the domestic fowl by the use of irradiated spermatozoa. They proposed that a 'pseudo-fertilization' took place when an irradiated nucleus penetrated the ovum. Transformation then occurred when fragments of the irradiated DNA were incorporated into the genome during a subsequent normal fertilization, effected by an unirradiated spermatozoon (Pandey, 1980; Pandey & Patchell, 1982). This hypothesis was based on the observation that fowl spermatozoa retain some motility, assessed subjectively *in vitro*, after exposure to 1000 Gy of radiation (Pandey, 1980), and that following much lower doses (50 Gy) many spermatozoa were capable of initiating abnormal development (Kosin, 1944).

The following work tests the validity of Pandey's hypothesis by a more rigorous assessment of sperm function both *in vitro* and *in vivo*.

Materials and Methods

Birds. Males and females were from a Rhode Island Red strain (Ross Poultry Ltd, Newbridge, Midlothian). They were caged individually, given 14 h light/24 h and fed a commercial breeders ration *ad libitum*.

Semen collection and irradiation. Semen, free of transparent fluid, was collected twice each week according to the method of Lake (1957). Collections from 15–20 birds were pooled to give one sample. Each sample was diluted 2-fold in a glutamate-based diluent, pH 7.1 (Lake & Ravie, 1979), and aliquants, one for each required dose of irradiation, were stored in sealed vials. These vials were transported to the radiation sources by placing them in a vacuum flask containing water at 5°C. Gamma radiation was provided by two sources of different intensities. Source A was a point source delivering 0.055 Gy sec⁻¹ at the chosen locus (1 Gy = 100 rads). This was used for doses of 50 to 200 Gy, each dose ranging by 18% between the top and bottom of the sample. Source B was

more active, delivering 0.2 Gy sec^{-1} . This was used for doses of 300 to 700 Gy. Dose variation within these samples was less than 0.1%. Irradiation was performed at room temperature and all samples, including controls (no irradiation), were held at room temperature for the time taken for the maximum dose (60.3 min for Source A and 60 min for Source B). Samples were then replaced in the flask at 5°C for the return journey. The total time in transit was 25 min and 40 min for the journeys to Sources A and B, respectively.

In-vitro semen tests. Motility was determined by a light-scattering method relying on the rheotactic and optically anisotropic properties of spermatozoa (Wishart & Ross, 1985). Motility was expressed as the constant '%(ΔOD)_m', which defines the maximum percentage fall in absorbance after stopping the movement of a suspension of spermatozoa through a flow-cell. This has been correlated with the percentage of motile fowl spermatozoa in diluted samples of semen (Wishart & Ross, 1985).

Sperm ATP concentrations were determined in boiled extracts using firefly luciferase (Wishart, 1982).

Sperm morphology was assessed by light microscopic appearance of spermatozoa as smears stained with nigrosin and eosin (Lake & Stewart, 1978).

Counting of spermatozoa trapped in the vitelline membrane. A square of vitelline membrane (2–3 cm per side) was removed from around the germinal disc area of eggs. This was then rinsed in Ca^{2+} -, Mg^{2+} -free Dulbecco's phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954) and spread on a glass microscope slide. The preparation was stained *in situ* by adding a few drops of a 1 µg/ml solution of 4,6-diamidino-2-phenylindole (DAPI), covered with a coverslip and sealed with nail varnish. The blue-fluorescing, comma-shaped nuclei were then examined by fluorescence microscopy between 1 and 3 h after staining. Numbers of spermatozoa in an area of 5.49 mm² were counted by scanning a 1 cm-wide gap, delineated with a marker pen, on the slide.

Counting embryonic cells. The vitelline membranes surrounding the yolk was cut in a 1 cm-diameter circle around the germinal area. The whole germinal area was then scooped out with a small spoon and made up to a 1 ml volume with PBS and 1% Triton X-100. This was dispersed with a glass-Teflon homogenizer and the resulting sample was diluted 2-fold with a solution of 1 µg DAPI/ml in PBS. Stained samples were transferred to a haemocytometer and the rounded somatic nuclei were observed by fluorescence microscopy. Numbers were counted by scanning the full 7 mm well of the haemocytometer with a 0.55 mm diameter microscope field, i.e. cells within a total volume of 3.9 µl were counted.

Fertility testing. Of 2-fold diluted semen, 0.1 ml containing about 200×10^6 spermatozoa was inseminated intravaginally into groups of randomly-selected hens. Fertility was assessed by candling with a fibre optic light source after 7 days of incubation. Apparently infertile eggs or those showing abnormal embryonic development were broken open for further examination.

With the unincubated eggs used for counting embryonic cells and spermatozoa caught in the vitelline membrane, 'fertility' was assessed by a macroscopic observation of the germinal area (Kosin, 1945).

Examination of utero-vaginal sperm-host glands. Longitudinal strips (5 × 1 cm) were cut from the dorsal side of the oviduct to include the utero-vaginal junction as well as some vaginal and shell-gland tissue. These were fixed in buffered formalin, embedded in paraffin wax and 6-µm sections were cut lengthwise. The numbers of sections of sperm-host glands in such preparations have been shown to be representative of the whole utero-vaginal area (Van Krey, Siegel & Leighton, 1971). Sections were stained with haematoxylin and eosin. Starting from the vaginal side, sections of glands in 5 mucosal folds were assessed for the presence or absence of spermatozoa.

Results

Spermatozoa were irradiated on two separate occasions, each with different semen samples and caesium sources. This was necessary since it was impractical to obtain lower doses of irradiation with the more active source, and preliminary studies showed that semen held for more than 1 h at room temperature tended to deteriorate. The effects of irradiation on sperm function *in vitro* were compared by describing values as a percentage of control values (Text-fig. 1). Sperm function values in control samples from the two treatments were different: the ATP content (nmol/10⁹ spermatozoa), motility (%(Δ OD)_m) and morphology (% normal) were respectively 44.9, 0.75 and 88, for Source A controls and 39.1, 0.58 and 88 for Source B controls.

Text-fig. 1. The effect of irradiation on sperm function *in vitro*. Samples of fowl semen were divided into aliquants, diluted, irradiated and subsequently incubated aerobically at 40°C. Each point represents the mean of two estimates.

Of 65 eggs laid by hens during 1 week after insemination with semen treated with 50 Gy irradiation, 2 eggs showed postoviducal growth during incubation for 1 week (Text-fig. 2). One had developed as far as the formation of blood islands (approximately 30 h), the other was the size of a 4-day embryo but had severe abnormalities.

Histological examination of the sperm-host glands showed that those taken 2 and 4 h after insemination with control spermatozoa had many spermatozoa in the uterine mucosa as well as in the sperm-host glands. These were not found in preparations from hens inseminated with spermatozoa irradiated with 300 Gy (Table 1).

Irradiated spermatozoa incubated *in vitro* for 4 h were indistinguishable from control spermatozoa when stained with haematoxylin and eosin or DAPI.

Discussion

Kosin (1944), Sarvella (1971) and Pandey (1980) found that the motility of irradiated fowl spermatozoa declined, with respect to control unirradiated samples, at doses greater than 85, 100

Text-fig. 2. The effect of irradiation on sperm function *in vivo*. Samples of semen were divided into aliquants, diluted and treated with different doses of radiation. Twenty hens were inseminated with semen for each dose. The eggs laid by 10 of these hens during 1 week were used for (a) and 3 eggs from each of the remaining 10 hens were used for (b), (c) and (d). Two γ -sources were used: one for 50 to 200 Gy (●); the other for 300 and 700 Gy (▲). When relevant, the s.e.m. is given.

Table 1. Status of the utero-vaginal sperm-host glands after insemination with control and irradiated fowl spermatozoa

Time after insemination (h)	Irradiation dose (Gy)	Bird no.	Total no. of glands counted	% of glands containing spermatozoa
2	0	1	113	87
		2	112	79
2	300	1	68	0
		2	55	0
4	0	1	204	89
		2	61	90
4	300	1	131	0
		2	179	0
24	0	1	50	46
		2	65	46
		3	127	59
		4	63	54
24	300	1	101	0
		2	71	0
		3	69	0
		4	64	0
24	700	1	56	0
		2	84	0
		3	54	0

and 250 Gy, respectively. Our results (Text-fig. 1b) showed that the motility of irradiated spermatozoa was unaffected at doses of up to 300 Gy. It is unlikely that our method of measuring motility is less sensitive because the above authors used a subjective microscopic 'scoring' method of motility estimation. More probably these differences reflect variable 'qualities' of semen: less vigorous spermatozoa from a highly inbred strain showed reduced activity after 300 Gy irradiation (G. J. Wishart, unpublished results). In the present work a dose of 700 Gy markedly reduced the motility, ATP content and morphology of spermatozoa measured *in vitro*. Since these characteristics have been correlated with the fertilizing ability of spermatozoa from individual males (Wishart & Palmer, 1985) it is unlikely that spermatozoa treated with the higher dose of irradiation would function 'normally' *in vivo*.

Normal embryos did not develop in eggs from hens inseminated with spermatozoa which had received the low dose of 50 Gy and at higher doses all eggs were infertile (Text-fig. 2a). These results agree with the work of Kosin (1944).

Following insemination after treatment with doses in excess of 100 Gy, no spermatozoa were found in the vitelline membranes from 107 eggs (Text-fig. 2d). With control semen, a total of 3768 spermatozoa were counted in membranes from 52 eggs. Since the secondary vitelline membrane, in which spermatozoa become caught (Bakst & Howarth, 1977), forms in the infundibulum directly after fertilization (Bain & Hall, 1969) it may be assumed that the irradiated spermatozoa did not reach the ovum. In fact, after irradiation with doses of 300 and 700 Gy, spermatozoa seem incapable of reaching as far as the utero-vaginal sperm-host glands which are recognized as the main oviducal storage site for fowl spermatozoa (Bobr, Lorenz & Ogasawara, 1964). Since spermatozoa could also be found lining the mucosal region of the utero-vaginal junction after insemination with control, but not 300 Gy-irradiated, semen, they probably do not even enter the uterus. Such irradiated spermatozoa, however, remain vigorously motile and appear normal for 6 h *in vitro* (Text-fig. 1). Presumably these spermatozoa can be discriminated and perhaps immobilized in the vagina.

How do these findings reconcile with published effects of irradiated spermatozoa on the development of eggs laid by inseminated hens? Irradiated spermatozoa have been reported to cause an increase in parthenogenetic membrane development (200 Gy: Sarvella, 1971) and to effect genetic transformation by 'pseudofertilizing' eggs (750 Gy: Pandey, 1980; Pandey & Patchell, 1982). Physiologically, these reports are justified on the basis of the measurement of sperm motility *in vitro* and on an extrapolation of the findings of Kosin (1944), that spermatozoa dosed with 50 Gy reach the ovum efficiently, to higher doses. The present results show that neither premise is justified. Other explanations of how their results were achieved are beyond the scope of this work.

Ova from hens inseminated with 50 Gy-dosed spermatozoa were clearly surrounded by significant numbers of spermatozoa at the time of normal fertilization, as indicated by those trapped in the vitelline membrane (Text-fig. 2d). However, these ova showed little sign of development, most having negligible numbers of embryonic nuclei (Text-fig. 2c) although 35% of the germinal discs of laid eggs did not have the lacunae (Text-fig. 2b) which are typical of degenerating infertile ova (Kosin, 1945). If degeneration of these 'ova' had been arrested, it might have resulted from penetration by an irradiated spermatozoon. Therefore, if irradiated spermatozoa are to be used as vectors for genetic transformation, then 50 Gy may be an appropriate dose to use.

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