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The improbability of irradiated spermatozoa as gene transfer vectors in chickens *

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Summary. It is concluded that chromatin fragments derived from irradiated chicken spermatozoa are not viable vectors for gene transfer. In three experiments conducted at sequential intervals over a period of 1½ years, no marker traits were found in 1,065 G_0 progeny from irradiated spermatozoa of Minnesota Dominant Marker males inseminated into recessive Rhode Island Red and White Leghorn females. The inability to secure transformants is ascribed to the following factors: a maximum of five and probably fewer potential vector fragments for each G_0 progeny because of irradiation effect on spermatozoan ability to enter the germinal disc; uncertainty of DNA integrity from highly irradiated chromatin; no known mechanism for release of chromatin fragments from irradiated spermatozoa supernumerary pronuclei; and the uncertainty of selective integration into the zygotic nucleus.

Key words: Irradiated sperm – Transfer vectors – Chickens

Introduction

The experiments reported here were undertaken to determine if chromatin fragments derived from irradiated chicken spermatozoa are viable vectors for gene transfer. Pandy and Patchell (1982), Baumgartner et al. (1986) and Tomita et al. (1988) reported transfers of single gene marker traits using such vectors. Bumstead et al. (1987a) reported transfer of an Ea-B blood group haplotype and an endogenous viral (ev) locus with the same technique. However, in a second experiment, Bumstead et al. (1987b) were unable to duplicate the Ea-B haplotype transfer.

The process of gene transfer begins with providing a dual population of genetically marked irradiated and nonirradiated spermatozoa with different alleles for concomitant entry into the germinal disc. Polyspermy is a normal occurrence in the meroblastic ovum of the chicken, resulting in numerous supernumerary pronuclei. According to the hypothesis, when the normal male and female pronuclei fuse, or shortly thereafter, free chromosomal fragments from a supernumerary pronucleus of irradiated origin could presumably be incorporated into the zygotic nucleus as a mutational event and become part of the genome.

The possibility of transformation by fragmented chromatin from irradiated spermatozoa has appeal because it is procedurally simple and, conceivably, chromosomal segments could contain all the elements necessary for expression. There are, however, disadvantages to this technique. Fragmentation of donor chromatin into segments of appropriate size containing an intact gene as well as controlling elements, with subsequent incorporation into the host genome, presumably would be rare and random events. For each desired gene transferred, it is probable that there would be a random cotransfer at the same frequency for other loci, including undesirable ones. In contrast, transformation with cloned recombinant DNA results in introduction of the single gene of choice. It is estimated that higher eukaryotes, including the chicken, have a minimum of 50,000 gene loci. If there were a 1% transformation frequency for the desired gene, there is an expectation that approximately 500 other loci would be cotransferred.

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We have examined gene transfer using irradiated sperm and report results contrary to those of Pandy and Patchell (1982), Baumgartner et al. (1986), Bumstead et al. (1987a), and Tomita et al. (1988). In three experiments conducted at sequential intervals over a period of about 1 1/2 years, no marker loci were found in 1,065 G_0 progeny from irradiated spermatozoa of the Minnesota Dominant Marker (MDM) males.

Materials and methods

Marker traits

The MDM stock carries 17 dominant morphological traits in varying frequencies. All traits are clearly recognizable in the F_1 crosses between MDM and the multiple recessive Rhode Island Red (RIR) breed. Except for the plumage color traits I, E, B, and S, the White Leghorn (WL) breed is recessive for 13 of the dominant traits found in MDM. The dominant traits and their frequencies are given in Table 1. If transformation occurs before, during, or after syngamy, these loci are capable of expressing in the resultant G_0 progeny.

In a preliminary trial, heterozygous MDM males used for semen irradiation were mated to RIR females and F_1 progeny were scored to determine average expected transmission of dominant traits. Five to 12 of the dominant traits, with an average of approximately 8 were expressed in the cross. The multiple markers also provide a check for possible procedural errors since, at a 1% transformation rate, it would require as many as 1×10^{10} progeny to obtain one chick with five or more of the traits. Thus, in our experiments recovery of a chick with five or more traits would undoubtedly be due to procedural error.

Table 1. Gene frequencies of dominant traits in Minnesota Dominant Markers (MDM), White Leghorn (WL), and Rhode Island Red (RIR) stocks

Traits	MDM	WL	RIR
Plumage colors			
Dominant white–I	80	100	0
Extended black–E	90	50	0
Barring–B	60	?	0
Silver–S	50	?	0
Comb type			
Rose–R	24	0	0
Pea–P	90	0	0
Duplex–D	25	0	0
Plumage variants			
Crest–Cr	25	0	0
Frizzling–F	25	0	0
Feathered shank–Fsh	80	0	0
Naked neck–Na	50	0	0
Muffs and beard–Mb	35	0	0
Skeletal			
Creeper–Cp	36	0	0
Polydactyly–Po	90	0	0
Multiple spurs–M	40	0	0
Double uropygial–U	40	0	0
Skin pigment–W^+	100	0	0

Semen collection

Semen was collected in each experiment from 10–12 MDM males. It was pooled and mixed 1:1 with MnA buffer[1] which is routinely used in our artificial insemination (AI) procedure. This buffer is capable of maintaining spermatozoa with good fertility for 8–24 h. Semen from the RIR and WL males was collected at the same time and mixed 1:1 with buffered diluent. Vials, 10 × 70 mm, containing about 4 ml of diluted semen were kept on ice until insemination.

Irradiation procedure

Four milliliter of diluted semen, in 10 × 70 mm vials, were irradiated for 120 min with a ^{137}Cs gamma irradiation unit (Landsverk-64, S.N. 438) producing 58.6 Gy/min. An exploratory irradiation pretrial test for effects of high irradiation on the semen from MDM males was made at 465 Gy and 698 Gy[2]. Microscopic observation indicated that irradiation caused no loss in motility nor increase in number of abnormal sperm. Recessive RIR females were inseminated once with 0.1 ml of semen diluted 1:1 with buffer. Eggs were collected for 10 days and incubated for 5 days, then broken out and examined for evidence of embryonic development. There was abnormal development in 23 of 106 eggs (Table 2), indicating that spermatozoa had reached the infundibulum and fertilized ova. The remainder of clear germinal discs was either not fertilized or development was stopped so early that it proceeded only for a few cell divisions, and thus could not be detected by visual observation. The dosage of 698 Gy was selected for the three experiments.

Artificial insemination

In Experiment I, 70 RIR multiple recessive females, 8 months of age, were artificially inseminated intravaginally with irradiated MDM semen diluted 1:1 with buffer. The following day they were artificially inseminated with recessive RIR semen. This AI schedule was repeated two more times on consecutive days (Table 3) in order to provide an ample supply of both types of spermatozoa.

Experiment II consisted of two trials with 22 WL females in Trial 1 and 37 WL females in Trial 2. White Leghorns have the dominant melanin inhibitor gene (I) and commonly carry other pigment traits hypostatically. Consequently, extended black (E),

[1] Minnesota Avian buffer (MnA) formula (g/1,000 ml): Tes [N-TRIS (hydroxymethyl) methyl-2-aminoethane sulfonic acid], 4 g; Hepes (N-2 hydroxyethylpiperazine-N'-2-ethane sulfonic acid), 4 g; [N,N-bis(2-hydroxyethyl)-2 aminoethane sulfonic acid], 3 g; potassium hydroxide, 1 g; glucose, 10 g; sorbitol, 0.7 g; potassium phosphate dibasic, 7 g; potassium phosphate monobasic, 1.6 g; sodium phosphate dibasic, 0.8 g; potassium citrate, 0.5 g; magnesium sulphate, 0.35 g; L-glutamic acid (monopotassium salt), 2.1 g; L-glutamic acid (monosodium salt), 6 g; sodium acetate, 2.5 g; potassium acetate, 2.1 g; pH 7.1; osmotic pressure 370 mOsm/kg

[2] Radiation is expressed as roentgens, rads, and Gray units (Gy). To make comparisons between the several reports where different terminology is used, we have converted all quoted dosages to Gray units (Gy). Roentgens indicate the amount administered and rads the amount absorbed. One roentgen equals 0.93 rads and 100 rads equals one Gy.

Table 2. Pretest for effect of semen irradiation on fertilizing capability of spermatozoa

Treatment	No. hens AI	No. eggs Set	Abnormal Develop-ment	Apparent Infertiles
465 Gy	12	52	11	41
698 Gy	12	54	12	42

Table 3. Experiment I. Insemination schedule, fertility, and hatchability for sequential AI of irradiated MDM semen and nonirradiated recessive RIR semen

Males	AI date	Egg date	Eggs set	%F	No. d_1	No. d_2	%H (f)	Chicks hatched
IRR MDM	10/15	10/16	108	0	0	0	0	0
RIR	10/16	10/17	107	0	0	0	0	0
IRR MDM	10/17	10/18	104	77	7	7	83	66
RIR	10/18	10/19	101	93	5	6	88	83
IRR MDM	10/21	10/20	111	97	5	10	86	93
RIR	10/22	10/21	112	100	10	5	87	97
		10/22	97	98	7	3	90	85
		10/23	119	98	7	3	92	107
			859	71	41	34	88	531

barring (B), and silver (S) were not considered reliable markers, since black flecking and ghost barring have been observed in WL stocks. Therefore, only 13 dominant traits exclusive of I, E, B, and S were utilized in these trials. To bypass the possibility that irradiation may have impaired the ability of treated spermatozoa to reach the infundibulum, hens were inseminated into the magnum after laparotomy. Irradiated MDM semen was combined 2:1 with nonirradiated WL semen in buffered extender, to provide a larger proportion of irradiated spermatozoa in the dual populations of spermatozoa available for fertilization. Two-tenths of a milliliter of the mixed diluted semen was placed into the magnum. Eggs were collected for 13 days following insemination in Trial 1 and for 21 days in Trial 2.

In Experiment III, 52 RIR females, 9 months of age, were inseminated with 0.2 ml of diluted, buffered semen by injection directly into the upper magnum, using a ratio of 2:1 irradiated MDM spermatozoa to nonirradiated RIR sperm. Eggs were collected for a period of 21 days following insemination.

Egg collection, incubation, and hatching

Hens were kept in individual laying cages. Eggs were collected daily, identified by cage number and date of lay, then stored at 18 °C for 7–10 days prior to incubation. Eggs were candled on day 7 and all clear eggs were removed to be examined for embryological development. On day 18, eggs were placed in pedigree baskets for identification of chicks at hatching. Chicks were wing-banded and carefully scrutinized at this time and again at 8 weeks of age for presence of dominant traits.

Fertility and embryo mortality observation

Eggs were candled at 7 days of incubation. Those without evidence of embryonic development were broken out and examined for stage of development, if any. Any stage beyond the unfertilized germinal disc was recorded. In practice, the normal fertilized ovum can be distinguished from an infertile one. However, those ova that are fertilized and only pass through a few divisions are indistinguishable from infertile ova. There is a distinct possibility that this may occur with highly irradiated spermatozoa. Consequently, some eggs classified as infertile may in fact have gone through a few cell divisions. Embryos that died up to 5 days of development are classified as dead in stage one (d_1). The remaining embryonic mortality is designated as stage two (d_2).

Table 4. Chick genotypes from successive AI of MDM and RIR semen into RIR hens of 306 chicks with fertility level of 95% and hatchability of 82%

Semen donor	Day after AI	MDM	RIR	Total
MDM	3	19	18	37
RIR	4	25	8	33
MDM	5	18	14	32
RIR	6	20	15	35
	7	14	16	30
	8	11	24	35
	9	12	19	31
	10	14	27	41
	11	15	17	32
		148	158	306

Results

Experiment I

The result of sequential intravaginal insemination of irradiated MDM semen followed by next-day insemination of recessive RIR semen is shown in Table 3. Five hundred thirty-one hatched chicks and 34, 18–21 day embryos, were observed for possible transformants, and all 656 had RIR phenotype. At 8 weeks of age, 527 chicks were reexamined and all were negative for dominant marker traits.

Detectable embryonic development for the first two days following insemination was zero, as expected. The fertility level from day 3 to day 13 was 95%, providing ample opportunity for chromatin contribution from irradiated sperm to be integrated into precleavage or early cleavage stage embryos. The 7% frequency of early dead (d_1) embryos is somewhat above that experienced in MDN, RIR, or F_1 embryos from nonirradiated semen. This suggests that a few irradiated sperm entered the germinal disc and united with a female pronucleus.

Following this experiment it was brought to our attention that in consecutive inseminations, spermatozoa from the last insemination tend to predominantly fertilize subsequent eggs, presumably because of the filling and emptying action of the uterovaginal sperm-host glands (Compton et al. 1978). In order to secure an estimate of the progeny ratio from the sequential insemination of the stocks involved, an indentical AI was made after the trial with MDM and RIR semen into RIR females. The results are given in Table 4 and indicate that

Table 5. Experiment II. Results of simultaneous insemination into the upper oviduct with 2:1 irradiated MDM and nonirradiated WL semen

Trial	No. hens	Days saved	Eggs set	No. fert.	d_1	d_2	Hatch	%F	$\%d_1$	$\%d_2$	%H(f)
1	22	13	157	113	9	11	93	72	8	10	82
2	37	21	407	270	44	117[a]	109	66	16	43	40
	59		564	383	53	128	202	68	14	33	53

[a] Excessive d_2 mortality due to overheated hatcher. Phenotypic descriptions were taken on late dead embryos

Table 6. Experiment III. Results of simultaneous insemination into the upper oviduct with 2:1 irradiated MDM and nonirradiated RIR semen

No. hens	Days saved	Eggs set	No. fert.	d_1	d_2	Hatch	%F	$\%d_1$	$\%d_2$	%H(f)
52	21	660	272	102	54	116	41	38	20	43

the latter insemination does predominate. For the entire schedule the ratios were nearly equivalent. However, the survival of the irradiated spermatozoa may have been different than observed in the subsequent test with nonirradiated semen.

Experiment II

The results are presented in Table 5. Three hundred thirty G_0 progeny were obtained and no dominant marker traits were found. The fertility for the first 10 days exceeded 80%, and although some fertility persisted for 21 days, it declined to 60% by the 12th day after insemination.

Experiment III

Table 6 gives the result of Experiment III in which RIR females were inseminated by upper oviduct injection. No dominant marker traits were found in 170 G_0 progeny at hatching nor in 116 8-week-old chicks. In both Experiments II and III, incubator conditions contributed to embryo mortality, so it was not possible to assess early embryo deaths due to radiation damaged sperm.

In both Experiments II and III, a majority of the hens exhibited intermittent infertile eggs in which it was not possible to detect embryonic development. This pattern indicates that some of the highly irradiated spermatozoa were uniting with a female pronucleus. It suggests that the disorganized genomic complement of the irradiated sperm made it impossible for development to progress beyond a few divisions. In three experiments there were a total of 1,065 G_0 progeny without evidence of a marker trait.

Discussion

Our empirical test of irradiated sperm as a vector to integrate exogenous DNA into the genome of the chicken was negative and at variance with the reports of others. An examination of the pivotal points in the procedure may explain why we were unable to make a successful gene transfer. The probability of a gene transfer via chromatin fragments and its recognition depends upon several factors, including: suitable marker genes, appropriate matings, number of marker loci, integrity of DNA in irradiated chromatin fragment vectors, number of irradiated sperm entering the germinal disc, a mechanism for release of chromatin fragments from supernumerary pronuclei, a mechanism for integration into the newly formed zygote, and positive proof of trait integration.

Appropriate donor marker loci

Genes expected to be expressed in the G_0 generation must be dominant or codominant loci that can be identified unambiguously in resultant transformed progeny. The marker loci used in this series of experiments are clearly identifiable in F_1 progeny from matings to recessive RIR stocks. Thus, we have confidence that integration of any chromatin segment with an intact functional sequence from dominant marker traits would be expressed in G_0 progeny.

Pandy and Patchell (1982) reversed their mating strategy and irradiated semen of a recessive genotype, which would not be expected to express in G_0 progeny. In their backcross test mating, a dominant White Leghorn (II) genotype was mated to possible G_0 transfectants Ii(i), which would not be expected to reveal a

recessive genotype in either II or Ii progeny. The appearance of four ii genotypes from this backcross could not be explained by any known genetic mechanism. The protoporphyrin pigment shell color used by Pandy and Patchell (1982) and Tomita et al.(1988) is an ambiguous trait. Some White Leghorn stocks characteristically segregate for variable amounts of pigment with resultant tinted eggs.

Physical effects of radiation on spermatozoa

Damage to the structural integrity of chromatin from irradiation includes breaks in both single and double DNA strands, destruction of hydrogen bonds and/or DNA bases, cross linkage between polynucleated strands, and breakage of internucleated bonds leading to discontinuities in the sugar-phosphate backbone of the DNA strand (Arnold 1970). All the above events may occur in intensely irradiated chicken spermatozoa chromatin, so that frequency of chromatin fragments with precise breaks containing functional sequences with undamaged internal structure, while unknown, would probably be of a very low order.

Irradiated spermatozoa are vehicles that carry the chromatin fragment vectors with marker gene sequences. In this study it was not possible to accurately determine what proportion of highly irradiated sperm introduced intravaginally reached the infundibulum and entered the germinal disc. The evidence for fertilization is based on visual observation of partial development in incubated eggs. Chromatin damage may be so severe that only a few cell divisions occur and thus fertilized eggs are indistinguishable from infertile ones.

Exposure to 700 Gy can in some cases severely reduce sperm motility, according to Wishart and Dick (1985). They postulated that lack of motility would be a significant barrier for irradiated sperm to reach the infundibulum and enter the germinal disc. However, muscular activity and ciliary movement of uterine cells and possibly sperm motility move sperm from the vaginal region to the infundibulum. Decrease in or absence of motility is correlated with infertility, primarily because it is an indication of impaired or dead spermatozoa that lack capability to enter the germinal disc. In Experiment I, initial sperm motility as judged by microscopic examination was unimpaired and, with alternative daily intravaginal inseminations, irradiated sperm most certainly were in the infundibular region. The intramagnal inseminations of motile sperm in Experiments II and III made certain that adequate numbers of irradiated spermatozoa were available for fertilization. Motility just prior to AI and 3 h following was excellent for all inseminations. We believe that reduced motility of irradiated sperm in our experiments was not a major factor for failure to secure transformants.

Penetration of the germinal disc by irradiated sperm is less certain. Some evidence, both direct and indirect, indicates that irradiated sperm did enter the germinal disc, form pronuclei, and unite with female pronuclei. Evidence by Baumgartner et al. 1986 (100–700 Gy), Wishart and Dick 1985 (50 Gy) and Kosin 1944 (85 Gy) indicated an incidence of early aberrant embryonic development from irradiated spermatozoa. The early abnormal development observed in our pretrial test of irradiated sperm (465 and 698 Gy) suggests that irradiated sperm could have entered the germinal disc.

Spermatozoa behavior in the fertilization process

By 1 h after ovulation, spermatozoa have penetrated the perivitelline membrane, entered the ovum cytoplasm and become transformed into pronuclei (Perry 1987). As many as 9 pronuclei were observed in the meroblastic ova by Perry (1987), and Favona (1965) reported a high of 60 with a mean of 10 male pronuclei. Assuming an average of ten spermatozoa entering the germinal disc with an equal number of irradiated and nonirradiated sperm, there would be an average of five vector spermatozoa in the ooplasm for each G_0 progeny.

When the spermatozoa penetrate the ooplasm, the sperm head enlarges and the previously tightly packaged chromatin is surrounded by vesicles that fuse and form a nuclear envelope around the spherical male pronucleus (Okamura and Nishiyama 1978). Packaging of the spermatozoa chromatin into a pronucleus is a function of the ooplasm, as an ooplasmic vesicle fuses around the chromatin to form a two-layer pronuclear envelope. If this is the case, as indicated in the Okamura and Nishiyama (1978) study, irradiation effects on the sperm would not be expected to interfere with chromatin packaging and no unpackaged chromatin would be available for integration. According to Perry (1987), the supernumerary pronuclei tend to move away from the juxtapositioned male and female pronuclei destined to become the zygote. This reduces opportunity for chromatin fragments to be incorporated into the zygotic nucleus.

Errors in meiosis and fertilization occur with varying frequencies in the reproductive process in the chicken (Thorne et al. 1987; Fechheimer et al. 1983). However, there has been no evidence in hundreds of controlled matings producing thousands of closely observed progeny that chromatin transfer originated from faulty packaging in a supernumerary pronucleus. In view of the long-time evolutionary polyspermy situation in birds, there must be some mechanism to prevent release of chromosomes from the supernumerary pronuclei vesicles or to keep free chromatin from integrating into the zygote.

In the hypothetical system proposed for integration of chromatin fragments, the pronuclear membrane has to

diverge from the normal packaging process and release fragments into the ooplasm. Several factors argue against this possibility. First, sperm irradiated by as much as 50 Gy (Wishart and Dick 1985) and 300 Gy (Baumgartner et al. 1986) were securely packaged in the pronuclear envelope until syngamy occurred with the female pronucleus. Second, radiation damage at high levels (698 Gy), severe enough to result in abnormal pronuclear envelope formation, would probably have adversely affected the sperm's capacity to enter the germinal disc. Third, according to the description of Okamura and Nishiyama (1978), the formation of the pronuclear envelope appears to be under control of the ooplasm and therefore independent of irradiation effects on formation of the pronucleus. On the basis of indirect evidence and no plausible mechanism for disintegration of irradiated sperm pronuclei vesicles, release of free chromatin into the ooplasm appears extremely improbable.

Mechanism for incorporation into the zygotic genome

Incorporation of free fragments into either zygote or male and female pronuclei involved in syngamy via entry through the membrane is potentially possible. Similar transfers have been accomplished under more stringent conditions where cell-free chromosomes in culture have entered a cell nucleus and become part of the genome (McBride and Ozer 1973; Burch and McBride 1975; McBride et al. 1978). Transfer efficiency was extremely low, in the range of 1×10^{-5} to 1.2×10^{-13}. Extra chromatin inclusions in cultured cells may not be detrimental as cells reproduce only themselves, whereas a differentiating zygote could be overwhelmed by the potential cotransfer of the 500–2,000 DNA fragments possible in reported transfer frequencies. In the absence of any conceivable mechanism that would restrict entry to a limited number of fragments including a specific marker gene, it would appear that this is another improbable event prohibiting gene transfer.

In our experiments with 1,065 G_0 progeny, an average of eight loci, and potentially as many as five irradiated sperm in the germinal disc, there could have been as many as 43,200 marker sequences in the ooplasm. In view of our results and the possibility of procedural error and misinterpretation in the reported experiments where only a single locus marker was used, and considering the physical and biological factors in the sequence of events needed for a successful gene transfer, we have to conclude that transformation by means of irradiated sperm is highly improbable if not impossible. To quote Dr. N. Bumstead (personal communication), "It is irritating that after all this work the situation remains largely that of balancing the integrity of the tests against the improbability of the results."

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