THE LABELLING OF LIVING RABBIT OVA WITH FLUORESCENT DYES

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Summary. Rabbit ova can be labelled with fluorescein isothiocyanate (FITC) or rhodamine blue isothiocyanate (RBITC) for use in mixed egg-transfer experiments. Both FITC and RBITC bind to the zona pellucida of the treated egg, allowing recognition of experimental and control groups in a mixed population. Labelling ova with one of these dyes is not detrimental to their penetrability or fertilizability and can be of significant value in a fertilization assay in which experimental and control ova are transferred to the same tubal environment.

The egg-transfer technique has been used extensively to assess the effects of various experimental manipulations on their fertilizability and developmental potential (Adams & Abbott, 1971). For the most part, these experiments have involved the transfer of control eggs to another recipient or to the contralateral oviduct of the same recipient. Such an assay is probably adequate for evaluation of the viability of developing embryos but it may lack sufficient sensitivity for a critical comparison of the fertilizability of two egg populations. Recent evidence has been presented which shows that the pattern of motility may differ significantly for the contralateral oviducts of the same female (Salomy & Harper, 1971). This suggests that variation in the transport of spermatozoa and ova, as well as in their interaction, may occur even between the two oviducts of the same animal. These considerations make it preferable that experiments designed to measure subtle differences in the fertility of ova should utilize an assay in which experimental and control ova are subjected to the same tubal environment. The present communication describes a technique for the fluorescent labelling of living ova which is not detrimental to their penetrability or fertilizability and which permits easy identification of one experimental group within a mixed egg population.

Does were induced to superovulate by treatment with a series of three daily subcutaneous injections of 0.5 i.u. porcine FSH (Sigma) in 1 ml saline, followed by an intravenous injection of 100 i.u. HCG (Pregnyl, Organon) on the 4th day. Eggs were recovered from the oviducts of these does 13 to 14 hr after HCG injection and were denuded of granulosa cells by brief exposure (5 min) to bovine testicular hyaluronidase (type I, Sigma) and gentle shaking for 2 min in 2% sodium citrate (Harper, 1970). The denuded eggs were transferred in groups of

two or three to flat-bottomed watch glasses containing 0.05 ml of the fluorochrome solutions. The dyes evaluated were: fluorescein isothiocyanate (FITC) (isomer 1, Sigma), acridine yellow, acriflavin HCl, auramine O, aurophosphine, berberine HCl, coriphosphine O, euchrysin, euchrysin 2GNX, primulin and rhodamine blue isothiocyanate (RBITC). All the dyes were purchased from K and K Laboratories. Each dye was dissolved in phosphate buffer (pH 7.4) at a concentration of 1 mg/ml. In the case of FITC, RBITC and berberine HCl, it was necessary to dissolve the dye first in a minimum volume (0.1 ml) of 0.1 N-KOH to which the buffer was added; acridine yellow, auramine O and coriphosphine O were similarly dissolved in 0.01 N-HCl before dilution with phosphate buffer. Primulin was soluble only in ether but when this solution was partitioned overnight with phosphate buffer, the fluorochrome activity appeared in the buffer. The final pH of the dye solutions ranged from 7.4 to 8.2.

After immersion in one of the dye solutions for 5 to 10 min at room temperature, the eggs were washed twice with saline and mounted between a glass slide

Table 1. Appearance of ova after exposure to fluorochrome dyes for 10 min

Fluorochrome	Colour of marked eggs			Cytotoxicity
	Phase contrast	Fluorescence		
		Zona	Vitellus	
Acridine yellow	Yellow	Yellow	Green	++
Acriflavin HCl	None	Dull blue	Bright green	+
Auramine O	Yellow	Dull green	Green	—
Aurophosphine	Yellow	Green	Green	++
Berberine HCl	None	Dull green	Dull green	—
Coriphosphine O	Dull green	Dull green	Dull green	++
Euchrysine	Orange	Orange	Orange	+
Euchrysine 2GNX	None	Orange	Bright green	++
Fluorescein isothiocyanate	None	Bright green	None	—
Primulin	None	Dull blue	Bright blue	—
Rhodamine blue isothiocyanate	Pink-red	Orange	Orange	—

and a coverslip for microscopic examination. The colour of each egg was evaluated with phase contrast and fluorescence microscopy (Reichert Zetopan microscope, Binolux IIB lamp, exciter filters KG1, UG1, barrier filter 1) and the condition of the ooplasm was examined for evidence of cytotoxic effects. Ova labelled with FITC, RBITC or primulin (0.2 mg/ml) were mixed with a like number of control ova, handled similarly but incubated in the buffer solution alone. Mixed populations of fluorochrome-labelled and control ova were transferred to the oviducts of recipients 12 to 15 hr after intravaginal insemination of 2 to 5×10^8 spermatozoa. These ova were recovered 24 hr after transfer and were examined for evidence of sperm penetration, fertilization, cleavage and the presence of the fluorescent marker.

Of the eleven dyes tested for their effect on living ova, FITC, RBITC, primulin, auramine O, and berberine HCl showed no obvious sign of toxicity to the egg cytoplasm (Table 1). Three of these, FITC, RBITC and primulin were chosen for further study because they displayed contrasting fluorescence under UV illumination, green, orange and blue respectively (Table 1, Plate 1).

PLATE 1

Unfertilized ova recovered from a rabbit 14 hr after HCG injection and subsequently denuded of granulosa cells. The ova have been labelled with RBITC (R), Primulin (P) or FITC (F). The fourth ovum is an untreated control (C). UV illumination $\times 200$.

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Following transfer of fluorochrome-treated eggs in a mixed population with unmarked control eggs and recovery from the oviduct 24 hr later, FITC-marked eggs were characterized by a bright green fluorescence of the zona pellucida and polar bodies, and RBITC-marked eggs emitted a red-violet

Table 2. Sperm penetration and fertilization of rabbit eggs treated with FITC

Experiment no.	No. of eggs transferred		No. of eggs recovered		No. of eggs fertilized		Mean no. of P.V.S. sperm./egg (range)	
	FITC	Control	FITC	Control	FITC	Control	FITC	Control
1	20	20	17	11	15	9	0.6 (0 to 4)	0.9 (0 to 3)
2	18	18	14	10	13	10	7.7 (1 to 25)	5.2 (0 to 10)
3	6	5	2	3	2	2	10.5 (9 to 12)	16.0 (12 to 21)
4	6	6	6	6	6	5	3.6 (1 to 6)	2.6 (1 to 6)
5	24	21	24	16	18	15	6.4 (4 to 9)	5.9 (3 to 9)
6	10	10	10	10	10	10	7.6 (4 to 15)	5.7 (2 to 8)
Totals	84	80	73	56	64	51	6.1	6.0

Experimental and control eggs were transferred as a mixed population into the oviducts of inseminated recipients and recovered 24 hr later. P.V.S.—perivitelline space.

Table 3. Sperm penetration and fertilization of rabbit eggs treated with RBITC

Experiment no.	No. of eggs transferred		No. of eggs recovered		No. of eggs fertilized		Mean no. of P.V.S. sperm./egg (range)	
	RBITC	Control	RBITC	Control	RBITC	Control	RBITC	Control
1	5	5	5	5	5	5	4.4 (1 to 9)	2.2 (0 to 5)
2	10	10	9	10	8	10	5.7 (3 to 8)	6.0 (1 to 9)
3	10	10	10	8	9	8	6.1 (2 to 10)	6.2 (4 to 10)
4	5	7	5	7	5	6	12.4 (9 to 15)	13.6 (10 to 15)
5	6	6	6	6	6	5	8.5 (5 to 15)	14.2 (5 to 18)
Totals	36	38	35	36	33	34	7.4	8.5

Experimental and control eggs were transferred as a mixed population into the oviducts of inseminated recipients and recovered 24 hr later. P.V.S.—perivitelline space.

fluorescence from the zona pellucida and vitellus. By contrast, primulin-treated eggs could not be distinguished from the unmarked controls. When transferred to inseminated recipients, the ova marked with FITC and RBITC were found to be as penetrable and as fertilizable as unmarked control eggs transferred to

the same oviduct (Tables 2 and 3). This finding was based on the observation that 24 hr after egg transfer, a similar proportion of fluorochrome-labelled and control eggs contained two well-formed pronuclei or had completed the first cleavage division. Similarly, there was no difference between the two groups in the mean number of spermatozoa in the perivitelline space of each egg.

The present experiments demonstrate the feasibility of marking eggs by fluorochrome conjugation for use in mixed egg transfer experiments. Both FITC and RBITC bind to the zona pellucida of the treated egg, allowing recognition of experimental and control groups in a mixed population. The marking technique is analogous to that of fluorochrome conjugation to living spermatozoa (Mellish & Baker, 1970; Overstreet & Adams, 1971; Baker & Degen, 1972) and may be used in combination with that or other techniques for sperm marking.

A method which allows critical evaluation of the penetrability and fertilizability of egg populations exposed to an identical environment can be of significant value for investigation of many aspects of fertilization which at present remain unclear. These include such problems as the existence of chemotaxis between mammalian gametes (Dickmann, 1963), the rôle of the egg vestments in promoting fertilization, and the factors underlying the species specificity of sperm-egg interaction. This method also provides a means for evaluating the response of the ovum and its vestments to treatment with specific enzymes or antisera, as well as to procedures for gamete preservation and storage. Further studies are being carried out in this laboratory at the present time and will be reported in detail elsewhere.

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