Chick production by *in vitro* fertilization of the fowl ovum

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The aim of this study was to produce viable chicks by *in vitro* fertilization and transfer of fertilized ova to the oviduct of recipient hens. Out of a total of 76 transferred ova, 53 were laid with fully calcified shells, 31 of which were fertile (58%). Despite the high rate of embryonic loss, six live chicks were hatched from 12 fertile ova exposed to 0.05 ml of semen (1:200 dilution). Nine healthy chicks were hatched from ten control ova which were recovered from the oviduct following artificial insemination and subsequent transfer to recipient hens. This experimental approach provides a useful model for production of transgenic chicks.

Introduction

Establishment of an *in vitro* culture method that would allow the fertilized ovum to develop into a hatchling could provide a useful means for genetic manipulation in domestic fowl. Freeman and Messer (1985) concluded that there were two outstanding problems, namely, establishing techniques for gene selection and devising methods for insertion of genetic material. Embryos (72 h) have been successfully cultured in surrogate eggshells to hatchlings by Rowlett and Simkiss (1987). Perry (1988) developed a complete culture system from embryo to hatching. Embryos of single-cell stage collected from the magnum were transferred to eggshells and cultured by three elaborate steps. Two healthy chicks were obtained (3%) and three weaklings (4%). Naito *et al.* (1990) improved this method and obtained a higher rate of hatchability (34.4%). Naito *et al.* (1991) incorporated the β-actin–lacZ hybrid gene, *MiwZ*, using the Naito–Perry system. They obtained day 4 embryos (64%) expressing *MiwZ*, but most of the embryos were of mosaic expression. The successful production of chicks by *in vitro* fertilization (IVF) could accomplish two important objectives. It would allow the production of transgenic fowl, as well as resolve the issue of capacitation of fowl spermatozoa. This could provide additional evidence to the observation by Howarth (1971) and Fujihara *et al.* (1973), who used a culture method and postulated that fowl spermatozoa did not require a period of capacitation within the reproductive tract of the hen.

The objective of the present study was to produce chicks by IVF, by returning the fertilized ovum into the oviduct of a recipient hen to complete the egg and shell formation, allowing subsequent incubation.

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Materials and Methods

Clean semen was collected by a one-man technique (Bogdonoff and Schaffner, 1954) from four Rhode Island Red roosters aged 7–14 months. The number of spermatozoa in the pooled semen was $29.2\ (\pm 1.4) \times 10^8\ ml^{-1}$. Since a phosphate buffer (Wilcox and Schaffner, 1958) was used as suitable diluent for the preservation of fowl semen, the pooled semen was diluted 1:1 with the phosphate buffer containing 1 g streptomycin sulfate l^{-1} and 1000 U crystalline penicillin l^{-1} (Meiji Co., Tokyo). A schematic presentation of IVF procedure is shown in Fig. 1. Virgin hens of White Leghorn strain, 6–10 months old, were killed with an overdose of Nembutal (Abbott Labs, Chicago, IL) 25 min after oviposition and the ovulated ovum was recovered from the abdominal cavity or from the infundibulum. Each ovum was placed in a 100 ml beaker containing about 80 ml of modified Ringer's solution which consisted of (in mmol l^{-1}) 145 NaCl, 2.68 KCl, 1.80 $CaCl_2$, 0.24 $NaHCO_3$, 1 g streptomycin sulfate l^{-1} and 1000 U crystalline penicillin l^{-1}. (Whenever reference is made to Ringer's solution or phosphate buffer, it always contained antibiotics as above.) The twofold diluted semen was further extended 1:100 with Ringer's solution just before application of 0.01–0.10 ml directly over the blastodisc of the immersed ovum (Howarth and Palmer, 1972), followed by incubation at 41°C for 15 min to allow for *in vitro* fertilization. The ova were then washed twice in 300 ml Ringer's solution to remove the spermatozoa, and they were kept at 41°C individually in 100 ml beakers containing fresh Ringer's solution.

Within a few minutes after oviposition of a midsequence egg, hens were anaesthetized with Nembutal (about 0.8 ml) and an incision, approximately 6 cm long, was made on the left side of the abdomen. The infundibulum was exteriorized and affixed with three clamps on a horizontal bar of a stand. The washed ovum was poured from a beaker into the open infundibulum. After removing the clips, the infundibulum was gently shaken to move the ovum into the anterior portion of the magnum.

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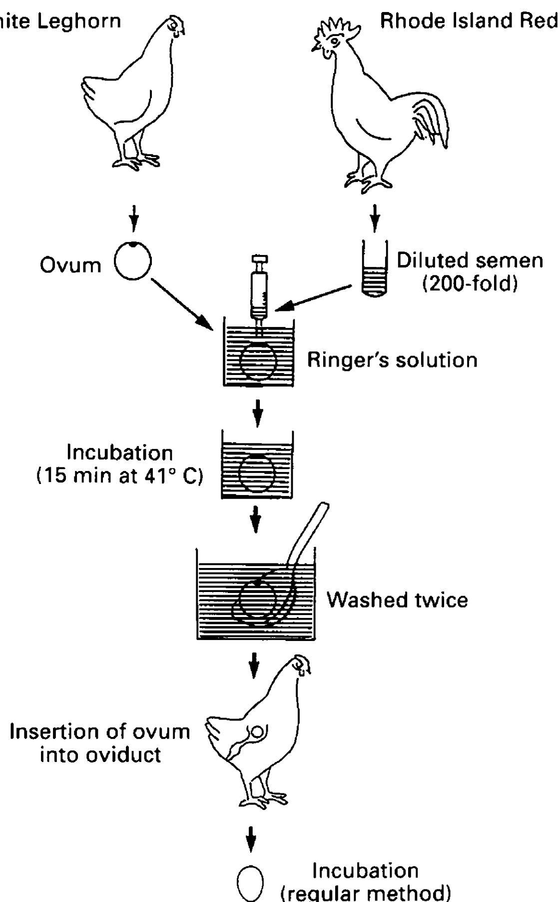

Fig. 1. Schematic representation of IVF procedure. Unfertilized ovum was placed in a beaker containing Ringer's solution and diluted semen (0.01–0.10 ml) was placed over the blastodisc. The ovum was then washed twice with Ringer's solution and inserted into the oviduct of a virgin hen shortly after oviposition. The egg laid on the following day was incubated.

The incision was then closed. Care was taken not to leave trapped air inside the oviduct, and to minimize the duration of the operation, to avoid undue stress to the hens. The entire procedure was performed under sterile conditions. Eggs laid on the day following the transfer of the ovum into the oviduct were completely normal in appearance, and were placed in an incubator for hatching under standard conditions. As a control, pooled semen collected from the same four roosters used in this study was diluted 1:1 with phosphate buffer and 0.1 ml was used for each of ten hens to inseminate intravaginally. Two days after insemination, the hens were killed by an overdose of Nembutal 1 h after oviposition and the ova were recovered from the infundibulum or the anterior part of the magnum. The ova were placed separately in a 100 ml beaker containing Ringer's solution until transfer to virgin recipient hens, as described above. On the next day all 10 hens laid fully calcified eggs, which were then placed in a standard incubator.

Results

Embryos that died during incubation were classified into three groups, according to their developmental stages (Hamburger and Hamilton, 1951) as shown (Table 1). Of a total of 86 transferred ova, 63 shelled eggs (73%) were produced on the day after transfer. The rates of production of shelled eggs and fertility were comparable among the three experimental groups using increasing volume of semen (0.01–0.10 ml). However, only in the group in which 0.05 ml semen was used were six fertilized eggs (50%) hatched. Of these, four chicks were healthy and two died during incubation (Table 1). There was a high incidence of early embryonic death between days 1 and 9 in all three experimental groups, but there was no significant difference in the embryonic mortality among the three incubation periods and among the three semen applications. By comparison, all ten ova transferred in the control group were laid shelled, of which nine eggs were hatched and one embryo died after incubation for 2 days. There was a significant difference in early embryonic mortality and hatchability between IVF and inseminated control groups ($P < 0.05$).

Discussion

This study demonstrated that fertile eggs can be obtained by IVF of ova removed shortly after ovulation and subsequently transplanted into the oviduct of recipient hens. It was important that the ovum was inserted into the infundibulum immediately or shortly after oviposition, otherwise shelled eggs were seldom laid. In addition, the ovum should be located in the anterior magnum before closing the incision, to avoid

Table 1. Percentage of fertilized eggs laid and viability of embryos following transfer of *in vitro* or *in vivo* fertilized ova to recipient hens

Semen (ml)	Number of ova transferred	Number of shelled eggs laid	Percentage of fertile eggs[a]	Viability of embryos			Number of chicks hatched day 21–22
				day 1–4	day 5–9	day 10–19	
0.01	16	11	55 (6/11)	3	2	1	0
0.05	27	22	55 (12/22)	2	2	2	6
0.10	33	20	65 (13/20)	3	9	1	0
Control[b]	10	10	100 (10/10)	1	0	0	9
Total	86	63	65 (41/63)	9	13	4	15

[a]Figures in parentheses indicate the number of fertilized eggs per shelled eggs laid.
[b]Oviductal ova ovulated following artificial insemination of hens were transferred to recipient hens and eggs laid were incubated.

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retrograde expulsion into the abdominal cavity. Although a fertility rate of 55–65% was obtained by IVF, only six of a total of 31 fertile eggs were hatched, because of losses during embryogenesis, particularly during the first 10 days of incubation. As eggs laid by hens in the control group had a 90% hatchability, such losses following IVF might have been due to excessive polyspermy, or to fertilization by damaged spermatozoa (Van Krey *et al.*, 1966; Lorenz and Ogasawara, 1968) or to microbial contamination (Tanaka and Koga, 1971), as the so-called 'sperm selection mechanism' (Lorenz and Ogasawara, 1968) is absent in the IVF system. In addition, longer manipulation *in vitro* compared with controls, such as semen application and washing of the ovum, might have affected the viability of embryos. Moreover, the results indicate that the relative volume of semen to number of spermatozoa has an important influence on the hatchability of *in vitro* fertilized eggs, as only in the group in which 0.05 ml of a 1:200 diluted semen was applied were live hatchlings obtained.

Howarth (1971) and Fujihara *et al.* (1973) showed that fowl spermatozoa do not require a period of capacitation within the reproductive tract of the hen. They cultured ova for 12–24 h after *in vitro* application of semen and examined the blastodisc of the ovum by histology. Our data support their observations and provide conclusive evidence that capacitation in the reproductive tract is not an essential feature of fertilization in fowl.

In a preliminary study, we attempted to incorporate *MiwZ* of linearized form into embryos of one-cell stage which were recovered from the anterior magnum, and transferred to recipient hens. Twenty shelled eggs were obtained of which three hatched and three embryos died during incubation. One embryo expressed *MiwZ*, but the hatchlings did not.

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