Studies on the functional activity of organotypically cultured mouse ovary

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Short-term maintenance of mouse and rat ovary in organotypic culture system is no longer a problem (Martinovitch, 1938; Gaillard, 1953; Trowell, 1959). Gaillard (1953) cultivated ovaries from 7- to 8-day-old and 21-day-old mice for a week on the plasma clot. Trowell (1959) maintained ovaries of 8-day-old mice on a synthetic medium in an O₂–CO₂ atmosphere for 9 days. He observed no histological differentiation in the tissues of the ovary. What needs confirmation and further investigation is the possibility of maintenance of functional activity of the ovary under culture conditions. A study was therefore undertaken to investigate if an ovary, cultivated in vitro for some time, shows hormonal activity when transplanted in vivo. In the present work cultured ovaries were grafted in the anterior eye-chamber of spayed female mice and the development of secondary sex organs such as mammary glands and uterus was studied.

MATERIAL AND METHODS

This study was carried out on ICRC mice, an inbred line of albino mouse of high breast-tumour incidence, developed at these laboratories and described before (Ranadive & Kanekar, 1963). Ovaries from 12- to 14-day-old mice were used for cultivation. Both the ovaries of an animal were dissected out and only polar cortical portions were cut out for cultivation. Ovarian pieces were explanted on the surface of plasma clot in embryologic watch-glasses as illustrated in the diagram (Text-fig. 1). Thus four ovarian explants from each of the eighteen females were cultivated separately in the culture vessels. A few ovaries were fixed in Bouin’s fluid for histological observations.

Intraocular transplantation

The technique for intraocular transplantation standardized by Martinovitch (1951) was adopted in these experiments with slight modification. The host animals were anaesthetized with Nembutal (660 μg/10 g body wt) and the cornea

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was pierced near the sclera, with a sharp cataract knife which may be bent at the tip for easy operation. The extruded aqueous humour was wiped off with moistened cotton wool. The corneal opening was enlarged to the desired size with iridectomy scissors. The size of the opening was ascertained and the inside space was cleared with a fine glass rod. The organ or tissue was introduced into the anterior chamber with the help of a fine scalpel and was slipped far inside with the blunt side of the scalpel. Care was taken not to damage the blood vessels. The recipients of the cultured ovaries were ICRC females spayed at the age of 21–28 days.

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Text-fig. 1. The sequence in the study of hormonal activity of a cultured ovary. (1) Cortical explants prepared from the ovaries of a 12-day-old female mouse. (2) The four ovarian explants from the same animal cultured for 15 days. (3) Three explants grafted for 5–10 days in the anterior eye-chamber of a spayed mouse. Fourth explant was fixed for histological study. Criteria to determine functional activity of ovarian transplants in castrate recipients: (a) cornification of the vaginal mucosa; (b) development of the uterus; (c) ductal proliferation in the mammary glands.

Design of the experiment

The experiment may be divided into three parts: (1) in vitro explantation of ovaries; (2) transplantation of cultured ovaries to the ocular chamber; (3) bioassay—to test functional activity of the transplanted ovary. Observations on target organs (vagina, uterine horns and mammary glands) of spayed hosts were used to determine functional activity of the cultured ovary.

RESULTS

(1) Explantation of ovaries in vitro

The cultivation of the ovarian explants was carried out in embryological watch-glasses at a temperature of 34–35 °C. The nutritive medium was composed of freshly prepared chicken plasma and chicken embryo extract in Tyrode’s balanced salt solution in equal proportion. Cultures were transferred to fresh nutritive medium every 3 days.
Fig. A. Ovary of a 13-day-old ICRC female. ×120.
Fig. B. Ovary of a 12-day-old female cultivated for 15 days. ×99.
Fig. C. Section of the same ovary culture as in Fig. B, showing growing follicles at higher magnification. ×330.
Fig. D. Section of the same ovary culture as in Fig. B, showing a dividing oocyte. ×660.

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Cultivated ovarian explants were transplanted into the anterior eye-chamber of spayed female mice on the 15th and 30th days of cultivation. A few explants and all transplants were fixed in Bouin's fluid for histological studies. The sections were stained with haematoxylin and eosin. Mammary glands (II and III pairs only) of recipients were fixed in 10% neutral formalin and stained with haematoxylin.

Histological observations on control and cultivated ovaries

Ovaries from 13-day-old mice (control group). The ovarian tissue of a 13-day-old mouse is mainly composed of cortical tissue in which Graafian follicles are developing (Plate 1, fig. A). The Graafian follicles are composed of 2–4 layers of epithelial cells in which mitotic figures are occasionally seen. The formation of the antrum by the widening of intercellular space is conspicuous in some follicles. The oocyte has a nucleus with one or two nucleoli. All oocytes are seen to be in the resting phase. Some of them look atretic. Clusters of primordial follicles are seen towards the periphery. Loose stromal cells with spindle-shaped nuclei are quite common. A thin germinal epithelium is present.

Ovaries from 12-day-old mice cultivated for 15 days. Explants of cortical tissue were prepared and cultivated for 15 days. During the first 24 h of explantation, the explants were encapsulated and grew as a unit organ. The central portion of the explant was found to be a little necrosed during the course of cultivation. The tissue of the explant was mainly composed of primordial and growing follicles (Plate 1, fig. B). Growing follicles contained a large oocyte with an eccentric nucleus. Cells of the epithelium were cuboid and the thecal cells were spindle-shaped (Plate 1, fig. C). At this stage of development follicular fluid had not been formed. Mitoses were observed in the germinal epithelium, stromal tissue and in the follicular epithelium. A few oocytes were also observed in division (Plate 1, fig. D).

Ovaries from 12-day-old mice cultivated for 30 days. The follicular protrusions which appeared in the ovary in the first 15 days of cultivation disappeared afterwards and the cultivated explants presented a rather blurred surface. In the histological preparations (Plate 2, fig. E) many Graafian follicles towards the centre were found to be atretic. However, primordial and growing follicles at the periphery were seen to be in a healthy condition. All follicles had a one- or two-cell-thick epithelium. Stromal cells were loose and spindle-shaped. Only occasional mitotic figures were observed in the follicular epithelium.

(2) Transplantation of cultured ovaries into the eye

(a) This part of the experiment was divided into two groups. In the first group, the grafted ovaries were cultivated for 15 days while in the other they were cultivated for 30 days. Cultivated explants of ovaries were transplanted into the anterior chamber of the eye of ICRC spayed females. The transplantations were done in a group of 7–8 mice, which were observed for 5–10 days. Daily vaginal smears of the spayed recipients were recorded carefully, and on killing the animals
the mammary glands and uterine horns were dissected out and fixed for morphological and histological studies.

Two groups of controls were maintained: one group received non-cultivated ovary while the other was maintained as spayed control.

Control groups

(b) Spayed recipients of non-cultivated ovaries. Normal ovaries from 13-day-old ICRC females were directly grafted to the anterior chamber of the spayed ICRC females. Only three animals with one control spayed mouse were used for this experiment. Six days after transplantation, the host females and the control spayed animals were killed and the target organs were studied.

(c) Spayed controls without ovarian transplants. An additional group of spayed mice was maintained as spayed controls without ovarian transplants.

Intraocular graft of cultivated ovary

Morphology and histology. During the in vivo period of 10 days in the intraocular chamber the transplanted ovaries showed advanced development of follicles appearing as translucent beads through the cornea of the host. On histological examination, these explants of ovarian cortex showed only cortical stroma with Graafian follicles (Plate 2, fig. F). Follicles were at different stages of development: primordial, growing and mature Graafian follicles. A mature follicle showed a stratified follicular epithelium, the basal layer of which consisted of columnar cells. The thecal layer surrounding the granulosa was a few cells thick. The cells were fibrocytic in shape with vacuolated cytoplasm. The oocyte was contained in the cumulus oophorus in the antrum. It had granular ooplasm and an eccentric nucleus. In between the follicles the stromal tissue simulated the appearance of interstitial glands (Plate 2, fig. G). Cells of these glands had round to oval nuclei and the cytoplasm was foamy and scanty. There were no mitotic figures. Islets of these cells were bounded by mesenchymal fibres.

Experimental group

(a) Spayed recipients of cultivated ovaries. The observations include both the groups: one receiving 12-day-old ovary cultivated for 15 days; the other receiving 30-day-old ovarian culture. Intraocular grafting was done on eight animals in the first group and on seven animals in the other. The responses of the target organs are summarized in Tables 1 and 2.

Vaginal smears. All spayed recipients with intraocular grafts showed cornification of the vaginal mucosa. Some of the females were in continuous oestrus.

Uterine horns. The uterus in these recipient females showed significant development (Plate 2, fig. H).

Mammary glands. In the spayed recipients of the cultivated ovaries ductal branching in the mammary glands was quite extensive. Some of the primary
Fig. E. Ovary of a 12-day-old female cultivated for 30 days. × 480.
Fig. F. Ovarian transplant in eye-chamber. × 29.7.
Fig. G. Section of the same transplant as in fig. F, showing Graafian follicles and interstitial glands. × 99.
Fig. H. Uterine horns of a spayed control (left) and recipient (right).
Figs. I, J. Second and third mammary glands respectively of a spayed recipient. ×4.2.
Figs. K, L. Second and third mammary glands respectively of a spayed control. ×4.2.
ducts, particularly those of the second pair, showed moderate dilatation. The ducts ended in club-shaped terminal buds, indicating the possibility of further ductal development (Plate 3, figs. I, J).

**Control groups**

(b) *Spayed recipients of non-cultivated ovaries.* This control group was made up of three animals. In this group vaginal smears showed continuous dioestrus, and the target organs—uterus and mammary glands—were not stimulated.

(c) *Spayed females with no ovarian grafts.* In these spayed controls vaginal smears showed continuous dioestrus. The uterine horns were thin and thread-like (Plate 2, fig. H) and the mammary glands presented only short slender ducts indicating stunted growth (Plate 3, figs. K, L).

Both the control groups (b) and (c) thus showed no indication of stimulation of target organs in contrast to the recipients of cultivated ovaries.

**DISCUSSION**

In order to study whether an ovary, cultivated *in vitro* for some time, attains its functional activity when transplanted *in vivo*, the cultured ovaries were grafted into the anterior chamber of the eye of female mice which had been spayed earlier and the development of the target organs such as mammary glands and uterine horns was studied.

Ovaries from embryonic or very young mice do not differentiate much histologically under culture conditions. Those from the weanling mice cannot be maintained *in vitro* sufficiently long. After consideration of the rate of development and the maintenance of the organ *in vitro*, ovaries of 12- to 14-day-old mice were selected and found to be the most useful material for long-term cultivation and the study of hormonal activity.

The ovaries of 12- to 14-day-old mice were very well maintained for about 30 days *in vitro*. During the first 15 days of cultivation, the explants showed organized growth in the tissues and increase in the number of layers of granulosa cells, but after that period growth stopped and only steady maintenance was possible for the next 15 days. All these explants, however, differentiated further in the ocular site. The cuboidal cells of the basal follicular epithelium developed into columnar cells. The stromal cells have also differentiated.

It is interesting to note here that the mesenchymal fibres and interstitial cells did not differentiate in the 15-day-old and 30-day-old ovary cultures. This differentiation was, however, possible when explants were transplanted *in vivo*. Theca interna was also differentiated in the ovary in the ocular chamber. According to Dubreuil (1957) granulosa cells induce the development of the theca interna. This thecal layer is believed to be responsible for the secretion of oestrogens. The cultured ovary may therefore attain hormonal activity when transplanted *in vivo*. 
Table 1. Response of spayed recipients to the isotransplants of ovaries cultivated for 15 days

<table>
<thead>
<tr>
<th>Serial no. of mice</th>
<th>Age at the time of transplantation (days)</th>
<th>Duration of transplantation (days)</th>
<th>Vaginal smears*</th>
<th>Weight of uterus (mg)</th>
<th>Mammary glands: ductal proliferation in II and III pairs†</th>
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<td>7</td>
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<tr>
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<tr>
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<td>7</td>
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<tr>
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<td>10</td>
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<tr>
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<td>.</td>
<td>DE DE DE DE</td>
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</table>

* PE, Prooestrus; OE, oestrus; M I, metoestrus I; MII, metoestrus II; DE, dioestrous; ± E, poor cornification.
† 4+, Ductal proliferation over tertiary branching; 3+, ductal proliferation to tertiary branching; —, absence of ductal proliferation.
Table 2. Response of spayed recipients to the isotransplants of ovaries cultivated for 30 days

<table>
<thead>
<tr>
<th>Serial no. of mice</th>
<th>Age at the time of transplantation (days)</th>
<th>Duration of transplantation (days)</th>
<th>Vaginal smears</th>
<th>Weight of uterus (mg)</th>
<th>Mammary glands: ductal proliferation in II and III pairs</th>
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<td>1</td>
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<td>5</td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>23</td>
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</tr>
<tr>
<td>7</td>
<td>42</td>
<td>5</td>
<td>OE</td>
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<tr>
<td>Spayed control without ovarian graft</td>
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<td>8</td>
<td>42</td>
<td></td>
<td>DE DE DE DE</td>
<td>18.7</td>
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</table>

Symbols as in Table 1.
The hormonal activity of the cultured ovaries had been determined from the response of target organs of the spayed female host. Three cortical explants of ovaries from one and the same animal were transplanted into the anterior eye-chamber of a female ICRC mouse which was ovariectomized at weaning. The host animal was thus totally devoid of its own ovarian hormones well before the ovaries were placed in the eye. During the course of 5–10 days in the eye the explants stimulated the growth of mammary glands and the uterus in the host. Continuous vaginal cornification was clearly observed. This stimulation of target organs was not found in the group of spayed females grafted with non-cultivated ovaries. The uterus and mammary glands instead showed regression. It can therefore be said that the ovarian explants cultivated \textit{in vitro} did attain a certain degree of tissue differentiation and there was evidence of functional activity of the organ. Maintenance of functional activity of C57 ovaries cultivated on hormone-enriched medium has recently been shown by Jacobs (1963).

The ovarian explants cultivated for 15 days caused stimulation in all the animals under experiment. Those cultivated for 30 days stimulated the organs only in about 40\% of animals under study. The failure of 60\% of the explants to stimulate the target organs may be due to the degenerating condition of the explants.

In some explants, cultivated for 15 days, a few oocytes showed metaphase. In mice, within 3–4 days of birth the oocytes enter the resting phase from diplo-tene. This phase continues throughout prepubertal and adult life until pre-ovulation maturation or pseudomaturation associated with atresia. The atresia in the ovary under cultivation may be due to sudden interruption in the blood supply or inadequate nutrition. In the present case the other signs of follicular atresia are not apparent. Ooplasm is not much granulated. The follicular epithelium is active, showing mitotic divisions. On the other hand, if the dividing oocyte is taken as going into preovulation maturation, the follicle does not appear to have attained that maturity. Metaphases observed in these oocytes are thus interesting but difficult to explain.

**SUMMARY**

1. Functional activity of cultured mouse ovary was studied by isologous transplantation into the ocular chamber of spayed mice. The experiments were carried out on ICRC mice. The ovaries were explanted from 12- to 14-day-old females.

2. The cortical explants of the ovaries were organotypically cultivated for 15 and 30 days on the coagulated medium composed of chick plasma and chick embryo extract in the embryological watch-glass. The transplant in the ocular chamber was maintained for 5-10 days.

3. The ovaries cultivated for 15 days stimulated the target organs in all the recipients that received the grafts. Those cultivated for 30 days did so in 3 out of 7 recipients.
Functional activity of mouse ovary

Résumé

Recherches sur l'activité fonctionnelle d'ovaires de souris en culture organotypique

1. On a étudié l'activité fonctionnelle d'ovaire de souris en culture au moyen de transplantations isologues dans la chambre oculaire de souris castrées. Les expériences ont été faites sur des souris ICRC. Les ovaires ont été prélevés sur des femelles âgées de 12 à 14 jours.

2. On a pratiqué la culture organotypique des explants corticaux d'ovaires pendant 15 et 30 jours, sur un milieu coagulé composé de plasma de poulet et d'extrait embryonnaire de poulet, dans des verres de montre. La transplantation dans la chambre oculaire a été maintenue pendant 5 à 10 jours.


It is a pleasure to acknowledge technical assistance rendered by Mr A. V. Bhat and Mr S. L. Naik.

Références


(Manuscript received 30 September 1965)