THE VAGINAL SMEAR OF SENILE LABORATORY RATS

BY D. L. INGRAM

From the Department of Anatomy, University of Birmingham*

(Received 16 April 1959)

SUMMARY

Rats aged 17 months or more did not undergo regular oestrous cycles during a period of 12 weeks when daily vaginal smears were taken. Vaginal cornification occurred at irregular intervals in most of the animals. Its incidence decreased following unilateral ovariectomy and temporarily increased following injections of gonadotrophin.

Acceptance of the male was rare and spasmodic and could not be correlated consistently with the presence of cornified cells in the vaginal smears.

Compensatory ovarian hypertrophy after unilateral ovariectomy occurred in all senile animals examined. The response of the vaginal epithelium to oestrogen did not appear to be less in senile than in young animals.

It has been shown that fertility, as measured by the number of young per litter, declines with age [Ingram, Mandl & Zuckerman, 1958]. The possibility that the loss of fertility may be at least partially due to a decline in the number of oocytes with increasing age is supported by the results of a study in which the number of oocytes was reduced by graded doses of X-rays [Ingram, 1958]. In this investigation it was shown that the mean litter-size declines step by step with the reduction in the number of oocytes. It was, however, also demonstrated that irradiated animals with only 1000–2000 oocytes were still capable of producing young, whereas the majority of normal animals at 18 months, although possessing 2000–3000 oocytes, are sterile [Arai, 1920; Mandl & Shelton, 1959; Shelton, 1959]. It is therefore apparent that while litter-size can be related to the numbers of oocytes in the ovary, some other factor which is also related to age and which operates before all the oocytes have been used up, must be involved for complete infertility to occur. The present study was designed to investigate three possible causes of infertility or irregularity in the oestrous cycle of old rats. First, that the pituitary fails to produce gonadotrophin; second, that the ovary no longer responds to gonadotrophin either by producing hormones, or by the maturation of follicles; and third, that the vaginal epithelium becomes insensitive to oestrogen.

Animals

The rats used in this experiment were derived from the Birmingham Colony. All were virgin animals which had been kept for the study of ageing. None of the animals had previously been subjected to any experimental treatment. At the start of the experiment, they were 17–20 months old, with the exception of one litter aged 12 months. Fourteen young animals aged 4–8 months were used as controls.

* Present address: The Hannah Dairy Research Institute, Ayr.
VAGINAL SMEAR OF SENILE RATS

183

Design of the experiments

Cyclic changes in the vaginal smear and mating behaviour in the senile rat

Vaginal smears were taken daily for 3 weeks from seventeen senile rats. Thereafter, the animals were housed with males of proven fertility; ten animals were left with the male for a further period of 9 weeks, and the remaining seven for 3 weeks. The latter group was then used for the next experiment (unilateral ovariectomy).

Compensatory ovarian hypertrophy

Seven animals were unilaterally ovariectomized and vaginal smears taken continuously for a further period of 6 weeks.

Response to exogenous gonadotrophin

Seven animals which had failed to become pregnant after being caged for 2 months with a fertile male were treated with gonadotrophin. Smears were taken from these animals for 14 days before treatment with doses of gonadotrophic hormones (10 i.u. pregnant mares' serum (PMS; Organon) followed 3 days later by 20 i.u. chorionic gonadotrophin (CG; Organon)) known to cause ovulation in the hypophysectomized rat [Rowlands & Williams, 1943]. A second course of injections was given 21 days after the end of the first course. Vaginal smears were taken for a period of 10 weeks.

After an interval of 6 months following the last injection, the animals that had been treated with gonadotrophin were ovariectomized and given 0.3 µg oestradiol monobenzoate (OB) in arachis oil. These animals were 23–26 months old at the time. Fourteen young adult females, from 4 to 8 months old, were ovariectomized and used as controls. Of these, seven received 0.3 µg and the remaining seven 0.2 µg OB/day for 10 days. Daily vaginal smears were taken for 10 days after operation.

Vaginal smears

Vaginal smears were taken in the usual way. The slides were fixed and stained by the method described by Mandl [1951a]. The smears were classified into the four commonly recognized phases of the oestrous cycle: early oestrus, oestrus, early dioestrus and dioestrus.

One or both ovaries were removed in the usual way through dorso-lumbar incisions.

RESULTS

Frequency of vaginal cornification

(a) Intact senile rats (ten). None of the animals showed regular oestrous cycles, but most underwent periods of 1 or more days during which the smear contained cornified epithelial cells and no leucocytes. The frequency with which cornification occurred was about the same throughout the experimental period (0.65 days/week).

(b) Effect of unilateral ovariectomy (seven rats). The removal of one ovary appears to reduce the frequency of vaginal cornification in the senile rats. While one animal which had not shown any cornification during the 6 weeks before operation did not do so afterwards, two animals which had had periods of cornification before unilateral
ovariectomy failed to show them after the operation. When the numbers of periods of vaginal cornification are compared (Fig. 2), it appears that significantly fewer periods occurred after unilateral ovariectomy than before ($P = 0.01$). A comparison of the number of periods of vaginal cornification in control senile animals (Fig. 1) during the first and second half of the experimental period reveals no significant difference ($P = 0.1$).

(c) Treatment with gonadotrophins (seven rats). During the 7 days of treatment with gonadotrophin (3 days following the first injection of 10 i.u. PMS and 4 days

![Fig. 1. Frequency of vaginal cornification in intact senile rats from three different litters (A, B, C) placed with males after the first 3 weeks. (Cornification shown by black columns: a single column represents a day on which smear contained cornified cells; $s =$ sperm in smear; L.B. = litter born).](image)

![Fig. 2. As above: senile rats from two litters (A, B) before and after unilateral ovariectomy (marked by ↓). Males introduced after first 3 weeks.](image)
following the second of 20 i.u. CG), vaginal cornification was recorded 1.3 times/week/animal (mean). In comparison, smears consisting of cornified cells were recorded only 0.65 times/week from each animal during the 14 days before treatment was started. The frequency of cornification fell off during the following 14 days (mean 0.55) and even after a second course of gonadotrophin injections did not regain the high level which had been observed during the first period of treatment (Fig. 3). None of these variations in incidence of vaginal cornification were statistically significant.

![Graph showing frequency of vaginal cornification](image)

**Fig. 3.** Frequency of vaginal cornification in intact senile females (not litter-mates) treated with gonadotrophin (cf. Fig. 1). Males present throughout whole period. (s = sperm in smear; ‡, injection of PMS; ‡, injection of CG.)

**Mating behaviour**

Presence of spermatozoa was found to be associated with various types of smears (e.g. smears of leucocytes and mucus, as well as those consisting of cornified cells only). In twelve of the sixteen instances in which spermatozoa were seen, the vaginal smear had been cornified on the previous day; in the other four instances, however, the oestrous type of smear was not observed, either before or after mating. One of the animals which became pregnant (see below) had not shown vaginal cornification for 7 days before it mated. Only two animals, one 14 and the other 20 months old, became pregnant and subsequently gave birth to litters of two and six young, respectively.

None of the unilaterally ovariectomized animals mated before or after the operation. Treatment with gonadotrophin did not appear to influence mating behaviour. Only one of the animals mated during the treatment. Four of the animals did, however, mate some time after the treatment was completed, one 11 days and the rest 4–5 weeks after the last injection.
Compensatory ovarian hypertrophy after unilateral ovariectomy

A comparison of the weights of the ovaries removed surgically and their fellows recovered at autopsy demonstrates that compensatory hypertrophy had occurred in every case (Table 1; mean difference between the two ovaries 25 ± 4 mg; P < 0.001).

Sensitivity of vaginal epithelium to oestrogen

None of the seven mature females which were spayed at the age of 4–8 months and received 0.2 µg OB/day showed a fully cornified vaginal smear. Smears consisting of nucleated epithelial cells (i.e. typical of early oestrus) were, however, observed. In contrast, both the young and senile animals which had received 0.3 µg OB showed fully cornified vaginal smears. In some instances, the smears taken from the senile animals contained mucus in addition to the cornified cells.

<table>
<thead>
<tr>
<th>Litter no.</th>
<th>Right ovary* (mg)</th>
<th>Left ovary† (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (4 rats)</td>
<td>36</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>58</td>
</tr>
<tr>
<td>2 (3 rats)</td>
<td>22</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>84</td>
</tr>
</tbody>
</table>

* Removed at operation. † Recovered at autopsy 6 weeks later.

DISCUSSION

In most of the animals used in the present investigation the vaginal epithelium became cornified at irregular intervals. There is little evidence, however, to suggest that this was always associated with 'oestrus', defined as the period characterized by ovulation and acceptance of the male. First, vaginal cornification often persisted for several days. Second, the presence of spermatozoa in the vagina did not consistently coincide with a period during which the vaginal epithelium was known to be cornified; and third, although in the young adult mating does not always occur at the first oestrus after pairing, in old animals mating was extremely infrequent in comparison with the incidence of vaginal cornification. Similar conditions occur after X-ray sterilization of the ovary [Mandl & Zuckerman, 1956; Mandl, 1959a]. The vagina of such animals becomes cornified for 2–14 weeks, but mating is hardly ever observed. One explanation which may apply equally to both irradiated and old animals (and to animals in which persistent cornification sets in at the age of about 6 months [Everett, 1939]) is that there is an absence or deficiency of progesterone at the time the vagina is cornified.

The presence of mucus in the vaginal smear was frequently observed in the present study, and occasionally it coincided with acceptance of the male. Wolfe [1943] has discussed the occurrence of spontaneous mucification of the vaginal smear in old animals, and suggested two possible explanations. One is that mucification is associ-
ated with the production of progesterone, and the second is that it is related to a secretion of a 'subthreshold' amount of oestrogen. The possibility that less oestrogen than normal is secreted by the ovaries of the senile rat is also suggested by the occurrence of vaginal smears consisting of both cornified cells and leucocytes. In the spayed animal this type of smear has been attributed to the secretion of 'subthreshold' amounts of oestrogen by the adrenal gland [Kostitch & Telebakovitch, 1929; Mandl, 1951b]. It is also worth noting that corpora lutea in very old rats frequently attain the size characteristic of corpora lutea of pseudopregnancy in younger animals [Mandl, 1959b]. The latter finding has led to the hypothesis that the pituitary of the senile rat may be able to secrete luteotrophic hormone in the absence of the mating stimulus. Whether or not these large corpora lutea are functionally active has not yet been determined.

A further possibility is that in the old rat the vaginal epithelium becomes less sensitive to oestrogen. Thung, Boot & Mühloch [1956] have reported that old mice are less sensitive to oestrogen than young ones. These workers found that in twelve senile and twenty-three young ovariectomized mice, the percentage of animals coming into full vaginal cornification increased as the dose of oestrogen was augmented. At the same time, they found that young animals reacted to a lower dose of oestrogen than did the old. In the present study, none of the young ovariectomized animals showed full vaginal cornification when given 0-2 µg OB/day, but both young and old animals reacted consistently to 0-3 µg. The vaginal smears taken from the old animals did, however, contain mucus in addition to the cornified cells, whereas those from the young animals did not. It would thus appear that while in mice the decrease in the frequency of oestrous cycles may be related to a fall in the sensitivity of the target organs to oestrogen, this does not apply to rats.

The prolonged periods of dioestrus observed in old animals may simply be related to a deficiency of oestrogen. On the other hand, persistent dioestrus can be produced experimentally by testosterone [Noble, 1939; Robson, 1936], androsterone [Browman, 1937] or progesterone [Phillips, 1937]. Prolonged periods of dioestrus might thus be attributed to the production of these hormones, either by the ovary or the adrenal glands. The possibility that the large corpora lutea frequently found in old rats [Mandl, 1959b] secrete progesterone clearly requires to be investigated.

The observation, made in the course of this study, that the removal of one ovary results in compensatory hypertrophy of its fellow indicates: (i) that the ovaries of these animals contain tissue which is capable of responding to an increased titre of gonadotrophin; and (ii) that the pituitary is still able to produce gonadotrophin. The failure of all these animals to undergo regular oestrous cycles of the length shown by the majority of young adults belonging to the same colony [Mandl, 1951a] cannot thus be attributed to an absolute failure of either the pituitary or the ovary. A possible explanation is that as the ovary ages and the number of oocytes diminishes follicles become atretic before ovulation.

Part of the costs of this study was defrayed from a grant made to Prof. Sir Solly Zuckerman by the Population Council, Inc.

The author wishes to thank Prof. Sir Solly Zuckerman and Dr A. M. Mandl for their helpful criticism.
REFERENCES

Everett, J. W. [1939]. *Endocrinology*, 25, 123.
Noble, R. L. [1939]. *J. Endocrin.* 1, 184.