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ON THE REPEATED USE OF MICE IN THE QUANTITATIVE BIOASSAY OF OESTROGEN WITH THE INTRAVAGINAL METHOD*

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It is known that evidence on the physiological action of oestrogen has been greatly advanced by the finding of the Allen-Doisy method (1). Later, the method of the oestrogen assay was improved as to sensitivity by Berger's success (2), in introducing the intravaginal application of oestrogen. Many useful modifications have been made since then (3—9), and the method has been extensively adopted in the field of reproductive physiology as well as that of clinical medicine.

However, despite its many profits this method, as well as other bioassay methods, is not be devoid of some disadvantages; namely, it required time, labour and expense. Differently expressed, the method requires some preparative treatments in making assay mice before starting the assay. Describing in detail, by daily examination of the smears, the following tests had to be carried out: i) ascertaining the existence of the oestrous cycle of the mice, ii) making certain of the disappearance of the cycle after ovariectomy, and iii) the mice thus treated could show positive results to certain amounts of oestrogen. These pretreatments needed at least 5 weeks.

Although some previous papers suggested that the assay animal was used in succession, it was not clearly reported how many times the animal could be used for assay (3, 5, 6). On this point, Biggers demonstrated (8) the secular variations in "median effective doses" of oestrogen in the course of 18 tests (done every second week up to 45 weeks), giving occasional primings, and found a temporal decrease in sensitivity of one colony, not in other one. Meanwhile

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Nagasawa (12) showed, in the course of his work, that the responsibility of the mice remained unchanged during three usages as far as his tests went.

The authors, hereupon, conducted an experiment to clarify whether the mice which were easily obtainable for us, could be employed in succession for the assay of oestrogen. If this is proved, improvements in time, labour and expense, as described above, could be expected, and then the assay be done more efficiently.

Materials and Methods

a) *Experimental Animals.*

Mice, dd-strain of Kasukabe, of 14—16g in body weight were bought and the occurrence of oestrus was confirmed by smear tests for 10 days. Some failed to show oestrus and these were omitted. Next, ovariectomy was carried out bilaterally and then for another 10 days the ascertainment of its effect, *i.e.*, stopping the oestrous cycle was examined.

A group of mice which had been prepared about five months previously (group A) was preliminarily used and results were compared with those of another group freshly prepared (group B). For one point of hormone concentration, more than three mice were allotted (3). The mice were fed on a laying hens' ration (No. 2, Yashima Sangyo, Tokyo) that was shortly cooked.

b) *Standard hormone suspension and the way of its administration*

As a hormone, synthetic oestrone (Tokyo Chemicals Mft.) was used. Oestrone was first dissolved into a small amount of *N*-NaOH ($2\mu g/ml$) and then was diluted with distilled water until the final concentrations ranging from 0.000025 to $0.0003\mu g/0.015$ *ml* (this is the total volume given to a mouse). The standard solutions were kept in the refrigirator throughout the experiment.

The instillation of the solutions were performed twice daily for two days with 1 *ml* Tuberculin injection syringe driven by a micrometer with screw gauge (6) (Natsume Mft. Co., Tokyo). Volumetric examination of each syringe had been previously checked. The needle for the instillation was cut at 0.5cm from the base and its top was ground smooth so as not to injure the vaginal wall. Otherwise, smear figures would be modified by the appearance of leucocytes and others which would give a false result. Careful attention was also paid to gentle insertion of the needle (6).

c) *Judging standard of the smear.*

Following the next morning of the final instillation, the smears were examined twice for two days. Positive or negative results was solely decided by the amount of the cornified epithelium which appeared in the smear. The amount of the epithelium was classified into four grades as follows:

(+++) ··· absolute dominance of the epithelium.

(++) ····· relatively dominant with the epithelium.

(+) ····· dominance in other kinds of cells, though the epithelium were also existent.

(−) ····· scarcity of the epithelium.

The first two (+++, ++) were regarded as a positive response.

d) *Repeated usage of the mice*

The second test was performed on the 13th day from the first instillation, examining daily the smears from the 6th to 12th day of the first test. Likewise the same procedure was repeated up to the seventh test.

Results and Discussion

The results obtained in the experiment are presented in Table 1. The individual response of group B is illustrated in Table 2.

Table 1. Response of mice used repeatedly for oestrogen determination.

	Oestrone μg / 0.015ml	1	2	3	4	5	6	7
	0.0003	5/6			3/3	3/3	0/2	1/2
	0.0002	5/6	2/3	3/3	3/3	3/3	1/3	1/3
A	0.0001	3/3	3/4	4/4	1/4	1/3	0/2	0/3
	0.00005		0/2					
	0.000025		0/4					
	0.0002	6/6	2/3	3/8	3/3	2/3	2/3	2/3
B	0.0001	3/5	3/4	2/4	2/4	1/4	2/3	1/3
	0.00005		1/4	0/4	1/4	1/4	1/3	0/3

Denominator and numerator indicate no. of mice used, and of positives, respectively.

As seen in Table 1, group A demonstrated a fairly high positive percentage with 0.0001 μg until the 3rd test and so it was with 0.0002 and 0.0003 μg until the 5th, although a long time had passed before it was used (5 months). The response seemed to be lowered with these doses since then. At doses below 0.0001 μg, though examined once at the 2nd test, slight response was shown.

As an experiment with group A, which started a little earlier than group B, was likely to indicate the minimum effective dose as 0.0001 μg, the dosage for group B was allotted at around 0.0001 μg with the exception of the first test. High response more than 2/3 was obtained with 0.0002 μg throughout the experiment until the seventh test. The same response appeared with 0.0001 μg until the 2nd test, and later, the sensitivity seemed to decline. With 0.00005 μg, as well as in group A, no value above 1/3 was entirely obtained.

Next, let us examine the individual records of the mice (group B) in response (Table 2). Among three mice which were given 0.0002 μg in all tests, one (No. 3) showed negative commencing with the 5th test. In the results with 0.0001μg, some fluctuation in response was observed.

Table 2. Record of response of individual mice at each test. (group B)

Animal no. \ Test no	1	2	3	4	5	6	7
1	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+
3	+	−	+	+	−	−	−
4	+	(+)	(+)	(−)	(−)	(−)	(−)
5	+	(−)	(+)	(+)	(−)	(+)	(−)
6	+	(+)	(−)	(+)	(+)	(+)	(+)
7	(+)	(+)	(−)	(−)	(−)	⊕	⊖
8	(+)	⊖	⊖	⊕	⊖	/	
9	(−)	⊖	⊖	⊖	⊖	⊖	⊖
10	(+)	⊖	⊖	⊖	⊖	⊖	⊖
11	(−)	⊕	⊖	⊖	⊕	/	

( ), and ○ indicate doses of 0.0001 and 0.00005μg, respectively.
/indicates death of mice.

Being different from our earlier anticipation that the minimum effective dose might be 0.0001 μg, it seemed better to compare the results adopting 0.0002 μg as minimum effective dose. Consequently the authors took this dose for analysis of the results obtained in the present experiment. In regards to the minimum effective dose, some reports (10, 13, 14) indicated as 0.0002 μg, and the other, as 0.0001 μg (6).

It can be said that the mice would be safely used for seven times as group B showed. However, it happened that some mice lost their responsibility in the later tests as seen in Table 2. Likewise, Biggers obtained (8) considerable variations of medium effective dose of oestrogen in secular usage of mice, using the mice every second week, and particularly a marked fall during 6th to 8th test in a colony. These evidences lead us to conclude that one can employ the mice in oestrogen assay without danger for 5 times repeatedly, using the first and 5th tests for qualification and approval of the mice with 0.0003 μg of oestrone, *i. e.*, the mice are to be used for the test of unknown samples three times.

Sulman mentioned that repeated usage of the animals led to more uniform and constant reaction in further test (6). He and Emmens (5) reported that the priming of the animals was effective for increasing the sensitivity and the regularity of the results. No priming was given to our mice in the present experiment, but our data have likely demonstrated that the response to oestrone was the same degree with the mice spayed 5 months previously (group A), as well the one freshly prepared (group B).

The reason why the mice of two groups in our test decreased in the response at later stages is obscure at present. However, considering from both

the earlier decrease in group A and no priming given, elapse of time or aging of the mice, may be the causes. Some deaths occurred in the mice during the progress of our experiment.

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Conclusions

Employing the quantitative bioassay of oestrogen according to Sulman, the possibility of repeated usage of mice for the assay was examined. The mice of dd-strain were intravaginally administered oestrone from 0.000025 to 0.0003 μg/0.015 *ml* water for seven times in succession with 12 day intervals. The results obtained in a group freshly prepared was likely to show the usage of mice to be permissible for seven times, regarding 0.0002 μg as the minimum effective dose. However, it happened that one mouse out of three ceased reacting to this dose from a later test (5th), as presented in the individual record (Table 2). The authors proposed, hence, a method of assay using the same mice three times, adding response test at the first and the 5th with 0.0003 μg of oestrone.

An evidence was obtained that mice prepared five months ago previously could be equally sensitive to the dose of 0.0002 μg up to the 5th time, although they had not been primed with a large dose of oestrogen at all.

The advantages of this method, repeated usage of mice, are in saving time, labour and expense in preparation of animals for the assay of oestrogen.

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