PROLONGED ADMINISTRATION OF DIETHYLSTILBESTROL (DES) AND SUBSEQUENT FECUNDITY

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ABSTRACT

George E. Glosson. PROLONGED ADMINISTRATION OF DIETHYLSTILBESTROL (DES) AND SUBSEQUENT FECUNDITY. (Under the direction of Dr. Everett C. Simpson). Department of Biology, October, 1976.

A total of 120 albino rats (Sprague-Dawley strain) were injected subcutaneously with 1.25 and 2.5 mg. DES for five, ten and fifteen estrous cycles at late estrus. Appropriate controls were injected with the oil vehicle. After completing treatment, the rats were bred and the incidence of pregnancy was determined by microscopic examination of daily vaginal lavages. Pregnant animals were sacrificed on day 15.

There was a significant reduction in the number of normal fetuses as the number of injections increased. This reduction in the number of normal fetuses continued to zero fertility in the fifteen cycle sequence groups. No products of conception were found in the DES-treated animals showing infections of the reproductive tract. Compared to the controls, there was a marked increase in uterine infections in all DES-treated groups except for the 1.25 mg. DES five cycle group. Examination of the reproductive tract revealed one uterine and five ovarian infections in the five cycle DES-treated groups. Of these infections, the uterine and two ovarian infections were seen in the high dosage level group. In the ten cycle DES-treated groups, there were two uterine infections and three ovarian infections observed at both dosage level groups. Two and three uterine infections were observed in the fifteen cycle low and high dosage level groups, respectively. There were three and six ovarian infections observed in the fifteen cycle

low and high dosage level groups, respectively.

There was a significant difference between the controls and their respective treated groups in the percent animals pregnant at day 15. There was a significant difference in percent of animals pregnant between dosage levels except between the ten cycle DES-treated groups. Animals not gravid at 15 days were allowed to breed as a follow-up of fertility; however, no products of conception were ever observed in these DES-treated animals.

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INTRODUCTION

The synthetic estrogen, 4,4-dihydroxy,-ab-diethylstilbene (stilbestrol or DES), has been intensively studied during the past several years. Most studies have dealt with the use of this drug as a post-coital agent since it is known to cause premature transport of ova into the uterus and thus interfer with implantation. However, in addition to the adverse effects of DES on implantation, there is some evidence that the drug has some harmful side effects (FDA, 1973).

The present study was designed to provide additional information on the long term effects of DES on the reproductive biology of the laboratory rat. Animals in each test group were followed through their reproductive cycles, and DES was injected subcutaneously within 24 hours following estrus. This regime was repeated according to the predetermined sequence of injections (5, 10 or 15) for each group. As an example, rats receiving ten injections of DES will have gone through ten estrous periods and injected with DES at the end of each estrus. They were placed with fertile males of proven fertility at their next return to estrus.

This study provides information on the influence of long-term administration of DES (1) on possible breeding complications and (2) on embryonic survival and possible anomalies of development.

REVIEW OF LITERATURE

Diethylstilbestrol was synthesized in 1938 (Dodds, et al., 1938) and was shown to be 2 to 3 times as potent as estrone in spayed rats. Later workers showed that the relative potency of DES varied according to the method of assay when compared to estrone (Leighty and Wicks, 1939; and Lee et al., 1942). The latter workers reported that the ratio of activity of DES to esterone by subcutaneous injections was approximately 32:1 and by mouth it was 12:1.

The rate of tubal transport of ova was shown to be increased by a single injection of DES in rabbits, (Whitney and Burdick, 1938) and in rats (Greenwald, 1959). Chang and Yanagimachi (1965) and Emmens (1970) showed that infertility after DES treatment was due to the premature explusion of the ova into the uterus before the endometrium is adequately prepared. When DES was fed at a dose level of 1.0, 0.5, and 0.1 mg. per rabbit, of the eggs recovered, the percent of recovered blastocysts was 0.0, 9.4, and 62 percent, respectively (Chang and Yanagimachi, 1965). Later Jacob and Morris (1969) reported the effective dose of DES in the rabbit was 0.005-0.25 mg./kg./day when it was administered from day 1 to day 3 of pregnancy. Morris and van Wagenen (1966) showed that DES given to monkeys for 6 days following mating prevented implantation and caused placental separation and death of the fetus. Kuchera (1971) found in a study involving 1000 women that when 25 mg. of DES was administered for 5 days commencing within 72 hours of unprotected sexual intercourse, no pregnancies resulted. It has also been reported that DES acts as an antifertility drug when used in cases of sexually assaulted females

(Morris and van Wagenen, 1966, Massey, et al., 1971). Because of its antifertility action this drug has been used as a morning-after contraceptive when used in a restricted manner (Food and Drug Administration, 1973). In all cases involved with human subjects, the daily dosage has ranged from 5 to 50 mg. and started before day 3 and lasted from 4 to 6 days post ovulation.

Although DES has been shown to be an antifertility drug at high dosages during or near the time of implantation, it is used to produce artificial cycles where estrogen secretion is too low to achieve sensitivity of the endometrium. Generally 5 mg. is administered for 20 days along with 10-20 mg. of a progestational substance. Restoration of endometrial sensitivity appears to be associated with an increase in hypothalamic center responsiveness so that spontaneous ovulation occurs after the treatment is stopped (Williams, 1968).

DES has been used by several investigators during various stages of pregnancy. Sommers, et al. (1949) reported that the weights of the baby and the placenta were increased in DES-treated full-term and premature births. Smith and Smith (1949) reported that fetal mortality was reduced in DES treated patients as was the incidence of late pregnancy toxemia. The overall improvement of pregnancy related complications reported in this study led to the widespread use of DES during the late 1940's and early 1950's. Ferguson (1953) and Dieckmann, et al. (1953) disagreed with the Smith's concept in that they reported no significant effect of DES administration on prematurity, post maturity, incidence of abortion, fetal weight or survival. The use of DES for pregnancy related complications had decreased markedly by the late 1950's.

Smith, et al. (1946) reported that DES administration during pregnancy would increase the level of pregnandiol in the urine. This was contrary to the findings of Davis and Fugo (1947) who reported that DES did not change the normal excretion of pregnandiol at any time during the first 16 weeks of pregnancy. Board and Bhatnagar (1972) reported on a group of regularly ovulating volunteers who began 25 mg. of oral DES treatment for five days beginning on the day of basal temperature rise. Plasma progesterone in the blood was lowered with DES.

Evidence of the harmful side effects of DES became known shortly after it was first synthesized. Lacassagne (1938) reported that injections of DES in male mice caused mammary carcinoma. Folkman (1971) reported carcinogenic properties of DES on the placenta and fetal tissues. In mice, mothers exposed to DES do not develop breast or genital tract neoplasms. The developing young are more sensitive to carcinogens than are the adult female mice. Tumors will appear in the infant mouse with 1/4000 the dose required to produce the same tumor in the adult mouse. Within the human, Greenwald, et al. (1972) reported on 48 cases of tumors of the breast and urogential organs excluding vaginal adenocarcinomas in males and females. They were compared to controls of unexposed cases of the 25 to 35 year age group. Their findings showed that DES does not contribute to the development of tumors other than the lower genital tract.

In the past several years, cancer of the reproductive organs has been reported in female offspring that were exposed to DES while in utero. Females 15 to 22 years of age whose mothers had DES therapy while pregnant have been shown to have adenocarcinoma. Although there

have been previous reports of adenocarcinoma, these have usually been in older patients (Herbst, et al., 1971). There is a highly significant association between mothers treated with DES during pregnancy and the subsequent development of adenocarcinoma of the vagina in their daughters (Herbst, et al. 1971). Similar findings were reported by Pomperance (1971) and by Kantor, et al., (1973); Herbst, et al., (1972); and Keiffer (1974).

From this review, it is obvious, what started out as a beneficial use of DES, may have adverse long-term effects. The widespread use of DES may have undetermined side effects on reproductive potential. The sequential use of this drug on subsequent pregnancy has not been investigated. It is with this in mind, that the present study is undertaken.

MATERIALS AND METHODS

Animals

Albino nulliparous female rats of the Spraque-Dawley strain were used in this investigation. Males of proven fertility were used for breeding purposes. The animals were housed in wire bottom cages, two or three animals to a cage. Animals were housed in a room with 14 hours of light (5:00 A.M.-7:00 P.M.) and 10 hours of darkness (7:00 P.M.-5:00 A.M.) and in a near constant temperature of 22°C. All animals had free access to water and received Wayne Laboratory Chow ad lib.

Vaginal lavages for all females in the investigation were obtained daily between 8:00 and 12:00 A.M. Only mature females having shown one or more 4-5 day estrous cycles were used in these experiments.

Design of Experiment

This investigation involved six groups of animals on treatment and three groups as controls. The treatments were divided into three groups based on time intervals (5, 10, and 15 cycles). Each interval was subdivided into a low (1.25 mg.) and a high (2.5 mg.) dosage of DES. Throughout this study, at the five cycle injection sequence, the control, the 1.25 and the 2.5 mg. DES treatment groups will be referred to as Groups 1, 2, and 3, respectively. Of the animals on the ten cycle sequence, the control, the 1.25 and 2.5 mg. DES treatment groups will be referred to as Groups 4, 5, and 6, respectively. At the fifteen cycle sequence, the control, the 1.25 and the 2.5 mg. DES treatment groups will be hereafter known as Groups 7, 8, and 9, respectively.

The controls were given sham injections of sesame oil in volume comparable to the high dosage of the animals on treatment (0.5 ml.). The sham injections were given for 5, 10, and 15 cycles. Daily lavages were taken for determining the various stages of their cycles. Following treatment, the females were confined to the breeding cages and daily lavages continued. With the appearance of sperm, lavages were continued daily for up to two weeks. All animals were laparotomized 15 days after the appearance of sperm or a copulatory plug if there was no subsequent cycle within that time. Those animals, that had either sperm or a copulatory plug, and cycled before 15 days, were allowed to remain caged indefinitely with fertile males. In the animals that were laparotomized, the numbers and the condition of the fetuses were observed and recorded. If, however, they were found to be infertile, they were sutured, allowed to recover and again placed with fertile males. A record of any abnormalities of the female reproductive tract was made.

Experimental Procedures

Vaginal Lavages

Vaginal lavages were taken on all animals. A small pipette containing 1/4 cc of warm tap water was inserted into the vagina and aspirated three or four times. This obtained an optimal amount of sloughed cells. The lavages from six to eight rats were placed on a microscope slide and were examined under 100X. This established the rats' stages of the reproductive cycle. See Appendix B for this determination.

At the termination of the predescribed injection regime, each animal

was placed in the breeding cage. The presence of spermatozoa in the vaginal lavages or of a copulatory plug established day 1 of pregnancy.

Injections

Injections of DES in sesame oil were administered with a 0.25 cc tuberculin syringe for low doses and 0.50 cc tuberculin syringe for high doses, using a 5/8 inch 25 gauge needle. The size of the needle used was recommended by Banik and Pincus (1962) to avoid leakage. All controls received sham injections of 0.50 cc sesame oil. All injections were made subcutaneously in the interscapular region between the hours of 8:00 A.M. to 12:00 noon.

Laprotomy Technique

The animals were anesthetized with an intraperitoneal injection of aqueous chloral hydrate (120 mg./cc; dosage = 30 mg./100 g. body weight). The injections were made with a tuberculin syringe (22 gauge needle). The lower abdomen was shaved and a mid-ventral longitudinal incision made about 2.5 to 3 cm anterior to the vaginal orifice. The uterine cornua were exteriorized and examined for fetuses. If no conception had occurred, the cornua were replaced in the abdominal cavity. The muscle wall was sutured every 1/4 inch and the skin incision was closed with surgical clips (11 mm). The animals were housed one per cage for post operative recovery. The clips were removed after seven days.

Autopsy

All the animals that were pregnant or had gross anomalies were autopsied 15 days after sighting of sperm. Each animal was weighed and

anesthetized by injection of aqueous chloral hydrate. A mid-ventral longitudinal incision was made from the diaphram to the pelvis. The uterine cornua were exteriorized. Reproductive organs were removed, stripped of fat and examined. The fetuses were separated from the fetal membranes and the umbilical cord cut near the abdomen of the fetus. All abnormalities of the uterus, oviducts, ovaries and all fetuses were preserved in formaldehyde.

The animals which had apparently not conceived approximately 45 days after the first breeding, were sacrificed at the termination of the experiment. Any fetuses, resorption sites, and reproductive organ infections were recorded.

Pictures

Photographs and slides of the reproductive organs in toto were taken with a "Nikon" 35 mm camera using Kodax "Pan-X" film (ASA 32).

Analysis

The students t-test was used in checking the significance of the number of normal fetuses surviving and percent animals pregnant at day 15. See Appendix C and E for statistical analyses.

RESULTS AND DISCUSSION

Part 1

Effects of Diethylstilbestrol Injected Subcutaneously at Late Estrus for Five Successive Estrual Cycles

Table I shows the values which were obtained on day 15 of pregnancy following subcutaneous injections of 1.25 and 2.5 mg. of DES and that of the control group. The results indicate a decrease in mean maternal weight between 1.25 and 2.5 mg., respectively. The mean maternal weight of the control group was 70.9 and 86.5 gms. greater than the 1.25 and 2.5 mg. dosage level groups, respectively. When the products of conception were removed, the maternal weights for the control group remained 71.4 and 91.8 gms. greater than the 1.25 and 2.5 mg. dosage level groups. Thus, the use of DES at the dosage levels given, had decreased the maternal body weight.

The controls (Group 1) had a higher number of normal fetuses as compared to Groups 2 and 3 (Figs. 2, 4, 5). The statistical analyses of the effects of DES on the number of normal fetuses is summarized in Appendix C. The differences between the control group vs. the DES-treated groups was statistically significant ($p < 0.01$). The difference between Group 2 and Group 3 was not statistically significant ($p > 0.05$). All controls in Group 1 became pregnant. Four out of sixteen and four out of twenty animals became pregnant in Group 2 and Group 3, respectively. The average litter size of each group may be determined by dividing the number of animals pregnant into the number of normal fetuses (Figure 1).

Based on the results presented in Table I, it can be said that: (1)

Table I. Values for 1.25 and 2.5 mg. diethylstilbestrol injected subcutaneously at late estrus for five successive estrual cycles. All values were obtained at sacrifice on day 15 of pregnancy.

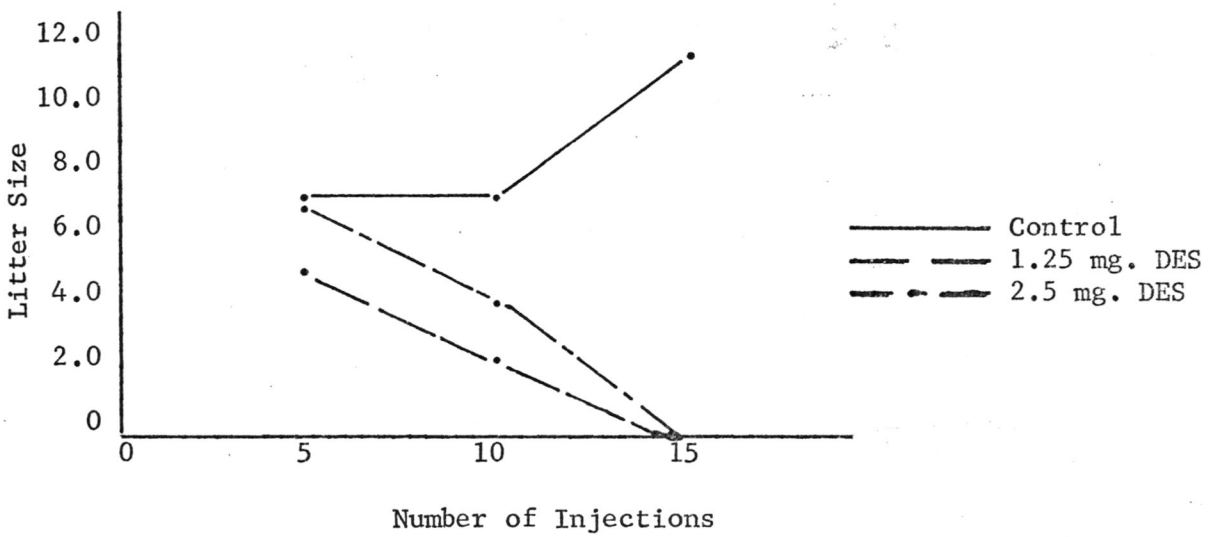
	Group 1 Control (5 cycle)	Group 2 Experimental (1.25 mg. - 5 cycles)	Group 3 Experimental (2.5 mg. - 5 cycles)
No. of Animals Bred	8	16	20
Mean Maternal Weight (grams) $\bar{X}$ - SE)	277.4 $\pm$ 10.47	206.5 $\pm$ 4.74	190.9 $\pm$ 3.95
Mean Maternal Weight - Fetal Weight (grams)	267.9	196.5	176.1
Percent Animals Pregnant	100.0%	25.0%	20.0%
No. Normal Fetuses	(8)* 56	(4)* 18	(4)* 27
Percent Normal Fetuses	81.1%	100.0%	84.3%
No. Resorptions	13	0	5
No. Animals with Uterine Infections	0	0	1
No. Animals with Ovarian Infections	0	3	2
No. Animals Died While on Test (Not included in above date)	0	2	0

*Number of animals pregnant

The reductions in the number of offspring for Groups 2 and 3 were statistically significant ($p<0.01$). The percent normal fetuses was based upon the number of implantations observed divided into the number viable young at 15 days. (2) The number of implantation sites was always equal to or greater than the number of young surviving at day 15. The differences between implantation sites and viable young at day 15 indicated the mortality of the fetuses. (3) The percentage for normal fetuses in the control was less than Groups 2 and 3. Obser-

Figure 1

Change in Litter Size with
Pre-coital DES Administration



vations of the uterine infection were made throughout the study. One uterine infection was observed at the five cycle time sequence, and that was in Group 3 (Fig. 3). Throughout this study, the ovaries were observed for any possible alterations from the norm. (4) In the five cycle time sequence, there were three ovarian infections observed in Group 2 and two in Group 3. (5) The percent animals pregnant was

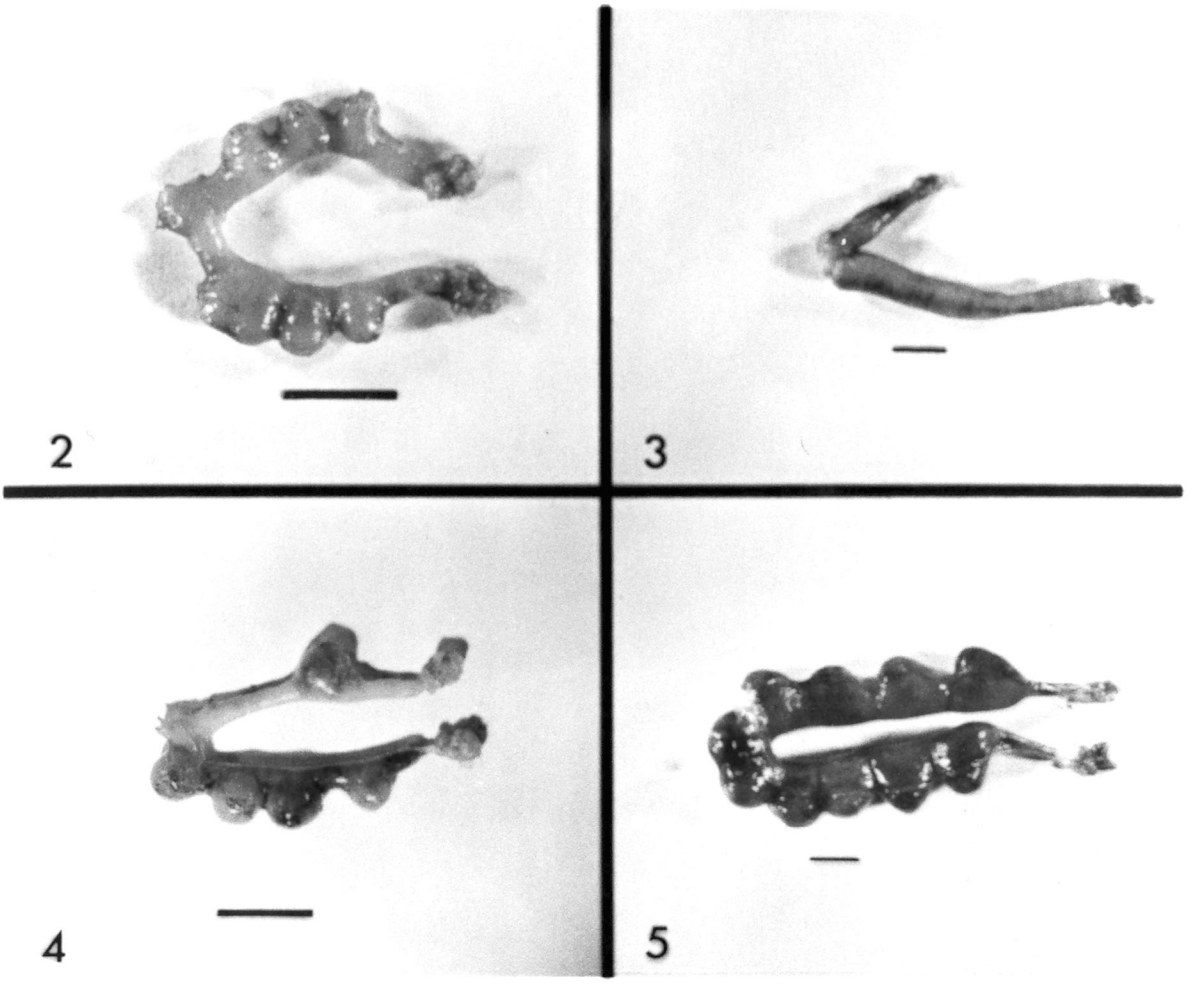
Figures represent the reproductive tracts which were removed 15 days after breeding. All animals were injected five times prior to breeding. The bar represents one cm.

Figure 2. The reproductive tract from an animal which received 1.25 mg. of DES per injection. Note reduced number of fetuses.

Figure 3. The reproductive tract from an animal which received 2.5 mg. of DES per injection. Note moderate degree of infection of the uterus. No products of conception were found.

Figure 4. The reproductive tract from an animal which received 1.25 mg. of DES per injection. Note reduced number of fetuses.

Figure 5. The reproductive tract of a control animal. Note the distribution and number of fetuses.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greatly reduced in the DES-treated groups. Values for the control vs. Groups 2 and 3 were 100%, 25% and 20%, respectively.

Part 2

Effect of Diethylstilbestrol Injected Subcutaneously at Late Estrus for Ten Successive Estrual Cycles

Table II shows the values which were obtained on day 15 of pregnancy following subcutaneous injections of 1.25 and 2.5 mg. of DES and that of the control group. The mean maternal weight of the control group is 67.1 and 79.3 gms. greater than the 1.25 and 2.5 mg. dosage level groups, respectively. When the products of conception were removed, the maternal weights for the control group was 58.5 and 74.8 gms. greater than the 1.25 and 2.5 mg. dosage level groups.

The controls (Group 4) had a higher number of normal fetuses as compared to Groups 5 and 6 (Table 2). The number of live normal fetuses of the controls was much greater than found in the experimental groups. The difference between the control group vs. the DES-treated groups was statistically significant ($p < 0.01$). The difference between Group 5 and Group 6 was not statistically significant ($p > 0.05$). Five of six controls became pregnant. Three out of seventeen and two out of eighteen animals became pregnant in Group 5 and Group 6, respectively (Table II).

Based on the results presented in Table II, it can be said that: (1) The reduction in the number of offspring present for Groups 5 and 6 was statistically significant ($p < 0.01$). (2) There were two uterine infections and three ovarian infections observed in Group 5 (Figs. 6,8)

Table II. Values for 1.25 and 2.5 mg. diethylstilbestrol injected subcutaneously at late estrus for ten successive estrual cycles. All values were obtained at sacrifice on day 15 of pregnancy.

	Group 4 Control (10 cycle)	Group 5 Experimental (1.25 mg.-10 cycles)	Group 6 Experimental (2.5 mg. -10 cycles)
No. of Animals Bred	6	17	18
Mean Maternal Weight (grams) $\bar{X}$ - SE	281.5 ± 4.31	214.4 ± 4.61	202.2 ± 8.91
Mean Maternal Weight - Fetal Weight (grams)	272.2	213.7	197.4
Percent Animals Pregnant	83.3%	23.5%	22.2%
No. Normal Fetuses	(5)* 38	(3)* 6	(2)* 7
Percent Normal Fetuses	97.4%	46.1%	53.8%
No. Resorptions	1	7	6
No. Animals with Uterine Infections	0	2	2
No. Animals with Ovarian Infections	1	3	3
No. Infections of Oviducts	0	1	2
No. Animals Died While on Test (Not included in above data)	0	3	1

* Number of animals pregnant

Figures represent the reproductive tracts which were removed 15 days after breeding. All animals were injected ten times prior to breeding. The bar represents one cm.

Figure 6. The reproductive tract from an animal which received 1.25 mg. of DES per injection. Note uterine outgrowth and infected ovary. No products of conception were found.

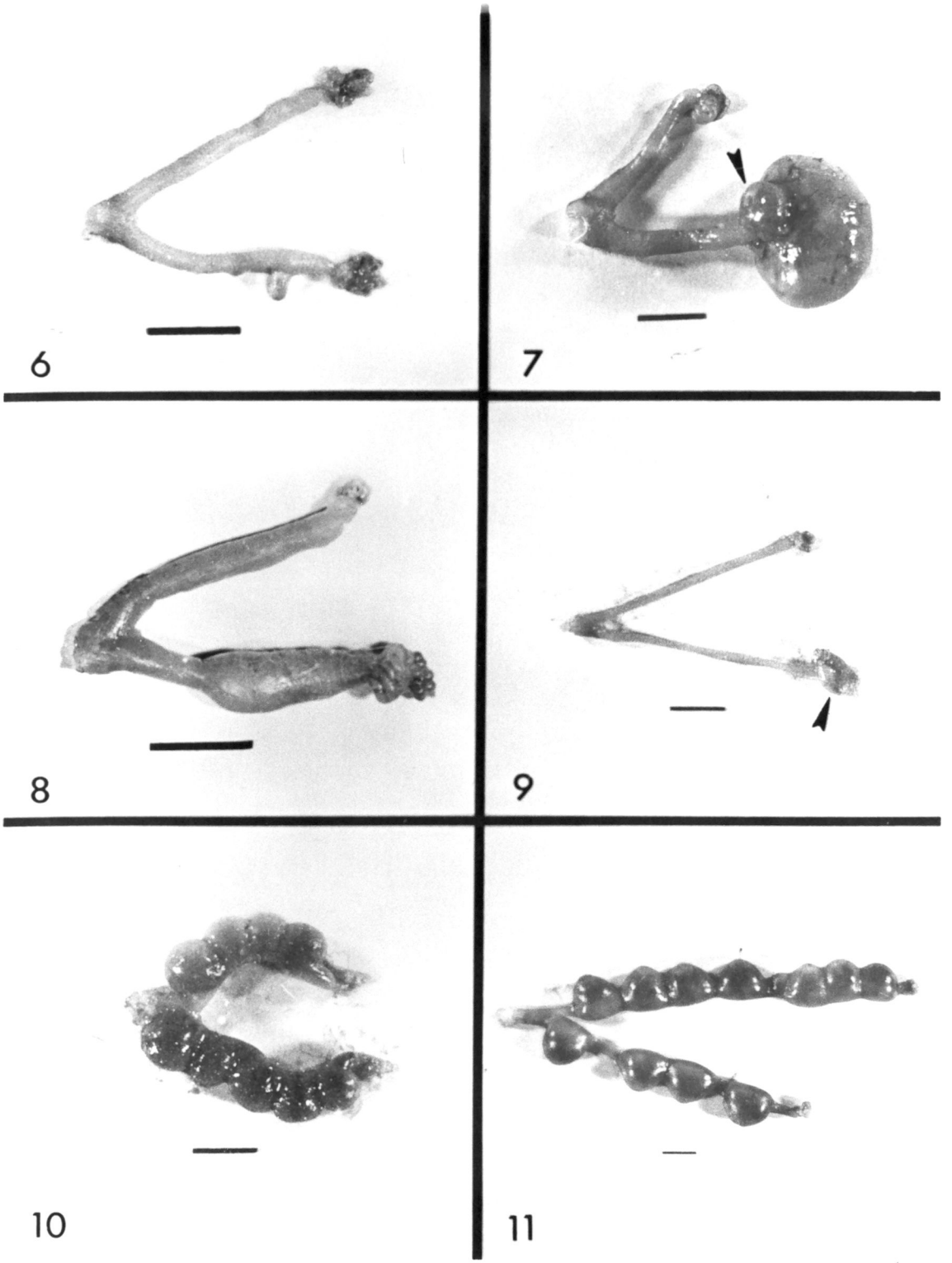
Figure 7. The reproductive tract from an animal which received 2.5 mg. of DES per injection. Note uterine and large ovarian infections. Arrow indicates hardened calcified body. No products of conception were found.

Figure 8. The reproductive tract from an animal which received 1.25 mg. of DES per injection. Note uterine, ovarian and oviduct infections. No products of conception were observed.

Figure 9. The reproductive tract from an animal which received 2.5 mg. of DES per injection. Arrow indicates hardened calcified body. There appears to have been no conception.

Figure 10. The reproductive tract from an animal which received 1.25 mg. of DES per injection. There were two normal fetuses and seven resorptions.

Figure 11. The reproductive tract of control animal. Eleven normal fetuses were found.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and two uterine (Fig. 7) and three ovarian infections observed in Group 6 (Figs. 7,9). One animal in both groups 5 and 6 had one uterine and one ovarian infection (Figs. 8, 7, respectively). (3) The percent animals pregnant was greatly reduced in the DES-treated groups (Fig. 10). Values for the controls vs. Groups 5 and 6 were 83.3%, 23.5% and 22.2%, respectively.

Part 3

Effects of Diethylstilbestrol Injected Subcutaneously at Late Estrus for Fifteen Successive Estrual Cycles

Table III shows the values which were obtained on day 15 of pregnancy following subcutaneous injections of 1.25 and 2.5 mg. of DES and that of the control group. The mean maternal weight of the control group was 75.9 and 77.7 gms. greater than the 1.25 and 2.5 mg. dosage level groups, respectively. When the products of conception were removed, the maternal weights for the control group was 64.4 and 66.2 gms. greater than the 1.25 and the 2.5 mg. dosage level groups.

The controls (Group 7) had 10.8 offspring per litter (Figs. 16, 17). No fetuses were observed in Groups 8 and 9. The differences between the control group vs. the DES-treated groups was statistically significant ($p < 0.01$). Four of five control animals became pregnant while the DES-treated groups had no offspring. Thus, it can be seen that 15 successive injections of DES at estrus resulted in zero fertility (Figure 1).

Based on the results present in Table III, it can be said that: (1) Since there were no offspring in the DES-treated groups, it is assumed that DES had a pre-coital effect on fecundity. (2) There were

Table III. Values for 1.25 and 2.5 mg. diethylstilbestrol injected subcutaneously at late estrus for fifteen successive estrual cycles. All values were obtained at sacrifice on day 15 of pregnancy.

	Group 7 Control (15 cycle)	Group 8 Experimental (1.25 mg. - 15 cycles)	Group 9 Experimental (2.5 mg. - 15 cycles)
No. of Animals Bred	5	19	18
Mean Maternal Weight (grams) $\bar{X}$ - SE	297.8 ± 15.51	221.9 ± 7.42	220.1 ± 6.39
Mean Maternal Weight - Fetal Weight (grams)	286.3	221.9	220.1
Percent Animals Pregnant	80.0%	0.0%	0.0%
No. Normal Fetuses	(4)* 43	(0)* 0	(0)* 0
Percent Normal Fetuses	91.4%	0.0%	0.0%
No Resorptions	4	0	0
No. Animals with Uterine Infections	0	2	3
No. Animals with Ovarian Infections	0	3	6
No. Infections of Oviducts	0	2	4
No. Animals Died While on Test (Not included in above data)	0	1	2

*Number of animals pregnant

Figures represent the reproductive tracts which were removed 15 days after breeding. All animals were injected for fifteen times prior to breeding. The bar represents one cm.

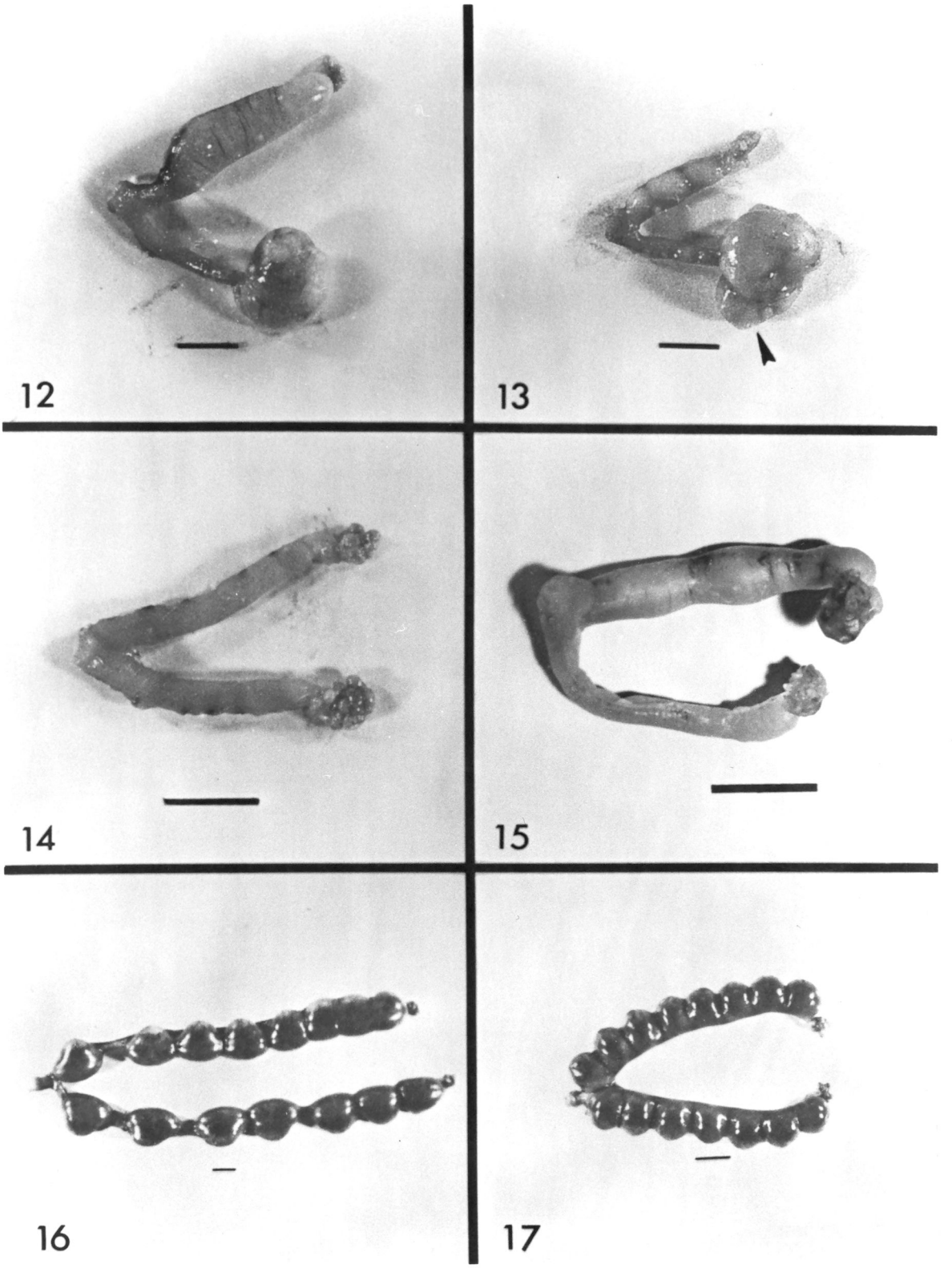
Figure 12. The reproductive tract from an animal which received 1.25 mg. of DES per injection. Note infected uterus and ovary. No products of conception were found.

Figure 13. The reproductive tract from an animal which received 2.5 mg. of DES per injection. Note infected uterus, ovary, and oviduct. Arrow indicates hardened calcified body.

Figure 14. The reproductive tract from an animal which received 1.25 mg. of DES per injection. No fetuses were observed, due to the uterine infection seen.

Figure 15. The reproductive tract from an animal which received 2.5 mg. of DES per injection. Note infected uterus and ovary. No products of conception were found.

Figures 16 and 17. The reproductive tracts from two control animals. Note the distribution and number of fetuses.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two uterine infections and three ovarian infections observed in Group 8 and three uterine infections and six ovarian infections in Group 9. One animal in Group 8 (Fig. 12) and two animals in Group 9 had both uterine and ovarian infections (Figs. 13, 15).

Part 4

Comparison of Results on the Effects of Diethylstilbestrol Injected Subcutaneously at Late Estrus for All Treatment Groups

The preceding tables (Table I, II, and III) indicate an increase in mean maternal weights between the control groups vs. the DES-treated groups. When the products of conception were removed, the maternal weights for the control groups remained greater than the DES-treated groups. Thus, the use of DES at the dosage levels given, decreased the maternal body weight.

The control groups had a higher number of normal fetuses as compared to all treatment groups. The percentages for normal fetuses in the control (Group 1) was less than in Groups 2 and 3. The percentage of normal fetuses was based on the total number of products of conception divided into the normal fetuses at 15 days of pregnancy. The controls of the five cycle group was the only control group to have a lower percentage of survival as compared to all treatment groups. The time lapse involved in this study may have influenced the survival rates seen when comparing the five cycle control group vs. the fifteen cycle control group. All treatment groups showed a progressive decrease in the numbers and percentages of fetuses surviving at day 15 as influenced by the increased length of treatment. This was shown at the 1.25 mg. and 2.5 mg. DES-treated levels. There appears to be a progressive increase

in the survival rates in the five, ten and fifteen cycle sequences. The differences between all the control groups vs. their respective DES-treated groups were statistically significant at the 0.01% levels. All treated groups were significantly different compared to those of the fifteen cycle sequence except between the ten and fifteen cycle 2.5 mg. dosage level. Within the five and ten cycle sequences, there was no significant difference of dosage level effects in the number of fetuses surviving at 15 days.

Effects of DES on Uterine Infections

It has been known for a number of years that prolonged treatment with estrogens increased the probability of uterine outgrowths (See Fig. 6). The infections seen in this study were characterized by milky fluid, thought to be pus, confined to the uterus (Figs. 3, 7, 8, 12, 13, 14, 15). This agrees with the findings of Meissner et al., (1957). Intramuscular injections of DES in rabbits resulted in enlarged uteri containing milky fluid. In the infected animals, of this study, no products of conception were found. There was a significant increase in uterine infections in all treatment groups except in Group 2 (Figs. 12, 13, 14, 15). The only marked difference at the low dosage level (1.25 mg.) was between the 5 and 15 cycle sequence. An increase in the number of uterine infections was seen between the high dosage levels (Groups 3, 6 and 9). Since the uterine infections were confined to the animals on treatment, it can be assumed that the problem was related to DES. Obviously these infections influenced fecundity.

Effects of DES on Ovarian and Oviduct Infections

Ovarian infections were confined to those animals on treatment with exception of one cystic ovary in controls (Group 4). All the infected ovaries were enlarged (Figs. 7, 8, 9, 12, 13, 15). Some of these appeared to have hardened bodies (Figs. 7, 9, 13) and are marked with arrows. Others appeared to have a milky like exudate. These hardened bodies may have been what Jabara (1959), defines as "psommona." She reported that the ovaries of DES-treated dogs had numerous calcified bodies. Jabara (1962) reported that ovarian changes in dogs were not related to the total amount of DES. Meissner et al. (1957) also observed gross ovarian enlargements following sequential intramuscular injections of DES in rabbits. There appeared to be no time lapse difference of DES on ovarian infections particularly at the lower dosage levels.

Salpingitis was confined to those animals on treatment. The number of animals with infected oviducts increased with the dosage and length of treatment. They were enlarged and fluid filled (Figs. 7, 8, 12, 13). Meissner et al. (1957) reported a similar condition in the oviducts of DES-treated rabbits.

There were no products of conception in any animal having an infected uterus, oviduct and/or ovary. All of the animals having an infection of the oviduct also had ovarian or uterine infections. Approximately 50% of those having ovarian infections, had either a uterine and/or infection of the oviduct. Of the animals with a uterine infection, 70% had either an infection of the ovary and/or salpingitis. See Appendix D for information on multiple infections.

Observations of Cycle Length During Treatment

The normal length of the estrous cycle of the white rat is four to five days. The control animals of the five cycle sequence (Group 1) averaged a cycle length of 5.0 days. The experimental animals of the fifteen cycle groups (Groups 8 and 9) were chosen to determine if DES administration altered the length of the estrual cycle while on treatment. The average cycle lengths for the first five and last five cycles of these groups were 4.86 and 5.26 days, respectively. During the last five cycles, it was observed that the injections did prolong the return to the next estrus. This prolongation increased to 13 days in one animal and 11 days in two animals. The increase in cycle length may be accounted for by the extended period of estrus following the last injection of DES. This may be due to liver impairment with a pharmacological effect of prolonged usage of DES. Under this condition, DES may have accumulated as cited by Elias et al, (1945). However, other possibilities may exist. Any alteration or change in release of FSH or alterations in the hypothalamic-hypophysial ovarian axis may have accounted for this elongation in the estrous cycle. Even a direct effect on the uterus cannot be ruled out. These possibilities loan themselves for further investigation.

Percent Pregnant at 15 Days

The percent of the control animals that were bred and pregnant at 15 days were 100%, 83%, and 80% for the five, ten, and fifteen cycle sequence, respectively. There was a significant difference between the controls and their respective treatment groups. All treated groups were

significantly different between dosage levels except for the treated groups of the ten cycle sequence. There was a significant difference between the treated fifteen cycle groups and the treated groups of the five and ten cycle sequence. The difference between the 1.25 mg. five and ten cycle sequence was highly significant ($p<0.01$). It would appear that the longer the treatment, the higher the probability of infertility.

Follow-up of Sexual Activity in the Treated Animals with Infertility Problems

Those animals, not found to be pregnant on day 15 following copulation, were placed with fertile males for further observation of sexual activity. The number of copulations of all experimental animals ranged between 2.0 and 2.5 for the five and fifteen 2.5 mg. DES sequences. It was observed that the animals of Group 9 had a longer phase between the estrual periods, thus reducing the number of copulations. No animals in this follow-up ever became pregnant.

SUMMARY

The effects of sequential use of diethylstilbestrol (DES) given to mature rats at estrus on subsequent fertility were studied. The experiment was divided into two dosages (1.25 and 2.5 mg. DES) within three intervals of time (5, 10, and 15 injections) Parts 1, 2, and 3, respectively. All injections were administered at or near the end of estrus. All bred animals were laparotomized at day 15 and examined for number and condition of fetuses if pregnant. The condition of their reproductive tracts was also noted. Those found to be non-pregnant were resubjected to fertile males for further study.

Part 1 was concerned with the effects of subcutaneous injections of DES (1.25 and 2.5 mg. DES) for five estrual cycles prior to breeding. The percent of females pregnant and the number of fetuses produced per female was significantly lowered in the treated groups. However the percentage of normal fetuses surviving per pregnancy was not affected. Examination of the reproductive tract revealed one uterine and five ovarian infections in the treated groups. Of these infections, the uterine and two ovarian infections were seen in the high dosage level group.

Those females, in Part 2, received similar dosages of DES for ten estrual cycles prior to breeding. The number of treated females pregnant, the number of normal fetuses per pregnancy, and the percent of normal fetuses per pregnancy were significantly lower in the treated group Dosage level between treated groups had no significant effect on these parameters. There were two uterine infections and three ovarian

infections observed at both dosage level groups.

High and low dosages of DES were given for fifteen estrual cycles prior to breeding (Part 3). The controls had 10.8 offspring per litter while no fetuses, normal or abnormal, were observed in the DES-treated groups, resulting in zero fertility. The length of treatment appeared to be more harmful than the dosage levels used. Two and three uterine infections were observed in the low and high dosage level groups, respectively. There were three and six ovarian infections observed in the low and high dosage level groups, respectively.

The sequential use of DES administered at late estrus has an adverse effect on subsequent breeding performance. This may be related to liver damage under prolonged usage of DES. If the metabolic processes of the liver are impaired, the drug or its metabolities may accumulate.

APPENDIX A

MATERIALS FOR MAINTENANCE AND TREATMENT OF ANIMALS

Wayne Laboratory Chow

Crude protein not less than 24.0%

Crude fat not less than 4.0%

Crude fiber not more than 4.5%

The ingredients of the food included corn and wheat flakes, corn, soybean meal and oil, fish meal wheat middlings, dried whey, brewer's dried yeast, animal liver meal, and vitamin supplements.

Obtained from: Granville Milling Company
Creedmoor, North Carolina

Diethylstilbestrol (DES)

The experimental solution was made by dissolving diethylstilbestrol in sesame oil to a concentration of 5.0 milligram per milliliter.

Obtained from: Sigma Chemical Company
St. Louis, Missouri

APPENDIX B

Stages in the rat estrous cycle and corresponding cell types in the vaginal lavage. (Turner, 1966)

STAGE OF CYCLE	CELL TYPE
Diestrus	Lavage dominated by leucocytes
Proestrus	Nucleated epithelial cells
Estrus	Large masses of cornified epithelial cells with degenerate nuclei
Metestrus	Many leucocytes with a few cornified epithial cells

APPENDIX C

"T" Values From Statistical Analysis of Data of Six Different Treatments on the Number of Normal Fetuses Surviving at Day 15

Groups	"T" value	Significance
(5 cycle) Control vs. 1.25 mg. DES	10.96	$p<0.01$
(5 cycle) Control vs. 2.5 mg. DES	9.79	$p<0.01$
(5 cycle) 1.25 mg. DES vs. 2.5 mg. DES	0.35	$p>0.05$
(10 cycle) Control vs. 1.25 mg. DES	5.42	$p<0.01$
(10 cycle) Control vs. 2.5 mg. DES	5.94	$p<0.01$
(10 cycle) 1.25 mg. DES vs. 2.5 mg. DES	0.18	$p>0.05$
(15 cycle) Control vs. 1.25 mg. DES	5.61	$p<0.01$
(15 cycle) Control vs. 2.5 mg. DES	5.55	$p<0.01$
(15 cycle) 1.25 mg. DES vs. 2.5 mg. DES	----	----
(5 cycle) 1.25 mg. DES vs. (15 cycle) 1.25 mg. DES	2.26	$p<0.01$
(5 cycle) 2.5 mg. DES vs. (15 cycle) 2.5 mg. DES	2.06	$p<0.05$
(10 cycle) 1.25 mg. DES vs. (15 cycle) 1.25 mg. DES	1.78	$p<0.05$
(10 cycle) 2.5 mg. DES vs. (15 cycle) 2.5 mg. DES	1.36	$p>0.05$
(5 cycle) 1.25 mg. DES vs. (10 cycle) 1.25 mg. DES	1.57	$p>0.05$
(5 cycle) 2.5 mg. DES vs. (10 cycle) 2.5 mg. DES	2.55	$p<0.01$

APPENDIX D

Females with Multiple Infections

Groups	Animal No.	No. Infections Uterine	Ovarian	Oviducts
10 cycles				
1.25 mg. DES	100	1	1	1
2.5 mg. DES	108	1	1+	1
	111	1+		1
15 cycles				
1.25 mg. DES	5		1	1
	13=	1+	1+	1+
2.5 mg. DES	22		1	1
	37		1	1
	24	1	1	1
	40	1	1+	
	35	1		1+
Total		7 out of 10	8 out of 21	9 out of 9

+Large infections observed
=Animal had a large infected left ovary and oviduct with a very large infected right uterus.

APPENDIX E

"T" VALUES FROM STATISTICAL ANALYSIS OF THE PERCENT ANIMALS PREGNANT AT DAY 15

Groups	"T" Value	Significance
(5 cycle) Control vs. 1.25 mg. DES	7.22	$p < 0.01$
(5 cycle) Control vs. 2.5 mg. DES	5.48	$p < 0.01$
(5 cycle) 1.25 mg. DES vs. 2.6 mg. DES	1.97	$p < 0.05$
(10 cycle) Control vs. 1.25 mg. DES	5.15	$p < 0.01$
(10 cycle) Control vs. 2.5 mg. DES	6.31	$p < 0.01$
(10 cycle) 1.25 mg. DES vs. 2.5 mg. DES	0.264	$p > 0.05$
(15 cycle) Control vs. 1.25 mg. DES	8.39	$p < 0.01$
(15 cycle) Control vs. 2.5 mg. DES	8.57	$p < 0.01$
(15 cycle) 1.25 mg. DES vs. 2.5 mg. DES	----	----
(5 cycle) 1.25 mg. DES vs. (15 cycle) 1.25 mg. DES	1.13	$p < 0.05$
(5 cycle) 2.5 mg. DES vs. (15 cycle) 2.5 mg. DES	1.18	$p < 0.05$
(10 cycle) 1.25 mg. DES vs. (15 cycle) 1.25 mg.DES	3.22	$p < 0.01$
(10 cycle) 2.5 mg. DES vs. (15 cycle) 2.5 mg. DES	1.15	$p < 0.05$
(5 cycle) 1.25 mg. DES vs. (10 cycle) 1.25 mg. DES	5.88	$p < 0.01$
(5 cycle) 2.5 mg. DES vs. (10 cycle) 2.5 mg. DES	1.18	$p < 0.05$

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