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The Effects of Ovariectomy and Supplemental Estrogen Therapy on the Growth and Development of Prepubescent Rats

Heather Hughbanks

ABSTRACT

The role of the ovaries in growth and development during puberty, and the therapeutic effect of estrogen in the absence of the ovaries was identified in this experiment. Growth rates, onset of puberty, and estrogenic effects on bone and the uterus were studied in sham and ovariectomized Sprague-Dawley rats. Ovariectomy and sham operations were performed before the onset of puberty, between 22 and 29 days of age. One ovariectomized group received estrogen injections of 10 ug daily. Growth rates were significantly increased (Scheffe's Test, $P<0.05$) in the OVAX group, 2.78g/d, compared to the other groups, 2.49g/d for Control, 2.23g/d for OVAX + E_2, and 2.26g/d for Sham. The OVAX group showed a significantly increased final weight compared to Sham and OVAX + E_2. Final weights were 204.20g, 188.35g, 172.00g, and 173.64g for OVAX, Control, OVAX + E_2, and Sham, respectively. The OVAX group also had an increased age (41.4d) and weight (129.67g) at vaginal opening compared to all other groups (33.17d, 34.00d, 35.17d in age, and 102.56g, 100.92g, 96.28g in weight for Control, OVAX + E_2, and Sham, respectively). Only two of the six rats in the OVAX group showed signs of an estrous cycle, and they gave irregular vaginal smears. One rat in the OVAX group still had an intact vaginal membrane at 55 days. OVAX + E_2 rats stayed in the estrus stage of the cycle constantly. The OVAX bone density (0.032g/cm^3) was significantly lower than the other three groups (0.058g/cm3, 0.063g/cm3, 0.050 g/cm3 for Control, OVAX + E_2 respectively) according to Scheffe's Test ($P<0.05$). The uterus in OVAX rats was infantile in comparison to the other three groups. These data suggest that ovariectomy causes an increased growth rate, delayed or retarded puberty, and decreased bone density in prepubescent rats. Estrogen restored the pubertal development, estrus stage of the cycle, and bone density, so it may serve as a replacement for some of the lost ovarian function.

INTRODUCTION

Ovariectomy permits study of the properties and characteristics of the ovaries. The gonads are removed from an organism, and development can then be compared to that of intact organisms. The differences between the ovariectomized and intact rat (*Rattus norvegicus*) should be due to the loss of ovarian function in the ovariectomized rat. Removal of the ovaries has a wide variety of effects on the experimental rat. Ovariectomy of prepubescent individuals may alter development of the secondary sexual characteristics, the onset of puberty, growth rates, and bone development. The following study analyzes developmental effects, such as those mentioned above, of removing the ovaries from prepubescent rats, and estrogen's (estradiol) role in the activities of the ovary.

It is well known that the ovaries are essential for the reproduction of species such as rats and humans. This gonad produces the egg that will unite with a sperm to form a new organism. Ovaries are also necessary for other activities in the organism. The ovaries release estrogen, which seems to take part in the initiation of puberty (Ramirez and Sawyer 1965), the formation and maintenance of bone (Kalu, et al., 1984), the onset of secondary sexual characteristics such as increased uterine weight (Ronnekleiv, et al. 1978), vaginal opening, and ovulation (Ojeda, et al., 1980). It also may have an effect on body fat and weight (Ronnekleiv, et al., 1978).

For female rats, puberty is the developmental stage in which cyclic ovulation occurs and the vaginal membrane opens. Estrogen may initiate brain maturity for the onset of puberty (Ramirez and Sawyer 1965). Levels of estrogen increase by four fold before puberty, causing a preovulatory surge of gonadotropins and prolactin (Parker and Mahesh, 1976; Ronnekleiv, et al., 1978). This surge initiates vaginal opening and cycling (Ojeda, et al.,1980).

Control rats experience vaginal opening at approximately 36 days of age and estrogen-supplemented intact rats at 29 to 30 days of age; vaginal estrous cycles usually begin the day following the rupture of the membrane (Ramirez and Sawyer 1965, and Ojeda, et al., 1980). At this time, uterine weight increases by three fold (Ronnekleiv, et al., 1978). When the ovary is removed, the major source of estrogen is gone, and puberty may not occur. ". . . Puberty is initiated only when the ovary becomes capable of secreting estrogen in amounts sufficient to trigger the first preovulatory surge of gonadotropins" (Ronnekleiv, et al., 1978). In intact rats, estrogen treatment advances the onset of puberty causing premature sexual development (Ramirez and Sawyer 1965).

Ovariectomy has been shown to have effects on the density, volume, and weight of bones. Kalu, et al.

(1984) state that bone loss results from ovarian hormone deficiency, and this deficiency leads to less calcium and phosphorus per unit volume of bone. The bones have decreased weight and density as compared to controls. No change was seen in the length or width. Reduced bone weight was also seen by Sherman, et al. (1989), but no bone loss was reported. Miller and Wronski (1993) state that gonadal hormones have substantial influence on bone metabolism, and that skeletal mass declines after ovarian function has ceased. Estrogen replacement is used often to slow the rate of bone loss in postmenopausal women (Miller and Wronski 1993). Also, following ovariectomy in rats, there is a rapid decrease in bone mass and increase in bone turnover (Miller and Wronski 1993).

Weight gain in rats after ovariectomy is a normal occurrence; in fact, it is a good indicator of a successful ovariectomy. On the other hand, when estrogen is given to intact rats, the final body weights are lower than controls, possibly due to early sexual maturity (Ramirez and Sawyer 1965). Ronnekleiv, et al. (1978) reveals another theory, a certain growth rate or body fat composition may be necessary for puberty to begin.

Rats are the organism of choice for this experiment because so much is known about their biology, and it is often comparable to human systems. These rodents are used frequently in the study of bone development and osteoporosis. They are representative of humans because gonadal hormone insufficiency results in enhanced bone turnover and osteopenia (Sherman, et al., 1989). Ojeda, et al. (1980) mentions that rats are used extensively to study the pubertal process due to ease of breeding and rapidity of pubertal development.

Ovariectomy is somewhat applicable to human research. Gonadal dysgenesis is a condition in humans where the gonads are not completely mature or they are absent (ovarian agenesis). In this situation, puberty is either delayed, not completed, or not initiated. Often, the uterus is infantile, there is a disproportionate cervix, the endometrial tissue shows sterility, and amenorrhea or retarded menarche is experienced (Botella-Llusia 1973). Stature, however is usually normal (Yen and Jaffe 1986).

The purpose of this experiment is to understand the developmental processes affected by ovariectomy in the prepubescent rat, and estrogen's role in the activities of the ovary. Ovariectomized rats will be compared to intact rats before and after an age at which puberty should occur. In order to study estrogen's role in these activities, one experimental group of ovariectomized rats will be given estrogen replacement therapy to define estrogen's role as opposed to the role of the entire ovary.

It is hoped that through this experiment, more will be understood about the total effects of the ovaries and the estrogen they produce upon the developing prepubescent rat. Examining for secondary sex characteristics, testing bone samples, and looking at growth rates should reveal a lot about ovarian and estrogen function.

MATERIALS AND METHODS

Experimental Design

Female Sprague-Dawley rats were obtained from Charles River Labs at 22 days of age. The rats were randomly assigned to the experimental and control groups, six in each of four groups, and housed in a laboratory setting of controlled temperature. Purina rodent chow and water were given ad libitum. Ovariectomy and sham operations were performed between 22 and 29 days of age. Twelve animals were ovariectomized (OVAX) through a procedure similar to the protocol in The Manual for Laboratory Work in Mammalian Physiology (D'Amour, et al., 1965). Ketamine HCl (0.05 ml/100g) was used as a general anesthetic. The ovaries were removed through a ventral incision. First, the uterus was located, and then the uterine horn was followed to the ovary. The uterus was sutured closed near the ovary, and the ovary was cut out. This procedure was repeated for both ovaries. Great care was taken to remove the entire ovary. The incision was closed by suture. Six rats were sham-operated in the same manner. The OVAX rats were divided into two groups of six. One group, OVAX + E_2, received estrogen on a daily basis, and the other group, OVAX, received no estrogen. The Control group was made up of six rats chosen randomly. Weight was taken weekly, and vaginal opening was checked daily. Bone analysis was done upon sacrifice of the rats at 55 days of age.

Estrogen Therapy

Six rats in the group OVAX + E_2 were injected daily with estradiol cypionate (β Estradiol cypionate) beginning on day 30. Injections were 10μg/kg of body weight, administered subcutaneously, diluted in 0.25 - 0.40 ml of vegetable oil. The treatment was given from 30 to 55 days of age. The OVAX + E_2 was the only group receiving injections.

Vaginal Smears

After vaginal opening, the presence of cycling was determined through vaginal smears. Estrus was confirmed by the presence of many flat, large cornified epithelial cells. Diestrus/anestrus showed very few cells, and was mainly composed of neutrophilic granulocytes (Schmid and Forstner 1986, and Turner 1966). In order to confirm non-cycling, smears were taken daily for five days, the duration of a normal cycle. Five consecutive smears were obtained for any abnormal results.

Bone Analysis

Blood samples were obtained after sacrificing the

rats at age 55 days. The blood was analyzed for calcium levels through the spectrophotometric method of Bausch and Lomb (1965). The right femur from each rat was removed and dried overnight at 100°C. The weight of the bone was determined, and the density was obtained through a comparison of the weight of the hollow shaft of the femur in water to its weight in air.

RESULTS

Growth Rates

The growth rates of the OVAX group (2.78g/d) were significantly increased compared to the OVAX + E_2 (2.23g/d) and Sham (2.26g/d) groups according to Scheffe's Test ($P<0.05$). The growth rate for the control group was 2.49g/d. A significant difference in average final weights (Fig. 1a) was seen using Scheffe's Test ($P<0.05$) between the OVAX (204.20g) and Sham (173.64g) groups and the OVAX and OVAX + E_2 (172.00g). Final weight for the Control group was 188.35g. No two other groups showed significant differences in final weight at the $P<0.05$ level.

Onset of Puberty

The onset of puberty was determined through vaginal opening and cycling. The average age (41.4d) and weight (129.7g) at vaginal opening for the OVAX group is increased compared to that of the other three groups (Figure 1b, 1c), which are all similar (33.17d, 34.00d, 35.17d in age, and 102.56g, 100.92g, 96.28g in weight for Control, OVAX + E_2 , and Sham, respectively). These data provide information that the OVAX group shows a delayed onset of puberty. Scheffe's testing shows that at a $P<0.05$ level, there is a significant difference between the weight at vaginal opening of the OVAX group and the Sham group (Fig. 1c). One rat in the OVAX group still had an intact vaginal membrane at 55 days of age, and was not included in the average of the group. No difference was seen in the ratio of weight at vaginal opening to the age at vaginal opening (3.11g/d, 3.09g/d, 2.96g/d, 2.74g/d for OVAX, Control, OVAX + E_2 , and Sham, respectively) (Fig. 1d). Rats in the OVAX group also showed differences in cycling. Only two of the rats in the OVAX group were showing signs of a cycle. Cornified cells observed in their vaginal smears were not present in the high numbers seen on the control slides. The remaining four rats did not experience an estrous cycle. The cells were mainly neutrophilic granulocytes, which are characteristic of a diestrus state. The smears changed very little from day to day for five consecutive days. Sham and Control smears showed all four stages of the normal cycle: estrus, proestrus, metestrus, and diestrus. The OVAX + E_2 smears showed an overabundance of cornified epithial cells each day for five days. These results reveal that the OVAX + E_2 rats stay in the estrus stage of the cycle constantly. Only differences in the number of cells were seen from day to day.

Estrogenic Effects on Bone and Uterus

Average calcium in serum levels are very similar for each group. They were 8.11mg/100mL, 9.82mg/100mL, 8.45mg/100mL, and 9.09mg/100mL (Fig. 1e). This data represented OVAX, Control, OVAX + E_2 , and Sham, respectively. At the level of $P<0.05$, in Scheffe's Test, no two groups are significantly different from one another. The average bone weights also show very little difference from group to group (Figure 1f), there is no significant effect of estrogen on the bone weight or calcium loss in one month. Bone weights were 0.35g for OVAX, 0.33g for Control, 0.35g for OVAX + E_2, and 0.33g for Sham. Bone density data (Figure 1g) prove that the OVAX group average (0.032g/cm^3) is significantly lower than the other three groups, which were somewhat similar in average (0.058g/cm3 for Control, 0.063g/cm3 for OVAX + E_2 , and 0.050g/cm3 for Sham). The estrogen injections created scar tissue under the skin which slowed the absorption of the oil/estrogen mixture.

Upon sacrificing the rats at 55 days of age, the uterus was viewed. In Sham and Control rats, the uterus was large and swollen with blood if it were in estrus, and somewhat smaller if the rat was in another stage of the cycle. In OVAX rats, the uterus was very small and infantile, with very little blood. All OVAX + E_2 rats showed uteri that were very large and swollen, as the uterus looks during an estrus stage of the cycle.

DISCUSSION

As expected, the OVAX group gained weight at a significantly faster rate than the other groups, with a growth rate of 2.78g/d compared to 2.49g/d, 2.23g/d, and 2.26g/d. Experiments by both Kalu, et al. (1984) and Sherman, et al. (1989) showed that rat weights were similar before the experiment, and ovariectomized rats weighed more at the termination of the experiment. This fact was confirmed by this experiment (Fig. 1a). The difference in average final weight was only significant in the Sham and OVAX + E_2 compared to OVAX. This data may seem irregular, but the Control group did not have surgery, which slows the growth during the healing process. More variability may have been evident in the final weights if the length of the experiment had been extended.

The OVAX group showed a delayed onset of puberty. Ramirez and Sawyer (1965) stated that estrogen was the limiting factor in the maturation of the brain for puberty. Not only were the rats older and weighed more on the average at vaginal opening than those in other groups (Fig. 1b, 1c), but cycling did not occur in four of the rats, and the other two rats

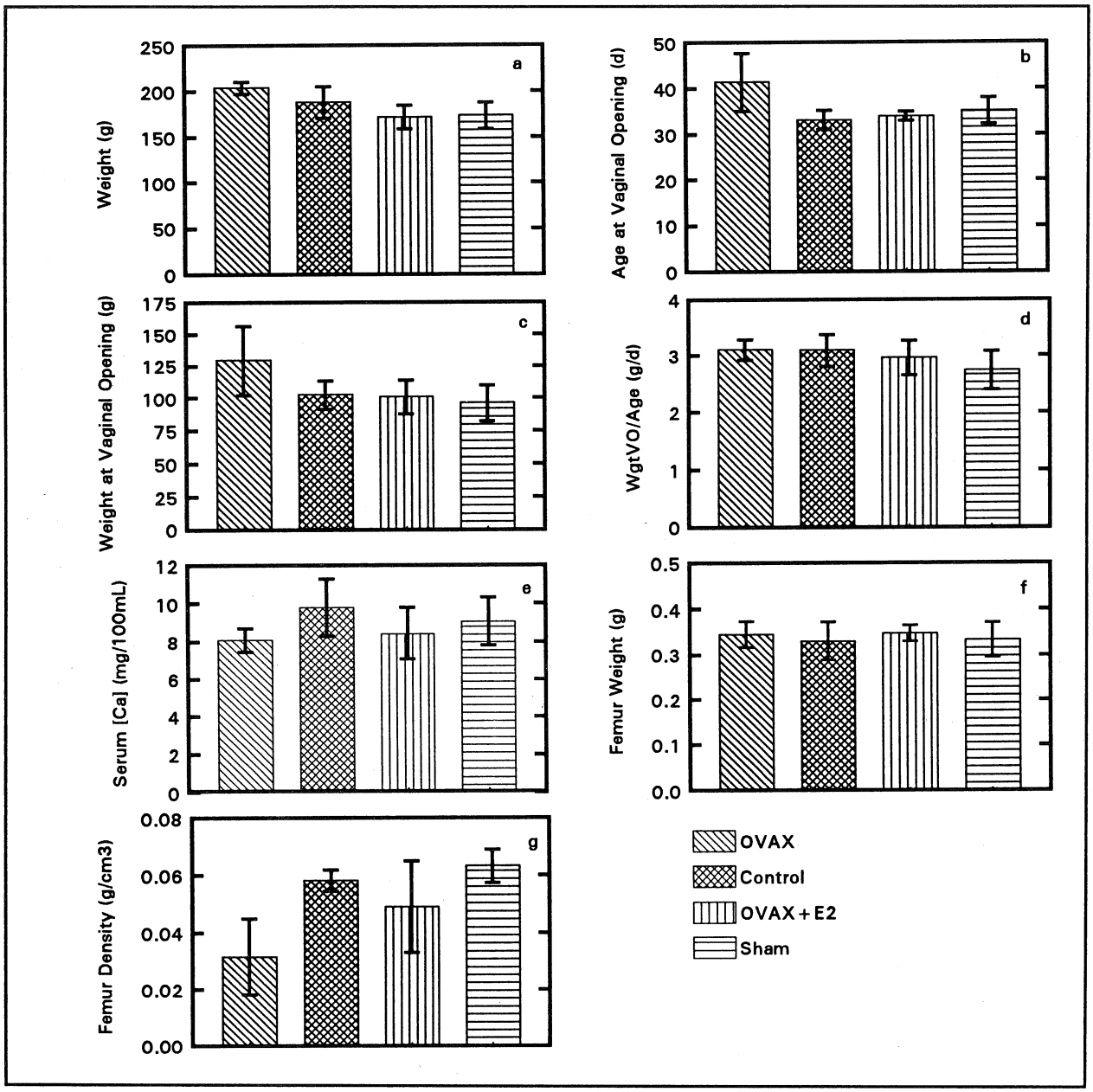

Figure 1. Comparison by average per group of (a) final weight, (b) age, (c) weight, (d) weight at age of vaginal opening, (e) calcium concentration in the blood serum, (f) dry weight of right femur, and (g) density of the shaft of the right femur.

showed abnormal stages in the cycle. Also, the vagina did not open on one of the rats. This data reveals that the OVAX rats, in some ways, were still considered to be juvenile, according to the definition of puberty. Ojeda, et al. (1980) states that puberty is a result of increased ovarian estrogen release, so when the ovaries are not present to produce estrogen, puberty should not occur. The variability within the OVAX group is not due to the date of ovariectomy; all OVAX surgeries were performed within approximately 30 hours of one another. The control rats had an average vaginal opening age of 33 days, Sham at 35 days, and the OVAX+E_2 at 34 days. All of these seem to fall within the normal range, which is 31-38 days for Sprague-Dawley rats (Steele 1977). Ramirez and Sawyer (1965) showed decreased age at vaginal opening for estrogen-supplemented OVAX rats, but the late start in giving estrogen may have caused other results in this experiment (Fig. 1b). The uteri of the OVAX rats were small and infantile in comparison to

the other cycling groups, just as expected when looking at the uteri of cycling versus non-cycling rats (Jackson Lab 1968). No specific weight was necessary for vaginal opening to occur because the weights were over a large range at vaginal opening (79.89g - 163.83g). Ronnekleiv, et al. (1978) supported this idea that no crucial body weight is necessary for puberty to occur.

Ovarian hormone deficiency seemed to have significantly affected only the bone density during the time allotted for this experiment (Fig. 1g). Kalu, et al. (1984) observed this also, but saw decreased serum calcium levels and slightly decreased bone weights. Sherman, et al. (1989) saw no change in serum calcium, but a difference in bone weight. However both sources agree that estrogen deficiency leads to bone loss. The weight of the femur was not significantly different between groups in this experiment, according to Scheffe's test (Fig. 1f). This could be due to the small amount of time in which this experiment was run; more time may have lead to greater treatment effects.

The effects of estrogen were decreased slightly by the method of administration. The estrogen injections were given sub-cutaneously, in volumes that were too large to be absorbed within a few days. As a result, pockets of oil/estrogen mixture formed under the skin. This created scar tissue which increased the pain for the rat, and prevented the estrogen from having the desired affect on the body. The estrogen therapy could also have been more effective if begun at an earlier age. Ramirez and Sawyer (1965) suggest no later than 26 days of age.

It is concluded from this study that developmental processes are altered by ovariectomy, and that estrogen plays an effective role in restoring ovarian function. Growth rates are increased in ovariectomized rats. Puberty is delayed through increased age and weight at vaginal opening. Vaginal cell smears are abnormal in these rats; showing few cornified cells. Most of the rats did not show cells characteristic of the estrus stage in the cycle. Bone density is decreased by ovariectomy. However, when estrogen therapy is given, growth rates and vaginal opening age and weight are similar to control values, and bone density is restored. Vaginal smears showed an abundance of cornified epithelial cells when estrogen is present, confirming that estrogen restores the estrus stage in the cycle.

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