Clomid or Nafoxidine Administered to Neonatal Rats Causes Reproductive Tract Abnormalities

Abstract. *A single injection of either Clomid or Nafoxidine in neonatal rats causes multiple abnormalities of the reproductive tract of the adult female animal. These anomalies include cystic ovaries, ovarian hypoplasia, hilus cell tumors, oviductal hyperplasia, pyometra, epithelial metaplasia, uterine cystic hyperplasia, and tumors of the uterus.*

These experiments were begun to test the hypothesis that estrogens are involved in masculinizing the hypothalamus. It has been suggested that androgens are converted to estrogens by the hypothalamus of the fetal or neonatal mammal and that these estrogens cause malculinization of the hypothalamic-hypophyseal axis (*1*). If estrogens were responsible for this action, it should be possible to use Clomid or Nafoxidine (*2*), which are long-acting atypical estrogens, to masculinize female rats (*3*). Since these compounds have no known androgenic function and can not be converted to androgens, a demonstration of masculinization by Clomid or Nafoxidine would provide strong evidence for the estrogen-induced masculinization theory.

Here we present evidence that treatment of neonatal rats with Clomid or Nafoxidine does induce effects which resemble those found in masculinized female rats. More important, however, we observed that this treatment causes a wide array of abnormalities of the reproductive tract.

Neonatal female rats of the Sprague-Dawley strain (Texas Inbred Mouse Company) were injected on day 1 of life with either Nafoxidine (1 to 100 μg per rat) or Clomid (10 to 500 μg per rat) (*4*). The animals were weaned at 21 days of age and the time of vaginal opening was noted. Vaginal smears were examined for 3 to 4 weeks before autopsy. The ovaries, oviducts, and uteri were removed between days 60 and 100 of life and were prepared for routine histological analysis.

Vaginal opening occurred in 86 percent of the rats injected with 100 μg of Nafoxidine or 500 μg of Clomid between days 26 and 34. Control rats have vaginal opening between days 35 and 50. The vaginal smears of treated rats did not show normal cyclic changes, and a high incidence of estrus smears was noted. Therefore, these animals are probably acyclic and in a state which outwardly resembles persistant estrus. These responses are typical of masculinized female rats; however, a more extensive evaluation of these animals is required before this can be stated with certainty.

Abnormalities of the reproductive tract involved a complicated array of anomalies. These included atrophic ovaries with accompanying atrophic uteri in some animals, while others showed cystic ovaries and enlarged uteri (Fig. 1A). Hypertrophy of the oviducts was a common observation. An examination of the histology of the uterine tissue revealed various stages of uterine hyperplasia and squamous metaplasia (Fig. 1, B and C). Tumors of the uterus were also observed in rats that received Clomid or Nafoxidine (Fig. 1D). Uterine tumors were observed in only a few animals; however, no animals older than 100 days were used in this study. The incidence of uterine tumors may increase considerably in older rats. Other abnormalities which are not shown include hypertrophied and hyperplastic oviducts; ovarian, oviductal, and uterine inflammation accompanied by pyometra; atrophic ovaries which contained few follicles, a condition that was usually accompanied by atrophic uteri; liquid-filled periovarian

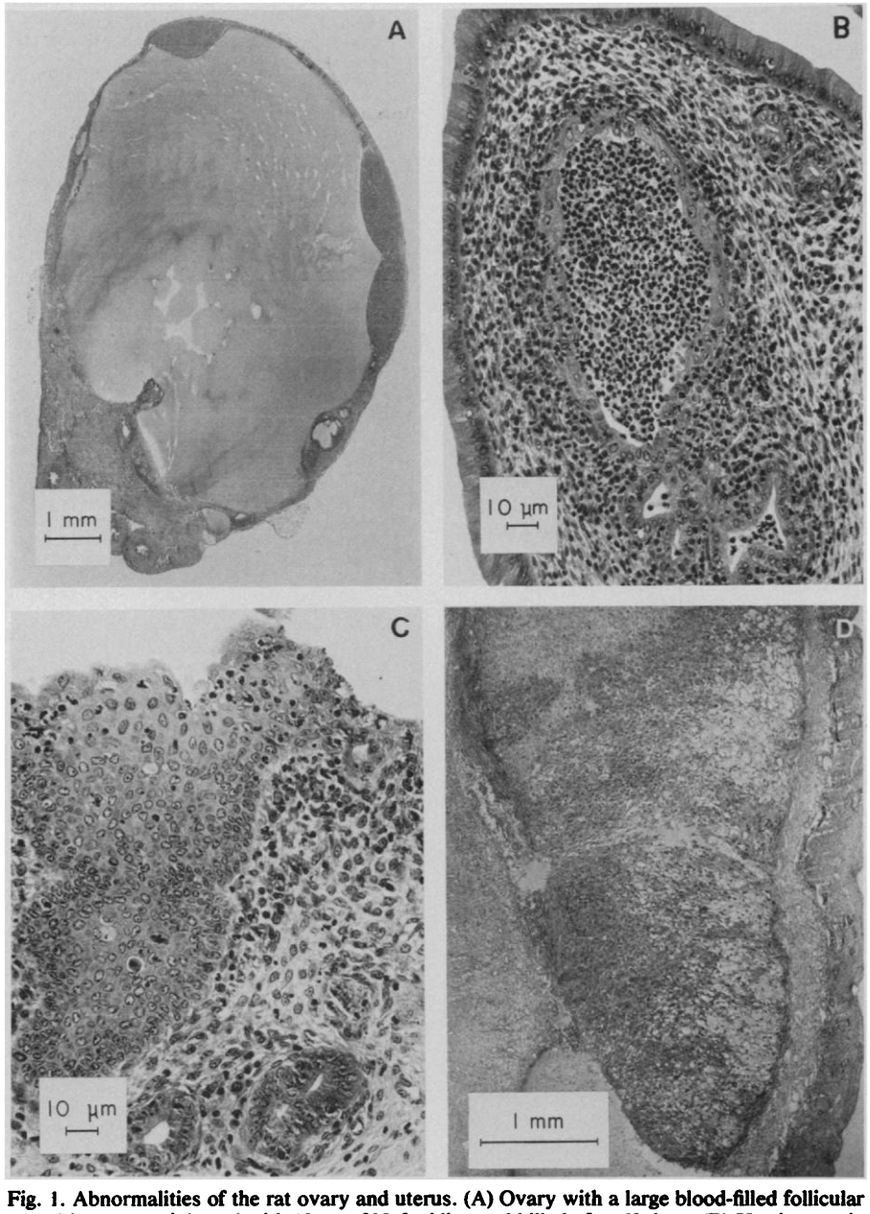

Fig. 1. Abnormalities of the rat ovary and uterus. (A) Ovary with a large blood-filled follicular cyst. The rat was injected with 10 μg of Nafoxidine and killed after 60 days. (B) Uterine cystic hyperplasia. The rat was injected with 50 μg of Clomid and killed after 100 days. (C) Uterine metaplasia and endometrial disorganization. The rat was treated as in (B). (D) Tumor in lumen of the uterus. The rat was injected with 2 μg of Nafoxidine and killed after 60 days.

sacs with small atrophic ovaries; and hilus cell tumors of the ovary. Control animals that received oil injections did not manifest any reproductive tract abnormalities and were cyclic. The types and frequency of abnormalities varied widely among the various treatment groups. The high dose of either Clomid (500 μg) or Nafoxidine (100 μg) produced some form of abnormality in 80 to 100 percent of the animals. Although intermediate and lower doses have not been completely evaluated, our results indicate that 10 to 50 percent of the animals will be adversely affected. The tumor that is shown in Fig. 1 was found in an animal that was injected with only 2 μg of Nafoxidine. Uterine metaplasia and infertility accompanied by polycystic degeneration of the ovary have been described by others (*5*).

The variation in the kinds of abnormalities probably relates to both the dose of the compound and the age of the animal to which it is administered. The ability of the reproductive organs to respond to estrogenic compunds depends, in part, on the presence of estrogen receptors, and the concentration of these receptors is known to increase with time in the neonatal rat (*6*). Therefore, the effectiveness of the compound, as well as its mode of action, may vary with time.

Although we have not ruled out the possibility of indirect effects of Nafoxidine and Clomid, it seems likely that these drugs are acting directly on the various target tissues. We have observed previously that Nafoxidine causes long-term retention of the estrogen receptor by uterine nuclei in immature rats (21 to 23 days old). This long-term retention is accompanied by a sustained stimulation of uterine growth, up to 19 days after a single injection (*3*). Our data indicate that this effect is also operating in the neonatal rat; therefore, we think that the abnormalities that we have observed are due to a similar long-term estrogenic stimulation. Whether such nuclear binding and estrogenic stimulation occurs in adult animals or in other species remains to be resolved.

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References and Notes

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2. Clomid, triethylamine, 2-[*p*-(-chloro-1,2-diphenylvinyl)-phenoxy]-citrate, and Nafoxidine, 1-(2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl) pyrrolidine hydrochloride, were obtained from Merrell-National Laboratories and the Upjohn Company, respectively.
3. Clomid and Nafoxidine have traditionally been considered to be estrogen antagonists; however, we have shown that they can function as long-acting estrogens in the immature rat uterus. [J. H. Clark, J. N. Anderson, E. J. Peck, Jr., *Steroids* **22**, 707 (1973)]; J. H. Clark, E. J. Peck, Jr., J. N. Anderson, *Nature (London)* **251**, 446 (1974); J. H. Clark, Z. Paszko, E. J. Peck, Jr., *Endocrinology* **100**, 91 (1977).
4. Each compound was dissolved in absolute ethanol, stirred into warmed (about 45°C) sesame oil until the ethanol had evaporated, and injected subcutaneously in the nape of the neck. Clomid is a mixture of *cis* and *trans* isomers and was used as such because this is the form which is administered to women.
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7. Supported by the American Cancer Society grant BC-92. We thank L. Swaine for assistance with the photography and G. Brady for technical assistance.

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Adriamycin: The Role of Lipid Peroxidation in Cardiac Toxicity and Tumor Response

Abstract. *The antitumor antibiotic, adriamycin, induces severe cardiac toxicity associated with peroxidation of cardiac lipids in mice. Both this lipid peroxidation and cardiac toxicity of adriamycin are reduced by prior treatment of the animals with the free radical scavenger tocopherol. Such treatment with tocopherol does not, however, alter the magnitude or duration of the adriamycin-induced suppression of DNA synthesis in P388 ascites tumor, nor does it diminish the antitumor responsiveness of P388 ascites tumor. These results suggest that adriamycin has at least two mechanisms of tissue damage: one, which involves lipid peroxidation, is blocked by tocopherol and results in cardiac toxicity; the other, which involves binding to DNA, is not antagonized by tocopherol and is responsible for tumor response.*

The antitumor antibiotic, adriamycin, is one of the most important new drugs in the field of cancer chemotherapy; it has exhibited activity against a wide spectrum of human neoplasms and in particular against solid tumors. Unfortunately, its clinical use has been compromised by an unusual and potentially lethal cardiac toxicity, which is thus far unexplained (*1*).

Handa and Sato (*2*) have recently demonstrated in microsomes exposed to anthracyclines a superoxide radical ion production that is dependent on reduced nicotinamide adenine dinucleotide phosphate. Previous investigators have shown that superoxide radical ions can decompose to yield hydroxyl radicals, peroxy radicals, and hydrogen peroxide (*3*). These, in turn, are known to initiate free radical mediated chain reactions which result in conversion of the membrane unsaturated fatty acids to lipid peroxides (*4*). We have recently reported that in mice treated with tocopherol, an effective free radical scavenger known to inhibit the formation of lipid peroxides, the toxicity of adriamycin is significantly reduced (*5*), a lethal dose for 85 percent of the animals being converted to a lethal dose for 10 percent. These results suggested that lipid peroxidation may play an important role in adriamycin toxicity.

In the present study, experiments

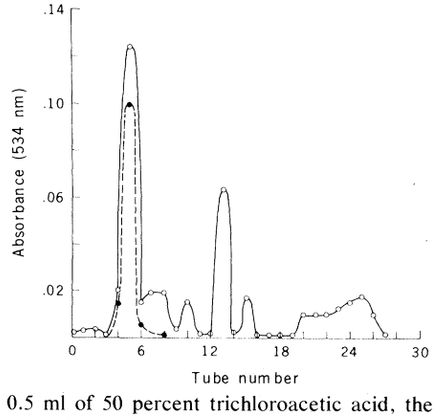

Fig. 1. Detection of malondialdehyde in murine cardiac tissue after the administration of adriamycin. Male mice (CDF_1; 20 to 25 g) were housed in a constant temperature environment, caged on hardwood bedding and fed Wayne F6 Lab-Blox. Each mouse (three groups of three mice each) received an intraperitoneal injection of adriamycin (15 mg/kg); 4 days later the animals were killed by cervical dislocation. The hearts were rapidly removed, washed in iced phosphate-buffered saline, *p*H 7.4, blotted dry, and placed in a weighed vial. After weighing, each group of three hearts was disrupted with a Polytron homogenizer in 2 ml of 0.02*M* potassium phosphate buffer, *p*H 7.4, containing butylated hydroxytoluene (0.5 mg/100 ml) to prevent further oxidation of lipids. After the addition of 0.5 ml of 50 percent trichloroacetic acid, the samples were heated to 90°C for 15 minutes. The samples were reduced to 0.5 ml by lyophilization, brought to *p*H 7.5, applied to a Sephadex G-10 column (0.9 by 40 cm), and eluted with 0.05*M* tris-HCP buffer, *p*H 7.4, containing 0.1*M* NaCl; 1-ml fractions were collected. After 300 μl of 1 percent 2-thiobarbituric acid (TBA) was added to 300 μl of each fraction, the mixture was heated to 90°C for 15 minutes and absorbance was read at 533 nm. The TBA-reactive material from tissue extracts (○) is compared with a malondialdehyde standard synthesized from tetraethoxypropane (K and K Chemical) (●) (*6*). The concentration of malondialdehyde was then calculated with a molar extinction coefficient of 1.5×10^5 being used.