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Ovarian Toxicity and Carcinogenicity in Eight Recent National Toxicology Program Studies

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Ovarian toxicity and/or carcinogenicity has been documented for at least eight chemicals recently tested in National Toxicity Program prechronic and chronic rodent studies. The chemicals that yielded treatment-related ovarian lesions were 1,3-butadiene, 4-vinylcyclohexene, vinylcyclohexene deipoxide, nitrofurantoin, nitrofurazone, benzene, Δ-9-tetrahydrocannabinol, and tricresylphosphate. Typical nonneoplastic ovarian changes included hypoplasia, atrophy, follicular necrosis, and tubular hyperplasia. The most commonly observed treatment-related neoplasms were granulosa cell tumors and benign mixed tumors. A relationship between antecedent ovarian hypoplasia, atrophy, and hyperplasia and subsequent ovarian neoplasia is supported by some of these National Toxicology Program studies. Pathologic changes in other tissues such as the adrenal glands and uterus were associated with the treatment-related ovarian changes.

Ovarian and Reproductivity Toxicity

Normal ovarian functional and morphologic integrity is inextricably associated with proper functioning of the HPOU (hypothalamus-pituitary-ovary-uterus) system (*1–3*). Consequently, reproductive perturbations can result from functional and/or morphologic compromise of any part of this system. The frequency of treatment-related ovarian lesions in general toxicity testing appears to be low. Thus, characterization and understanding of ovarian lesions when they do occur pose difficulties because of the lack of substantial knowledge about the ovary as a target tissue for toxicity. Even in rodent reproductive toxicity studies, significant ovarian pathology is usually not found. This is largely a consequence of the greater emphasis given to functional perturbations in reproductive performance in these studies. Specific ovarian lesions such as oocyte necrosis and premature atresia have been produced by exposure to ionizing radiation (*4*) or to chemicals such as some polycyclic aromatic hydrocarbons, alkyl halides, and nitrosamines (*4–6*). Follicular cysts and reduction in number of corpora lutea have been associated with estrogenic action of DDT and its analogs (*7*).

In the human clinical setting, occupational exposure of women to chemicals is not frequently associated with specific ovarian lesions. In a review of 270 reports relating to clinical manifestations of female reproductive toxicity (*8*), approximately 45% of the reports deal with toxicity to the embryo, fetus, or placenta. Another 40% discuss perturbations of the integrated functioning of the HPOU axis. Ovarian toxicity per se represents a small proportion of the alterations, and these usually involve hormonal dysfunctions. An expanded discussion of the association between ovarian toxicity and occupational exposure to chemicals is contained in a recent review (*3*). Similarly, specific functional or morphologic effects on the ovary represent a small proportion of untoward effects of chemicals on the rodent reproductive system as judged by the low frequency of occurrence of ovarian lesions in standard toxicity studies such as those conducted by the National Toxicology Program (NTP) or in noninvasive reproductive toxicity studies (*9*). Functional deficits in reproductive performance and altered development of reproductive capacity in rodents have been associated with a wide spectrum of chemicals (*10,11*). Ovarian lesions have not been specifically documented in these latter studies.

Chemicals that are toxic to the female reproductive system act either directly or indirectly (*12*). Direct-acting toxins are often structurally similar to biologically important molecules or are chemically reactive compounds that behave nonspecifically. Examples of the former include nutrients or hormones that act as agonists or antagonists of endogenous hormones. Examples of chemically reactive nonspecific compounds include alkylating agents, denaturants, and chelators. Indirect-acting toxins exert their toxicity either by being metabolically activated to active toxins (e.g., cyclophosphamide, dibromochloropropane, polycyclic aromatic hydrocarbons) or by induction or inhibition of reproductively important enzyme systems. Since the ovary

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contains microsomal monooxygenases, epoxide hydrases, and transferases (*5,12*), the ovary may metabolically activate chemicals with consequent production of ovarian lesions.

Ovarian Neoplasia

Based upon epidemiological studies in nulliparous women, it appears that endogenous hormones are associated with the induction or growth of ovarian tumors (*13–15*). Further, it has been postulated that gonadal failure may be a common perturbation for both infertility and ovarian neoplasia (*16*). Based upon the known presence of specific hormone receptors in ovarian tissue, it is reasonable to anticipate that endogenous hormones (e.g., gonadotropins, estrogens, progesterones, androgens, corticosteroids) would influence the clinical behavior of benign and malignant ovarian neoplasms in humans and animals.

From results of experimental studies, a sequence of events has been postulated relative to the development of granulosa cell tumors and tubular adenomas in rodents (*17–21*). These investigators hypothesize that degeneration of follicular granulosa cells subsequent to oocyte destruction stimulates a compensatory increase in pituitary gonadotropins which, in turn, stimulates cell proliferation and eventual development of neoplasia. While such a mechanism may be associated with development of ovarian neoplasia in mature rodents, ovarian tumors in pubertal rodents can develop in the presence of a normal population of growing oocytes and follicles (*22*). A similar mechanism associated with interruption of the normal hormonal feedback system between ovary and hypothalamus/pituitary is seen in experiments where normal ovarian tissue is grafted to the spleen of ovariectomized rodents (*23*). Since venous drainage from the spleen passes through the liver, hormones produced by the grafted ovary are metabolized, and thus, the feedback necessary to prevent release of pituitary gonadotropins is interrupted. The result is continued production of pituitary gonadotropin, which stimulates cell proliferation in the grafted ovary and leads to development of granulosa cell tumors.

Hormonally mediated induction of ovarian tumors in rodents has also been demonstrated following neonatal thymectomy (*24*), as well as following parabiosis with a castrate, transplantation of a gonadotropic pituitary tumor, or chronic exposure to hormones (*23*).

Ovarian Toxicity and Carcinogenicity in NTP Studies

Ovarian toxicity and/or carcinogenicity has been observed for a least eight chemicals in recent NTP toxicity and/or carcinogenicity studies. Three of these chemicals are closely related structurally: 1,3-butadiene, 4-vinylcyclohexene, and vinylcyclohexene diepoxide.

1,3- Butadiene

1,3-Butadiene is a four-carbon aliphatic used as an intermediate in production of elastomers and polymers. Its largest use is in synthetic rubber production, and in that setting, it is primarily an air contaminant. Occupational exposure is estimated at 62,000 workers annually.

1,3-Butadiene also occurs in urban atmospheres as a combustion product of fossil fuels. 1,3-Butadiene is clearly carcinogenic, as shown by a recently completed inhalation study in B6C3F1 mice (*25,26*). This study was prematurely terminated because of high mortality secondary to neoplasia after 61 weeks of inhalation exposure to 625 or 1250 ppm 1,3-butadiene. In addition to causing ovarian tumors, 1,3-butadiene caused hemangiosarcomas of the heart, malignant lymphomas, lung tumors, and forestomach tumors in male and female mice and mammary gland tumors and liver tumors in female mice (*25*).

Ovarian lesions in mice exposed to 1,3-butadiene for up to 60 weeks included loss of follicles, tubular hyperplasia, and several different tumor types (Tables 1 and 2). One of the granulosa cell tumors in the 1250 ppm group was malignant. Neoplasms with both a tubular cell component and a granular cell component were diagnosed as "benign mixed tumor." Epithelial hyperplasia refers to a proliferation of tubular structures in the ovarian parenchyma and is regarded as a precursor to tubular adenoma in the context of this specific 1,3-butadiene study. Significant microscopic lesions were not present in the ovaries following either 15 days or 14 weeks of inhalation exposure to 625, 1250, 2500, 5000, or 8000 ppm 1,3-butadiene.

4-Vinylcyclohexene

4-Vinylcyclohexene is a dimer of 1,3-butadiene. It is a high-volume chemical used in the production of epoxy

Table 1. Findings of 1,3-butadiene study.

Study	Species	Ovarian pathologic findings	Other pathologic findings in females
90-day inhalation	Mouse	None	None
61-week inhalation	Mouse	Atrophy Hyperplasia Neoplasia	Liver neoplasia Heart neoplasia Lymphoma Lung neoplasia Forestomach neoplasia Mammary neoplasia

Table 2. Numbers of mice with neoplastic and nonneoplastic ovarian lesions in the 61-week inhalation study of 1,3-butadiene.

	Exposure group		
	Control	625 ppm	1250 ppm
No. of mice examined	49	45	48
Lesion			
Granulosa cell tumor	0	6	13
Tubular adenoma	0	2	0
Benign mixed tumor	0	0	2
Cystadenoma	0	1	0
Granulosa cell hyperplasia	0	2	0
Epithelial hyperplasia	0	3	0
Atrophy	2	40	40

resins and is present in gases discharged during the curing of synthetic rubber. It was administered by gavage to rats and mice in a 90-day toxicity study and a 2-year carcinogenicity study (doses: 200 and 400 mg/kg body weight) (*27*). While there were no significant microscopic lesions in females in the 90-day rat study, ovarian atrophy (reduction in the number of primary and mature graafian follicles) and mild gastritis were documented in treated mice. Following 2 years of exposure, 4-vinylcyclohexene produced ovarian hyperplasia and neoplasia in mice (Tables 3 and 4) but not in rats. In addition, there was treatment-related cytologic degeneration in the adrenal cortex, as well as adrenal cortical neoplasia, in female mice. 4-Vinylcyclohexene produced clitoral gland neoplasia in female rats but was concluded to be an inadequate study in female rats because of extensive early mortality in both dose groups.

Vinylcyclohexene Diepoxide

Vinylcyclohexene diepoxide is a potential metabolic product of 4-vinylcyclohexene. Its primary use is as a reactive diluent for epoxy resins. Occupational exposure is chiefly by the dermal route. Vinylcyclohexene diepoxide has been tested by gavage and dermal exposure routes for 90 days in rats and mice and is currently being tested in a 2-year dermal study in rats and mice. While treatment-related ovarian lesions were not observed in either 90-day rat study, treatment-induced ovarian atrophy was found in the 90-day mouse dermal study (Table 5). At the 65-week interim sacrifice of the 2-year dermal study, ovarian atrophy, tubular hyperplasia, and neoplasia were present in mice. No ovarian lesions were found in rats at the interim sacrifice. While final conclusions regarding ovarian carcinogenicity must await completion of the 2-year study, based upon 65-week data, it is probable that vinylcyclohexene diepoxide will be considered an ovarian carcinogen in B6C3F1 mice.

Studies in progress on two related chemicals, nitrofurantoin and nitrofurazone, have shown a neoplastic response in the ovary (Tables 6 and 7). Nitrofurantoin is a commonly used urinary antibiotic. Nitrofurazone is a topical antibiotic dressing. The ovarian responses in

Table 3. Findings of 4-vinylcyclohexene study.

Study	Species	Ovarian pathologic findings	Other pathologic findings in females
90-day gavage	Rat	None	None
90-day gavage	Mouse	Atrophy	Gastritis (minimal)
2-year gavage	Rat	None	Clitoral gland neoplasia
2-year gavage	Mouse	Hyperplasia Neoplasia	Adrenal cortical degeneration Adrenal cortical neoplasia

Table 4. Numbers of female mice with selected ovarian lesions in the 2-year gavage study of 4-vinylcyclohexene.[a]

	Exposure group		
	Control	200 mg/kg	400 mg/kg
No. of mice examined	49	48	47
Lesions			
Benign mixed tumor	0	25	11
Tubular adenoma	0	2	1
Tubular hyperplasia	1	10	13
Granulosa cell tumor	1	9	11
Granulosa cell carcinoma	0	1	2
Granulosa cell hyperplasia	0	5	1

[a]A few other neoplastic and hyperplastic lesions were present in a small number of mice (*27*).

Table 5. Findings of vinylcyclohexene diepoxide study.

Study	Species	Ovarian pathologic findings	Other pathologic findings in females
90-day gavage	Rat	None	Forestomach hyperplasia, toxic nephrosis, pancreatic atrophy, uterine atrophy,
90-day gavage	Mouse	None	Forestomach hyperplasia, uterine atrophy
90-day dermal	Rat	None	Skin irritation
90-day dermal	Mouse	Atrophy	Skin irritation, uterine atrophy
2-year dermal	Rat	at 65 weeks: None	Skin irritation
2-year dermal	Mouse	at 65 weeks: Atrophy, hyperplasia, neoplasia	Skin irritation

Table 6. Findings of nitrofurantoin study.

Study	Species	Ovarian pathologic findings	Other pathologic findings in females
90-day feed	Rat	Follicular epithelial cell necrosis	None
90-day feed	Mouse	Follicular epithelial cell necrosis	None
2-year feed	Rat	None	None
2-year feed	Mouse	Atrophy, hyperplasia, neoplasia	Adrenal hyperplasia, liver neoplasia

Table 7. Findings of nitrofurazone study.[a]

Study	Species	Ovarian pathologic findings	Other pathologic findings in females
90-day feed	Rat	Pale, vacuolated interstitial cells	Uterine hypoplasia, bone lesions, muscle lesions
90-day feed	Mouse	None	Thymic atrophy
2-year feed	Mouse	Hyperplasia, neoplasia	None

[a]Data for 2-year feed study in rats not yet available.

the carcinogenicity studies of these two compounds is important because of the widespread use of these chemicals and also because the studies represent examples of carcinogenicity test results wherein the neoplastic effect is primarily restricted to the ovary. Although the results of toxicity and carcinogenicity testing should be regarded as preliminary until all aspects of the studies are peer reviewed, it is apparent at this time that the ovary was a primary target tissue for both chemicals.

Nitrofurantoin

Nitrofurantoin was administered by dosed feed to rats and mice. In the 90-day toxicity study, necrosis of ovarian follicular epithelial cells was documented in both rats and mice and was the principal pathologic finding in the 90-day studies. Results (not peer reviewed) from the 2-year carcinogenicity studies indicate no treatment-associated lesions in the ovaries of rats. However, ovarian atrophy, tubular hyperplasia, and neoplasia were observed in treated mice (Table 6). In addition, adrenal hyperplasia and hepatic neoplasia were seen in treated female mice.

Nitrofurazone

In the 90-day nitrofurazone dosed feed toxicity study, there was pallor and vacuolization of the ovarian interstitial cells in rats, but no ovarian changes were associated with treatment in mice. Uterine hypoplasia was also documented in the 90-day rat study. While data are not yet available from the 2-year feeding study in rats, treatment-associated ovarian hyperplasia and neoplasia were observed in the 2-year carcinogenicity study in mice (Table 7).

Pathologic changes in the ovaries have been documented in studies on at least three additional unrelated chemicals: benzene, Δ-9-tetrahydrocannabinol, and tricresylphosphate.

Benzene

In 90-day and 2-year studies, benzene was administered by gavage. While there were no pathologic findings in the ovaries of rats or mice in the 90-day study or in rat ovaries in the 2-year study, ovarian atrophy, cysts, hyperplasia, and neoplasia were observed in mice treated for 2 years (Table 8). The diagnoses and frequencies of the ovarian tumors in mice are detailed in Table 9. It should be noted that benzene was considered carcinogenic in both rats and mice, producing a wide spectrum of epithelial tumors in several tissues (*28*). The observation of significant ovarian neoplasia in mice was an unexpected finding at final sacrifice.

Δ-9-tetrahydrocannabinol

At the present time, Δ-9-tetrahydrocannabinol has been tested by gavage administration for 90 days in rats and mice. Two-year studies in both species are planned.

Table 8. Findings of benzene study.

Study	Species	Ovarian pathologic findings	Other pathologic findings in females
90-day gavage	Rat	None	None
90-day gavage	Mouse	None	None
2-year gavage	Rat	None	Uterine neoplasia, Zymbal gland neoplasia, oral cavity neoplasia
2-year gavage	Mouse	Atrophy, cysts, hyperplasia, neoplasia	Lymphoma, mammary neoplasia, lung neoplasia, Zymbal gland tumors, adrenal capsular hyperplasia, forestomach neoplasia, Harderian gland neoplasia

Table 9. Numbers of mice with selected ovarian lesions in the 2-year gavage study of benzene.

	Exposure group			
	Control	25 mg/kg	50 mg/kg	100 mg/kg
No. of mice examined	47	44	49	48
Lesions				
Atrophy	15	35	32	22
Epithelial hyperplasia	12	39	31	29
Papillary cystadenoma	0	0	2	1
Granulosa cell tumor	0	1	6	8
Luteoma	0	2	3	2
Tubular adenoma	0	0	3	3
Mixed tumor (benign)	0	1	12	7

While treatment-associated uterine hypoplasia was documented in both rats and mice, ovarian changes were seen only in rats at the end of the 90-day study. The principal pathologic effect in the rat ovaries was hypoplasia characterized by a decrease in the size and number of maturing ovarian follicles. Diagnostic nomenclature for the observed ovarian changes in rats is problematic in that it is not possible to reliably distinguish between hypoplasia and atrophy by examination of tissues only at the conclusion of a 90-day study. Atrophy implies that the organ was fully formed and then lost some of its mature structure. Hypoplasia implies that the ovary is being well formed but is smaller than normal (a form of ovarian dysgenesis). There was reversibility of ovarian lesions within 60 days after the conclusion of the 90-day dosing period.

Tricresylphosphate

Tricresylphosphate has been tested for 90 days by gavage as well as dosed feed administration in both rats and mice. While results have not been peer reviewed and, thus, should be regarded as preliminary, there was a treatment-associated hypertrophy and cytoplasmic

vacuolization of interstitial cells in the ovaries of both species. In addition, cytologic alterations were noted in the adrenals of treated female rats and mice. Tricresylphosphate will ultimately be tested for carcinogenicity in 2-year studies.

Conclusions

Based upon review of the completed and uncompleted toxicity and carcinogenicity studies for the eight chemicals described, some general conclusions and considerations regarding pathologic evaluation of ovarian tissues seem justified. First, the occurrence of treatment-associated ovarian lesions in 90-day and 2-year rodent studies is not common. To date, more than 300 chemicals have been tested in 90-day and/or 2-year carcinogenicity studies. Significant treatment-related ovarian effects were found in only eight of these studies. This probably represents an underestimate of the true incidence of treatment-associated ovarian pathologic changes, since in past years, 90-day studies were used primarily to set doses for 2-year studies rather than to define subtle toxicity. Consequently, in the absence of grossly visible changes, one would not expect ovarian tissues to have been examined as critically in the past as is currently done. Despite the possibility that there may be more than eight studies in which ovarian lesions were induced by treatment, it is still reasonable to conclude that the frequency of ovarian target tissue toxicity is not high in conventional rodent toxicity and carcinogenicity studies.

Ovarian function and dysfunction are intimately linked with the hypothalamus, the pituitary, the uterus, and other endocrine organs. Thus, the observation that treatment-associated ovarian changes were frequently associated with pathologic alterations in other tissues (e.g., adrenal, uterus) in the eight NTP chemical studies is not surprising. In fact, the low probability that ovarian lesions would occur in isolation should prompt the pathologist to closely examine all parts of the HPOU and endocrine systems whenever a lesion is found in one component of this system. Closer examination of these other tissues may reveal subtle but important changes not noted on the initial pathologic examination.

The ovarian lesions documented in the NTP studies covered by this paper provide supportive evidence that alterations noted in ovaries of treated rodents at the conclusion of a 90-day study may herald the ultimate development of ovarian neoplasia upon continued treatment. A relationship between antecedent ovarian hypoplasia, atrophy, and hyperplasia and subsequent ovarian neoplasia has been previously proposed (*17–21*). Consequently, when ovarian changes are observed in treated animals in prechronic toxicity studies, special study design considerations (e.g., adding extra females, interim sacrifices, vaginal cytology, hormone measurements) might be warranted in subsequent carcinogenicity/chronic toxicity studies. Definitive reproductive studies would also be warranted when ovarian lesions occur in a 90-day study.

Histopathologic distinction between ovarian atrophy and hypoplasia in rodents cannot be reliably made on the basis of examination of tissues only at the conclusion of a 90-day study. Atrophy implies that the organ was fully formed and then lost some of its mature structure. Hypoplasia implies that the ovary is being well formed but is smaller than normal (a form of ovarian dysgenesis). At the end of a typical 90-day study, the rats or mice are approximately 20 weeks of age, and ovarian tissues would be expected to be fully developed and functional. The observation of atrophy or hypoplasia at this point is problematic in that the pathologist does not know if the ovary became fully developed and then underwent atrophy or if the ovary never fully developed in the first place and was thus hypoplastic. Since the distinction between atrophy and hypoplasia may be mechanistically important, it would be appropriate either to repeat the study and to sample ovarian tissue at earlier times or to incorporate appropriate interim sacrifices early in subsequent chronic toxicity/carcinogenicity studies. If ovarian changes are anticipated in advance, it is recommended that recovery groups be incorporated into the standard toxicity study.

Histopathologic evaluation of the ovary is frequently frustrated by sampling limitations. Since the ovary is small, a typical single histologic section may not contain all tissue elements. Thus, assessment of the number of follicles or corpora lutea may be compromised. If ovarian changes are anticipated, serial sections from both ovaries would help ensure adequate amounts of tissue for examination. By observing similar changes in both ovaries, the conclusion that the effect is systemic will be supported. Such observations, combined with careful examination of other hormonally related tissues and the use of interim sacrifices and recovery studies, should provide appropriate material for a comprehensive histopathologic assessment of ovarian toxicity.

The issue of combining tumors for purposes of assessing carcinogenicity is controversial. If the practice of combining related tumors is followed, it may be inappropriate to combine some ovarian tumors. The NTP-recommended nomenclature for rodent ovarian neoplasms is based on the cell of origin of the neoplasms. Tumors arising from diverse cells of origin should not be combined for purposes of assessing carcinogenicity.

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