PROTECTIVE ROLE OF COMPENSATORY GROWTH IN THE DEVELOPMENT OF MAMMALIAN INNER EAR

Pages with reference to book, From 308 To 315

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Abstract

The inner ear in rats is ectodermally derived. It appears as a placode on day 8 of a 22-day gestational period. In the developing inner ear most of the cells undergo terminal mitosis between gestational days 13 and 15. During this critical period of development exposure to toxic substances may result in malformations of the adult inner ear. To test this hypothesis, rat fetuses were exposed to 5-f luoro-2'deoxyuridine (FUdR), an antimitotic substance, on gestational days 12-16. The animals also received one injection of ^{3}H-thymidine 1 h prior to autopsy. The inner ear specimens were prepared for morphological observations and bioche mical assays. Four hours after the administration of FUdR there was a substantial decrease in the number of otic cells. This trend was seen until 24 h after treatment. When examined on day21 of gestation, the inner ears from the fetuses exposed to FUdR during gestational days 12-15 almost completely recovered from the toxic effects of the drug. The phenomenon of compensa ory growth observed during embryonic development may represent an important protective feature. During early stages of organogenesis, the otocyst perhaps has a remarkable capacity for recovery resulting in relatively fewer congenital anomalies of the inner ear. (JPMA 36: 308, 1986).

INTRODUCTION

The development of murine and rodent inner ear has been well documented. In a classical work Wada[1] correlated the anatomic development and functioning of the inner ear in fetal and neonatal rats. Ultrastructure studies on development suggested that innervation was not responsible for morphologic differentiation of sensory receptors and supporting cells[2,3] In vitro studies showed that otocyst explant from fetal mice can differentiate into sensory and supporting cells even when the statoacoustic ganglion complex was extirpated[4].

In an earlier study it was suggested that the entire cochlear duct developed from a growth center located at the junction of the primitive saccule and the evaginating cochlea[5]. With the use of an antimitotic substance coichicine, a zone of mitosis was shown for the developing cochlear duct at its junction with the saccule[6].

According to a recent study[7] it was shown that most of the cells of the inner ear undergo terminal mitosis during gestational days 13 and 15. Thus during this critical period of development the inner ear would be most vulnerable to environmental stress, and exposure to potentially toxic substances may result in malformations of the adult inner ear.

In order to test this hypothesis rat fetuses between gestational days 1 2 and 16 were exposed to 5-fluoro-2'-deoxyuridine (FUdR), an analog that inhibits the enzyme thymidylate synthetase, thus blocking DNA replication[8,9]. The effects of FUDR are observable as early as 4 h after its administration, The nuclei appear pyknotic, and the cells exhibit chromatolytic degeneration and eventually die[10,11]

In this report data on effects of a toxic substance on inner ear during a critical stage of development are

presented. Possibility of a reserve mechanism in this developing system is discussed which may perhaps be a reason for relatively low incidence of congenital anomalies of the. mammalian inner ear.

MATERIAL AND METHODS

Timed-pregnant Sprague-Dawley rats were obtained from a commercial source. Four litters with an average litter size of ten was examined and analyzed under each condition.

On the mornings of days 12-16 of gestation pregnant rats were given a single intraperitoneal injection of FUdR at a dose of 100 mg/kg of body weight (Sigma Chemical Co.)[11]. Fetuses were delivered by caesarean section under ether anaesthesia at 4,8,16 & 24 h after the injection. Another group of rats was given one injection of FUdR on the mornings of gestational days 12-16 and the fetuses from these rats were delivered by caesarean section on the morning of day 21 of gestation. The fetuses obtained from uninjecte4 pregnant rats served as controls. One hour before delivery, all rats were injected intraperi toneally with 1.0 uCi/g of body weight of ^{3} Hthymidine (^{3} H-TdR) (New England Nuclear, specific activity 20 Ci/m M).

MORPHOLOGICAL METHODS

The fetuses were transected at the level of forelimbs, and the heads were bisected. The specimens were fixed for 4 h in 2.5% glutaraldehyde (4°C) in 0.1 M cacodylate buffer, pH 7.4, washed in the same buffer, dehydrated in ethanol, and embeded in Epon 812. The specimens of gestational days 19-21 were dissected under a stereomicroscope in the fixative to obtain adequate fixation. These specimens were decalcified in 4.13% EDTA for 24 to 48 hours. The specimens were oriented in an anteroposterior direction in flat embedding molds to facilitate sectioning of the otocysts/inner ears in the coronal plane. Sections were cut at a thickness of 5 umusing glass knives on LKB Pyramitome and LKB Ultrotome. Sections were individually floated on small drops of water on glass slides in a serial order, and the slides were placed on a hot plate to adhere the sections. Sections were stained with 1% aqueous toluidine blue 1% borax for 5-10 seconds, examined on Zeiss photomicroscope III and photographed on Kodak Panotomic X film(ASA 32).

Biochemical Methods.

The otocyst/inner ear specimens were analysed to determine total DNA content and ^{3}H-TdR incorporation. Specimens were dissected out by removing the surrounding mesenchyme. Statoacoustic ganglionic complex was not removed during dissection, and the cartilagenous otic capsule from specimens older than day 15 of gestation was also left intact.

The specimens were homogenized in 15% cold trichloroacetic acid (TCA), washed twice in 5% TCA, extracted in ethanol and ether, and dried at room temperature. Deoxyribonucleic acid (DNA) was extracted with 5% TCA at 90°C for 20 minutes and used for the determination of DNA content and incorporation of ^{3}H-TdR[7].DNA was determined by the spectrofluorometeric method of Hinegardner[12]. Equal amounts of the sample (TCA extract) and a 40% solution of diaminobenzoic acid (Aldrich Chemical Company) were mixed and heated in a water bath at 60°C for 45 minutes, cooled to room temperature, neutralized with 1 N HCL, and read in a spectrofluorometer (Farrand Optical Company, Model 801, 420 pm excitation and 510 pm fluorescence).

To determine the ^{3}H-Tdr in corporation aliquots of the TCA extracts were added to Aquasol) New England Nuclear) and counted in Beckman liquid scintillation counter (Model LS 150). The specific activity of 3H-TdR incorporation was expressed as cpm/pg DNA.

RESULTS

At the 11th day of gestation the otocyst was an ovoid veiscle separated from the surface ectoderm and

completely surrounded by loose mesenchyme. It exhibited a pseudostratified columnar epithelium of uniform thickness. A small endolymphatic duct arose from the medial wall. The otocyst of 12th day of gestation developed a utriculosaccular dilatation that terminated in a small cochlear appendage (Figure 1).

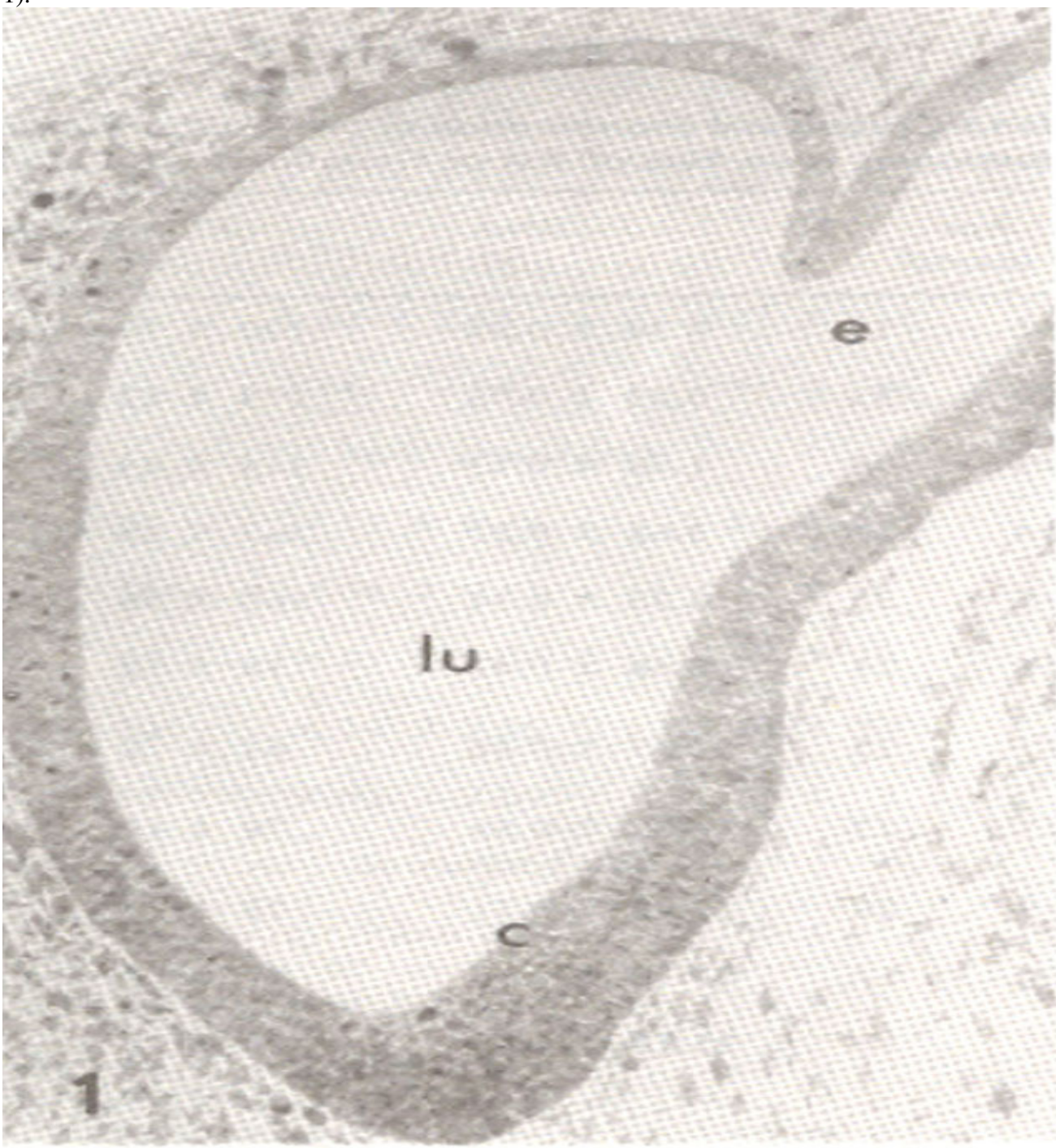

Figure1.Light micrograph of a day 12 control otocyst. The otocyst is ovoid with pseudostratified columnar epithelium. A conspicuous endolymphatic duct (e) and the developing cochlear appendage (c) are present. lu, lumen. X 220.

The endolymphatic duct was further developed and the surrounding mesenchyme become more dense. On the 13th day of gestation the otocyst assumed configuration that gave the first indication of the shape of the adult membranous labyrinth. The superior semicircular canal was fully formed and the posterior semicircular canal could also be identified. The endolymphatic duct was further elongated and developed dorsally to reach the developing brain. The cochlear appendage was pushed ventrolaterally from its junction with the saccule. The mesenchymal bed was more compact and the cytoplasm of these cells stained metachromatically with toluidine blue. The inner ear exhibited all the adult features about the 18th day of gestation. The sensory areas had morphologically identifiable hair cells. In the organ of Corti the tectorial membrane was formed and the hair cells showed typical arrangement of a single row of inner hair cells and three parallel rows of outer hair cells.

Another characteristic of the developing otic epithelium was the presence of metachromatically stained areas seen as early as the placodal stage and present at least until the 15th day of gestation. The cells had pyknotic nuclei, large granules surrounded by a clear halo, and numerous vacuoles (Figure 2).

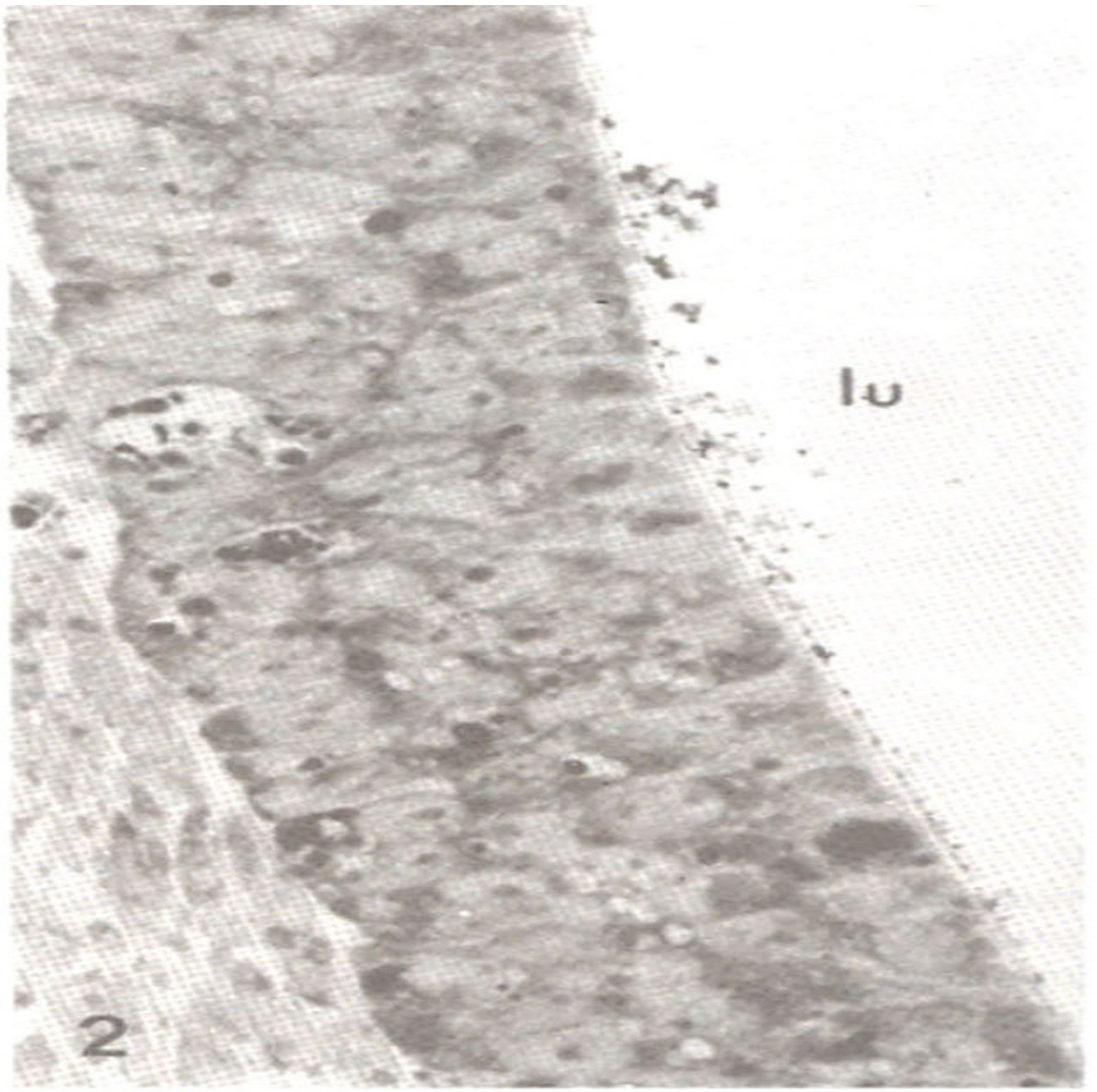

Figure 2. Higher magnification of the posteromedial wall of a day 13 otocyst showing the necrotic zone. This zone, characterized by pyknotic nuclei, metachromatic granules, and numerous vacuoles, is poresent in the plane of the endolymphatic duct at the junction of the primitive saccule and the cochlear duct. Dead cells are frequently seen inside the otocystic lumen. lu, lumen. X 560.

This circumscribed zone of necrosis was present at the junction of the saccule and the developing cochlear duct in the plane of the endolymphatic duct.

When FUdR was administered on day 12 of gestation and morphologic observatione made 4 h after the injection, antiluminal cells on the medial wall of the otocyst at the sacculo-cochlear junction were

observed to contain vacuoles, many of which had dark-staining granules within. The nuclei of these cells had irregular contours (Figure 3).

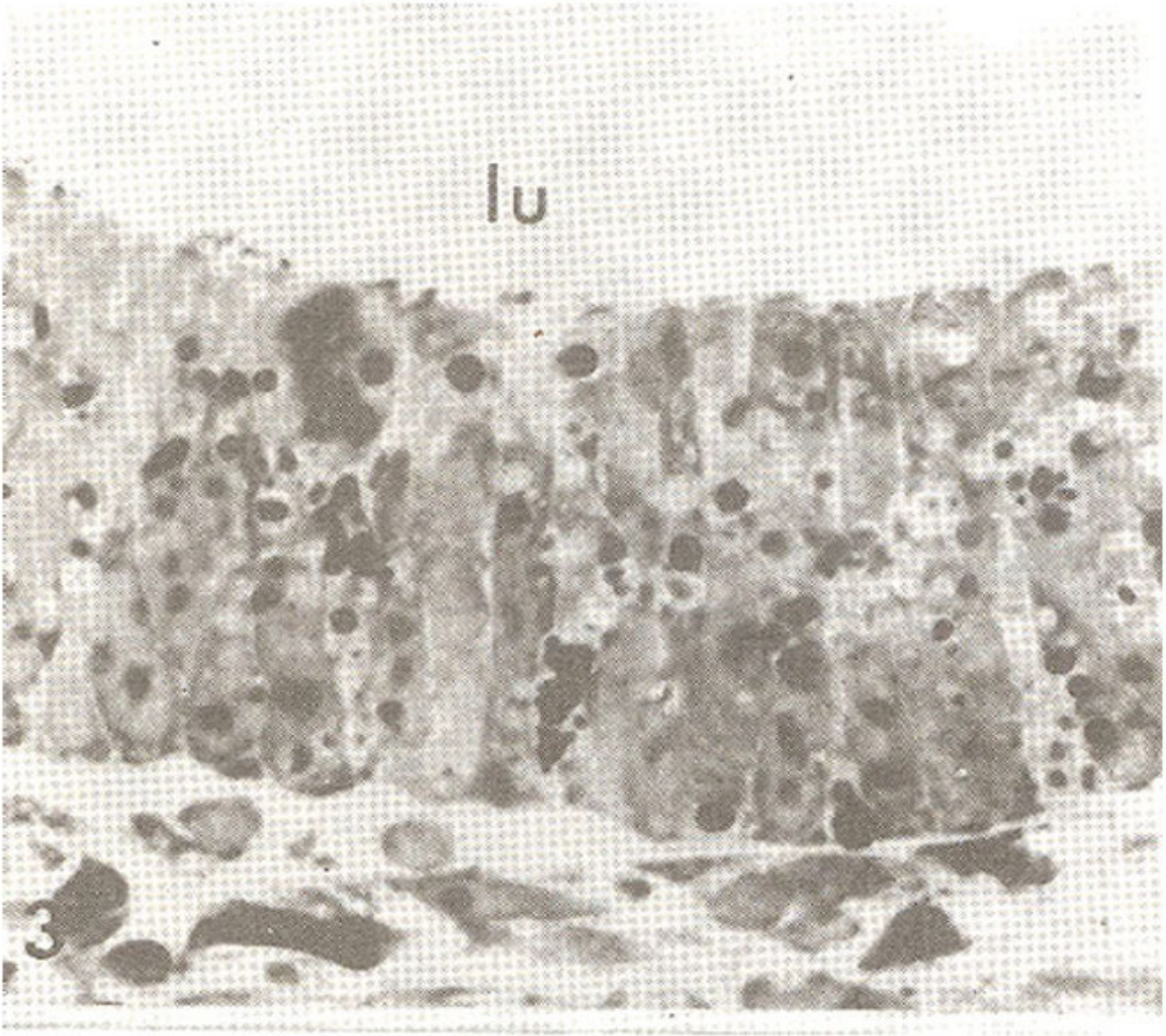

Figure 3. Light micrograph of an otocyst from a day 13 fetus showing the effect of FUdR 4 h after treatment. Most of the affected cells are present in the abluminal region. Note the difference between normally occurring necrosis seen in Figure 2 and the damage caused by FUdR. lu, lumen. X 560.

This zone of degeneration extended for a greater distance than the normally occurring zone of cellular degeneration (Figure 2). Longer exposures to FUdR resulted in more extensive cellular necrosis. The

maximum effect of FUdR was seen 8 h after the administration of the drug. The degenerated cells were present in the otic epithelium for at least 24 h after the administration of FUdR.
The effect of FUdR on the developing inner ear can be appreciated by measuring the total DNA content of the otic tissue at regular intervals after the administration of the drug.

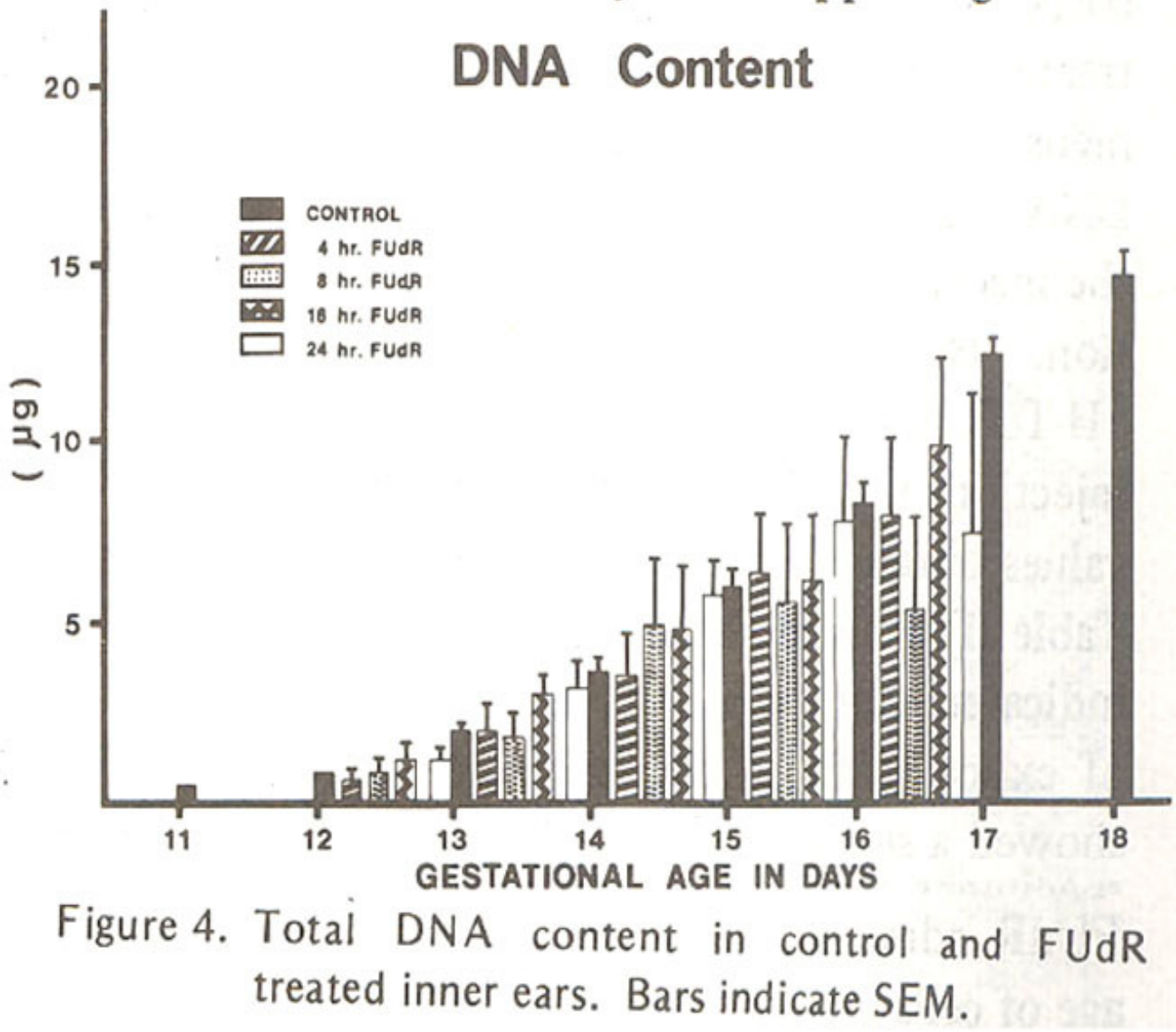

Figure 4. Total DNA content in control and FUdR treated inner ears. Bars indicate SEM.

Figure 4 shows the changes in total DNA content in control and FUdR-treated specimens (Tables I and II).

TABLE - I

DNA Content and Incorporation of ^{3}H–TdR in (Control) developing inner Ears of Rat on Gestational Days 11–21.

Age (days)	N	DNA (ug)	CPM/ug DNA
11	16	0.34 ± 0.03	120 ± 19
12	33	0.73 ± 0.05	175 ± 22
13	34	1.98 ± 0.13	315 ± 44
14	34	3.68 ± 0.27	262 ± 28
15	33	6.23 ± 0.34	260 ± 36
16	24	8.46 ± 0.47	113 ± 12
17	25	12.57 ± 0.48	64 ± 6
18	22	14.85 ± 0.59	78 ± 10
19	28	19.58 ± 0.71	29 ± 4
20	27	21.78 ± 0.89	11 ± 1
21	17	25.18 ± 1.43	21 ± 2

Means and standard error of means are shown.

TABLE - II

DNA Content and incorporation of ^{3}H–TdR in FUdR Treated Developing inner Ears of Rat on gestational Days.

12—16

Age (days)	N	DNA (ug)	CPM/ug DNA
		4 h after FUdR	
12	22	0.67 ± 0.23	186 ± 47
13	22	2.07 ± 0.82	195 ± 42
14	22	3.65 ± 1.24	378 ± 108
15	22	6.33 ± 2.23	417 ± 108
16	16	7.99 ± 2.38	16 ± 1
		8 h after FUdR	
12	22	0.74 ± 0.32	390 ± 104
13	22	1.79 ± 0.53	484 ± 126
14	22	5.04 ± 2.18	636 ± 176
15	16	5.50 ± 2.58	622 ± 18
16	14	7.34 ± 2.61	391 ± 66
		16 h after FUdR	
12	21	1.17 ± 0.43	85 ± 18
13	15	3.08 ± 0.61	214 ± 57
14	22	4.85 ± 2.00	136 ± 30
15	22	6.14 ± 2.29	140 ± 30
16	16	10.06 ± 2.69	20 ± 3
		24 h after FUdR	
12	15	1.11 ± 0.28	73 ± 5
13	16	3.26 ± 0.73	77 ± 2
14	16	5.74 ± 1.04	42 ± 1
15	16	7.83 ± 2.72	52 ± 2
16	15	9.56 ± 3.85	564 ± 50

Means and standard error of means are shown.

The amount of DNA was consistently less in FUdR-exposed inner ears than in the control specimens. However, when the total DNA content of the specimens exposed to FUdR from gestational days 13, 14 and 15 to day 21 gestation, no significant difference was noted (Figure 5).

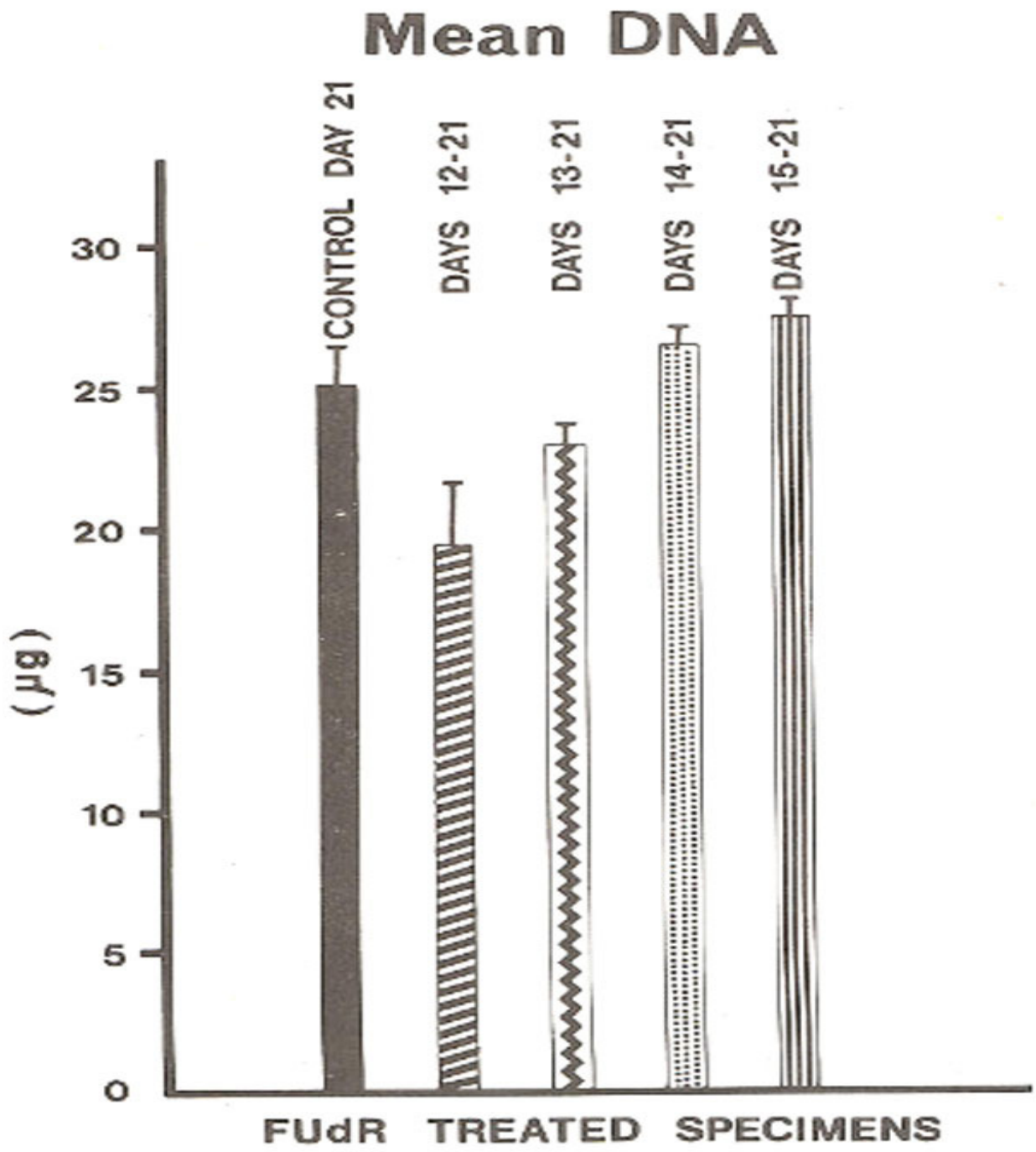

Figure 5. Total DNA content on day 21 of gestation in control specimens and in the inner ears exposed to FUdR during gestational days 12-21, 13-21, 14-21 and 15-21. Bars indicate SEM.

The DNA content of the inner ears of day 21 control specimens and the inner ears of fetuses exposed to FUdR between gestational days 12-21 showed a significant difference (Table III).

TABLE - III

DNA Content and Total Number of Cells in the Inner Ears of control Specimens on Day 21 of gestation and in the Inner ears of Fetuses treated with FUdR between gestational Days 12–15 and 21.

Day of injection	N	DNA(μG)	Total Cells	No. 1 $X\ 10^{11}$
	FUdR treated specimens			
12	10	19.38 ± 1.90[2]	29.45	± 2.89
13	10	23.06 ± 0.51	35.05	± 0.78
14	10	26.64 ± 0.44	40.49	± 0.66
15	10	27.53 ± 0.49	41.84	± 0.74
	Control Specimens			
–	17	25.18 ± 1.43	38.30	± 2.17

Morphologic observations confirmed the result of biochemical assays for DNA. On day 12 of gestation, the otic epithelium showed cellular degeneration at 8 h following FUdR injection. The base of the evaginating cochlear appendage exhibited pyknotic nuclei and the cells had many cytoplasmic vacuoles. When the inner ears of the fetuses exposed to FUdR on day 12 were examined on day 21 of gestation, the organ of Corti exhibited normal morphology, and the vestibular structures also appeared normal. A similar picture was obtained when the fetuses were exposed to FUdR on days 13, 14 and 15 of gestation. The only difference between these specunens was that with increasing age a longer segment of the cochlear was free from the effect of the drug when looked at 8 h after FUdR administration. When examined on day 21 of gestation, the organ of Corti and other areas of the inner ears exposed between days 13-21, 14-21 and 15-21 showed normal arrangement of the sensory and supporting cells.

The DNA synthetic activity, i.e. the incorporation of ^{3}H-TdR was altered in FUdR treatecT inner ears as compared to control specimens. In control specimens the increase in total DNA content (Figure 4) paralleled an increase in the incorporation of H-TdR until day 15 of gestation. However, in FUdR-exposed specimens, ^{3}H-TdR incorporation peaked at 8 h after the injection and then fell significantly below the values obtained from control specimens (Figure 6, Table II).

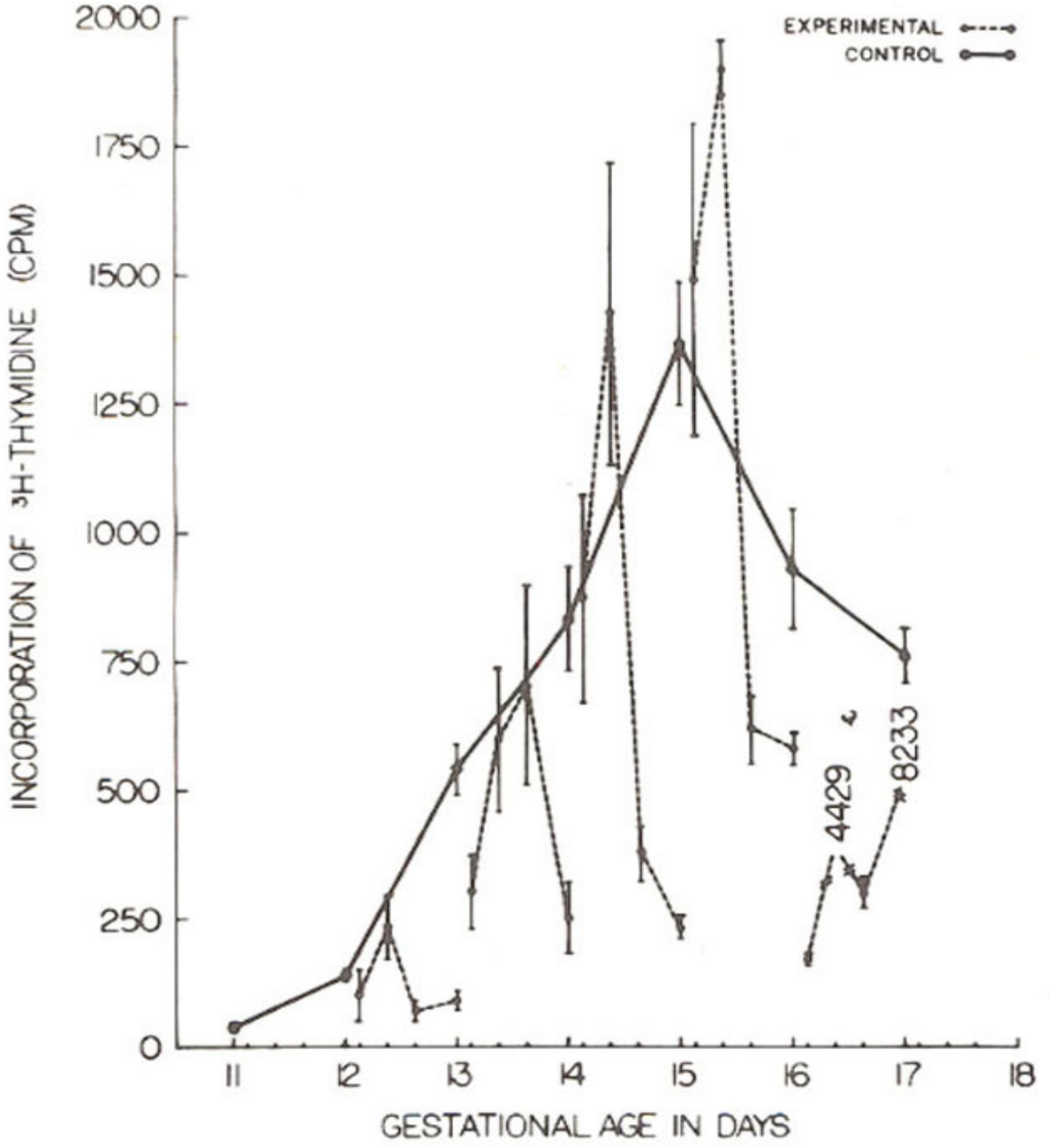

Figure 6. Medial wall of day 12 otocyst 8 h after FUdR administration. Note the dark granules and pyknotic nuclei. lu, lumen. X 560.

This increase in incorporation of ^{3}H-TdR indicated stimulation of DNA synthesis after 8 h of exposure to FUdR. Specific activity of DNA showed a significant elevation from that of control specimens (Figure 7).

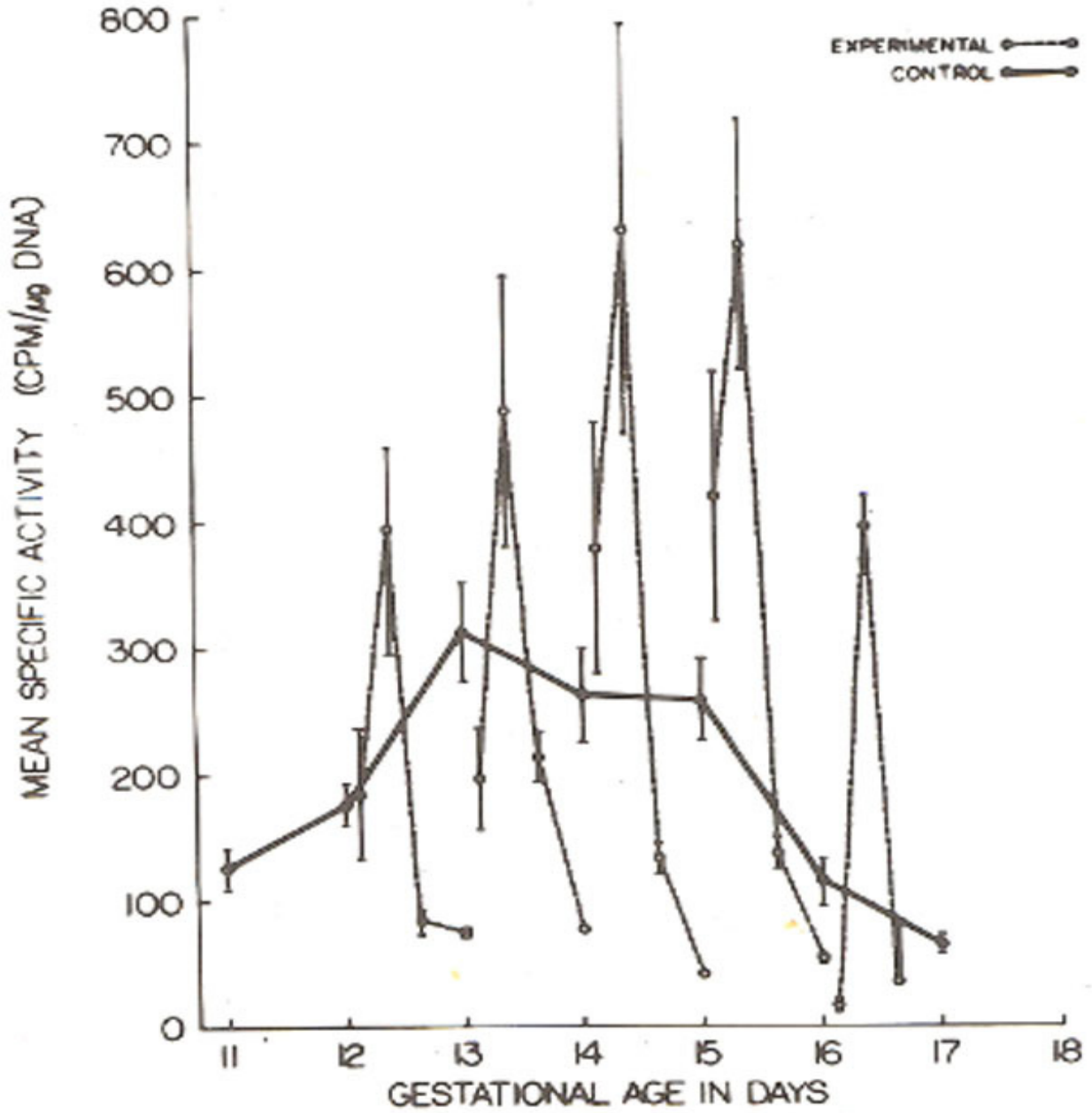

Figure 7. Cross section through Organ on Corti of a fetal inner ear on day 21 of gestation. FUdR was injected into the mother on day 21 of gestation. FUdR was injected into the mother on day 12 of gestation. Note the normal morphology of the outer hair cells (ohc) and the inner hair cells (arrow); lu, lumen of the cochlear duct; tm, tectorial membrane. X 560.

The peaks seen at 8 h after FUdR administration indicated a higher percentage of cells involved in DNA synthesis.

DISCUSSION

There are innumerable teratogenic agents and it is generally acknowledged that the effect of any one agent is dependent in part on the dose and period of gestation during which it is administered[13] In an attempt to identify a teratogen, the compound is administered to a pregnant female, followed by autopsy about 24 h before parturition. This system maximises the period during which abnormalities may develop and circumvents loss by canabalisation of dead or deformed newborn animals by their mother. This methodology is simple and efficient but provides no information about the immediate response of the embryo to the drug and any capability it may have for regulation or recovery from damage. Thus developmental disturbances could pass unnoticed. The present study demonstrates the non-uniform susceptibility of the developing inner ear in rats to FUdR and discusses the reasons about the capacity for recovery from severe damage and developmental retardation induced during early stages of development.

FUdR blocks DNA synthesis by inhibiting the enzyme thymidylate synthetase[14,15] In the experiments reported here, the first morphologic effect of the drug became apparent about 4 h after administration, when pyknotic nuclei and dark granules were noted in the DNA synthesis zone of the otocyst. Since the effect of FUdR is on cells in the S phase[11], it is expected that morphologic effects of the drug would be seen in cells in the DNA synthesis zone. The majority of the cells labelled at 4 h were antiluminal and near the basement membrane, which is the area shown to be the synthetic zone of the otocyst[16]. A zone of necrosis at the junction of the saccule and the cochlear appendage has described for the normally developing inner ear[17] However, area of necrosis in the FUdR-exposed specimens were extended for a far greater distance than the normally occurring zone of cellular degeneration.

It is not quite certain how long FUdR is effective in the fetus, but as long as it is present in certain critical concentration, it will presumably prevent or slow down the cells from entering and leaving the S phase. Since the number of affected cells 8 h after treatment was relatively small in comparison to the total number of DNA synthesizing cells, it seems likely that some of the cells arrested in the S phase remain morphologically normal and viable for a considerable period of time. By 24 h after treatment, FUdR was clearly no longer inhibiting the cells in the S phase and many mitotic figures were present at the lumen. The total DNA content of the otic tissue 24 h after treatment also indicated that FUdR was no longer effective (Figure 4).

The amount of total DNA and the morphologic observation of the inner ears on day 21 of gestation clearly indicate that the inner ear recovers from the toxic effect of an earlier exposure to FUdR. It is interesting that the pregnant rats given FUdR on day 12 of gestation carried fetuses that were grossly malformed when observed on day 21 of gestation. The malformations included cleft palate, abnormal skull, and deformed fore- and hindlimbs. in spite of these abnormalities, the inner ears of these fetuses were structurally normal. However, the total number of cells showed a significant decrease from that of controls (Table III). The fetuses exposed to FUdR between gestational days 13, 14, 15 and day 21 of gestation were free from any malformations. The inner ears of these fetuses were also structurally normal and contained close to normal number of cells. The increase in DNA content and in number of total cells observed in the inner ears of the fetuses given FUdR on gestational days 14 and 15 and examined on day 21 was within the normal range of variability present in different litters. A difference of few hours in insemination, hence fetal age, makes an appreciable difference in the size of fetuses of different litters when delivered at the same time by caesarean section. These findings suggest that the inner ear has a mechanism whereby it over comes the toxic effects of teratogenic agents during early development.

Programmed cell death, which is now an established correlate of embryogenesis and cellular differentiation, has been reported to occur in a variety of tissues[18-20] . This phenomenon was also observed to occur in the developing rat otocyst and it was suggested to be a normal process to occur in an organ system that undergoes remodelling during development[17] The occurrence of programmed cell death indicates that more cells are produced during development than are required for the definitive

adult structures. This may also represent an important protective feature. The redundant production of cells perhaps allows the developing otocyst to respond to an environmental stress by subtotal destruction from the pool of undifferentiated cells.

The significant peaks of ^{3}H-TdR incorporation at 8 h after FUdR treatment (Figures 6 and 7) show that most of the surving cells undergo DNA synthesis to compensate for the earlier loss of cells due to FUdR. This phenomenon of compensatory growth has also been observed in murine fetuses after a single injection of a known teratogen mitomycin C during early stages of organogenesis[21] It may also be possible that this accelerated development is one of many genetically determined control mechanisms to overcome the adverse conditions during early development. This protective feature perhaps is another reason because of which we see relatively few congenital anomalies of the inner ear in mammals.

ACKNOWLEDGEMENTS

Thanks are due to Ms. Shahinda Jaffarali for typing this manuscript. This work was supported by grant from Deafness Research Foundation, U.S.A. and by funds from the Department of Otolaryngology, The Mount Sinai Medical Center, New York, U.S.A.

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