A Histopathological Study of the Effect of Ethylnitrosourea on HA(ICR) Mice Testes

Rodriguez

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A HISTOPATHOLOGICAL STUDY OF THE EFFECT OF ETHYLNITROSOUREA ON HA(ICR) MICE TESTES

Mildred Rodriguez, M.S.
Western Michigan University, 1982

Ethynitrosourea (ENU) is very strongly mutagenic, carcinogenic and teratogenic in mice. In the present study ENU was used to induce germ cell damage in the mouse testis. Mice were given single injections of vehicle, 50, 100, or 200 milligrams of ENU per kilogram of body weight and killed at various times over a fifteen week period. Microscopic examination of the testes showed that extensive killing of spermatogonial cells and therefore maturation depletion had occurred in the seminiferous tubules. The histopathology was dose dependent and the testicular weight decreased with an increasing ENU concentration.
ACKNOWLEDGEMENTS

I would like to thank everyone who contributed to making my research successful. I would like to thank, Dr. Gyula Ficsor, for all of his help and advice. I would also like to thank the members of my graduate committee; Dr. Cecil L. McIntire, Dr. Leonard Beuving, and Dr. Leonard C. Ginsberg. I thank Dr. Brahma B. Panda, Greg Oldford and Tony Torres for their technical assistance. I give a warm thanks to my parents and Tim for their confidence and constant support.

Mildred Rodriguez
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WESTERN MICHIGAN UNIVERSITY, M.S., 1982

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................ ii
LIST OF TABLES ........................................ iv
LIST OF FIGURES ......................................... v
INTRODUCTION ........................................... 1
MATERIALS AND METHODS ..................................... 7
   Animals .............................................. 7
   Chemical .......................................... 7
   Injection and Killing .................................. 7
   Histological Evaluation ................................ 10
   Statistical Analysis .................................. 11
RESULTS .................................................. 12
   Histopathology ...................................... 12
   Testicular Weight .................................... 28
   Non-Motility ........................................ 29
DISCUSSION ............................................... 32
CONCLUSIONS ............................................ 40
APPENDIX A ............................................. 41
APPENDIX B ............................................. 42
APPENDIX C ............................................. 43
BIBLIOGRAPHY ........................................... 44
LIST OF TABLES

1. Correlation of Dose of ENU and the Induced Histopathology of the Testes..................13
2. Correlation of the Effect of ENU on Testicular Weight...........................................30

iv
LIST OF FIGURES

1. The 200 mg/kg dosage two weeks after treatment.................................15
2. The 200 mg/kg dose four weeks after treatment.................................16
3. Sterile seminiferous tubules in the 200 mg/kg dose four weeks after treatment.................................18
4. Control seminiferous tubules four weeks after treatment.................................19
5. Pre-spermatozoa cells in the 200 mg/kg animals two weeks after treatment.................................20
6. The 200 mg/kg dose after two weeks.................................21
7. Mitotic figures in the 50 mg/kg animals four weeks after treatment.................................22
8. Mitotic figures in the 100 mg/kg animals four weeks after treatment.................................23
9. Control animals showing vacuolation one week after treatment.................................24
10. The 50 mg/kg animals showing vacuolation four weeks after treatment.................................25
11. Control animal with intertubular spaces.................................26
12. Control animals with intertubular spaces.................................27

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INTRODUCTION

All living cells have DNA and/or RNA, the material responsible for transmitting hereditary traits. Mutagenesis is the result of a change in transcription of DNA. This can be a naturally occurring event or as a result of a chemical modification of this polymer.

In the present study ethylnitrosourea (ENU) was used to induce germ cell damage in the testis. ENU was first prepared in 1919 by Warner reacting N-ethylurea and nitrous acid (IARC, 1978). This agent does not require metabolic activation. The ethyl-carbonium ions (the alkylating group) which are formed from the decomposition of ENU are considered the ultimate carcinogen which react with nucleophilic sites of cellular macromolecules. The alkylation of DNA yields $O^6$-ethylguanine, $O^2$-ethylcytosine, $O^2$- and $O^4$-ethylthymidine, and ethylphosphotriesters. These adducts result in altered secondary structure and perturbation of base-pairing interactions that may affect serious aspects of DNA function including replication and transcription. Wei et al. (1980) proved this using template DNA from transducing, temperate bacteriophage, $\lambda$h80dlacp, which contains the entire lac operon from E. coli. The DNA was transcribed to messenger RNA which in turn was translated to functional proteins, including beta-galactosidase. DNA pretreated with ENU resulted in
a proportionally decreased beta-galactosidase activity with an increased ENU concentration.

ENU was found to be a strong mutagen and carcinogen. One day old strain A, C57BL, DBAf, and IF mice given single subcutaneous (s.c.) injections of ENU at dose levels from 10 to 160 milligrams per kilogram body weight (mg/kg bw) developed a high incidence of tumors. Especially common were hepatomatas and hepatocellular carcinomas in the C57BL and DBAf mice, lung adenomas and adenocarcinomas in A mice, and tumors of the nervous system in C57BL, DBAf, and IF mice (Searle & Jones, 1976). Vesselinovitch et al. in 1974 (IARC, 1978) found that mice given single intraperitoneal (i.p.) injections of 60 or 120 mg/kg bw ENU at 1, 15, or 42 days of age died by the 90th week from benign and malignant tumors at multiple sites. Rice in 1969 (IARC, 1978) found that the offspring of female strain A/J, A/He, C3Hf/He, C57Bl/6, and GP mice given single injections of ENU at doses ranging between 29 and 117 mg/kg bw between the 12th and 19th day of gestation were found to have pulmonary adenomas and occasionally lymphocytic leukemias at twelve weeks of age. Hepatomas were seen in strain A and C3Hf mice after forty weeks. It is important to mention at this point that Grasso and Crampton (1972) comment that it is doubtful that the use of the mouse, without data from a second species, is a reliable model for detecting carcinogenic activity because mice
have a natural incidence for some of the tumors mentioned above.

There have been various studies done to evaluate the effect of ENU on germ cells in mice. Johnson and Lewis (1981) state that the detection of mutations in the offspring of mutagen exposed animals is the only way to determine whether an agent causes germinal mutations in mammals. Russell et al. (1979) using a morphological specific-locus test demonstrated an exceedingly high frequency of morphologically-expressed germinal mutations induced by ENU in mouse spermatogonia. A single i.p. injection of 250 mg/kg of ENU induced mutations at a rate five times greater than the highest rate induced by acute exposure to 600R of irradiation. Johnson and Lewis (1981) tested for electrophoretic variants, produced by mutations at specific loci. The offspring of male mice treated with a single i.p. injection of 250 mg/kg ENU were killed and erythrocyte lysates and kidney homogenates were prepared and analyzed for electrophoretic variants of several enzymes. The parent males and the $F_1$ progeny were examined using the same set of techniques. They found ENU to be a potent inducer of electrophoretically expressed mutations. There were tenfold more mutations induced among the ENU treated mice as compared with the controls.

The dominant lethal test is another method of assessing mutations. In this test males are injected and then
mated to females. The loss of offspring is attributed to dominant mutations resulting in lethality. Ramaiya (1968) treated male mice with a single i.p. injection of 100 mg/kg ENU and used these to inseminate untreated females. The females were changed every 7-10 days. The sequence of matings took place in such a way that the sperm that eventually took part in fertilization had been in different stages of spermatogenesis at the time of ENU treatment. Matings with spermatozoa derived from spermatids and early spermatocytes exposed to ENU showed a significant increase in total death percent in embryos as compared with controls. While there is a small percent of undeveloped embryos in the controls the decreased fertility of the males treated with ENU could be partially attributed to the dominant lethal effect of this compound.

In the present study ENU was used to induce germ cell damage in the testis. Ever since the work of von Ebner in 1888 it has been known that germ cell development in rodents proceeds in regular cell associations. These cell associations move in wave form along the seminiferous tubules. The length of a tubule occupied by a whole sequence of characteristic cell associations is described as a wave length (Schleiermacher, 1972). There are sixteen stages of spermiogenesis in the mouse testis. The first twelve of these stages correspond to one cycle of the seminiferous epithelium, which is the series of changes occur-
ring between two successive appearances of the same cell association in one area (Leblond & Clermont, 1952). Four cycles of the seminiferous epithelium were shown to occur during the interval required for a type A₁ spermatogonia to become a mature spermatozoon, which is approximately 34.5 days (Oakberg, 1956). The type A spermatogonia are subdivided into A₁ to A₄ spermatogonia. These cell types are not easily separated by morphological differences but can be distinguished by the stage of the cycle of the seminiferous tubule in which they are found. Spermatogonia have an ovoid, pale nucleus with a thin nuclear membrane and are found in linear groups of four or eight cells along the basement membrane of the tubule. The type A₀ spermatogonia occur singly or paired and exhibit very low mitotic activity. These cells do not participate in the production of spermatocytes but may serve to repopulate the seminiferous tubules that have been depleted of all other types of spermatogonia (Dym & Clermont, 1970). The A₄ spermatogonia divide mitotically and give rise to intermediate spermatogonia which are transitional between type A spermatogonia and type B spermatogonia. Type B spermatogonia may be identified by thickening of the nuclear membrane, a result from "attaching" chromatin. Type B cells are known as "crust-like" spermatogonia because of the presence of coarse chromatin masses in the small spherical nucleus. These cells divide mitotically and be-
come resting primary spermatocytes. Resting spermatocytes are round and much smaller than type B spermatogonia, non-dividing, and also possess "crust-like" chromatin in the nucleus. These cells terminate their dormancy and become spermatocytes, which are found in the seminiferous tubules two cell layers inward from the basement membrane. Spermatocytes undergo mitosis and meiosis to become spermatids. Spermatids are the germ cells nearest the lumen and are found interspersed between the spermatozoa. A spermatozoa has a large elongated nucleus with very little cytoplasm which is mostly located near the tail insertion. These are found in the lumina of the seminiferous tubules, alongside the spermatids or in bundles associated with the Sertoli cells. The nuclei of the Sertoli cells are found near the basement membrane and are not active mitotically, these cells make up the blood testis barrier.

Because spermatogonia undergo seven mitotic divisions before becoming spermatocytes these cells are a good target for ENU. HA(ICR) male mice treated with a single i.p. injection of 100 mg/kg ENU showed a decrease in testicular weight (Ficsor, 1982). The present study was done to determine the extent of histopathology in the testis related to this decrease in testicular weight.
MATERIALS AND METHODS

Animals
The animals used were 25 to 30 gram male HA(ICR) mice from Harlan Industries Inc. They were kept in 28x13 cm polypropylene cages, three animals in each cage, bedded with wooden shavings. Water and Purine Mouse Chow were supplied ad libitum. The animals were allowed one week to adjust to their new environment before treatment. The animal room was kept at a twelve hour light-twelve hour dark cycle, the light period going from 0700hr to 1900hr. The temperature of the room was 25.5 °C and the relative humidity was 41 percent. The mice were weighed as soon as they arrived and then again one half hour before they were killed.

Chemical
The ENU was donated by the Upjohn Co., Kalamazoo, Michigan; through Dr. David Swanson.

Injection and Killing
One hundred and eight mice were injected with either 0 (vehicle), 50, 100, or 200 mg/kg ENU. There were twenty-seven mice in each dosage group. The total ENU for each dosage level was dissolved in 3 mls of saline plus 0.3 ml of dimethyl sulfoxide (vehicle). The ENU solutions were prepared on the day of injection and all of the animals were treated within one hour of preparation,
within 7 days after their arrival. ENU in saline has a half life of approximately 1.5 hrs. (IARC, 1978) while in vivo it is five to eight minutes. The animals were injected i.p. with a 1 ml tuberculin syringe using a 26G, ⅛ inch needle. Injection volume was adjusted to the weight of the individual animals in order to achieve the desired ENU dosage. The animals remained under a ventilated hood for 48 hrs. after which they were transferred to the animal room. The contaminated bedding was collected and properly disposed.

Three mice from each dosage group were killed by cervical dislocation 1, 2, 4, 5, 7, 9, 11, 13, or 15 weeks after treatment. The vas deferens were removed with forceps and placed in a petri dish containing 1 ml of sperm Ringer's solution (Appendix A). The sperm were freed from the vas by holding one end of the vas with one forceps and squeezing the duct longitudinally with another forceps while keeping the vas submerged in the Ringer's solution. The clumps of sperm were mixed by aspiration using a Pasteur pipette. From this mixture 0.1 ml was transferred to a 1.5 ml microsample centrifuge tube and centrifuged at 1500 g for three minutes. The pellet was reconstituted with one drop of skim milk (Appendix A). A drop of the sperm suspension was added to a Makler counting chamber (Israel Electrophoretic Industry Ltd.) and the motile and non-motile sperm counted (Makler, 1978).
For the histological processing, both testes were removed and freed of adipose tissue, leaving the tunica albuginea intact. Once removed they were weighed on a petri dish. The testes were placed in metal cassettes and fixed in 10% neutral buffered formalin for 24 hrs. The tissues were then processed in an automatic tissue processing machine (Autotechnicon: Model 2A, The Technicon Co). During this time the tissues were dehydrated and infiltrated with TissuePrep (a mixture of purified paraffin and synthetic polymers) at 56 °C (Humason, 1979). The tissues were embedded longitudinally in TissuePrep using tissue Tek embedding rings. The TissuePrep was allowed to solidify and then the blocks were placed in a -1 °C freezer. One day later the blocks were cross-sectioned at five microns using an American Optical Co. "820" rotary microtome. Three slides with four cross-sections each for each pair of testes were prepared. Each slide was separated from the next by approximately thirty microns. The slides were stood on edge in a slide rack and placed in an oven at approximately 57 to 60 °C. There they remained for a minimum of two hours until the sections became properly affixed. Afterwards the remaining TissuePrep was removed with xylene and the slides were stained with PAS-Hematoxylin (Appendices B & C). One or two drops of Permount mounting medium were applied to each slide and then the sections were coverslipped.
Histological Evaluation

A light microscopic examination of the testes was done to determine the extent of any histopathology that was present. The cross-sections were examined at random using blind scoring.

The assessment was done in levels. The first level consisted of examining the gross appearance of the tissue. At this level various observations were made as follows: (1) Were there spaces devoid of any substance between the seminiferous tubules? (2) Was there interstitial tissue present? (3) Were there any sterile seminiferous tubules (tubules void of all germ cells excluding reserve stem cells)?

The next assessment level was used to determine the percentage of seminiferous tubules containing mature spermatozoa. This was done by dividing the number of seminiferous tubules containing spermatozoa by the total number of seminiferous tubules counted in one cross-section. The tubules considered were those that approximated a circular or slightly ovoid shape; the tangential sections were not used.

The third level of assessment determined the following: (1) Which germ cell types were absent? (2) Whether there was a breakdown of the continuity of the seminiferous epithelium present? (3) Whether pre-spermatozoa cells were present in the lumina? (4) Whether mitotic
Statistical Analysis

The data from the mean percent of seminiferous tubules with sperm present, the mean for the testicular weights, and the mean for the body weights were analyzed with the student’s t-test, comparing each treated animal with the controls of the same time interval. Statistical significance was assumed at $P$ equal to or less than 0.05.
RESULTS

Animals treated with vehicle, 50, 100, or 200 mg ENU per kg bw were killed over a fifteen week period. Determination of damaged germ cell types after treatment was made.

Histopathology

Spermatogonial cells were absent one week after treatment in all of the groups which received ENU (Table 1). The duration of the effect was dose dependent beyond one week. Two, four, and five weeks after treatment the spermatogonial cells were absent in the 200 mg/kg animals. The number of animals which exhibited this condition decreased from two animals two weeks after treatment to one animal four and five weeks after treatment (Table 1).

Spermatocytes were absent in one and two of the 100 and 200 mg/kg animals, respectively, two weeks after treatment (Figure 1). Four weeks after treatment spermatocytes were absent in only one of the 200 mg/kg animals.

Spermatids were absent four weeks after treatment in all of the 200 mg/kg animals (Figure 2). This cell type was present throughout the other time intervals and throughout all other dosage groups.

The mean percent of seminiferous tubules containing mature spermatozoa decreased with an increased dose of ENU. At four weeks, but not at five weeks, the animals
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<th>Dose of ENU (mg/kg)</th>
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<th>Mean % of ST with Sperm</th>
<th>Sterile ST</th>
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<th>MF</th>
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a-Three animals sacrificed for each dose weekly. Sa-Spermatogonia. Sc-Spermatocytes. St-Spermatids. ST-Seminiferous tubules. PS-Pre-spermatozoa cells. MF-Mitotic figures in spermatocytes. * P≤ 0.05
Continuation of TABLE 1

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<th>Dose of ENU (mg/kg)</th>
<th>Time Interval (wks)</th>
<th>No. of animals with absent:</th>
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</table>

b-One of the animals in this group died. Sa-Spermatogonia. Sc-Spermatocytes. St-Spermatids. ST-Seminiferous tubules. PS-Pre-spermatozooa cells. MF-Mitotic figures in spermatocytes. * P ≤ 0.05
Figure 1. The 200 mg/kg dosage two weeks after treatment. Spermatogonia and spermatocytes are absent while spermatids (st) and spermatozoa (sz) are present.
Figure 2. The 200 mg/kg dose four weeks after treatment. Spermatids were absent.
treated with 50 mg/kg ENU had a significant reduction of mature spermatozoa. The 100 and 200 mg/kg animals also had a significant reduction of mature spermatozoa in the fourth and fifth weeks post-treatment. Seven weeks after treatment the 50 and 100 mg/kg animals showed a non-significant reduction of mature spermatozoa in the tubules while the 200 mg/kg animals showed significant reduction.

Some sterile seminiferous tubules were still present in the 100 and 200 mg/kg animals four to seven weeks after treatment (Figures 3 & 4).

An increase in the number of pre-spermatozoa cells in the seminiferous tubules' lumina was observed one to five weeks after treatment in all of the ENU animals (Figures 5 & 6).

Mitotic figures were absent in some of the treated animals. Specifically, there was a total absence in the 50 mg/kg animals one week after treatment, whereas, the 100 and 200 mg/kg animals suffered a complete loss of mitotic figures in the second and fourth weeks after treatment, respectively. Seven weeks after treatment mitotic figures were absent in the control animals. In all of the other time intervals mitotic figures were present, irrespective of treatment (Figures 7 & 8).

Vacuolation of the germinal epithelium was observed but there was no correlation between dosage and the appearance of this condition (Figures 9 & 10). Intertubular
Figure 3. Sterile seminiferous tubules of 200 mg/kg four weeks after treatment. Sertoli cells (sc) present.
Figure 4. Control seminiferous tubules four weeks after treatment.
Figure 5. The 200 mg/kg dose exhibiting pre-spermatozoa cells (ps) in a seminiferous tubule two weeks after treatment.
Figure 7. Mitotic figures (MF) present in the 50 mg/kg animals four weeks after treatment.
Figure 8. Mitotic figures present in the 100 mg/kg animals four weeks after treatment.
Figure 9. Control animals show vacuolation (v) in the ground substance of the seminiferous epithelium one week after treatment.
Figure 10. The 50 mg/kg animals show vasculature four weeks after treatment.
Figure 11. Spaces (g) between the seminiferous tubules of control animals one week after treatment.
Figure 12. Spaces between the seminiferous tubules of control animals two weeks after treatment.
spaces separating the seminiferous tubules were also noted in all animals including controls, and the condition was designated a histological artifact (Figures 11 & 12). Interstitial tissue and Sertoli cells were present in all of the cross-sections.

Testicular Weight

With ENU a direct correlation with the total dose of the drug and a decrease in testicular weight with respect to time intervals was determined (Table 2). One week after treatment the testicular weights did not vary significantly. The testicular weight/body weight (TW/BW) ratio did not vary significantly either. Two weeks after treatment the mean testicular weight of the 200 mg/kg animals was significantly reduced as compared with the controls but the TW/BW ratio did not vary significantly. Four and five weeks after treatment the mean testicular weights of the ENU treated animals continued to be reduced as compared to the controls and this was supported by the TW/BW ratios which decreased considerably as compared to the controls. At five weeks after treatment the mean body weight value for the 200 mg/kg animals varied significantly from the controls thus invalidating the TW/BW ratio because the animal suffered a decrease in body weight. The decrease in testicular weight could be attributed to this decrease in body weight. Seven weeks after treatment the mean testicular weights of the 100 and 200 mg/kg
animals varied significantly from the controls but only the TW/BW ratio of the 200 mg/kg animals showed a noticeable decrease as compared with the controls. Nine and eleven weeks after treatment the mean testicular weights and the TW/BW ratio of the 200 mg/kg animals varied significantly from the controls.

Non-Motility

The percentage of non-motility for each animal was determined by dividing the number of non-motile sperm by the total number of sperm (motile and non-motile). Then the percent value for each of the three animals was used to calculate a mean percentage for a dosage group within one time interval. Ficsor et al. (1981b) found that ENU decreased sperm motility, sperm number, and the frequency of sperm with proteolytic activity when pre-leptotene spermatocytes or spermatogonial cells were treated. In the present study the percent of non-motility in the control animals was just as high as in the ENU treated animals, irrespective of the germ cell type present at the time of treatment. It is likely that a procedural error took place. Consequently, non-motility is considered to be a non-reliable assay in the present study.
TABLE 2

Correlation of the Effect of ENU on Testicular Weight

<table>
<thead>
<tr>
<th>Dose of ENU (mg/kg)</th>
<th>Time Interval (wks)</th>
<th>Mean Body Weight (BW) (g)</th>
<th>Mean Testicular Weight (TW) (mgs)</th>
<th>TW/BW Ratio ($10^{-3}$)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>29.3 ± 2.6</td>
<td>250 ± 40</td>
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<tr>
<td>50</td>
<td>1</td>
<td>27.5 ± 1.8</td>
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<tr>
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<td>210 ± 30</td>
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</tr>
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<td>2</td>
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<td>240 ± 46</td>
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</tr>
<tr>
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<td>2</td>
<td>27.0 ± 2.8</td>
<td>200 ± 5.8</td>
<td>6</td>
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<tr>
<td>100</td>
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<td>28.7 ± 0.6</td>
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<tr>
<td>200</td>
<td>2</td>
<td>29.3 ± 1.5</td>
<td>150 ± 20.8*</td>
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</tr>
<tr>
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<td>270 ± 26.5</td>
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<tr>
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<td>29.3 ± 9.4</td>
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<tr>
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<tr>
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<td>33.7 ± 1.2</td>
<td>180 ± 32*</td>
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<tr>
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<td>34.5 ± 1.2</td>
<td>210 ± 17</td>
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</table>

a-Three animals killed for each dosage weekly. * P $\leq$ 0.05
Continuation of TABLE 2

<table>
<thead>
<tr>
<th>Dose of ENU (mg/kg)</th>
<th>Time Interval (wks)</th>
<th>Mean Body Weight (BW) (g)</th>
<th>Mean Testicular Weight (TW) (mgs)</th>
<th>TW/BW Ratio (10^-3)</th>
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</tr>
<tr>
<td>200</td>
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<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15</td>
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<td>210 ± 35</td>
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<td>200</td>
<td>15</td>
<td>28.3 ± 2.6</td>
<td>260 ± 35</td>
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</tr>
</tbody>
</table>

<sup>b</sup>-One animal died in this group.  *P < 0.05
DISCUSSION

In this study sterile seminiferous tubules were encountered two through seven weeks after treatment in the 200 mg/kg ENU animals. Shaver (1953) demonstrated that the mitotic divisions of type A spermatogonia were temporarily suppressed in adult rats for approximately 30 days following 500R of total body irradiation. The more mature type B spermatogonia continued to differentiate leading to a maturation depletion of the germinal epithelium at four weeks after irradiation. Oakberg in a personal communication (Russell et al., 1979) stated that a 250 mg/kg i.p. injection of ENU induced a long period of sterility. The mice were fertile for approximately the first two weeks after treatment and then suffered a temporary sterile period which was shown to be a result of extensive spermatogonial killing. The first fertile matings did not occur until after a thirteen week period.

Similarly, the animals in this experiment, had no sperm in the seminiferous tubules. Type A spermatogonia present at time of ENU treatment were damaged. These cells would have normally become spermatozoa within five weeks.

One week after treatment, the 50, 100, and 200 mg/kg dosage groups had at least one animal which exhibited an absence of $A_1$ to $A_4$ spermatogonia. Dym and Clermont
(1970) found a type A spermatogonia that they called reserve stem cells \( (A_0) \). These cells were radioresistant and inactive mitotically. After the seminiferous epithelium lost all of its spermatogonia and other germ cells, the \( A_0 \) spermatogonia which rarely divide in normal adult rats were capable of an increased mitotic activity and of repopulating the type A spermatogonia. In the present study the 200 mg/kg group continued to exhibit an absence of spermatogonia in some of its animals two through five weeks after treatment. There were, however, cells which were assumed to be reserve spermatogonia because they occurred singly in seminiferous tubules which only contained Sertoli cells.

On the average, 8.8 days are required for spermatogonia to become primary spermatocytes (Oakberg, 1956). In the present experiment two weeks after treatment one and two animals of the 100 and 200 mg/kg dosage group, respectively, suffered a loss of spermatocytes. Four weeks after treatment only one of the 200 mg/kg animals had an absence of spermatocytes. Two of the 50 mg/kg animals suffered a loss of spermatogonia one week after treatment, therefore, these animals were expected to suffer a loss of spermatocytes. It was postulated that the 50 mg/kg dose may have allowed an immediate repopulation of the spermatogonia and thus the presence of spermatocytes. Spermatids were absent in the 200 mg/kg animals be-
gaining four weeks after treatment. One of the 100 mg/kg animals was expected to suffer a spermatid depletion because there had been an absence of spermatocytes. Apparently, the 100 mg/kg dose allowed a repopulation of the germinal epithelium by the fourth week after treatment. The 200 mg/kg dose of ENU proved very effective in inducing prolonged histopathology in the testes of mice.

The decreased testicular weight was positively correlated with the increased concentration of ENU and the consequent presence of histopathology. Comparing the control animals and the 200 mg/kg animals, during the time interval of three to nine weeks after treatment, there was a significant difference between the testicular weights and the testicular weight/body weight ratio of these two groups. Five weeks after treatment there was, however, a significant difference between the body weight average of the controls and the 200 mg/kg animals. Consequently, the significant difference between the testicular weights of these two groups may have been due to loss of weight caused by other factors (Table 2). During these time periods there was also a difference in the appearance of the germinal epithelium, in the controls the cell associations were intact while the ENU treated animals had interrupted cell associations with one, two, or all cell types absent. In rodents ninety percent of the volume of the testes are seminiferous tubules (Shire & Bartke, 1972), consequently,
a reduction of germ cells would reduce the testicular weights as seen in the present study. Decreased testicular weight was also seen with a histologic analysis of the testes and epididymides of mice five weeks after treatment with mitomycin C. This drug caused an extensive emptying of the tubular germinal elements into the epididymides in comparison with control animals. This accounted for a fifty percent decrease in testicular weights (Ficsor et al., 1982). Two and four weeks after treatment the control animals of the present study exhibited a healthy epithelium, none of the cell types were absent and there was an average of approximately seventy-two percent of the seminiferous tubules containing sperm. There is a possibility that a small percent of the seminiferous tubules are emptied of their sperm before a completed fixation.

The animals receiving 200 mg/kg of ENU showed an absence of spermatogonia and spermatocytes during the same time period mentioned above. During the second week there were pre-spermatozoa cells in the lumina of the tubules and during the fourth week two of the animals had sterile seminiferous tubules. There was an average of forty-seven percent of the seminiferous tubules containing sperm in the 200 mg/kg animals two and four weeks after treatment. Four and five weeks after treatment the 50 mg/kg animals suffered a decreased percent of seminiferous tubules with sperm present; twelve and fifty-two percent, respectively.
This was an unexpected result because there was not a loss of spermatocytes nor spermatids. There is a possibility that some of the spermatogonia present at the time of treatment were not lethally damaged. These spermatogonia were then repaired and remained in the cell associations but may have been eliminated at a later time or they may have become faulty spermatozoa. These spermatogonia possibly occurred as pairs and were assumed to be type A₀ cells. Five weeks after treatment there was no histopathology in the controls whereas one of the 200 mg ENU per kg treated animals had an absence of spermatogonia and all three animals had cells in the lumina of the tubules and sterile seminiferous tubules. There were no spermatozoa present in the tubules of these 200 mg/kg animals. During the seventh through ninth week periods there was a partial absence of some of the germ cells in the ENU treated animals. Both time periods showed at least one 200 mg/kg animal with pre-spermatozoa cells present in the lumina of the tubules and in the seventh week one animal had sterile seminiferous tubules. Twenty-four percent of the seminiferous tubules contained sperm in the seventh week and this increased to eighty percent in the ninth week. This indicated that seven and nine weeks after treatment the 200 mg/kg animals had started recovering from the ENU treatment.

During the eleventh through fifteenth week periods
the histopathology in all of the ENU treated animals, irrespective of dose, was comparable to the controls. The average percent of seminiferous tubules containing sperm throughout this time period in the controls was eighty-seven percent. The average percent of tubules containing sperm in the 200 mg/kg animals was eighty-six percent.

Interstitial cells and Sertoli cells were present in all of the cross-sections examined regardless of treatment. The pathology observed in the testes was probably not due to decreased anterior pituitary gland function. Interstitial cells produce testosterone when stimulated by luteinizing hormone (LH) which is secreted by the anterior pituitary gland. Testosterone supports the maturation and function of the accessory reproductive structures. The more easily observed accessory structures such as the epididymis, vas deferens, seminal vesicles, scrotum and penis were apparently normal in the ENU treated animals. Consequently, endocrine disturbances are not a likely explanation for the testicular histopathology seen here.

Soukup and Au (1975) did a study to determine the effect of ENU in vivo on rat and mouse chromosomes in bone marrow. The in vivo study was done by injecting animals i.p. with 100 or 200 mg of ENU per kg. The bone marrow from the femurs was used to examine the chromosomes. A high number of chromosome aberrations was detectable in
bone marrow preparations six hours after injection. In one rat and one mouse six hours after treatment with 200 mg/kg no metaphases were seen. Since none of the other 34 treated or untreated animals failed to show metaphases, it was concluded that ENU could have produced mitotic inhibition. Goth-Goldstein and Painter (1981) using a clonal derivative of M3-1 male Chinese hamster bone marrow-derived cells found that ENU inhibited DNA synthesis at a concentration of 1, 2.5, or 5 mM. At the highest concentration of ENU the DNA synthesis of the treated cells dropped as low as twenty-seven percent of the controls. In the present study when examining the cross-sections, the mitotic figures were attributed to dividing spermatocytes. Absence of mitotic figures was correlated with the loss of spermatocytes in the 100 and 200 mg/kg animals, two and four weeks after treatment respectively. Mitotic figures were absent in the 50 mg/kg animals one week after treatment and in the controls seven weeks after treatment. Consequently, the mitotic figures assessment is non-significant.

Vacuolated seminiferous tubules were seen at all dosages. This was classified as a degenerative characteristic due to chemical treatments by Block (1981). In the present study vacuoles in the ground substance of the seminiferous epithelium were seen throughout all of the cross-sections including the controls. Much of the frag-
mentation in the tissue was due to the histologic preparation and this contributed to the apparent vacuolation in the controls so as to resemble the ENU treated animals. This histologic artifact may have been caused by incomplete fixation due to the intactness of the tunica albuginea. The intertubular spaces seen in the cross-sections could have been caused by the water bath temperature, which may have approached the melting point of the Tissue-Prep and thus the cross-sections were spread excessively creating spaces between the seminiferous tubules.
CONCLUSIONS

The decreased testicular weights and selective elimination of germ cells from the seminiferous tubules of ENU treated animals were correlated at a highly significant level with the drug treatment.

If the present study were repeated it is suggested that time intervals of 1, 2, 3, 4, 5, 6, 21, and 42 days after treatment be included so that greater continuity may be obtained on the toxicity over time curve and so that a more detailed picture of the earliest histotoxic changes may be obtained.

Tissue fixation should be extended beyond twenty-four hours in an attempt to eliminate unnecessary vacuolation and/or Helly's fluid should be considered because of the rapid penetrating property of the mercuric chloride. The water bath temperature should be monitored very closely to prevent the occurrence of intertubular spaces.
APPENDIX A

Sperm Count Solutions

Tris-Sperm Ringer
Distilled water............................................. 1000 ml
Magnesium Sulfate........................................ 0.30 gm
Sodium Chloride.......................................... 1.70 gm
Sodium Bicarbonate....................................... 0.20 gm
Potassium Chloride...................................... 0.36 gm
Potassium Dihydrogen Phosphate....................... 0.16 gm
Tris-Base.................................................. 2.40 gm

Buffer to 7.4 pH with concentrated HCl and then add 4.0 gms of fructose (Ginsberg & Hillman, 1974).

Skim Milk
Add 9.5 gms of Dairy fresh flavor Samalac instant non-fat dry milk to 100 mls of distilled water. Use at room temperature (Almquist et al., 1954).
APPENDIX B

Dehydration and Staining Procedure

Xylene (two changes)........................................ 2 min.
Absolute ethyl alcohol (two changes)........................ 2 min.
95% ethyl alcohol (two changes)............................ 2 min.
Rinse in distilled water.
1% Normal Hydrochloric Acid at 60°C...................... 15 min.
PAS............................................................ 30 min.
0.5% Sodium Metabisulfite in distilled water
(three changes).............................................. 2 min.
Running tap water.......................................... 5 min.
Harris Hematoxylin.......................................... 1 min.
Rinse in tap water.
1% Hydrochloric Acid in 70% Alcohol...................... 3-10 dips
Rinse in tap water.
Dip in saturated lithium carbonate until the sections
are bright blue.
Running tap water.......................................... 10-20 min.
95% ethyl alcohol (two changes)............................ 2 min.
Absolute ethyl alcohol (two changes)........................ 2 min.
Xylene (two changes)....................................... 2 min.
Coverslip with permount.
APPENDIX C

Staining Solutions

"Cold" Periodic Acid Schiff

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic Fuchsin</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Sodium Metabisulfite</td>
<td>1.9 gm</td>
</tr>
<tr>
<td>Hydrochloric Acid (0.15 N)</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Mix until clear and yellowish. If the solution is not the proper color allow it to stand in the dark overnight. Add 500 mg of fresh activated charcoal and agitate for 1-2 minutes. Use a double layer of no. 1 filter paper and filter into a volumetric cylinder; wash the residue with distilled water to restore the original 100 ml. Store in the refrigerator (Lillie & Fullmer, 1976).

Harris Hematoxylin

<table>
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<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoxylin crystals</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>95% ethyl alcohol</td>
<td>50 ml</td>
</tr>
<tr>
<td>Ammonium or Potassium Alum</td>
<td>100 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Mercuric Oxide</td>
<td>2.5 gm</td>
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</tbody>
</table>

Dissolve the hematoxylin in the alcohol, the alum in the water aided by heat. Mix. Bring the mixture to a boil as rapidly as possible and then remove from heat and add mercuric oxide. Reheat solution until dark purple, about one minute, and remove container from heat and place in cold water. Use when cool. Add 2-4 ml of glacial acetic acid to 100 ml if desired (Humason, 1979).
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