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# *In Vivo* and *in Vitro* Evaluations of Spermatotoxicity Induced by 2-Ethoxyethanol Treatment[1,2]

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*In Vivo* and *in Vitro* Evaluations of Spermatotoxicity Induced by 2-Ethoxyethanol Treatment. OUDIZ, D., AND ZENICK, H. (1986). *Toxicol. Appl. Pharmacol.* **84,** 576–583. 2-Ethoxyethanol (EE), a member of the glycol ethers, has been shown to produce testicular atrophy in laboratory animals. The present study further identified the spectrum of effects on the male reproductive system *in vivo* and initiated *in vitro* studies on possible mechanisms of action. Adult, male rats were treated (po) with 0 or 936 mg EE/kg, 5 days/week for 6 weeks. Semen samples were collected on a weekly basis during the exposure period from ovariectomized, hormonally primed females immediately following mating and analyzed for sperm count, sperm morphology, and sperm motility. Sperm count and percent normal morphology were decreased at Weeks 5 and 6, and sperm motility was decreased at Week 6. These data, along with other studies, indicated that the pachytene spermatocyte was the most sensitive target for EE. *In vitro* studies monitored $O_2$ consumption and ATP concentrations in isolated pachytene spermatocytes which were treated with 10 mM EE or 1 or 10 mM ethoxyacetic acid (EAA), the reported active metabolite of EE. An increase in respiratory ratio for the lactate rate/endogenous rate and a decrease in the 2,4-dinitrophenol rate/lactate rate were observed only for cells treated with 10 mM EAA. Additionally, a decrease in ATP concentration was seen only with 10 mM EAA. These results indicated that EAA interfered with energy metabolism in the pachytene spermatocyte. This effect may, in part, explain the testicular toxicity produced by this compound. 

Concern over the reproductive toxicity of the glycol ethers has increased during the last several years due to their widespread use in industry. 2-Ethoxyethanol (EE; ethylene glycol monoethyl ether) is of particular interest since this compound accounts for almost 40% of the glycol ethers used in industry with approximately 400,000 workers exposed annually. This compound has been shown to have a marked toxic effect on the testes in a number of species (Morris *et al.*, 1942; Stenger *et al.*, 1971; Nagano *et al.*, 1979). These testicular effects included edema, an absence of germ cells (particularly the more mature stages), and testicular atrophy. Following a single exposure to EE, immature male rats displayed degeneration and loss of spermatocytes (Foster *et al.*, 1983). In a detailed histological study, Creasy and Foster (1984) specifically identified the pachytene spermatocyte as the germ cell most at risk to EE.

In this laboratory we found a temporary condition of azoospermia between 4 and 7 weeks in male rats exposed to 1872 or 2808 mg EE/kg following a 5-day exposure period (Oudiz *et al.*, 1984). A significant decrease in sperm counts and an increase in abnormal

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sperm morphology were also seen in rats exposed to 936 mg EE/kg.

The present subchronic *in vivo* studies were undertaken to further evaluate the effects of EE on spermatogenesis. To accomplish this, semen from male rats was evaluated on a weekly basis as previously described (Zenick *et al.*, 1984; Oudiz *et al.*, 1984). *In vitro* experiments were initiated employing isolated pachytene spermatocytes to determine the ability of EE or its active metabolite, ethoxyacetic acid (EAA), to interfere with energy metabolism. EAA has been identified as the major metabolite of EE and is considered to be the active agent producing the testicular toxicity (Jonsson *et al.*, 1982; Foster *et al.*, 1983; Cheever *et al.*, 1984; Gray *et al.*, 1985). An alteration in energy metabolism was pursued as a hypothesis because the spermatocyte has been shown to preferentially utilize lactate and pyruvate and to have limited capabilities for energy metabolism (Jutte *et al.*, 1981, 1982; Nakamura *et al.*, 1984). These cells have also been observed to be sensitive to compounds which interfere with energy metabolism (Paul *et al.*, 1953; Mazzanti *et al.*, 1964; Sullivan *et al.*, 1979). The purpose of these experiments, therefore, was to investigate the time course and effects of a subchronic treatment with EE on sperm parameters in the rat. Furthermore, *in vitro* studies were performed on isolated pachytene spermatocytes in order to begin to elucidate possible mechanisms of action.

## METHODS

### *In Vivo Experiments*

Male and female Long Evans hooded rats (Charles River, Portage, Mich.) were purchased at 70–80 days of age. All animals were kept on a reverse 10:14 light–dark cycle with the lights turned out by 0900. Purina Lab Chow 5001 and tap water were available *ad libitum*. Upon arrival in the laboratory, the male rats were allowed a 3-week adjustment period. During this period, they were mated several times with ovariectomized, hormonally primed females in order to gain mating proficiency. The male rats were then intubated with water for 5 days and, on the fifth day, pretreatment baseline analyses were made of copulatory behaviors and semen parameters (see below). Male rats with total sperm counts of less than $20 \times 10^6$ sperm were eliminated from the study. The males were divided into two groups (10 males/group), that were matched on two sperm parameters (sperm count and percent sperm motility). The animals received either 0 or 936 mg EE/kg. The selection of this dose was based upon the data collected in the previous study which employed a 5-day exposure to EE (Oudiz *et al.*, 1984). It was believed that this dose would not be overtly toxic to the animal during the course of the subchronic treatment yet would produce a spectrum of effects on the male reproductive system. Treatment was initiated 2 days after the baseline semen evaluation, and the male rats were gavaged 5 days/week for 6 weeks. 2-Ethoxyethanol (Aldrich) was determined to be 98% pure by gas chromatography. EE was diluted in water and both groups received equivalent volumes (2 ml/kg). Semen samples were collected and analyzed at weekly intervals. All males were terminated 3 days after cessation of treatment. Reproductive organ weights (testis, epididymis, vas deferens, prostate, and seminal vesicles) were recorded. Cauda epididymal sperm counts were performed and slides were prepared for sperm morphology.

### *Semen Analyses*

Semen analyses and assessment of copulatory behaviors (mount latency, number of mounts, number of intromissions, and ejaculation latency) were performed as described in previous publications (Zenick *et al.*, 1984; Oudiz *et al.*, 1984), with the exception of sperm motility. Motility assessments were made from the uterine samples, which were diluted with medium (Paz-Frenkel *et al.*, 1978). The medium was kept at 37°C and the microscope stage was prewarmed by an air curtain incubator. A 15-$\mu$l aliquot of the diluted sperm suspension was placed on a slide, observed under 100× magnification on dark field, and three independent fields were videotaped for 10 sec each. Subsequently, tapes were replayed in stop action on a video cassette recorder, and all the sperm in a field (approximately 50–100) were assessed for motility. The data were expressed as percent motility. Swimming speeds were determined on samples collected on Week 6 by employing a digitizing cursor interfaced into a microcomputer to track individual sperm (50 sperm/sample) across the video screen. The absolute distance travelled by a spermatozoon was divided by the elapsed time to yield swimming speed (Zenick *et al.*, 1984). For morphological assessment, sperm heads were classified into one of five categories: normal, banana (the angle of the head is opened up), hook (the angle of the head is more pronounced), truncated, and amorphous.

### *In Vitro Experiments*

Single-cell suspensions were prepared from the testes of two adult Long Evans hooded rats as described by Meis-

trich *et al.* (1981). The animals were terminated by decapitation and their testes immediately removed and placed in a petri dish at room temperature. After the tunica had been removed, the testes were minced into 1-mm$^3$ blocks and placed in a flask with 100 ml of phosphate-buffered saline (Dulbecco's PBS with calcium and magnesium, GIBCO) with 8.3 mM glucose, 3 mM sodium lactate, 0.1% trypsin (Code TRL, Cooper Biomedical, Malvern, Pa.), and 17 μg/ml DNase (DN-25, Sigma). Penicillin (31 μg/ml) and streptomycin (50 μg/ml) were added to this buffer and to all subsequent buffers. All buffers were adjusted to pH 7.4. The tissue was placed in a 32°C shaking water bath for 30 min, after which 8% fetal calf serum (GIBCO) was added. The cell suspension was filtered through a 300-μm nylon mesh screen, an 80-μm nylon mesh, and finally through glass wool. The cell suspension was centrifuged at 500*g* for 15 min and resuspended in 50 ml of cold PBS containing 8.3 mM glucose, 3 mM sodium lactate, 0.02% soybean trypsin inhibitor (Code SI, Cooper Biomedical), and 2 μg/ml purified DNase (Code D, Cooper Biomedical). The samples were placed on ice and pachytene spermatocytes were separated by centrifugal elutriation (Grabske *et al.*, 1975; Meistrich, 1977; Meistrich *et al.*, 1981). After isolation, the spermatocytes were centrifuged twice (500*g*, 15 min), resuspended in phosphate-buffered saline without glucose or lactate, and placed on ice until use. The spermatocyte fraction contained 85–90% pachytene spermatocytes with the remaining cells composed primarily of round spermatids. Viability, determined by erythrosin B exclusion, was greater than 90% for up to 6 hr after separation. The combined yield of spermatocytes from four testes was approximately $4 \times 10^7$ spermatocytes. The spermatocytes were subsequently treated with 2-ethoxyethanol, ethoxyacetic acid (Aldrich), or vehicle.

Energy metabolism was monitored in these cells by oxygen utilization and changes in ATP concentrations. Both of these indices have been used to assess alterations in energy metabolism in germ cells (Storey, 1978; Nakamura *et al.*, 1984). Oxygen consumption was measured by an oxygen monitor (Yellow Springs, Ohio) with a Clark-type polarographic electrode. The system was maintained at 32°C by a circulating water jacket. Three milliliters of the spermatocyte suspension ($4–7 \times 10^6$ spermatocytes) was placed in the chamber for a 30-min preincubation period. The cells were preincubated in one of the following solutions: 1 mM EAA, 10 mM EAA, 10 mM EE, or control. EAA was buffered to pH 7.2 with NaOH before addition to cells. Preliminary experiments indicated that these concentrations did not affect the viability of the spermatocytes, as assessed by erythrosin B exclusion, after 1 hr. Following the incubation period, oxygen utilization was monitored to determine the endogenous rate (no exogenous substrates) and the subsequent changes in rate with consecutive additions of lactate (5 mM) and 2,4,-dinitrophenol (DNP, 30 μM). DNP was added as a positive control. Respiration was monitored for 10 min for each condition and the results calculated by standard procedures (Estabrook, 1967). The rates were standardized per $10^6$ cells and expressed as ratios: lactate/endogenous and DNP/lactate (Storey, 1978).

ATP concentrations were determined on separate samples by the luciferin–luciferase bioluminescent reaction (Webster *et al.*, 1980; Spielman *et al.*, 1981) using firefly extract (Fle-50, Sigma). EE (10 mM), EAA (10 mM), EAA (1 mM), or vehicle control was added to 3 ml of spermatocyte suspension ($4–7 \times 10^6$ cells) and the sample was placed in a 32°C shaking water bath. After 30 min, lactate (5 mM) was added to all samples and they were incubated for another 30 min. DNP (30 μM) was added to some control samples to serve as a positive control. After incubation, the samples were centrifuged at 500*g* for 10 min and the pellet resuspended in 1 ml of hot (90°C) Tris buffer (10 mM). The samples were placed in a boiling water bath for 3 min, after which 2 ml of cold (4–10°C) 10 mM Tris was added. The samples were assayed for ATP immediately on a JRB ATP photometer.

### *Statistical Analyses*

A two-way repeated-measures analysis of variance (ANOVA) with one between factor (treatment) and one within factor (weeks) was used for the analyses of the semen parameters, copulatory behaviors, and body weights. Mount latency, ejaculation latency, number of mounts, and number of intromissions were transformed using a logarithmic transformation in order to normalize distributions. The sperm morphology data were transformed by an arc sine transformation. All significant trends were further tested using *t* tests. The data collected at sacrifice were analyzed by *t* tests. Data were analyzed for both the respiratory rates and ATP concentrations by a one-way analysis of variance and significant trends were further tested by Duncan's multiple range test.

## RESULTS

### *In Vivo Experiments*

*Semen evaluations.* None of the animals died during the study; however, one control male and one treated male were eliminated from the study due to highly variable, inconsistent sperm counts which appeared to be independent of treatment. All of the animals showed significant increases in body weights over the course of the experiment, and there were no overt signs of toxicity in any of the males. Analyses of copulatory behaviors

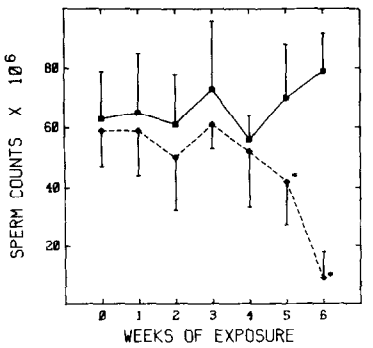


FIG. 1. Weekly sperm counts (mean ± SD) during 2-ethoxyethanol (EE) treatment. Semen was recovered from ovariectomized, hormonally primed females 15 min after mating. (■) Control, (♦) 936 mg EE/kg. *$p < 0.001$.

(mount latency, number of mounts, number of intromissions, and ejaculation latency) and seminal plug weights indicated no significant treatment differences (data not shown).

The EE-treated males had significantly decreased sperm counts at Weeks 5 and 6 ($p < 0.001$; Fig. 1). There was a 30–40% decline in sperm counts at Week 5 and by Week 6, three of the males in the EE-treated group were azoospermic and the remaining males had severely reduced sperm counts ranging from 5 to 30 $\times$ $10^6$ sperm.

The three azoospermic males were eliminated from the analyses of sperm morphology and motility, since the absence of sperm obviated the evaluation of these indices. There were significant increases in abnormal morphology for the treated males at Weeks 5 and 6 which paralleled the sperm count data (Fig. 2). Photomicrographs of normal and abnormal sperm shapes are shown in Fig. 3. The shapes depicted in Fig. 3d–f were only seen in males treated with EE. A significant decrease in percent sperm motility was seen in the treated males at Week 6 (Fig. 4); however, swimming speeds of the motile sperm from the treated animals at this time did not differ from control values (47.6 $\mu$m/sec ± 7.4 and 49.3 $\mu$m/sec ± 9.8, respectively).

There were significant decreases in testes, epididymides, and caudae epididymides weights in the treated males (Table 1). In addition, there were significant decreases in cauda epididymal sperm counts and percent normal sperm morphology which were consistent with the data obtained on ejaculated sperm.

### *In Vitro Experiments*

The respiratory rates of cells and their responses to different biochemical probes are indicative of their metabolic state (Storey, 1978; Nakamura *et al.*, 1981, 1982). When the spermatocytes were treated with 10 mM EAA, there was a significant increase in the lactate/endogenous ratio ($p < 0.05$) and a significant decrease in the DNP/lactate ratio ($p < 0.05$) compared to control cells (Table 2). The increase in the lactate/endogenous rate indicated an increased utilization of oxygen by the treated spermatocytes. The subsequent decrease in stimulation by DNP in these cells, reflected in the decrease in the DNP/lactate ratio, suggested that the spermatocytes were, at least partially, in an uncoupled oxidative state. These observations were further substantiated by a significant decrease in ATP concentration in cells treated with 10 mM EAA (Table 3). The integrity of this system was verified by adding DNP to control samples which produced a significant decline in ATP con-

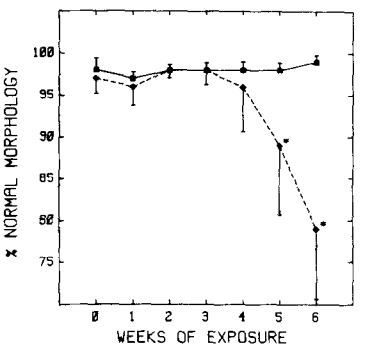


FIG. 2. Percent normal sperm morphology (mean ± SD) assessed on weekly semen samples recovered from ovariectomized, hormonally primed females 15 min after mating during 2-ethoxyethanol treatment. (■) Control, (♦) 936 mg EE/kg. *$p < 0.01$.

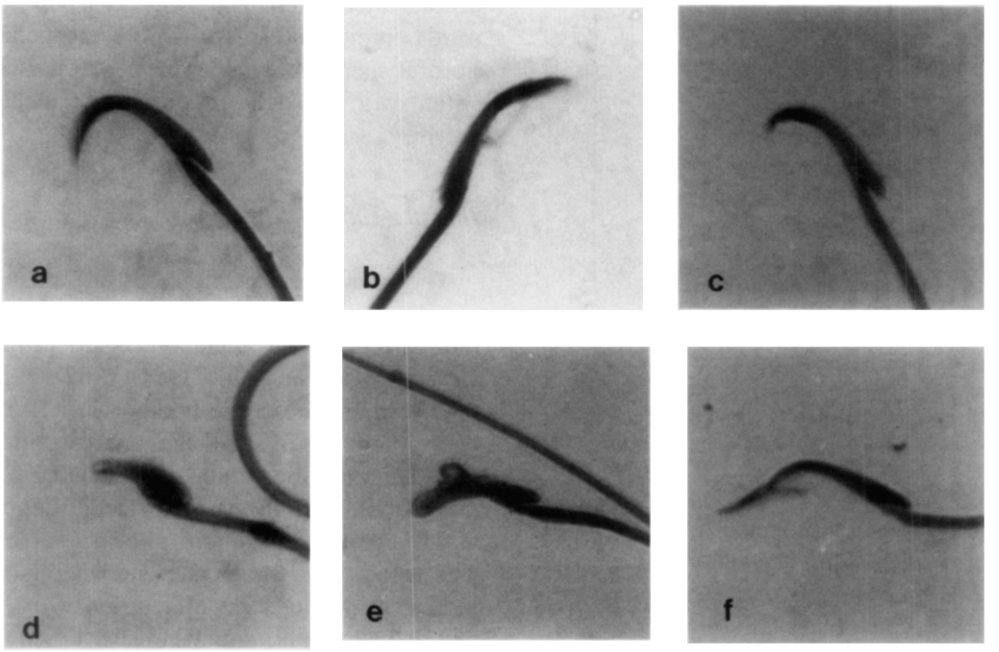


FIG. 3. Photomicrographs of different types of sperm head morphology seen during subchronic 2-ethoxyethanol exposure. (a) Normal sperm shape, (b) banana, (c) hook, (d) truncated, (e, f) amorphous sperm shapes. The most common abnormal shapes seen in both treated and control males were b and c. Abnormal shapes seen only in treated animals were d–f. ×750.

centrations. No significant treatment-related effects were seen for the respiratory rates or ATP concentrations with the addition of 1 mM EAA or 10 mM EE.

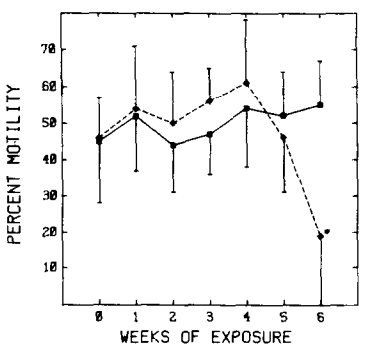


FIG. 4. Percent sperm motility (mean ± SD) assessed weekly on semen samples collected from ovariectomized hormonally primed females 15 min after mating during 2-ethoxyethanol treatment. (■) Control, (♦) 936 mg EE/kg. *$p < 0.01$.

## DISCUSSION

The effects produced by EE on the sperm parameters occurred at Weeks 5 and 6. From the known transist time for sperm to appear in the ejaculate from a given developmental stage (Gomes, 1970), the changes in sperm parameters evident at Weeks 5 and 6 are indicative of damage to the spermatocyte. This is in agreement with other authors who have also identified the spermatocyte (Foster *et al.*, 1983; Oudiz *et al.*, 1984) and, more specifically, the pachytene spermatocyte (Creasy and Foster, 1984) as the target cell for EE. It is interesting to note that even with prolonged exposure to EE, the cells which were spermatozoa, elongated spermatids, or round spermatids at the initiation of treatment were not affected as assessed by the sperm parameters. The time course and spectrum of sperm effects in this study were not markedly different from the

TABLE 1

REPRODUCTIVE ORGAN WEIGHTS AND SPERM PARAMETERS OBTAINED AT TERMINATION (MEAN ± SD) FOLLOWING SUBCHRONIC 2-ETHOXYETHANOL EXPOSURE

| Treatment (mg/kg) | Prostate | Seminal vesicle | Vas deferens | Testes | Epididymides | Cauda epididymis | Caudal sperm count ($\times 10^6$) | % Normal morphology |
|---|---|---|---|---|---|---|---|---|
| 0 | 0.84 ± 0.15 | 2.21 ± 0.24 | 0.26 ± 0.03 | 3.33 ± 0.36 | 1.22 ± 0.11 | 0.15 ± 0.03 | 114 ± 26 | 95 ± 2.5 |
| 936 | 0.70 ± 0.14 | 2.41 ± 0.31 | 0.24 ± 0.03 | 1.37 ± 0.03* | 0.75 ± 0.08* | 0.09 ± 0.01* | 11 ± 7* | 61 ± 14* |

* $p \leq 0.01$.

effects seen in the previous acute exposure study (Oudiz *et al.*, 1984); however, the magnitude of the effects in this study were greater. The similar temporal sequencing of the sperm count and sperm morphology data suggests a relationship between these two parameters. This relationship may also apply to the effects seen on sperm motility. The decrease in this parameter at Week 6 was probably a reflection of viability rather than a direct effect upon motility, since there was no treatment difference for the swimming speeds of the motile sperm population. The data collected at sacrifice on the reproductive parameters (organ weights and sperm parameters) substantiate the results seen in the semen evaluations. Similar effects of EE on mouse sperm parameters have recently been reported by Lamb *et al.* (1984). Epididymal sperm evaluations indicated a significant increase in abnormal sperm shapes and a decrease in both sperm counts and sperm motility.

The *in vitro* experiments, in which isolated spermatocytes were treated with EE and EAA, were performed to address the question of direct toxicity to these cells. Spermatocytes have limited capabilities for energy metabolism and these cells are dependent on lactate or pyruvate as an energy substrate (Jutte *et al.*, 1981, 1982; Nakamura *et al.*, 1984). Because of these factors, the spermatocyte may be a particularly sensitive target for agents which affect energy metabolism. There are several compounds known to affect energy metabolism which do have adverse effects upon the testes. These compounds include nitrofurans (Paul *et al.*, 1953), 5-thio-D-glucose (Zysk *et al.*, 1975), fluoracetamide (Mazzanti *et al.*, 1964), and fluoracetate (Sullivan *et al.*, 1979). In the present study, spermatocytes treated with 10 mM EAA exhibited a significant increase in lactate/endogenous respiratory ratios and a significant decrease in DNP/lactate respiratory ratios. These findings indicated that the functional

TABLE 2

RESPIRATORY RATIOS (MEAN ± SE) FOR ISOLATED PACHYTENE SPERMATOCYTES TREATED WITH 2-ETHOXYETHANOL (EE) OR ETHOXYACETIC ACID (EAA)

| Treatment | No. of experiments | Lactate rate[a]/ endogenous rate | DNP rate/lactate rate |
|---|---|---|---|
| Control | 14 | 1.91 ± 0.07 | 1.52 ± 0.05 |
| EE, 10 mM | 10 | 1.77 ± 0.16 | 1.38 ± 0.07 |
| EAA, 1 mM | 12 | 1.94 ± 0.08 | 1.41 ± 0.05 |
| EAA, 10 mM | 12 | 2.43 ± 0.23[b] | 1.27 ± 0.03[b] |

[a] nmol $O_2$/min/$10^6$ cells.

[b] Significantly different from control value, $p < 0.05$.

TABLE 3

PERCENT ATP LEVELS (MEAN ± SE) IN ISOLATED PACHYTENE SPERMATOCYTES TREATED WITH 2,4-DINITROPHENOL (DNP), 2-ETHOXYETHANOL, OR ETHOXYACETIC ACID (EAA)[a]

| Treatment | No. of experiments | Percent of control |
|---|---|---|
| DNP, 30 μM | 9 | 55.30 ± 5.00[b] |
| EE, 10 mM | 8 | 97.75 ± 7.42 |
| EAA, 1 mM | 13 | 90.73 ± 6.66 |
| EAA, 10 mM | 9 | 66.70 ± 4.83[b] |

[a] Mean and SE for control values were 0.71 ± 0.05 nmol ATP/$10^6$ spermatocytes.

[b] Significantly different from control value, $p < 0.01$.

integrity of these cells has been compromised and that EAA created a partially uncoupled respiratory state in these cells as seen by the overall increased respiration and the decreased stimulatory effect of DNP. Since DNP is a known uncoupler of oxidative phosphorylation, its addition to the cell suspension should produce an increased oxygen consumption in cells that are tightly coupled. This supposition was further supported by the significant decrease in ATP levels seen after incubation with EAA. Decreased ATP levels were also seen with DNP. Decreases in ATP concentrations have been reported in other cell preparations following the addition of DNP (Ford and Harrison, 1981; Nakamura *et al.*, 1981).

While EAA produced metabolic changes in the spermatocytes, 10 mM EE did not produce effects on these cells. Gray *et al.* (1985) also found that up to 50 mM EE did not produce toxicity in Sertoli–germ cell cultures, while 5 and 10 mM EAA produced degeneration and loss of pachytene spermatocytes after 72 hr. Several other reports also support that the alkoxyacetic acid derivatives are the ultimate toxicant for this class of agents (Miller *et al.*, 1982; Foster *et al.*, 1983; Gray *et al.*, 1985). The lack of toxicity seen in the spermatocytes when treated with EE suggests that these cells are deficient in the necessary enzymes to metabolize EE to EAA.

The closely related 2-methoxyethanol (ME) and its metabolite, methoxyacetic acid (MAA), have produced similar effects on spermatocytes *in vitro*. MAA (10 mM) was shown to increase $^{14}CO_2$ production from [$^{14}$C]lactate while ME did not have an effect (Beattie, 1984). ME and EE have the same spectrum of effects and are thought to exert their toxicity via the same mechanisms (Miller *et al.*, 1981; Foster *et al.*, 1983; Chapin *et al.*, 1984, 1985; Gray *et al.*, 1985).

In conclusion, 2-ethoxyethanol did not alter respiration or ATP levels in pachytene spermatocytes; however, the active metabolite, ethoxyacetic acid, did affect these two parameters in a manner consistent with an encoupled oxidative state. Further studies need to be performed to determine if this effect has a major role in the observed testicular toxicity caused by EE.

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