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REPRODUCTIVE TOXICITY OF ETHYLENE BISDITHIOCARBAMATES AND THE ROLE OF FREE RADICALS IN THE FERTILIZING ABILITY OF SPERMATOZOA

By

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ABSTRACT

REPRODUCTIVE TOXICITY OF ETHYLENE BISDITHIOCARBAMATES AND THE ROLE OF FREE RADICALS IN THE FERTILIZING ABILITY OF SPERMATOZOA

By

Li Chen

Ethylene bisdithiocarbamates (EBDCs) are a class of fungicides first developed in the 1930s. Existing information indicates that EBDCs at high doses cause infertility and neonatal death in animals but there is not adequate information to assess the reproductive toxicity due to long-term low-level dietary exposure to the most widely used fungicides in this country. The mechanisms of EBDC toxicity remain unknown. This project was designed based on the hypothesis that ethylene thiourea (ETU), a common metabolite of EBDCs, decreases sperm production and fertilizing ability by perturbing the homeostasis of reactive radicals in germ cells. The experimental protocol emphasized sperm production in pubertal and aging mice, as well as in adult mice.

The specific aims were to examine (1) the reproductive performance of mice treated with maneb/zineb- or mancozeb-containing diets, (2) the fertilizing ability and the free radical activity of sperm either treated with ETU in vitro or collected from EBDC-treated mice, (3) sperm intracellular free-calcium concentration as a possible marker for sperm

injury, and (4) mitochondria as a source of free radicals.

Mancozeb at dietary concentrations of 300 ppm and above decreased litter sizes in mice. The fertilizing ability of sperm collected from males treated with a mixture of 3000 ppm maneb and zineb or 300 ppm mancozeb was decreased. In vitro exposure of mouse gametes to 5 ppm ETU decreased fertilization.

Lipid peroxyl radical production in non-exposed sperm appeared to increase as sperm capacitation progressed and decreased with the completion of sperm capacitation. A second surge of peroxyl radical production, coinciding with decreases in sperm motility, may have been a result of lipid membrane degradation in dying spermatozoa. In the presence of 1000 ppm ETU in vitro, sperm peroxyl radical production increased without any changes in mitochondrial function. Peroxyl radical and H_2O_2 production in the spermatozoa of mancozeb treated mice, however, were lower when compared to controls.

Increase in sperm intracellular free calcium concentration was a very sensitive indication of low-dose EBDC exposure. Mancozeb, at concentrations of 30 ppm and above, decreased sperm production in the offspring at 6 weeks of age, but increased sperm production at 8 weeks of age. Changes in the progress of spermatogenesis in the pubertal mice were revealed by relative distribution of DNA content in testicular germ cells. In aging mice, exposure to mancozeb did not alter sperm production.

To

my **MOTHER** *Ruifeng Chen,*

my **HUSBAND** *Yonggang Feng* **and** *my* **SON** *Justin Linjie Feng.*

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PREFACE

This dissertation is divided into nine chapters. Chapter one provides an overview of the dissertation project. Chapters two through four are the literature reviews of three subjects: the role of free radicals and lipid peroxidation in biological function; the relationship between sperm function and lipid peroxidation; and the toxicity of ethylene bisdithiocarbamates.

Chapters five through eight present the four phases of the laboratory studies. Each chapter includes four sections: an introduction, the materials and methods used, the results, and a final discussion. An overall conclusion covering all four-phase studies and perspectives are presented in chapter nine.

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CHAPTER I

INTRODUCTION

Man's use of synthetic chemicals to improve his living environment has led not only to improvements in human health and agricultural production, but also to certain unforeseen negative consequences. A major example is the widespread use of pesticides in controlling pests and plant diseases. Without pesticides, the global annual lost in agricultural production could amount to $74.9 billion (Spynu, 1989).

Along with important economic advantages, these chemicals have also created environmental and human health concerns. Ethylene bisdithiocarbamates (EBDCs) have been widely used in fruit and vegetable production for the past thirty years. Most EBDCs, such as zineb, maneb, or mancozeb, have low acute toxicity with LD_{50s} ranging from 5 to 8 gm/kg body weight in rats and mice. At relatively high doses, these fungicides have been recently implicated as mutagenic, teratogenic, and carcinogenic agents in laboratory animals, thus becoming a major human health concern (Lentza-Rizos, 1990).

Previous studies suggested possible adverse effects of EBDCs and a metabolite -- ethylene thiourea (ETU) -- on fertility in rats (Ryazanova, 1967; Shtenberg *et al.*, 1969; Peters *et al.*, 1982) and sperm morphology in mice (Hemavathi and Rahiman, 1993; Jablonicka and Kobzova, 1988). The mechanisms by which EBDCs and ETU effect fertility are yet to be understood.

Among the key events that regulate a fertilizing ability of spermatozoa are capacitation and the acrosome reaction.

Mammalian sperm capacitation is required by freshly ejaculated or epididymal sperm before mammalian egg fertilization occurs. Capacitation is accompanied by changes in the membrane's lipid and protein composition, and in protein location on the sperm head (Langlais and Robert, 1985; Myles *et al.*, 1987; Wolf, 1987). After capacitation, the upper acrosome membrane fuses with the plasma membrane to form numerous pores and vesicles. The released acrosomal enzymes are necessary for sperm-egg fusion.

Free radicals have been implicated in the capacitation and the acrosome reaction processes. They can cause degradation of membrane phospholipids in spermatozoa followed by a progressive loss of motility and structural integrity, and thus this may lead to infertility (Jones and Mann, 1976). Nevertheless, there is also evidence that free radicals contribute to the normal processes of maturation, capacitation and sperm-zona pellucida binding (Aitken *et al.*, 1989; Kumar *et al.*, 1989; Bize *et al.*, 1991).

How EBDCs exert their toxic effects on sperm cells at the cellular level is still unknown. Hayes (1982) hypothesized that the toxicity of ethylenethiourea (ETU), a common metabolite of all EBDCs, is related to a highly reactive form of atomic sulfur released during the metabolic formation of ethyleneurea (EU). The reactive sulfur may initiate lipid peroxidation by directly interacting with the sperm membrane components. Another possible mechanism involves the

interaction between the reactive sulfur and oxygen free radicals, which in turn causes membrane lipid peroxidation. Spermatozoa provide an excellent model for studying lipid peroxidation because they are available as free intact cells and their motility may be used as an index of their viability (Jones and Mann, 1976).

The objectives of this study were: (1) to investigate the effects of EBDCs on animal reproduction and (2) to study the role of free radicals and lipid peroxidation in sperm cells.

The specific aims were to examine (1) the reproductive performance of mice treated with maneb/zineb or mancozeb containing diets, (2) the fertilizing ability and the free radical activity of spermatozoa treated with ETU in vitro or collected from EBDC-treated mice, (3) the possibility of using sperm intracellular free-calcium concentration as a marker for sperm injury, and (4) mitochondria as a source of free radicals.

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CHAPTER II

LITERATURE REVIEW I: THE ROLE OF FREE RADICALS AND LIPID PEROXIDATION IN BIOLOGICAL FUNCTIONS

Free radicals in biology

A radical is defined as an atom or group of atoms with an unpaired electron. Radicals may be positively charged, negatively charged, or neutral (Pryor, 1976). Because free radicals are missing an electron in their outer shells, they are highly reactive. Even at extremely low concentrations, they exist for only a fraction of a second.

A biradical is a species with two unpaired electrons. Molecular oxygen (O_2) is a biradical containing two unpaired electrons with parallel spins. Incoming electrons must have a parallel spin in order to enter the vacant orbital spaces. This restriction on reactivity requires that a spin inversion of one electron must occur. This spin restriction slows down the reaction of oxygen with nonradicals. In addition, electrons must arrive one at a time.

A partial reduction of oxygen by addition of electrons forms short-lived oxygen radicals. The normal tetravalent reduction of O_2 produces water (Reaction 1), while the univalent reduction of O_2 produces superoxide (Reaction 2). The divalent reduction of O_2 produces hydrogen peroxide (Reaction 3) and trivalent reduction of O_2 produces hydroxyl radical (Reaction 4):

$$O_2 + 4e + 4H^+ \rightarrow 2H_2O \qquad (1)$$

$$O_2 + e \rightarrow O_2^{-\cdot} \quad (2)$$

$$O_2^{-\cdot} + e + 2H^+ \rightarrow H_2O_2 \quad (3)$$

$$H_2O_2 + e + H^+ \rightarrow H_2O + \cdot OH \quad (4)$$

Although O_2 is essential for aerobic organisms, too much O_2 or the inappropriate metabolism of O_2 may be toxic. Mammals derive most of their cellular ATP by the controlled, four-electron reduction of O_2 to form H_2O in the mitochondrial electron transport system (Reaction 1). Approximately 98 percent of all O_2 consumed by cells enters the mitochondria where it is reduced by the terminal oxidase, cytochrome oxidase. During the course of normal metabolism, however, O_2 can accept less than four electrons to form reactive O_2 intermediates (Reactions 2, 3, and 4) that may be toxic to cells (Grisham and McCord, 1986). Some partially reduced oxygen is released as a superoxide radical by a leaky electron-transport chain. Mitochondria produce superoxide and hydrogen peroxide by generating an electron via NADH dehydrogenase or a semiquinone radical (Loschen *et al.*, 1974; Boveris and Cadenase, 1975; Boveris *et al.*, 1976; Cadenase *et al.*, 1977; Boveris, 1977; Nohl and Hegner, 1978).

Microsomes (Auclair *et al.*, 1978; Kuthan *et al.*, 1978, 1982; Talcott *et al.*, 1979) and nuclei (Patton *et al.*, 1980) also produce superoxide radicals. Furthermore, several enzymes produce $O_2^{-\cdot}$, such as aldehyde oxidase (Rajagopalan *et al.*, 1962; Badwey *et al.*, 1981), cytochrome P-450 reductase (Bosterling and Trudell, 1981) and xanthine oxidase (McCord

and Fridovich, 1968, 1969). Some immune cells, such as granulocytes and macrophages, produce $O_2^{-}\cdot$ during the respiratory burst that follows the activation of these specialized cells (Babior *et al.*, 1973; Markert *et al.*, 1980; Nakagawara *et al.*, 1981).

H_2O_2 is formed by either the two-electron reduction (Reaction 3) or by the $O_2^{-}\cdot$ dismutation reaction (Reaction 5):

$$2O_2^{-}\cdot + 2H^{+} \rightarrow H_2O_2 + O_2 \qquad (5)$$

H_2O_2 is stable in the absence of trace metals. It may directly oxidize biologically important molecules or form the highly reactive ·OH radical during the metal-catalyzed decomposition (Reaction 6). The $O_2^{-}\cdot$ can provide an electron to reduce ferric iron to ferrous iron, which may then reduce H_2O_2 to yield ·OH (Reaction 7).

$$H_2O_2 + Fe^{2+} \rightarrow HO\cdot + OH^{-} + Fe^{3+} \qquad (6)$$

$$O_2^{-}\cdot + Fe^{3+} \rightarrow O_2 + Fe^{2+} \qquad (7)$$

In biological systems, the ·OH radical is among the most reactive radicals. It reacts rapidly with most organic molecules with a diffusion-limited reaction rate of 10^7-10^{10} M/s (Brigham and Owen, 1975; Green *et al.*, 1984). If a metal, such as Fe^{2+}, is sequestered with a vital molecule, the ·OH formed from H_2O_2 in the proximity quickly binds to the molecule and alters its biological functions.

In general, the effects of reactive oxygen species on cellular function may be summarized in three categories: DNA and RNA damage (Ames, 1983), protein and enzyme dysfunction,

and membrane lipid peroxidation (Riley and Behrman, 1991). Free radicals may also unleash proteolytic enzymes and other destructive agents, thus causing normally harmless chemicals to become toxic and/or carcinogenic.

However, free radicals may also have essential roles in biological functions or have beneficial effects, such as the destruction of invading microorganisms and cancer cells. They also stabilize (through protein cross-linking) the spermatozoal cellular membrane during sperm maturation in the epididymis (Shalgi *et al.*, 1989; Seligaman and Shalgi, 1991). Free radicals also promote cross-linkage on the vitelline membrane of eggs following sperm entry (Shapiro, 1991).

Lipid peroxidation in biomembrane

Autoxidation of lipids in artificial and biological membranes is generally termed "lipid peroxidation" (Ivanov, 1985). Lipid peroxidation occurs in the membranes of all normal living cells (Voskresensky *et al.*, 1970; Koslov *et al.*, 1972; Vladimirov *et al.*, 1972). In normal cells, lipid peroxidation may facilitate the metabolism of cell components, synthesis of new phospholipids in membranes (Bulakova *et al.*, 1982), and to disassemble intracellular membrane structures (Koslov *et al.*, 1972; Bulakova *et al.*, 1982). Lipid peroxidation involves the generation of free radicals derived mainly from polyunsaturated fatty acids in the

phosphoglycerides of the plasma membrane (Rosen *et al.*, 1980). Lipid peroxidation is a radical chain reaction often described in three steps: initiation, propagation, and termination (Mead, 1976) (Reactions 8-16):

Initiation	$LH + R\cdot \rightarrow L\cdot + RH$	(8)
Propagation	$L\cdot + O_2 \rightarrow LOO\cdot$	(9)
	$LH + LOO\cdot \rightarrow LOOH + L\cdot$	(10)
	$LOOH + R\cdot \rightarrow LOO\cdot + RH$	(11)
	$LOOH + M^n \rightarrow LO\cdot + OH^- + M^{n+1}$	(12)
	$LOOH + M^{n+1} \rightarrow LOO\cdot + H^+ + M^n$	(13)
Termination	$L\cdot + L\cdot \rightarrow LL$	(14)
	$LOO\cdot + LOO\cdot \rightarrow LOOL + O_2$	(15)
	$LOO\cdot + L\cdot \rightarrow LOOL$	(16)

Initiation is thought to occur either by a radical addition to a double bond or by the abstraction of an allylic hydrogen by a reactive radical ($R\cdot$). A free radical with sufficient energy to abstract a hydrogen atom from a methylene carbon of an unsaturated fatty acid (LH) can initiate a chain reaction of lipid peroxidation.

Once initiated, lipid peroxidation may propagate through a multiple reaction (Reactions 9-10). The carbon-centered radical ($L\cdot$) reacts rapidly with molecular oxygen (10^9-10^{10} M/s) (Pryor, 1976) to form a peroxyl radical ($LOO\cdot$), which may then abstract a hydrogen atom from an unsaturated fatty acid to form a lipid hydroperoxide (LOOH) and another lipid free radical ($L\cdot$). In the presence of certain trace-metal ions,

such as Fe^{2+}, Cu^{2+}, and Co^{2+}, the reduction-oxidation of lipid hydroperoxide may lead to the formation of alkoxyl and peroxyl radicals (Reactions 11-13) (Halliwell and Gutteridge, 1984). In addition, when the dissociation energy of R-X is higher than that of LOO-H (90 kcal mol^{-1}), a radical may abstract a hydrogen atom directly from the hydroperoxyl group of a lipid hydroxide. Examples of such radicals include hydroxyl radicals and lipid alkoxyl radicals (Terao, 1990).

The free-radical chain reaction propagates quickly unless two free radicals collide with each other to terminate the chain reaction (Reactions 14-16).

The mechanisms of the lipid peroxidation initiation in living cells are still under investigation. Some in vitro experiments suggested that a hydroxyl radical ($HO\cdot$) can peroxize lipids in the chemical system. The superoxide radical ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), transition metals, and semiquinone radicals are involved in the production of hydroxyl radicals (Reactions 3, 4, 6, 7, 17-19) (Turrens and Boveris, 1980; Kappus and Sies, 1981; Gutteridge, 1982; Pryor, 1982; Yagi, 1982; Gutterigde and Wilkins, 1982; Rowley and Halliwell, 1983; Norh and Jordan, 1983):

$$O_2^{-}\cdot + Cu^{2+} \rightarrow O_2 + Cu^{+} \quad (17)$$

$$H_2O_2 + Cu^{+} \rightarrow HO\cdot + Cu^{2+} \quad (18)$$

$$\text{Semiquinone}\cdot + H_2O_2 + H^{+} \rightarrow \text{quinone} + HO\cdot + H_2O \quad (19)$$

These in vitro experiments on hydroxyl radical formation do not prove that the above reactions are really involved in

lipid peroxidation in living organisms. It is generally assumed that the superoxide radicals and transition metal ions play a major role in initiating lipid peroxidation (Kappus, 1985).

Biological relevance. Lipid peroxidation reactions destroy the physical structure and the biological function of cellular membranes. Plasma membranes, as well as intracellular membranes, lose their ultrastructural architecture, leading to changes in fluidity and permeability (Hicks and Gebicki, 1978; Yau and Mencl, 1981). This could result in an altered influx or efflux of various cellular and extracellular components. Lipid peroxidation may also inactivate membrane enzymes and protein receptors through conformational changes of proteins. The best known examples are the losses of cytochrome P-450 and glucose-6-phosphatase in microsomes and the inactivation of the respiratory chain in mitochondria (Nielson, 1981; Natabayashi *et al.*, 1982; Marshansky *et al.*, 1983). Reactions of nucleic acids, especially DNA, with products of lipid peroxidation could lead to mutagenic, carcinogenic, cytostatic or lethal effects (Floyd and Schneider, 1991; Joenje *et al.*, 1991).

Results from cell culture studies suggest that lipid peroxidation is responsible for regulating cellular growth (Poot, 1991; Burlakova *et al.*, 1991). The hypothesis that lipid peroxidation is responsible for aging purports that

aging results from cellular damage induced by lipid peroxidation (Harman, 1956). This hypothesis is supported by the accumulation of pigment (lipofuscin) in the cellular matrix during the aging process (Brunk and Sohal, 1991). These pigments are the product of the peroxidation of polyunsaturated fatty acids. The pigments' formation depends on malonaldehyde, a product of lipid peroxidation. Malonaldehyde acts as a cross-linking agent with amino groups of proteins in aging-related pigments (Rothstein, 1982).

In general, lipid peroxidation is considered a highly destructive process, altering or destroying diverse types of biomolecules. As a result, cells, organs or whole organisms may lose their biochemical functions. The role of lipid peroxidation in sperm function is described in Chapter III.

Methods of measuring lipid peroxidation

The direct measurement of lipid peroxidation relies on both qualitative and quantitative detection of free radicals. Capturing these highly reactive and extremely short-lived molecules is the major barrier in studying lipid peroxidation in all organic matter. A few indirect assays were developed in the past, but most of these methods measured the relatively stable products of lipid peroxidation, such as alkanes, malondialdehyde, and conjugated dienes. The electron spin resonance (ESR) methods take advantage of nitrone or nitroso

compounds to "trap" the highly reactive free radicals. The free radical-spin trap adducts, also radicals in nature, are more stable than the original radicals. Signals recorded by the ESR spectrometer reflect the natural intensity of the magnetic moment generated by free radicals in a magnetic field. The justification and limitations of these methods are discussed next.

1. Generation of volatile hydrocarbons. Ethane and pentane, two volatile alkanes originating from the monohydroperoxides of ω-3 or ω-6 unsaturated fatty acids in peroxidation, were used to measure the rate of lipid peroxidation in live animals (Cohen, 1982; Tappel, 1980). These alkanes were collected from respiratory air and measured via gas chromatography. The amount of alkanes formed during lipid peroxidation is very small, so the sensitivity of the gas chromatograph is a limiting factor of this assay. This method, however, is useful in human and animal studies that demand non-invasive methods. (Fentone and Ward, 1982; Freeman and Crapo, 1982; Wispe *et al.*, 1985).

The problem with this method is that it cannot determine the alkane origin; some bacteria in the intestinal track may also produce pentane (Gelmont *et al.*, 1981). Alkanes are only minor reaction products of lipid peroxidation. For every molecule of ethane or pentane produced, there are 100-500 molecules of malondialdehyde. In addition, malondialdehyde is

only about 10% of the total peroxidized unsaturated fatty acids (Kappus, 1985). In general, measuring alkanes is a specific qualitative assay, but only semi-quantitative for lipid peroxidation. This method is used only when a large amount of alkanes are produced by host animals. Negative results do not exclude the possibility of low levels of lipid peroxidation. The origin of the alkanes from intestinal bacteria as well as from already peroxidized food lipids must also be taken into account.

2. Malondialdehyde formation. Malondialdehyde is formed in lipid peroxidation after the rupture of the carbon chain of unsaturated fatty acids. Malondialdehyde is a toxic compound, which cross-links lipids and proteins (Riley and Behrman, 1991). Measuring malondialdehyde by a thiobarbituric acid (TBA) method has been used extensively as an index of lipid peroxidation in a variety of organs. Essentially, thiobarbituric acid is added to tissue samples obtained under acidic conditions. Malondialdehyde, released from peroxidized lipids, reacts with thiobarbituric acid resulting in formation of a red-colored reaction product. The absorption of the color is measured by the spectrophotometer at 535 nm.

This assay has several drawbacks. First, the conversion of lipid hydroperoxides to malondialdehyde in the TBA assay is less than 5% (Smith and Anderson, 1987; Van Kuijk and Dratz, 1987). The assay thus measures only a small fraction of

membrane lipid peroxidation. Second, this assay is not a specific indicator of fatty acid oxidation. In acidic conditions, thiobarbituric acid also reacts with sugars and lipid peroxides, forming compounds that interfere with absorbance at 525 nm (Taylor and Townsley, 1986). Third, malondialdehyde is volatile, subject to be further metabolism, and reactive with other cellular lipids and proteins (Freeman and Crapo, 1982). Although numerous publications on lipid peroxidation in biological systems are based on this method, the above quantitative limitations are still a concern (Kappus, 1985).

3. Conjugated diene formation. When radicals react with polyunsaturated fatty acids, the subsequent hydrogen abstraction and bond migration result in conjugated diene formation (Reaction 20). This is measured as an increase in absorbance at 233 nm (Freeman and Cropo, 1982; Rechnagel and Glende, 1984).

Diene conjugation:

$$\begin{array}{l} R\text{-}CH{=}CH\text{-}CH_2\text{-}CH{=}CH\text{-}\dot{C}H\text{-}CH{=}CH\text{-}R' \\ \qquad\qquad\qquad\qquad\qquad\qquad \downarrow \\ R\text{-}CH{=}CH\text{-}CH_2\text{-}\dot{C}H\text{-}CH{=}CH\text{-}CH{=}CH\text{-}R' \end{array} \qquad (20)$$

Theoretically, this product is relatively specific for lipid peroxidation. In practice, however, this method has a

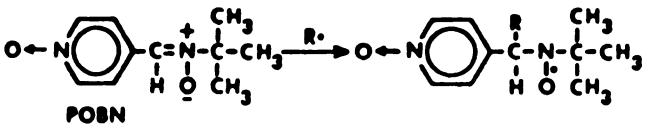
number of limitations. First, the peroxidized lipid must be extracted into an organic phase. Caution must be taken to prevent lipid peroxidation caused by the extraction procedure. Second, the measurement depends on the difference between the spectra of the control and test samples, but appropriate control samples are not available in most of the biological systems tested. Third, the differences in absorption intensity between the control and test samples are usually quite small. In summary, the measurement of conjugated dienes is a specific, semi-quantitative method for lipid peroxidation, but not very sensitive (Kuppus, 1985).

4. Electron spin resonance (ESR) and spin trapping. The mechanism for lipid peroxidation processes involves free radical formation. In a magnetic field, the magnetic moment of free radicals is directly detected by an ESR spectrometer. Since the ESR can detect only free radicals, it is considered the best technique available for directly detecting and identifying free radicals (Schaich and Borg, 1980). Its high degree of selectivity renders the ESR useful in complex biological systems (Rosen and Rauckman, 1981).

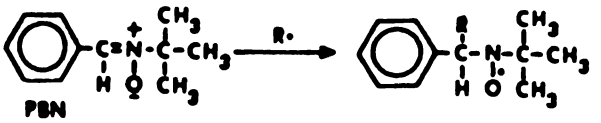
Most free radicals in a biological system are so reactive that they never reach the necessary steady-state concentration detected by ESR methodology. Spin trapping compounds overcame this difficulty by forming a stable nitroxide covalently with the radicals. In this manner, the unstable radical is

"trapped" as a long-lived adduct.

Because the adducts are also radical in nature and possess a paramagnetic resonance spectrum, they can be kept at room temperature and measured with conventional ESR equipment. The characteristic spectra of different adducts, such as the number of peaks and the value of splitting constants, provide a means not only for detecting free radicals, but also for determining the quantity of their production. In some cases, the characteristic of the spectrum provides a means for identifying radicals (McCay, 1987). For example, spin-trapping agents, α-4-pyridyl 1-oxide-N-tert-butyl nitrone (4-POBN) and phenyl-N-tert-butylnitrone (PBN) can be used to trap lipid free radicals, both in vivo and in vitro, to produce spin-trap adducts (Reaction 21-22), and a characteristic electron spin resonance spectra (Figure 1-1) (Rosen *et al.*, 1980; Rosen and Freeman, 1984).



(21)



(22)

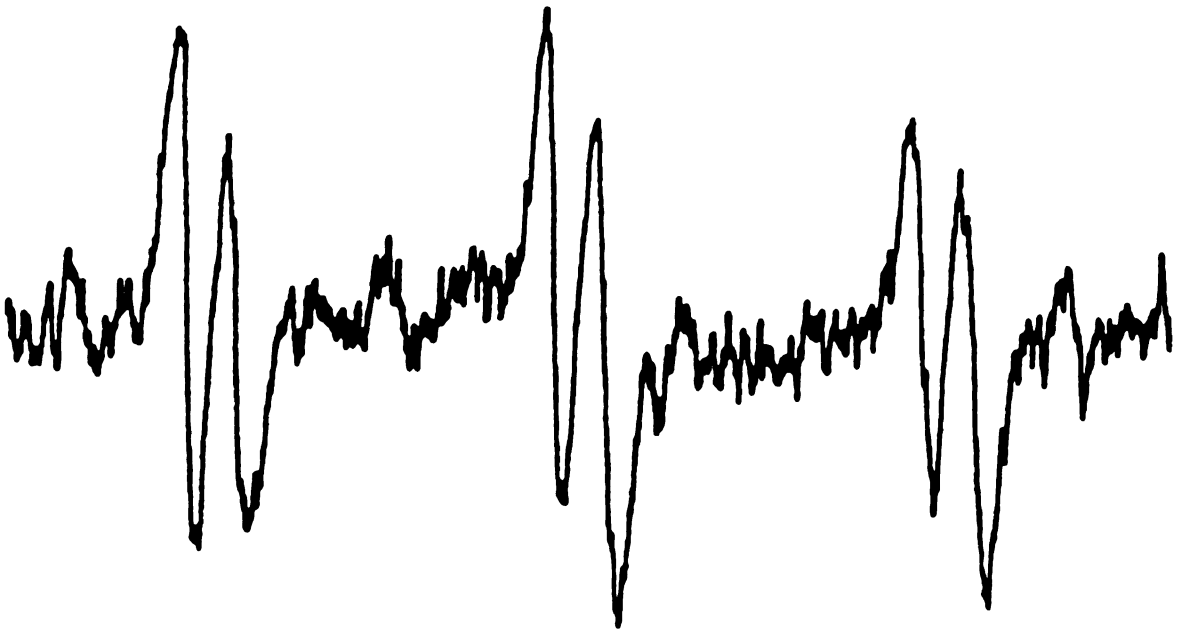


Figure 1-1. The ESR spectrum obtained with normal intact mouse sperm and the spin trap, α-(4-pyridyl-1-oxide)N-t-butylnitron (4-POBN) after 1 hr incubation. It is the spectrum for peroxyl-4-POBN adducts.

In order to measure lipid peroxidation products, promoters such as ascorbate, Fe^{2+}, Co^{2+} and Cu^{2+} are used in measurement procedures. Ferrous iron is a strong catalytic promoter. Ferrous iron donates electrons and acts either by initiating the formation of free radicals (Reactions 6-7) or by increasing the rate of breakdown of peroxides (Reactions 23-24) (Halliwell and Gutteridge, 1989):

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO\cdot + OH^- \quad (23)$$

$$Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO\cdot + H^+ \quad (24)$$

where Fe^{2+} produces an alkoxy radical and Fe^{3+} produces a peroxyl radical. Trapping lipid peroxyl radicals ($LOO\cdot$) by the spin trap 4-POBN has been used in many studies examining lipid peroxidation in biological samples (Connor *et al.*, 1986). The 4-POBN-peroxyl radical adducts are stable at room temperature in an aqueous solution (Connor *et al.*, 1986).

The major advantage of the ESR and the spin trap is that they can provide unequivocal evidence for free radical formation in biological systems. It avoids the extrapolations and assumptions that other methods discussed above encountered in investigating free radical phenomena in animal tissue (McCay, 1987).

Very few people, however, have used the ESR and the spin trap to study free radical production and lipid membrane peroxidation in spermatozoa. In the literature there has been only one study which used the ESR and the spin trap PBN to measure superoxide radical production from human spermatozoa

(Sinha *et al.*, 1991). The author believes that applying ESR and spin trap will provide an improved method for studying the free radical activity and lipid peroxidation in spermatozoa.

Summary

A free radical is a chemical species that contains an odd number of electrons. Partial reduction of oxygen (addition of electrons) forms short-lived oxygen radicals. Lipid peroxidation is a free-radical reaction that requires radicals for initiation and propagation. Certain trace metals, such as Fe^{2+}, Co^{2+} and Cu^{2+}, play an important role in free-radical reactions. They can initiate the formation of free radicals, propagate lipid peroxidation, and increase the rate of breakdown of peroxides. Lipid peroxidation results in structural and functional alterations in cell membranes. The current knowledge, however, is still insufficient to define the mechanisms by which oxidative stress induces lipid peroxidation in vivo. The lack of information is mainly due to a lack of routine specific methods for measuring lipid peroxidation. The ESR and spin trap techniques are currently the best methods for the most direct measurement of free radicals including lipid peroxyl radicals in lipid peroxidation.

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CHAPTER III

LITERATURE REVIEW II. THE RELATIONSHIP BETWEEN SPERM FUNCTION AND LIPID PEROXIDATION

Mammalian spermatozoa, when first released from the male reproductive tract, are not fertile until they acquire the ability to fertilize eggs through the capacitation process in the female tract (Austin, 1951; Chang, 1951) or a specifically designed culture media in vitro. The modifications of the spermatozoon that bring about this functional transformation are termed collectively "capacitation." Capacitation is accompanied by changes in membrane lipid and protein composition and their locations on the sperm membrane (Langlais and Robert, 1985; Myles *et al.*, 1987; Wolf, 1987). Only capacitated sperm are able to undergo the acrosome reaction in which the upper acrosome membrane fuses with the plasma membrane to form numerous pores and vesicles. The acrosome reaction is triggered, in part, by an influx of the calcium ion (Langlais and Roberts, 1985; Yanagimachi, 1988). The released acrosomal enzymes are necessary for sperm-egg fusion.

Sperm membrane composition

The key events that regulate fertility -- sperm capacitation and the acrosome reaction -- interrelate membrane structure and function. Biochemical events regulating the structure and fluidity of the sperm cell membrane during capacitation and the acrosome reaction may be classified as: (1) decreases in net negative charges on the sperm surface

(Vaidya *et al.*, 1971; Rosado *et al.*, 1973); (2) decreases in the cholesterol to phospholipid ratio (Davis and Gergely, 1979; Langlais *et al.*, 1981); and (3) the degradation and methylation of membrane phospholipids (Hartree and Mann, 1961; Soupart *et al.*, 1979;. Llanos and Meizel, 1983).

The lipid composition of human spermatozoa (Nissen and Kreysel, 1983) is quite similar to that of the plasma membrane of human erythrocytes (Van Deenen and De Gier, 1974). Phophospholipids make up about 70% of the total plasma membrane lipid in boar sperm. The major phospholipids of boar spermatozoa are phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylserine, phosphatidylinositol, and lysophosphatidylcholine. (Nikolopoulau *et al.*, 1985).

Sterols are the second most abundant lipid in sperm membrane. The cholesterol/phospholipid ratio of ejaculated, pre-capacitated spermatozoa is 0.99 for human (Darin-Bennet and White, 1977) and 0.12 for boar (Eddy, 1988). A portion of the steroid is sulfoconjugated (Lalumiere *et al.*, 1976). Cholesteryl sulfate, representing 2.2% of the total sterol fraction of spermatozoa, is located mostly on plasma membranes overlying the acrosome (Lalumiere *et al.*, 1976; Langlais *et al.*, 1981). Cholesteryl sulfate accounts for 20% of the surface of the head of human spermatozoa.

Glycolipids are less abundant than phospholipids and sterols in spermatozoa. Although diglycerides and triglycerides account for only 16% of the lipid composition

(Minassian and Terner, 1966), they play an important role in sperm-ovum interaction (White *et al.*, 1976).

Free fatty acids make up a relatively small amount of the lipid in boar spermatozoa (Nikolopoulau *et al.*, 1985). The major phospholipid-bound fatty acids are palmitic acid (16:0), which is a predominantly saturated fatty acid, while docosahexaenoic acid (22:6) is a major constituent of the sperm unsaturated fatty acids (Ahluwalia and Holman, 1969; Nissen and Kreysel, 1983).

The cholesterol level in sperm membrane is associated with membrane stability and permeability. Ejaculated, pre-capacitated human spermatozoa have a relatively stable membrane with a 0.99 cholesterol/phospholipid ratio and a 1 polyunsaturated/saturated fatty acid ratio (Darin-Bennet and White, 1977). The plasma membrane overlying the acrosome is essentially impermeable to calcium ions. During capacitation, the cholesterol/phospholipid ratio decreases, and the membrane permeability to calcium increases (Langlais and Roberts, 1985).

Phospholipase A_2 activity has been characterized in hamster, guinea pig, mouse, and human sperm (Llanos *et al.*, 1982; Ono *et al.*, 1982; Thakkar *et al.*, 1983, 1984). Mammalian phospholipase A_2 is an ubiquitous enzyme that hydrolyzes phospholipids at the *sn*-2 position to generate *cis*-unsaturated fatty acids and 1-acy lysophospholipids. Both products perturb the phospholipid bilayer and may act as endogenous fusogens

(Poste and Allison, 1973).

In spermatozoa, phospholipid A_2, which is most active at the neutral pH, is calcium-dependent and regulated by zinc (Thakkar *et al.*, 1984). At high concentrations, zinc inhibits sperm phospholipase A_2 activity; at low concentrations, zinc stimulates the enzyme. Removing zinc from the sperm surface by albumin has been proposed as a major event of capacitation leading to the destabilization of the plasma membrane overlying the acrosome (Johnsen and Eliasson, 1978).

Reactive oxygen species and lipid peroxidation in sperm - An overview

Spermatogenesis, the period during which dormant spermatogonia differentiate into spermatozoa, occurs over a period of about 34.6 days in mice, 49 days in rams and 64 days in humans (de Kretser and Kerr, 1988; Riley and Behrman, 1991). Maturation of mammalian spermatozoa continues while passing through the epididymis. In rats, the spermatozoa become motile and fertile in the cauda epididymis (Seligman *et al.*, 1992). After deposition in the female tract, survival of spermatozoa is limited from 30 to 45 hrs (Johnson and Everitt, 1980). If fertilization is to occur during this period, three important changes appear in the spermatozoa: capacitation, acrosome reaction and zona-sperm binding. Reactive oxygen species have been implicated in the maturation of spermatozoa,

capacitation, acrosome reaction and sperm-zona pellucida binding (Aitken *et al.*, 1989; Kumar *et al.*, 1989b; Bize *et al.*, 1991).

Mammalian spermatozoa undergo spontaneous lipid peroxidation, while producing $O_2^{\cdot -}$ and H_2O_2. Incubation of human spermatozoa at 37°C in a modified high Na^+ medium equilibrated with air resulted in the production of malonaldehyde. Spontaneous lipid peroxidation in spermatozoa increased linearly as a function of time (Alvarez *et al.*, 1987). During aerobic incubation of washed ram spermatozoa at 37°C in the presence of ascorbic acid (0.1 mg/ml) and ferrous iron (2.8 μg Fe/ml), peroxides became detectable in 30 min. Their concentration reached a maximum after 60 min (Jones and Mann, 1976).

There are diverse opinions among researchers on the endogenous source of reactive oxygen species in spermatozoa. Most evidence is that mitochondria are the origin of free radicals (Holland and Storey, 1981; Capman *et al.*, 1985); but, the endoplasmic reticulum, peroxisomes, cytoplasm, and the plasma membrane can also generate free radicals (Chance *et al.*, 1979; Ramasarma, 1982; Aitken and Clarkson, 1987; Aitken *et al.*, 1989). Holland and Storey (1981) used exogenous glucose oxidase to produce H_2O_2 in rabbit sperm. Based on this study, the role of the electron transport chain in free radical production was suggested because of the involvement of ferro-cytochrome *c*/cytochrome *c* peroxidase activity in H_2O_2

production. The mitochondrial H_2O_2 production increases with increasing amounts of substrates such as ADP, lactate, and malate. In other words, mitochondrial H_2O_2 production is modulated by ATP and ADP concentrations, and the availability of ADP (Holland and Storey, 1981). It is, therefore, hypothesized that reactive oxygen species produced in mitochondria may be a cause for membrane destabilization during capacitation.

Electron leakage from rabbit sperm mitochondria have been demonstrated using the spin label TEMPONE (Chapman *et al.*, 1985). Free radical production was decreased by rotenone in the incubation medium and increased with the presence of antimycin A and potassium cyanide, suggesting that the source of electrons was ubiquinol.

Nevertheless, Aitken and Clarkson (1987) reported that the reactive oxygen species generated in response to ionophore A23187 were not of mitochondrial origin. When mitochondrial inhibitors oligomycin, antimycin A, and rotenone were added to suspensions of human spermatozoa for 5 min before the addition of A23187, no changes in production of reactive oxygen species were observed, indicating an extra-mitochondrial source of oxygen species. Superoxide anions generated by human spermatozoa appear to be derived from an NADPH oxidase system located in the sperm plasma membrane (Aitken *et al.*, 1989). H_2O_2 can be produced by NADH oxidoreductase located at the plasma membrane (Ramasarma, 1982). Chance *et al.* (1979)

suggested the endoplasmic reticulum, peroxisomes, and cytoplasm as possible sources of H_2O_2.

Other investigators, although agreeing on the source of free radicals, speculated that sperm mitochondria act as a biological clock timing cell aging (Bize *et al.* 1991) as presented in the free radical theory of aging (Harman, 1981).

Non-motile spermatozoa produce more malonaldehyde than the motile sperm. In a sperm culture, there is a linear correlation between malonaldehyde production and the percentage of non-motile spermatozoa (Alvarez *et al.*, 1987). The rate of lipid peroxidation in human non-motile spermatozoa, assessed by malondialdehyde production, is 4 times that of sperm before a complete loss of motility. The maximum malonaldehyde production is 0.4nmol/10^8 sperm cells. Lipoperoxidative lethal endpoint, defined as the malonaldehyde produced at the time when 100% of the cells lose motility, is 0.1 ± 0.03 nmol/10^8 cells for human, 0.5 nmol/10^8 cells for rabbit, and 0.8 nmol/10^8 cells for mouse spermatozoa (Alvarez *et al.*, 1987).

Production of reactive oxygen species in spermatozoa of infertile males could exceed 40-fold of that in normal human spermatozoa. Superoxide anion is a principal component in the reactive oxygen species (Aitken and Clarkson, 1987).

Using thin-layer and gas-liquid chromatography, Jones and Mann (1976) examined the phospholipid and neutral lipid fractions obtained in spermatozoa. Lipid peroxidation caused

an approximately 50% loss of plasmalogen, 20:4 and 22:6 fatty acids, and palmitaldehyde. Lipid peroxidation decreased sperm motility and increased in sperm agglutination. The decline of motility may be due to increased in cellular permeability resulting from changes in membrane-bound lipids, or due to a direct toxic effect of lipid peroxides on the spermatozoa (Jones and Mann, 1976). A study by Aitken *et al.* (1989), however, revealed a dose-dependent induction of lipid peroxidation by A23187 without any concomitant change in motility, nor did lipid peroxidation increase agglutination of sperm in the absence of a zona pellucida.

To answer the question whether the production of reactive oxygen species influences lipid peroxidation or lipid peroxidation influences the production of reactive oxygen species in spermatozoa, Altken *et al.* (1989) examined the free-radical-generating activity of spermatozoa exposed to ferrous ion. The results suggested that peroxidation was not the primary instigator of enhanced oxygen radical production but, most likely, its consequence.

The role of free radicals in sperm maturation

As sperm mature in the epididymis, there is increased production of superoxide radicals and decreased in the activity of superoxide dismutase (Kumar *et al.*, 1989a). Studies on normal human spermatozoa revealed that there is a

low superoxide dismutase activity and high proton secretion with the acquisition of motility (Kumar *et al.*, 1989b). Spermatozoa of oligospermic individuals have a remarkably high superoxide dismutase activity and extremely low superoxide radical generation compared to normal spermatozoa (Sinha *et al.*, 1991). The production of superoxide anion radicals, which trigger oxidation of saturated phospholipids to unsaturated ones, is favored by the low activity of superoxide dismutase. A decrease in the saturated/unsaturated phospholipid ratio is necessary for an increase in fluidity of sperm membrane during epididymal maturation (Kumar *et al.*, 1989a).

In addition, during epididymal maturation the surface thiol groups are oxidized to disulfide (Shalgi *et al.*, 1989; Seligman and Shalgi, 1991). Blocking thiol groups by cobaltous ion in vitro results in an instantaneous, 100% loss of motility in the ejaculated human spermatozoa (Kumar *et al.*, 1990). This suggests that surface thiols are involved in sperm motility; they are the source of protons in motile spermatozoa. Spermatozoa from oligospermic individuals have less thiol groups on the plasma membrane than those from fertile individuals (Sinha *et al.*, 1991).

The role of free radicals in sperm capacitation

Time-course studies indicated that the production of oxygen species spontaneously changes during capacitation. In

human spermatozoa, peak production of reactive oxygen species occurs within the first two hours of incubation, followed by a progressive decline (Aitken and Clarkson, 1987). Epinephrine and albumin, two agents that cause lipid peroxidation, accelerate capacitation (Alvarez and Storey, 1983). H_2O_2 may play a physiological role during capacitation. Spermatozoa generate H_2O_2 when incubated aerobically, owing to their lack of catalase and the conversion of $O_2^{-\cdot}$ to H_2O_2 by superoxide dismutase (SOD). Incubation of sperm with exogenous catalase, which removes H_2O_2, substantially delays the acrosome reaction.

It was also found that exogenous H_2O_2 or glucose oxidase, a H_2O_2 generator, accelerated the progress of capacitation (Bize *et al.*, 1991). Reduction of membrane thiols by dithiothreitol inhibits the acrosome reaction in guinea pig spermatozoa (Flemin *et al.*, 1982). Controlled oxidation of membrane thiol groups by endogenous H_2O_2 is likely an integral part of sperm capacitation (Bize *et al.*, 1991). These observations supported the hypothesis that during capacitation H_2O_2 facilitates thiol oxidation of the sperm membranes.

The role of free radicals in sperm acrosome reaction

The acrosome is a large, flattened, secretory granule at the anterior end of the sperm head containing proteolytic and lipolytic enzymes essential for zona penetration and sperm-oocyte membrane fusion (Riley and Behrman, 1991). During the

acrosome reaction, the anterior plasma membrane and the outer acrosomal membrane fuse and the acrosomal contents are released. This membrane fusion is triggered by a calcium-ion influx. Calcium ionophore A23187, a rapid inducer of the acrosome reaction, also induces membrane lipid peroxidation (D'Agata *et al.*, 1990). Local lipid peroxidation may result in the creation of a multiphasic phospholipid arrangement within the plasma membrane, which then enhances the binding of phospholipase A_2 to its substrate (Ungemach, 1985). While the acrosome reaction has not been well characterized at the molecular level, phospholipase A_2 is believed to play a role in the enzymatic hydrolysis of fatty acids in the plasma membrane overlying the acrosome. Studies have shown that oxidants activate phospholipase A_2 by inactivating its inhibitor, lipocortin (Chakraborti *et al.*, 1989). Inhibitors of phospholipase A_2 block the acrosome reaction in vitro (Meizel, 1984;), while lysolipids (lysophosphatidyl choline, lysophosphatidyl ethanolamine, unsaturated fatty acids), products of phospholipase A_2 accelerate or induce the acrosome reaction (Langlais and Roberts, 1985). In addition, H_2O_2 may facilitate the acrosome reaction by activating phospholipase A_2 (Hirata *et al.*, 1984; Koshio *et al.*, 1988), increasing the content of lysophospholipids in, thus the fusogenicity of, the plasma membrane (Fleming and Yanagimachi, 1981).

Lipid peroxidation through activation of phospholipase A_2 increases the ability of human spermatozoa to bind to the zona

(Llanos *et al.*, 1982; Bennet *et al.*, 1987; Aitken *et al.*, 1989). Lipid peroxidation also enhances the activity of phospholipase A_2 by increasing substrate availability and affinity (Robison *et al.*, 1990). Ionophore A23187 is a potent inducer of membrane lipid peroxidation. The relationship between lipid peroxidation and sperm-zona binding was demonstrated by incubating spermatozoa with ionophore A23187. Vitamin E reversed ionophore induced the sperm-binding ability (Aitken *et al.*, 1989). However, phospholipase A_2 activity appears to be required for the generation of superoxide by NADPH oxidase (Henderson *et al.*, 1989). The enhancement of sperm-zona interaction following the induction of lipid peroxidation in vitro suggests superoxide has a normal biological role in regulating sperm function.

Sperm defense system against oxidative damage

The relative lack of vigorous cellular defense mechanisms against oxidative agents in spermatozoa, particularly after ejaculation, may be related to functional roles of peroxidation in capacitation and sperm-zona binding (Riley and Behrman, 1991). Spermatozoa are notoriously poor in catalase, and at the same time, extremely sensitive to H_2O_2 (Mann, 1958; Wales *et al.*, 1959).

In the glutathione cycle, glutathione (GSH) is oxidized to the disulfide form (GSSG) by glutathione peroxidase while

hydrogen peroxide and organic peroxides are reduced. In reverse, GSSG is reduced to GSH by glutathione reductase, a membrane-bound enzyme (Brown *et al.*, 1977; Li, 1975; Smith *et al.*, 1979). This cycle offers an effective defense mechanism against oxidative damage in several types of mammalian cells.

The efficiency of glutathione redox cycle in sperm cells varies. Mouse sperm respond to exogenous H_2O_2 with a greater rate of lipid peroxidation than human and rabbit spermatozoa, despite the highest concentration of glutathione and the greatest activity of glutathione peroxidase in the mouse spermatozoa when compared with human and rabbit spermatozoa (Alvarez and Storey, 1989). These observations indicate that the glutathione cycle provides a major protection in mouse spermatozoa against peroxidation (Chance *et al.*, 1979). Lacking glutathione and the redox enzymes, rabbit spermatozoa rely mostly on superoxide dismutase to scavenge superoxide anion (Alvarez and Storey, 1984).

Human seminal plasma has been shown to possess antioxidant properties. Peroxidative reactions and the resultant loss of motility in washed sperm, caused by incubation with exogenous superoxide generators or lipid peroxides, can be prevented but not reversed by seminal plasma (Kocak *et al.*, 1990). The protective action in seminal fluid is heat stable and associated with multiple components. Furthermore, the antioxidant activity of seminal plasma is not sperm specific; it also protects brain homogenates against

spontaneous autoxidation (Kocak *et al.*, 1990).

Summary

After the completion of spermatogenesis, sperm maturation, capacitation, and the acrosome reaction make up a sequence of additional membrane changes required for all mammalian spermatozoa to gain fertilizing ability. These changes, occurring under fine regulatory controls, are sensitive to chemical and physical perturbation, whether from an intrinsic source or an extrinsic environment. A fine-tuned interaction between free radical activity and the defense systems results in an appropriate amount of lipid peroxidation to regulate sperm capacitation and acrosome reaction. The nature of the lipid peroxidation reaction, that occurs in all living cells, can be most conveniently studied in spermatozoa; these cells are available as free intact cells, and their motility and in vitro-fertilizing ability may be used as an index of their viability.

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CHAPTER IV

LITERATURE REVIEW III: THE TOXICITY OF ETHYLENE BISDITHIOCARBAMATES (EBDCs)

Over the past four decades, enormous improvements in food supply and nutrition have been accomplished through advances in agricultural practices including use of pesticides. Use and the impact of pesticide on applicators and farm workers, and pesticide residues in food are regulated by the Environmental Protection Agency (EPA), under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Recently, a new dimension has also been added to public concerns about the use of chemicals on farms: the detection of agrochemicals in food and potable groundwater (Anon, 1988a, 1988b).

Sources of exposure and general concerns over EBDCs

Dithiocarbamate compounds are an important class of organic fungicides. They are not only used all over the world in agriculture to control many diseases in a variety of crops, but they also have important uses in the rubber industry, and the field of medicine (Hill, 1992). In agriculture, a few dithiocarbamates are used for soil sterilization; while others are used as bird and animal repellents. The most widespread use of dithiocarbamates has involved protectant fungicides.

Frequently used ethylene bisdithiocarbamates (EBDCs) include maneb, mancozeb, metiram, nabam and zineb. The global market was estimated at $525 million in 1984. In the United States, more than 30 million pounds are used annually to control a wide variety of fungal diseases on fruits,

vegetables, field crops, seeds, and ornamental plants. Approximately one-third of all fruits and vegetables in the United States are treated with EBDCs (BANRC 1987).

This class of fungicides has been regarded as relatively harmless because of its low acute toxicity to mammalian. The oral LD_{50} in rats is 6,750 mg/kg bw for maneb, >5,200 mg/kg bw for zineb, and >8,000 mg/kg bw for mancozeb. The LD_{50} in mice is 8,000 mg/kg bw for maneb, and 7,600-7,700 mg/kg bw for zineb (Hayes, 1982; Lentza-Rizos, 1990). Some subchronic and chronic toxicity studies showed that EBDCs cause leucopenia in rabbits. In rats, EBDCs also change the permeability of blood-tissue barriers and decrease weights of several internal organs. The affected organs include the kidneys, adrenal glands, and ovaries. Changes in the immunological structures of the spleen and goitrogenic activity in albino rats, and increases in thyroid weight in Wistar rats have also been reported (Lentza-Rizos, 1990). No tumorigenicity was reported for maneb, zineb, or nabam in chronic feeding studies utilizing three strains of mice (Fishbein, 1977).

The concern over EBDCs as a residue of agricultural products is intensified by the recently recognized toxicity of their common metabolite and degradation product, ethylene-thiourea (ETU). ETU is present in nearly all commercial formulations of EBDCs. It is formed during environmental degradation and heat processing of food containing EBDC residues (Meneguz *et al.*, 1987). Because of the report of

their carcinogenic, goitrogenic, and teratogenic effects in laboratory animals, EBDCs have become a major human health concern among some consumer groups (Lentza-Rizos, 1990).

Absorption, distribution, metabolism and excretion of EBDCs

In rats, about 55% of maneb, delivered by stomach tube in a single dose of 333 to 390 mg/kg bw, was excreted in feces and urine within 5 days. The metabolites included ethylenediamine, ethylene-bis-thiuram monosulfide, and ethylene-bis-thiourea (Seidler *et al.*, 1970). In mice, the percentages of delivered ^{14}C-maneb found in the feces and urine within 48 hours were 91 to 93% and 7 to 9%, respectively. In the urine, 8 to 16% of the radioactivity was ETU. Other metabolites were more polar than ETU (Jorden and Neal, 1979). No maneb, as the parent compound, was found in the tissues of rats fed at levels as high as 2,500 ppm for 2 years, nor was any found in the tissues of dogs receiving 75 mg/kg bw/day for one year (FAO/WHO, 1968).

In rats, only 11-17% of an oral dose of zineb was absorbed from the gastrointestinal tract (Smith *et al.*, 1953), while about 50% of mancozeb was absorbed. Mancozeb is extensively metabolized in rat. ETU was the major metabolite found in the rats' feces and urine. Of the total radioactive dose, approximately 18.76% was identified as ETU in the urine and 5.2% in the bile. The amount of bioavailable ETU was

estimated to be 23.96% of the total dose (EPA, 1989).

EBDC interaction with ethanol

Ethanol intake after treatment with disulfiram increases the concentration of acetaldehyde in the blood, which causes illness, such as apnea, and decreases blood pressure in humans. The illness may also be caused by the accumulation of disulfiram metabolite, carbon disulfide (Stromme, 1965). Aversion to ethanol has been noted in humans occupationally exposed to carbon disulfide (Novak *et al.*, 1969).

Antithyroid effects of EBDCs

In rats, maneb at a concentration of 5,000 ppm in the diet for 6 weeks reduces iodine-uptake in the thyroid function test (Bankowska *et al.*, 1970). Calves fed zineb at 200 mg/kg body weight daily for 80 days had decreased concentrations of triiodothyronine (T3) and thyroxine (T4) in the serum and an increased weight of the thyroid gland.

Pathologic changes of the thyroid gland are epithelial vacuolization and foci of hyperplasia (Nebbia, 1991). Maneb and zineb may inhibit the synthesis of thyroid hormones by blocking the conversion of inorganic iodide into organically bound iodine (Ivanova *et al.*, 1967).

In another rat study, treatments with either 2,500 ppm

maneb in water for two years or 300 to 10,000 ppm zineb in water for 30 days decreased iodine uptake by the thyroid (Ivanova *et al.*, 1967). Early thyroid hyperplasia caused by a dietary level of 1,000 ppm of zineb is reversible. A complete recovery occurs less than 2 weeks after the treatment was discontinued (Bankowska *et al.*, 1970).

The most prominent aspect of ETU toxicity in the adult rat is its action as a goitrogen. It causes reversible hyperplasia of the thyroid and decreases circulating thyroid-hormone levels (Daston *et al.*, 1987). Kurttio *et al.* (1986) examined the altered function and morphology of thyroid glands in ETU-treated rats. After treatments of 0 to 300 mg/kg bw/d ETU in the drinking water for 28 days, ETU inhibited T3 and T4 secretion. This inhibition was dose-dependent. ETU also increased the basal TSH secretion 10-fold due to a lack of negative feedback by T4 and T3.

Reproductive toxicology and teratogenicity of EBDCs

Maneb, zineb, and mancozeb are maternally toxic at high doses in rats and less toxic in mice, hamsters, and guinea pigs (FAO/WHO, 1981). No-observable-effect levels (NOEL) were 250 mg/kg diet, equivalent to 12.5 mg/kg bw/d, for maneb in rats; 100 mg/kg diet, equivalent to 5 mg/kg bw/d, for mancozeb in rats; and 2,000 mg/kg diet, equivalent to 50 mg/kg bw/d, for zineb in dogs. Based on these levels, participants of the

1980 Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues (JMPR) derived the Acceptable Daily Intake (ADI) of 0 to 0.05 mg/kg bw for humans, of which no more than 0.002 mg/kg bw may be present as ETU. This ADI applies to maneb, mancozeb, and zineb individually or the sum of any combination of them (FAO/WHO, 1981).

Maneb and zineb cause infertility in laboratory animals (Shtenberg *et al.*, 1969). Both male and female rats receiving maneb at 39 mg/kg bw/d for 11 or 12 months showed decreased fertility and increased stillbirths and neonatal deaths. In a standard three-generation rat study, maneb at dietary levels of 0, 125, and 250 ppm, caused no effect on fertility, gestation, viability, lactation, or litter size, and no gross abnormalities were found in the animals (Hayes, 1982). Ryazanova (1967) reported that oral doses of zineb at 100 mg/kg/day for 2 months or longer produced sterility, resorption of fetuses, and anomalous tails in newborn rats.

The development toxicity of mancozeb in rabbits was also reported (Solomon and Lutz, 1989). Mancozeb was administered by gavage to artificially inseminated New Zealand white rabbits at doses of 10, 30, or 80 mg/kg bw on days 7 to 19 of gestation. On day 29 of gestation, rabbits were examined for reproductive and other pathologic changes. Body weight, feed consumption, and clinical signs were not different among the control, the 10 mg/kg bw, and the 30 mg/kg bw treated groups.

No treatment-related malformation in the fetuses was detected. Two treatment-related deaths and 5 abortions were noted in the 80 mg/kg bw group. The NOEL was 30 mg/kg bw for the doe and 80 mg/kg bw for the conceptus.

The teratogenicity of ETU in rats and other species was reviewed by Khera (1987). ETU has a low acute toxicity with a LD_{50} of 1,832 mg/kg bw for male rats and 545 mg/kg bw for female rats. The LD_{50} is 4,000 mg/kg bw for the adult mice of both sexes (Khera, 1987). The no-maternal-toxic dose in rats is less than 80 mg/kg bw and the no teratogenic dose is 10 mg/kg bw (FAO/WHO, 1981). ETU produces hydrocephalus in fetal rats through a single maternal oral dose of 30 mg/kg bw, which is three times lower than the oral NOEL dose in the dam and about 18 times lower than the LD_{50} for pregnant rats (Lentza-Rizos, 1990).

ETU is also a neuroteratogen; it caused necrosis of the neuroblast when a single oral dose of 30 mg/kg bw or higher was administered between day 13 and 20 of pregnancy. A single dose ranging from 30 to 60 mg/kg bw delivered on any day from the 15th to the 19th day of pregnancy produced dose-related postnatal hydrocephalus and death. An increase in the dose from 80 to 480 mg/kg bw resulted in additional teratogenic effects on the alimentary tract, and the skeletal and the urogenital systems (Lentza-Rizos, 1990).

Carcinogenicity

Non-tumorigenicity was reported for maneb, zineb and nabam in chronic feeding studies on three strains of mice (Lentza-Rizos, 1990). Mancozeb produced skin tumors in mice at 100 mg/kg bw, 3 times per week for 31 weeks. Histological examination revealed that these tumors were mostly benign squamous-cell papillomas and keratoacanthomas (Shukla *et al*, 1990). ETU at the oral dose of 215 mg/kg bw/day was tumorigenic in mice (Innes *et al*., 1969). McGregor *et al*. (1988) reported that ETU was a potent mutagen in L-5178Y $tk^{+/-}$ mouse lymphoma cells.

Ethyleneurea (EU) is a main degradation product of ETU (Rhodes, 1977). EU by itself is an innocuous oxidation product. In the presence of nitrite, however, it readily forms dimethylnitrosamine, a potent tumorigenic agent in rats (Marshall, 1978). When ziram and nitrite solutions were introduced simultaneously into rats' stomachs, dimethyl-nitrosamine was detectable within 15 minutes (Eisenbrand *et al*., 1974).

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CHAPTER V

DIETARY MANEB AND ZINEB IN B_6D_2-F_1 MICE: REPRODUCTIVE PERFORMANCE AND THE FERTILIZING ABILITY OF SPERMATOZOA IN VITRO

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INTRODUCTION

Maneb and zineb are two of the ethylene bisdithiocarbamate (EBDC) fungicides widely used in this country. About one-third of all fruits and vegetables in the United States are treated with EBDCs (BANRC, 1987). In rats, thiram, maneb and zineb have antithyroid effects (Hayes, 1982). Both maneb and zineb cause malformations and infertility in laboratory animals. With a single oral dose of 770 mg/kg bw on day 11 of pregnancy, maneb produced malformations in all pups observed (Larsson *et al.*, 1976). Infertility was observed in rats when both female and male were treated orally with zineb at 100 mg/kg bw/day for 2 months. The same dose caused fetal resorption and tail abnormalities in the offspring (Ryazanova, 1967). Rats showed decreases in fertility and increases in stillbirth and neonatal deaths after receiving 30 mg maneb/kg bw/day for 11-12 months. The no observable effect level for long-term oral exposure in rats was 5 mg/kg bw/day for maneb (Shtenberg *et al.*, 1969), and 50 mg/kg bw/day for zineb (Ryazanova, 1967).

Studies of EBDC effects on fertility in mice are lacking, although a few developmental studies have been reported. Larsson *et al.* (1976) reported that 770 mg/kg bw of maneb, the oral dose that caused malformation in rats, did not cause

reproductive or developmental abnormalities in mice. However, increased incidence of abortions and stillbirths was observed in mice treated with two intraperitoneal injections of zineb at 320 mg/kg bw during the second half of gestation (Kvitnitskaya and Kolesnichenko, 1971). After 10 oral gavage doses of 1200 mg/kg B.W., from day 6 to 15 of gestation, increases in bent tails, reduced ossification, and high fetal mortality in mice were observed (Beck, 1990).

We report in this study effects of mixtures of maneb and zineb on reproduction in mice. Treatment doses were higher than previously reported no-observable effect levels, but lower than known toxic levels. The objective of this study was to examine the possible reproductive toxicities of mixtures of maneb and zineb in mice.

MATERIALS AND METHODS

Chemicals and media. Maneb and zineb (95.0 % purity) were obtained from Chem Service, West Chester, PA. Brinster's medium (BMOC-3) for gamete collection and fertilization was purchased from Gibco (Grand Island, N.Y) and adjusted to a pH of 7.34 and an osmolarity of 288 mOSM. All other chemicals were from Sigma Chemical Co. (St. Louis, MO). BMOC-3 without bovine serum albumin (BSA) was prepared with 119.4 mM sodium chloride, 4.78 mM potassium chloride, 1.70 mM calcium chloride, 1.19 mM potassium phosphate, 1.19 mM magnesium

sulfate heptahydrate, 25.1 mM sodium bicarbonate, 5.56 mM glucose and 1 mM sodium pyruvate. The media for gamete collection and fertilization were equilibrated for 12-18 h in 5% CO_2 in air and at 37°C immediately before use.

Animals and treatments. Twenty four male B_6D_2-F_1 mice, which were the first generation of C57BL-6J females and DBA-2J males, were randomly divided into four groups at 6 weeks of age. Treatment diets contained equal weights of maneb and zineb, at the total concentration of 0, 30, 300 and 3000 ppm in ground Mouse Chow #5015 (Purina Mills Inc., St Louis, MO). Mice were treated with the maneb/zineb diet for 15 weeks. Body weights were recorded weekly. Fresh feed was supplied daily. Feed intake was recorded by weighing the remaining from the previous day.

After 9 weeks of treatment, males were individually paired with previously non-treated, 12-week-old females for 7 days. Females received the corresponding diet at the beginning of breeding and through gestation and lactation. After weaning at 3 weeks of age, offspring were fed the corresponding diet for an additional 4 weeks. Litter size, sex ratio, mortality and body weight of the offspring were recorded on day 1, 4, 21 and 49 postpartum. The animal room was maintained at 21°C, 55 ± 5 % humidity, and 14h/10h light/dark cycle. Treatment diets and water were provided *ad libitum*.

In vitro fertilization. After 15 weeks of treatment, the fertilizing ability of cauda epididymal sperm was examined by in vitro fertilization of eggs from non-treated females. Each pair of epididymides was excised and punctured with a 25 G needle in 1 ml BMOC-3. Sperm were released and incubated at 37°C and 5% CO_2 in air in an organ culture dish (Falcon 3037, Cockeysville, MD) for 2 h before insemination. Eggs were obtained from 4- to 12-week-old B_6D_2-F_1 females that had been superovulated by intraperitoneal injections of 10 IU of pregnant mare's serum gonadotropin (PMSG) followed by 10 IU of hCG 48 to 50 h later. Thirteen hours after human chorionic gonadotropin (hCG) injection, cumulus enclosed eggs were collected from oviducts into 1 ml BMOC-3 in an organ culture dish and inseminated with 50 ul sperm suspension (1 to 2.4 x 10^7 cells/ml). The final sperm concentration was 0.5 to 1.2 x 10^6 cells/ml. In previous studies in our laboratory, maximum percentage of in vitro fertilization, 80 to 100%, was achieved by sperm concentration of 0.1 x 10^5 cells/ml. Sperm from each male inseminated eggs from two females. Twenty-four h after insemination, nuclei were stained for 30 min with 50 ul 0.375 mM bisbenzimide trihydrochloride. Eggs were then washed in 0.5 ml BMOC-3 without BSA and transferred to glass slides for assessing fertilization with an inverted phase-contrast microscope (200x). Eggs at the two-cell stage or one-cell stage with two pronuclei and a second polar body were considered as fertilized.

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Statistical analyses. Body weight gain, feed intake and litter size were analyzed using the one-way ANOVA procedure. Body weight and body length of offspring were analyzed using the General Linear Models (SAS institute, Inc., Cary, NC, $p < 0.05$). Where significant differences between treatment responses were noted, multiple comparison tests were conducted using the Least Significant Differences test. Statistical analyses of fertility, in vitro fertilizing ability, mortality of offspring and sex ratio of offspring were performed using Chisquare procedures (MINITAB Inc., State College, PA, $p < 0.05$). Logarithmic transformations were made for the ratios of testis weight/body weight. Statistical analyses of the transformed values were made with one-way ANOVA ($p < 0.05$).

RESULTS

After the 15-week treatment, only the 300 ppm treatment group showed a marginal decrease in total body weight gain relative to the controls (Table 5-1, $p < 0.1$).

Treatments did not affect feed intake, number of females giving birth or mortality of offspring (Table 5-2). Litter size tended to decrease and mortality before weaning tended to increase in the 3000 ppm treatment group (Table 5-2).

TABLE 5-1. Body Weight Gain of Male Mice Treated with Mixtures of Maneb and Zineb for 15 weeks

Treatment (ppm)[a]	No. of mice	$\bar{x}$[b]
0	6	11.1 ± 0.9
30	6	10.8 ± 0.7
300	6	8.9 ± 0.7[c]
3000	6	9.5 ± 1.3

[a] Mixtures of maneb and zineb (ratio = 1:1, w/w) in the diet.

[b] Values represent the means of body weight gain ± SE during the treatment period.

[c] $p < 0.10$ vs controls.

TABLE 5-2. Reproductive Performance of Female Mice Bred with Males Pretreated with Maneb/Zineb

Treatment (ppm)[a]	No. of females giving birth/No. of females paired	Litter size[b]	Offspring mortality (%)[b]
0	5 / 6	8.8 ± 1.6	2.0 ± 1.8
30	5 / 6	9.8 ± 0.3	4.0 ± 3.6
300	6 / 6	8.8 ± 1.4	4.2 ± 3.8
3000	5 / 6	7.2 ± 1.2	8.9 ± 4.9

[a] Male mice were treated for 9 weeks before pairing with previously non-treated females. Both males and females received treatment diet during breeding and the females were treated throughout gestation and lactation.

[b] Values are means ± SE of 5 or 6 litters from birth to day 4. No statistically significant differences from the control ($p < 0.05$) were observed.

Body weight and body length of offspring were frequently increased in all treatment groups before weaning (Table 5-3). At week 7 postpartum, offspring from the 300 and 3000 ppm treatment groups were 10% and 8% heavier than controls ($p < 0.05$), yet the body length was similar among all treatment groups. Although testis weights for all treatments were similar, there was a decrease ($p < 0.1$) in the testis/body weight ratio in the high-dose group (Table 5-4). the sex ratio of offspring was not different among treatments.

The in vitro fertilizing ability of epididymal sperm from mice treated with 3000 ppm maneb/zineb was 67% of controls (Table 5-5, $p < 0.01$). The number of degenerating eggs increased in the 3000 ppm groups. There were no differences in the first embryonic division in all treatments. Twenty four h after insemination, more than 95% of fertilized eggs were at the 2-cell stage.

DISCUSSION

This study suggests the maneb/zineb at 3000 ppm in the diet of mice affects sperm fertilizing ability. Testis weights of the offspring were similar among treatments. The ratio of testis to body weight decreased, reflecting the larger size of offspring from dams exposed to test chemicals. The increase in body weight and body size of offspring from treated dams is not surprising, since this is frequently observed in animals treated with low doses of an antimicrobial agent.

TABLE 5-3. Body Weight and Body Length of Offspring from Maneb and Zineb Treated Mice[a]

	day 1		day 4		day 21		day 49	
Treatment (ppm)	B.W.[b] (g)	B.L.[b] (mm)	B.W. (g)	B.L. (mm)	B.W. (g)	B.L. (mm)	B.W. (g)	B.L. (mm)
0	1.3±0.03 (44)	28±0.2 (44)	2.1±0.04 (44)	31±0.5 (44)	9.1±0.2 (43)	56±0.7 (43)	23.8±0.5 (43)	80±0.6 (43)
30	1.4±0.03[c] (49)	28±0.2 (49)	2.4±0.05[c] (48)	33±0.2[c] (48)	10.6±0.2[c] (47)	59±0.5[c] (47)	24.4±0.5 (47)	80±0.5 (47)
300	1.3± 0.03 (58)	27±0.2 (58)	2.2±0.04 (55)	32 ± 2 (55)	10.8±0.1[c] (55)	58±0.5[c] (55)	26.2±0.7[c] (55)	81±0.5 (55)
3000	1.4± 0.03[c] (36)	29±0.2[c] (36)	2.1±0.06 (36)	32 ± 2 (36)	9.9±0.3[c] (32)	55±0.6 (32)	25.8±0.8[c] (32)	80±0.5 (32)

[a] Male mice were treated for 9 weeks before pairing with previously non-treated females. Both males and females received treatment diet during breeding and the females were treated throughout gestation and lactation. The offspring received the corresponding diet from weaning to 49 days of age.

[b] Values are means ± SE of offspring observed. Number of observations are indicated in the parentheses.

[c] Denotes statistically significant difference from control ($p < 0.05$)

TABLE 5-4. Testis Weights (TW) and Testis Weight/Body Weight (TW/BW) Ratios of Offspring at 7 Weeks of Age

Treatment[a] (ppm)	No. of male observed	TW (g)[b]	TW/BW ratio[c] (10^3)
0	20	0.1844 ± 0.0320	7.0 ± 0.3
30	22	0.1792 ± 0.0216	6.6 ± 0.2
300	32	0.1968 ± 0.0223	6.8 ± 0.2
3000	16	0.1883 ± 0.0415	6.5 ± 0.4[d]

[a] After 9 weeks of treatment, males were individually paired with previously non-treated, 12-week old females for 7 days. Females received the corresponding diet at the beginning of the breeding and through gestation and lactation. After weaning, the offspring were on the corresponding diet for additional 4 weeks.

[b] Values are means ± SD of testis weights.

[c] Values are means ± SE of testis weight/body weight ratios.

[d] $p < 0.10$ vs controls.

TABLE 5-5. In Vitro Fertilizing Ability of Epididymal Sperm from Male Mice Treated with Mixtures of Maneb and Zineb for 15 Weeks

Treatment (ppm)	No. of eggs observed[a]	No. of eggs fertilized	No. of eggs degenerated	$\bar{x}$ of fertilization (%) ± SE	$\bar{x}$ of degeneration (%) ± SE
0	100	70	1	69 ± 5	1 ± 1
30	95	59	0	64 ± 2	0 ± 0
300	142	100	3	66 ± 7	3 ± 3
3000	90	44	15	46 ± 10[b]	17 ± 7[b]

[a] Data were pooled from 4 experiments.
[b] Denotes statistically significant difference from the control ($p < 0.01$).

Based on feed intake, 30, 300 and 3000 ppm maneb/zineb in the diet are equivalent to 4, 40 and 400 mg/kg bw/day, respectively. Smith *et al.* (1953) demonstrated that 11-17% of ingested zineb was absorbed from the gastrointestinal tract of rats. At least 50% of ethylene bisdithiocarbamate in maneb was absorbed from the gastrointestinal tract, while no detectable manganese was absorbed. Since simultaneous administration of Fe^{3+}, Zn^{2+} and Cu^{2+} reduced the maneb absorption (Brocker and Schlatter, 1979), the absorption of maneb from the maneb/zineb diet is likely to be less than that of maneb alone in the diet.

In this study, in vitro fertilization is a more sensitive method of testing sperm fertilizing ability than pregnancy rate and litter size. The mean litter size of dams bred to males receiving 3000 ppm maneb/zineb in the diet was 7.2, which is 1.6 less than the control value. This magnitude of change in litter size would require an experiment with 50 or more mice per treatment to demonstrate a statistically significant difference at $p < 0.05$. However, when sperm quality was measured in vitro, only 4 epididymides per treatment were necessary to demonstrate a 33% decrease in sperm fertilizing ability. This significant decrease in sperm fertilization ability, consistent with marginal decreases in litter size, suggests effects of maneb/zineb on male fertility. The results, however, do not rule out the possibility that maneb/zineb could decrease early embryonic

survival. The no observable effect level for sperm fertilizing ability obtained in this study is 300 ppm.

The concern over EBDCs as a residue from agricultural application is heightened by knowledge of the toxicity of its metabolite and degradation product, ethylenethiourea (ETU). ETU is present in commercial formulations of EBDCs. In addition, ETU is formed during biotransformation, environmental degradation, and heat processing of food containing EBDCs (Kurttio *et al.*, 1990; Nebbia, *et al.*, 1991; Meneguz *et al.*, 1987). ETU has been shown to possess carcinogenic, goitrogenic, and teratogenic activities in animals (Lentza-Rizos, 1990).

It has been hypothesized that ETU toxicity is related to the highly reactive form of atomic sulfur released during the metabolic formation of ethyleneurea (Hayes, 1982). The reactive sulfur may cause excessive oxidation of sperm membranes. The hypothesis relating maneb/zineb effects to oxidative homeostases of sperm membranes, however, is complicated by the presence of Zn^{2+} in the formulation. The three treatment diets in this study contained 3.6, 36, and 360 ppm Zn^{2+}, respectively. Approximately 20-30% ingested zinc is absorbed (Goyer, 1991). Zinc ion has been demonstrated as an inhibitor of $O_2^{\cdot}/H_2O_2$-mediated cellular membrane lysis in erythrocyte ghosts (Girotti *et al.*, 1985). Zinc ion inhibits sperm capacitation and fertilization in vitro (Aonuma *et al.*, 1981). When zinc was added to capacitation medium, sperm

failed to acquire fertilizing ability. However, the fertilization rate was not affected when eggs were inseminated with precapacitated sperm in the presence of zinc. An excessive amount of Zn^{2+} may also inhibit sperm phospholipid A_2 (Thakkar *et al.*, 1984), which functions, during capacitation, by destabilizing the plasma membrane overlying the acrosome (Johnsen and Eliasson, 1978).

There is growing evidence pointing to a physiological role of H_2O_2 in sperm capacitation and fertilizing ability. Both exogenous H_2O_2 and glucose oxidase, an enzyme that generates H_2O_2, accelerate the onset of the acrosome reaction (Bize *et al.*, 1991). Earlier studies also suggest that H_2O_2 induces membrane lipid peroxidation, which facilitates membrane fusion in acrosome reaction and sperm-zona interaction (Aitken *et al.*, 1989; Gopalakrishna and Anderson, 1989; Riley and Behrman, 1991). The overall effect of maneb/zineb exposure on the oxidative status of sperm is, therefore, uncertain.

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CHAPTER VI

ELECTRON SPIN RESONANCE AND IN VITRO FERTILIZATION STUDIES OF ETHYLENE THIOUREA TOXICITY IN SPERMATOZOA

INTRODUCTION

Ethylene bisdithiocarbamates (EBDCs) are a group of widely-used fungicides for fruits and vegetables. The common metabolite of EBDCs, ethylene thiourea (ETU), has been found in exposed animals and plants, as well as in commercial EBDC formulations (Nebbia *et al.*, 1991; Kumar and Agarwel, 1993; Kurttio *et al.*, 1990; Engst and Schnack, 1974). In addition to biotransformation, heat also facilitates the conversion of EBDCs to ETU during food processing (IUPAC, 1976). Animal studies have shown that ETU caused thyroid-gland hyperplasia (Griepentrog, 1962; Ivanova *et al.*, 1967; Stanisstreet *et al.*, 1990) and increased in thyroid and liver tumors in mice and rats (Innes *et al.*, 1969; Fitzhugh *et al.*, 1948). Maternal exposure to ETU also causes hydrocephalus in rat fetuses without observable effects on the dams (Khera, 1987; 1989). In the study described in the previous chapter, the sperm fertilizing ability decreased in male mice exposed to 3000 ppm mixtures of maneb and zineb (w:w=1:1) in the diet. The treatment diet was provided *ad libitum* from 6 to 21 weeks of age. A marginal decrease in litter sizes was observed when non-treated females were bred by males treated with 3000 ppm maneb/zineb.

It has been hypothesized that ETU toxicity is related to

a highly reactive form of atomic sulfur released during the metabolic formation of ethyleneurea (Hayes, 1982). Therefore, the objectives of this study were to examine the ETU effects on fertilization in vitro and free radical production in ETU treated spermatozoa.

MATERIALS AND METHODS

Animals. B_6D_2-F_1 mice, which were the first generation of C57BL/6J females and DBA-2J males, were used in this study. The animal room was maintained at 21°C, 55 ± 5% humidity, and 14h/10h light/dark cycle. Mouse Chow #5015 (Purina Mills Inc., St Louis, MO) and water were provided ad libitum.

In vitro fertilization. Sperm were collected from epididymides of mature males. Each pair of epididymides were excised and submersed in 1.0 ml Brinster's medium (BMOC-3), a capacitation supporting medium. Sperm were released by poking epididymides with a 25G PrecisionGlide needle and incubated for 1 h at 37°C and 5% CO_2 before insemination. Eggs were obtained from 4 to 12 week-old B_6D_2-F_1 females superovulated with intraperitoneal injections of 10 IU of pregnant mare's serum gonadotropin (PMSG) followed, 48 to 50 hrs later, by 10 IU of human chorionic gonadotropin (hCG). Thirteen hours after hCG administration, cumulus enclosed eggs were collected into 1 ml BMOC-3.

For insemination, eggs in cumulus masses and 50 ul sperm suspension (1.0 to 2.5 x 10^7 cells/ml) were transferred into an organ dish containing 1 ml BMOC-3. The final sperm concentrations were 0.5 to 1.25 x 10^6 cells/ml. In previous studies in our laboratory, maximum percentage of fertilization, 80 to 100%, was achieved by sperm concentration of 0.1 x 10^5 cells/ml. ETU, at the concentration of 0, 1, 5, 10, 25, 50, 100, 200, 400 or 800 ppm, was present in the media for gamete collection and insemination. After 25 to 27 hr incubation, 50 ul 0.375 mM bisbenzimide trihydrochloride (Sigma Chemical Company) were added and the gametes were incubated for additional 30 min for nuclear staining. Eggs were then washed in 0.5 ml BMOC-3 without bovine serum albumin (BSA). Eggs were assessed for fertilization with an inverted phase-contract microscope (200 x). Eggs at the two-cell or at one-cell stage with two pronuclei and a second polar body were considered fertilized. Fragmented, degenerated, and one-cell eggs without multiple nuclei were considered non-fertilized.

Sperm motility. Sperm from two epididymis of each male mouse were collected, as described in the methods for in vitro fertilization, into separate culture dishes: sperm from the left epididymis were collected into BMOC-3 containing 1000 ppm ETU, while those from the right one into BMOC-3 as controls. Sperm motility was measured with a CellSoft Sperm Analyzer at 0, 1, 2, 3, 4, and 5 hours after sperm collection. A Nikon-

OPTIPHOT microscope equipped with a 20x objective lens and a 10x eyepiece was fitted to the sperm analyzer. A threshold curvillinear velocity of 20 microns/sec was used to define a motile sperm. The pixel scale was set at 2.880 micron. Minimum and maximum sizes to exclude an object from analysis were 5 and 27 pixels, respectively. At least 100 cells were analyzed in each sample.

Sperm mitochondrial function. The sperm mitochondrial function was determined by rhodamine 123 (R123, Sigma, St. Louis, MO) uptake using fluorescence flow cytometry, as described by Graham *et al.* (1990), who applied R123 to assess mitochondrial function in bull sperm based on the studies by Evenson *et al.* (1982) and Evenson and Ballachey (1986). Sperm, collected as described for motility analysis, were incubated for 15 min at 37°C before being transferred into a FALCON 2054 polystyrene, round-bottom tube (BECTON DICKINSON). Rhodamine 123 (20 μl of 0.01mg/ml in water) was added to 1 ml sperm suspension and spermatozoa were incubated for additional 20 min before centrifugation (300 xg, 5 min) and resuspension in 1 ml BMOC-3. Final sperm concentrations were 2 to 5 $x10^6$ cells/ml.

A Becton Dicksen Vantage flow cytometer equipped with an ILT air-cooled argon laser was used to analyze the fluorescent intensity at 0, 1, 2, 3, 4, and 5 hr after sperm collection. Sperm cells were excited by 488 nm line and detected with a

530 ± 30 nm band pass filter. Linear scaling of green fluorescence was used and the mean of fluorescence intensity was calibrated as the sample fluorescence intensity. Data was collected on an NP Consort 32 computer and analyzed using PC Lysis (version 1.0) on an NP Vectra PC. Ten thousand cells were analyzed in each sample.

Detection of free radicals. Sperm lipid-peroxyl radicals were examined by an Varian E-4 ESR spectrometer, using spin trap [α-(4-pyridyl-1-oxide) N-t-butylnitrone] abbreviated as 4-POBN. Free radical production from intact sperm was measured by adding 0.03 ml, 1 M 4-POBN and 0.03 ml, 10 mM $FeCl_2$ into 0.24 ml sperm suspension. The final reaction solution contained 8 to 10 x 10^6 sperm cells/ml. After being gently stirred for 2 to 3 sec, the reaction solution was transferred into a flat quartz cell and ESR spectra were recorded. The operation condition was set at microwave power, 20 mW; modulation intensity, 0.8 G; time constant, 1.0 s; scan time, 8 min; modulation frequency, 100 kHz; microwave frequency, 9.508 GHz; scan range, 100 G; temperature, 20°C; and receiver gain, 2.5 x 10^4.

Statistical analyses. In vitro fertilizing ability and sperm motility were analyzed by the Chisquare procedure (MINITAB Inc., State College, PA) at the 0.05 significance level. The relative values of the mitochondrial function were transformed

according to arcsin root transformations and analyzed by a Paired T-test (SigmaStat, Jandel Scientific, San Rafael, CA).

RESULTS

Effects of ETU on fertilization in vitro

No inhibitory effects of 1 ppm ETU on fertilization were observed. At 5, 10, 25 and 50 ppm, fertilization was inhibited to about 80 % of that seen in the controls (Figure 6-1). At 100 ppm, fertilization was inhibited to 59%. At 800 ppm, no eggs were fertilized. In the controls, 80 to 90% of the eggs were fertilized. No differences in the numbers of degenerated eggs were observed among all treatment groups. All fertilized eggs observed were at the two-cell stage except the two at one-cell and 2-pronuclei stage: one was treated with 1 ppm ETU and the other with 400 ppm ETU.

Effects of ETU on sperm motility

In the non-treated spermatozoa, motility decreased steadily from 74% at the second hr to 50% at the fifth hr. The effects of ETU on sperm motility varied, depending on the sampling time. At 1 hr, sperm motility in the 1000 ppm ETU group was 25% lower than that of the controls ($p < 0.05$). At 3 hr, however, motility was 23% higher. No differences between

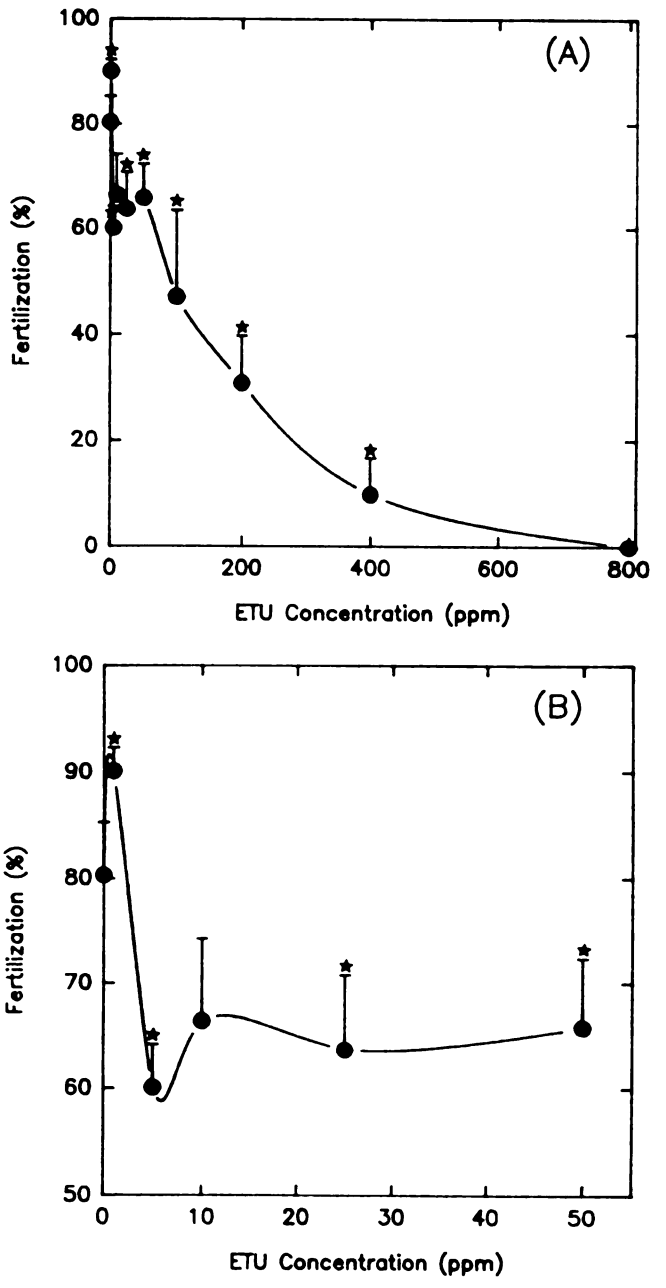

Figure 6-1. The in vitro fertilizing ability of epididymal sperm in the presence of ETU. ETU was present in both capacitation and insemination media. Values are means ± SE of the percentage of eggs fertilized. (A) represents the overall in vitro fertilization results. (B) represents the results of in vitro fertilization in the presence of ETU at the concentrations of 1 to 50 ppm.
* Denotes statistically significant difference from the control ($p < 0.05$).

control and treatment groups were observed at 0, 2, and 4 hrs (Figure 6-2). At 5 hr, the motility of sperm treated with ETU again was 14% lower than the control.

Effects of ETU on mitochondrial function

In the control spermatozoa, the fluorescence intensity of R123 declined continuously during the 5 hr observation period (Figure 6-3). At the end, only 25% of the original intensity remained. The rate of decline for the R123 uptake was divided into 3 phases: 0 to 1, 1 to 3, and 3 to 5 hrs. The highest of decline rate occurred during the second and third hrs, which was about 4 to 5 times faster than the initial rate and the rate at the fourth and the fifth hr. The initial decline rate and the decline rate of the first two hrs were about the same, or about 6% per hour. The fluorescence intensity in ETU-treated sperm also declined, to 21% of its original. Comparisons between the control and the treated spermatozoa showed no statistical differences in the R123 uptake at any sampling point. The decline of fluorescence intensity in ETU-treated spermatozoa, however, does not appear to follow the same 3-phase pattern as in the control. Instead, 4 phases were observed in the treated spermatozoa: 0 to 2, 2 to 3, 3 to 4, and 4 to 5 hrs. During the first and third phases, a rapid decline of R123 uptake, about 26-29% per hr, was observed. In the second and fourth phases, few changes were observed.

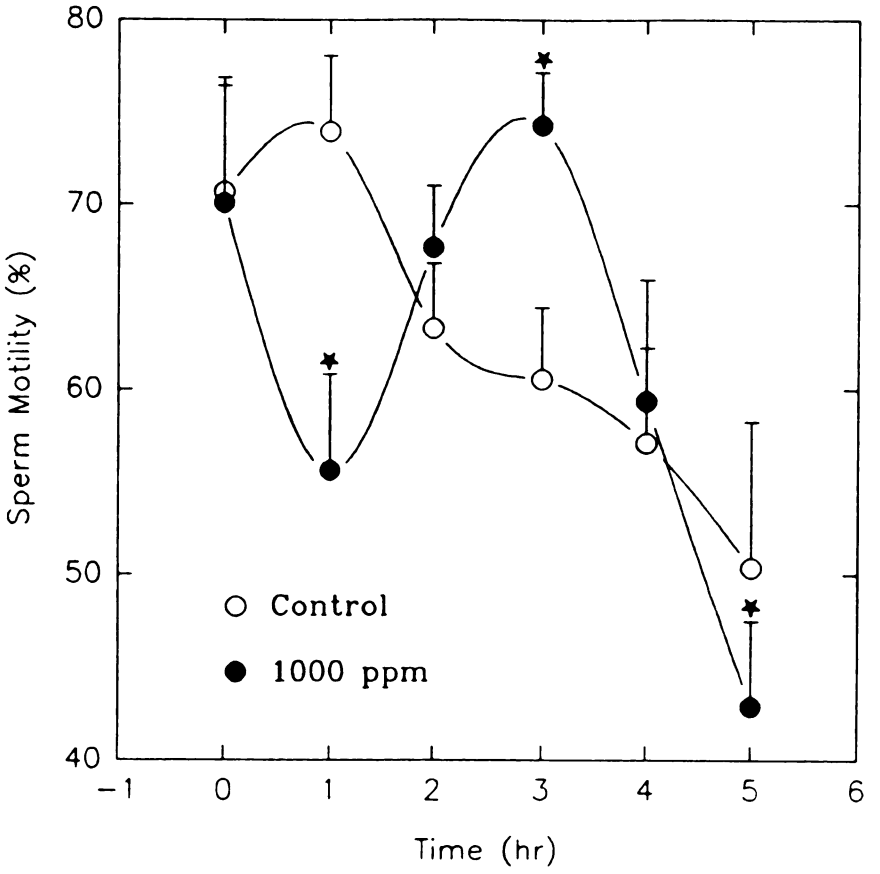

Figure 6-2. **Time course changes of sperm motility in the presence of ETU. Values are means ± SE of 4 experiments.**
*** Denotes statistically significant difference from control ($p < 0.05$).**

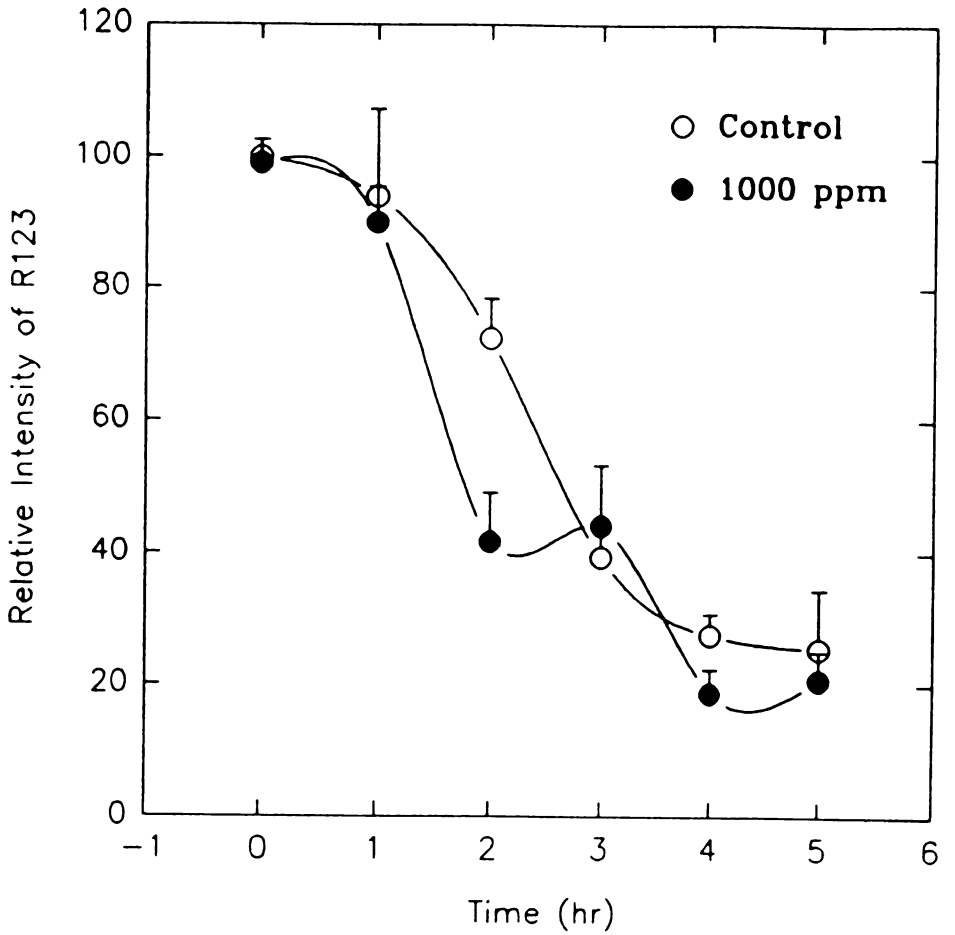

Figure 6-3. **Time course changes of the reletive intensity of rhodamine 123 fluorescence. Values are means ± SE of the fluorescent intensity relative to the control value at 0 hr, which is arbitrary set up at 100. Data are means of 3 experiments. No significant difference from the control was observed, $P > 0.05$.**

Effects of ETU on ESR signal

The ESR spectra of sperm labeled with POBN consisted of a triplet of doublets (Figure 6-4). The triplet represents interactions of unpaired electrons with the N nucleus of 4-POBN and the doublet represents splits by interactions with a single H of 4-POBN. The observed hyperfine couplings (a^N=15.8 G, a_{HB}=2.6 G) are inherent characteristics of the 4-POBN radical adduct of lipid peroxyl radicals (Conner *et al.*, 1986, Rosen *et al.*, 1980).

The relative height of the spectrum was used to compare the intensity of lipid peroxyl radical production. In the control spermatozoa, the production of lipid peroxyl radicals surged at two sampling points: one peaked at the first hr and the other at the fourth hr (Figure 6-5). Spermatozoa incubated in the ETU produced more lipid peroxyl radicals than the controls at all sampling points. The time-course changes in the amount of radical production appeared to be a mirror image of those in the non-treated spermatozoa. Instead of two surges, ETU-treated spermatozoa showed only one peak production of peroxyl radicals.

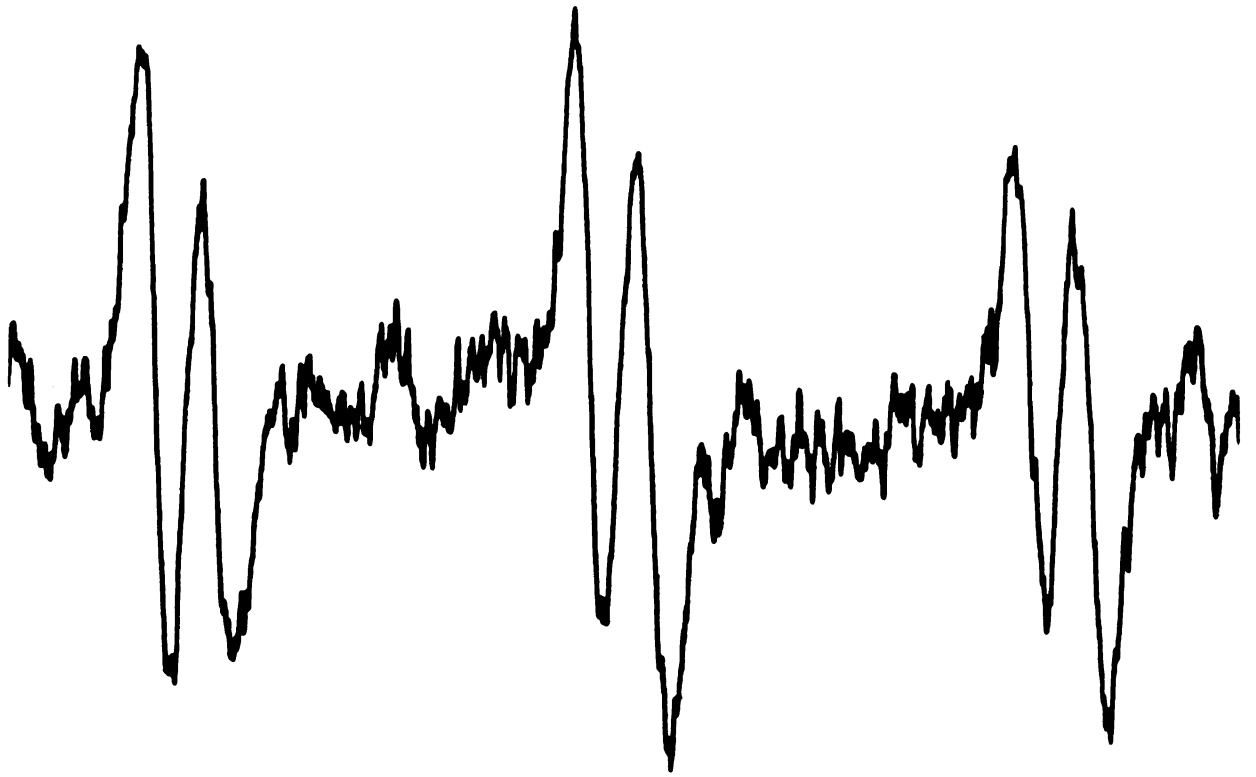

Figure 6-4. The ESR spectrum was obtained with intact mouse sperm and the spin trap, α-(4-pyridyl-1-oxide)N-t-butylnitron (4-POBN), after sperm were incubated in BMOC-3 for one hour at 37°C and 5% CO_2 in air.

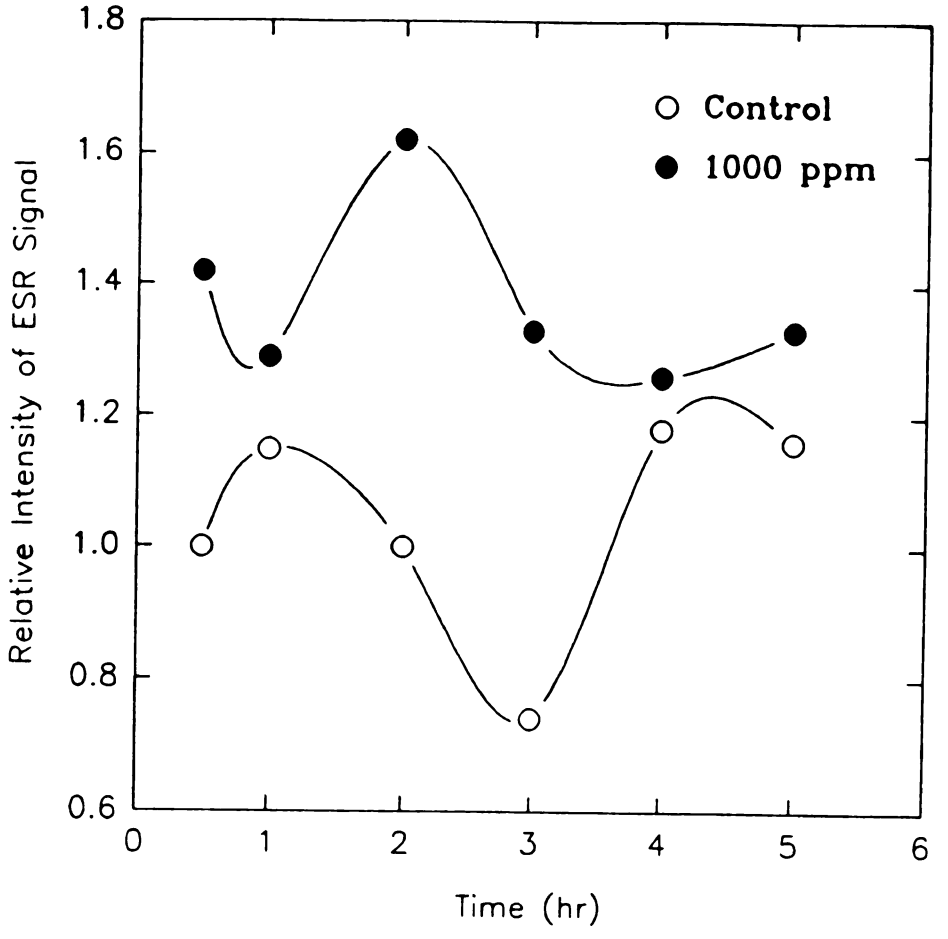

Figure 6-5. **The time-course changes of relative intensity of ESR signals from ETU treated spermatozoa. Epididymal spermatozoa were collected and incubated in BMOC-3 medium. Values are means of two experiments. The ESR signal intensity is expressed as signal heights relative to the signal heights of the controls at 0.5 hr.**

DISCUSSION

In the past decades, many studies have pointed to several possible roles of reactive oxygen species in sperm motility, capacitation, acrosome reaction and sperm-zona pellucida binding (Aitken and Clarkson, 1987; Alvarez and Storey, 1983; Bize *et al.*, 1991; Flemine *et al.*, 1982; D' Agata *et al.*, 1990; Aitken *et al.*, 1989; Llanos *et al.*, 1982). Some results suggested that excessive free-radical production and lipid peroxidation decrease sperm motility and cause infertility (Mann *et al.*, 1980; Jones and Mann, 1976; MacLeod, 1943; Aitken and Clarkson, 1987). Other studies provided evidence for the biological function of the reactive oxygen species and lipid peroxidation in sperm function (Kumar *et al.*, 1989; Bize *et al.*, 1991; and Aitken *et al.*, 1989).

Results in this study demonstrated temporal changes in free-radical production in capacitating spermatozoa. In the non-treated spermatozoa, higher production of lipid peroxyl was detected at 30 min and 1 hr than at 2 and 3 hr. The first peak of free-radical production in the control spermatozoa coincided with the timing of sperm capacitation. Under these culture conditions, most of spermatozoa initiated their capacitation process during the first two hrs. The second peak of lipid peroxyl radical peroxidation is probably associated with membrane damage or mitochondria dysfunction in sperm near

death. Jones and Mann (1976) measured peroxide production in ram spermatozoa. Similar to the results in this study, peroxides in ram spermatozoa became detectable after a 30 min incubation with ascorbic acid. The activity of lipid peroxidation reached its maximum after 60 min. Aitken and Clarkson (1987) made similar observations in a time dependent study of human spermatozoa. Washed spermatozoa from 6 individuals were resuspended in a BWW medium with luminol. Luminol is a sensitive chemiluminescent probe that reacts with H_2O_2, $O_2^{-\cdot}$, $\cdot OH$, and $^1O_2$. Oxygen radical production was measured by luminescence, which reached its maximum intensity within the first two hrs of incubation, followed by a progressive decline (Aitken, and Clarkson, 1987).

The relationship between lipid peroxidation and sperm motility was studied previously. Jones and Mann (1976) reported that peroxide formation coincides with the decline of sperm motility. In this study, time-course changes of sperm motility were positively correlated with the sperm peroxyl radical production during the first two hrs, in both the control- and the ETU-treated samples (Figure 6-2 and 6-5). This correlation, however, diminished over the next 3 hrs.

Upon exposure to ETU, spermatozoa quickly lost 15% of their motility within 1 hr (Figure 6-2). Despite the gradual recovery of motility in the following two hrs, these spermatozoa however completely lost their fertilizing ability (Figure 6-1). The overall increases in lipid peroxyl radical

production in ETU-treated spermatozoa supported the hypothesis that ETU generates reactive radicals that facilitate lipid peroxidation in sperm cells.

Researchers today have diverse opinions on the source of free radicals in normal spermatozoa (Holland and Storey, 1981; Bize *et al.*, 1991; Aitken and Clarkson, 1987; Aitken *et al.*, 1989). The sources suggested include mitochondria, the endoplasmic reticulum, peroxisomes, cytoplasm and the plasma membranes (Chance *et al.*, 1979; Ramasarma, 1982; Aitken and Clarkson, 1987; Aitken *et al.*, 1989). In our study, sperm mitochondrial function decreased during the time course in both non-treated and ETU-treated samples. Unlike the production of lipid peroxyl radicals, the mitochondrial function remained similar in both the control and ETU-treated spermatozoa. The lack of correlation between membrane lipid peroxidation and sperm mitochondrial function indicated that the increase in lipid peroxyl radicals in ETU-treated spermatozoa was not caused by a dysfunction of the mitochondria.

In conclusion, ETU at a concentration of 5 ppm and above decreased fertilization of mouse gametes in vitro. This study could not exclude possible inhibitory effects of ETU on egg fertilizing ability, but showed that ETU altered the time course changes of motility and peroxyl radical production in the treated spermatozoa. The overall increases in peroxyl radical production in ETU-treated spermatozoa did not coincide

with altered mitochondrial functions measured by the R123 uptake.

Results of this study also suggested that moderate increase of lipid peroxidation during capacitation were associated with the sperm function. Excessive lipid peroxidation, however, were associated with a lack of fertilizing ability. Furthermore, multiple phases of biochemical changes took place during sperm capacitation and acrosome reaction.

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CHAPTER VII

EFFECTS OF MANCOZEB ON REPRODUCTION AND SPERM FREE RADICAL PRODUCTION IN MICE

INTRODUCTION

Mancozeb is one of the ethylene bisdithiocarbamate (EBDC) fungicides. It became the most important EBDC remaining on the market after makers of the chemicals voluntarily halted the production of maneb and zineb in 1990. Our previous studies described in Chapter V showed decreases in the fertilizing ability of spermatozoa collected from animals fed a mixture of 3000 ppm maneb and zineb in their diet from 6 to 21 weeks of age. Increases in sperm intracellular free calcium concentration were evident in all treatment groups; the lowest dose of maneb and zineb tested was 30 ppm.

The study described in Chapter VI showed that in the presence of 1000 ppm ethylene thiourea (ETU), a common metabolite of EBDC fungicides, lipid peroxidation in the incubation medium increased in mouse spermatozoa. The in vitro fertilizing ability of mouse gametes incubated in ETU decreased; the percentage of eggs fertilized decreased from 80% in the control to 60% in 5 ppm, 47% in 100 ppm, and 10% in 400 ppm of ETU. At 800 ppm, no eggs were fertilized. Several recent reports addressed the diverse physiological effects of free radicals and reactive oxygen species in sperm function. In our electron spin resonance studies, the signal intensity of lipid peroxyl radical/4-POBN adducts varied during the

progress of capacitation. In non-treated spermatozoa, peroxyl radical production surged at 1 hr and 4 hr. In 1000 ppm ETU-treated spermatozoa, the peroxyl radical activities were higher than those in the control sample at all sampling points.

The objectives of this study were to examine the long-term mancozeb exposure on mouse reproduction and on sperm H_2O_2 and free radical production, with an emphasis on aging animals. biochemical changes. Based on the hypothesis that mancozeb may alter the function of lipid membranes and macromolecules in male reproductive cells due to increased free-radical activities, the following parameters were chosen: intracellular free calcium concentration, H_2O_2 production, the free-radical activity and the relative DNA content of testicular germ cells.

MATERIALS AND METHODS

Chemicals and media. Mancozeb is a complex of zinc-and-manganese-ethylene bisdithiocarbamate ion ($C_4H_6N_2S_4$) containing 16% manganese and 2% zinc and 62% EBDC ions. Dithane M-45 (Rohm and Haas, Philadelphia), a fungicide grade mancozeb containing 80% active ingredient, was used in this study.

For in vitro fertilization and ESR procedures, Modified Tyrode's medium was prepared in our laboratory with 99.23 mM sodium chloride, 2.68 mM potassium chloride, 1.8 mM calcium

chloride, 0.36 mM sodium phosphate monobasic, 0.49 mM magnesium chloride, 25.0 mM sodium bicarbonate, 25.0 mM sodium lactate, 0.25 mM sodium pyruvate, 5.56 mM D-glucose, 0.5% bovine serum albumin (BSA), with the pH adjusted to 7.30. Brinster's medium (BMOC-3) was purchased from Gibco (Grand Island, NY) and adjusted for pH (7.34) and osmolarities (288 mOSM) before use. BMOC-3 without BSA was prepared in our laboratory with 119.4 mM sodium chloride, 4.78 mM potassium chloride, 1.7 mM calcium chloride, 1.19 mM potassium phosphate, 1.19 mM magnesium sulfate heptahydrate, 25.1 mM sodium bicarbonate, 5.56 mM glucose and 1 mM sodium pyruvate. The media used for gamete collection and culture were equilibrated for 12 to 18 hr, in 5% CO_2 in air, at 37°C, immediately before use. Pregnant mare's serum gonadotropin (PMSG), human chorionic gonadotropin (hCG), bisbenzimide trihydrochloride and all ingredients of the culture media were obtained from Sigma Chemical Co. (St. Louis, MO).

For the measurements of intracellular free calcium concentration ($[Ca^{2+}]_i$), a modified BMOC-3 with 0.1% was prepared in our laboratory for sperm collection and incubation. The fluorescent Ca^{2+} chelator, Fura 2/AM, was purchased from Molecular Probes (Eugene, OR). EGTA, Tris, HCL, $CaCL_2$ and digitonin were obtained from Sigma Chemical Co. (St. Louis, MO).

To measure H_2O_2 and free radical in sperm, NADPH, trichloroacetic acid, ferrous ammonium sulfate, Spin trap α-

(4-pyridyl-1-oxide)-N-t-butylnitrone (4-POBN), and $FeCL_2$ were obtained from Sigma Chemical Co. (St. Louis, MO).

To measure the relative distribution of DNA content in testicular germ cells, phosphate buffered saline (PBS) with 5% or 0.5% BSA was prepared in our laboratory, with the pH adjusted to 7.4. Triton X-100 was purchased from the Research Products International Corp. (Elk Grove Village, IL). An intercalating dye propidium iodide (PI) reagent was prepared with 1 mg/ml PI (Sigma) and 0.5 mg/ml RNase (Boehringer Mannheim Corporation, Indianapolis, IN) in PBS.

Animals and treatments. One hundred and forty male C57BL/6J mice were randomly divided into five groups at 3 weeks of age. Treatment diets contained 0, 5, 30, 300 and 3000 ppm of mancozeb in ground Mouse Chow #5015 (Purina Mills Inc., St Louis, MO). The animal room was maintained at 21°C, 55 ± 5 % humidity, and 14h/10h light/dark cycle. Treatment diets and water were provided ad libitum for total of 70 weeks. During the treatment period, clinical signs were observed daily. Food consumption and body weight were recorded.

Breeding study. The reproductive performance of mancozeb-treated males were tested by mating with previously non-treated C57BL/6J females at 9, 13, and 29 weeks to produce 3 litters. For the breeding at 9 weeks, males and females were paired for 5 days. For the breeding at 13 weeks and 29 weeks,

mice were paired for 7 days. Males continuously received the treatment diets, while females received the corresponding treatment diets during breeding and throughout gestation and lactation.

An additional breeding study conducted at 13 weeks. Male and female mice were paired individually for 5 nights from 8:00 PM to 10:00 AM, during which time no feed was provided. During the day time, the males continuously received the treatment diet. The females were provided with Mouse Chow #5015. These females were never exposed to the treatment diets. Fecundity and litter sizes were recorded. Body weight and sex ratio of offspring were also recorded.

In vitro fertilization. At 29 and 43 weeks of the study, the fertilizing ability of cauda epididymal sperm from treated males was examined by in vitro fertilization with eggs from non-treated females. Each pair of epididymides were excised, submersed in 1 ml modified Tyrode' medium, punctured with a 25G needle, and incubated in the center well of an organ culture dish (Falcon 3037, Cockeysville, MD) for 2 hr before the sperm were used for insemination. The incubation was maintained at 37°C with 5% CO_2 in air. Mouse eggs were obtained from 4-to-12-week old B6D2-F1 females superovulated with intraperitoneal injections of 10 IU of PMSG followed, 48 to 50 hr later, by 10 IU of hCG. Thirteen hours after the hCG injection, cumulus-enclosed eggs were collected from oviducts

into 1 ml Tyrode's medium in the center well of an organ culture dish, and transferred into the center well of another organ culture dish, in which the outer well contained BMOC-3 without BSA.

For insemination, 50 ul sperm suspension (1 to 2.4 x 10^7 cells/ml) was transferred into the egg-containing dish. The final sperm concentrations were 0.5 to 1.2 x 10^6 cells/ml. In previous studies in our laboratory, maximum percentage of in vitro fertilization, 80 to 100%, was achieved by sperm concentration of 0.1 x 10^5 cells/ml. Sperm from each male were used to inseminate eggs from two females. After a 24-hr incubation, 50 ul 0.375 mM bisbenzimide trihydrochloride was added for nuclear staining and cultured for another 30 minutes. Eggs were then washed in 0.5 ml BMOC-3 without BSA and transferred to glass slides, for assessing fertilization with an Nikon Optiphot microscope (magnification = 200 x). Eggs at the two-cell stage or one-cell stage with two pronuclei and a second polar body were recorded as fertilized. Egg fragments or at one-cell stage without multiple nuclei were recorded as non-fertilized. At 29, 43, and 70 weeks, epididymal sperm were collected for concentration and motility analysis.

Measurement of intracellular free calcium concentration. Spermatozoa from a pair of epididymides were collected into 1 ml BMOC-3 medium containing 0.1 % BSA. Sperm cells were loaded

with fura-2/AM with a final concentration of 2 μM/ml and incubated at 37°C, in 5% CO_2 in air, for 60 min. After being washed twice and resuspended in BMOC-3, their relative fluorescence intensity was measured with a Perkin-Elmer luminescence spectrometer, model LS-5B. The excitation wavelength was set at 339 nm with 5 nm slits and emission recorded at 500 nm with 10 nm slits. Intracellular free Ca^{2+} concentration was calculated from the relation:

$$[Ca^{2+}]_i = K_d\ (F - F_{min})/(F_{max} - F)$$

where K_d = 224 nM is the dissociation constant of Ca^{2+} binding to fura-2.

Measurement of H_2O_2 in mouse sperm. Mouse sperm were collected from a pair epididymides and released into 1 ml phosphate buffer (138 mM NaCl, 7 mM Na_2PO_4, 3 mM NaH_2PO_4, pH 7.4), and incubated at 37°C, in 5% CO_2 in air, for 45 min. The H_2O_2 production was initiated by adding 10 μl 0.6 mM NADPH and terminated 15 min later by 15% trichloroacetic acid in water. After removal of precipitated protein by centrifugation at 500 x g for 5 min, 0.2 ml of 10 mM ferrous ammonium sulfate and subsequently, 0.1 ml of 2.5 M potassium thiocyanate were added to a 1.0 ml supernatant. The absorption of the red ferrithiocyanate complex formed in the presence of peroxides was measured at 480 nm in Gilford 2400-S spectrophotometer and compared to H_2O_2 standards (Yu *et al.*, 1990 and Thurman *et al.*, 1972).

Measurement of free radical in mouse sperm. Pooled sperm from two pairs of epididymides were collected into 2 ml BMOC-3 after a 14-week treatment or into Tyrode's medium after a 44-week treatment and incubated at 37°C, 5% CO_2 in air. Free radical production was measured at 0.5, 1, 2, 3, 4, or 5 hours after sperm collection. To prepare the reaction solution, 0.03 ml of 1 M 4-POBN and 0.03 ml of 10 mM $FeCl_2$ were added to a 0.24 ml sperm suspension. After gently stirring for 2 sec using a Vortex, the reaction solution was transferred to a flat quartz cell and ESR spectra were recorded with Varian E-4 ESR spectrometer. Operating conditions were microwave power, 20 mW; modulation intensity, 0.8 G; time constant, 1.0 s; scan time, 8 min; modulation frequency, 100 kHz; microwave frequency, 9.508 GHz; scan range, 100 G; temperature, 20°C; and receiver gain, 2.5×10^4.

Measurement of relative distribution of DNA content in mouse testis. Testes were placed in the central well of cell culture dish containing 1.0 ml PBS with 5% BSA, stripped of the tunica albuginea, and minced with scissors until reduced to small fragments. The preparations were transferred into Eppendorf centrifuge tubes, and vortexed for 1 min to release germ cells from seminiferous tubules. The cellular suspensions were sieved through a 35-μm nylon mesh, centrifuged (250 Xg) for 2 min, resuspended in 0.4 ml PBS with 0.5% BSA, and fixed in 1.2 ml of ice cold 70% ethanol overnight. Testicular cell

suspensions of 1-2 x 10^6 cells/ml were treated with 0.1% (v/v) Triton X-100 for 10 min, then washed and stained with PI solution at room temperature, in the dark, for 1 hr before storage at 4°C. Before analysis with the flow cytometry, cells were again filtered through a 35-μm nylon mesh.

Relative distribution of DNA content in the testicular cells were analyzed with a flow cytometer Ortho Diagnostics Cytofluorograph 50-H Fluorescence-activated cell sorter (FACS)/2150 computer system equipped with the Ortho DNADISC protocol, in which singlet and doublet cells were discriminated using area and peak fluorescent processing of the PI signals. PI was excited using the 488 nm line of an argon laser and emission was detected at 620-700 nm. The singlet events ranged from 60 to 80% of the total populations analyzed. The number of fluorescent events collected per sample depended on cell yield. At least 1500 fluorescent events per sample were analyzed. In most samples this number exceeded 2000. The relative DNA content within each sample was determined using the data analysis software of the flow cytometer. The mouse diploid spleen was used as a ploidy reference marker.

Statistical analyses. Statistical analyses of body weight gain, dietary intake, litter size, sperm concentration, sperm intracellular free calcium concentration, sperm hydrogen peroxide concentration and the body weight of offspring were

made using the General Linear Models (GLM) procedure (SAS institute, Inc., Cary, NC) at the 0.05 significance level. Where significant differences were noted between treatment responses, multiple comparison tests were conducted using the Least Significant Differences (LSD) test. Statistical analyses of fertility, in vitro fertilizing ability, sperm motility and sex ratio of offspring were made using the Chisquare procedure (MINITAB Inc., State College, PA) at the 0.05 significance level. Arcsin root transformations were made for the relative DNA content. Statistical analyses of the transformed values were made using the GLM procedure (SAS institute, Inc., Cary, NC) at the 0.05 significance level.

RESULTS

Body weight and feed intake

After one week of mancozeb treatment, males in the 3000 ppm treatment group had lower body weights than the controls ($p < 0.05$) (Table 7-1). The decrease in body-weight gain in the 3000 ppm group persisted through the study. The lack of statistical differences from the control from week 16 to week 32 was due to the small sample size. No decreases in feed intake in the treatment groups were observed (Tables 7-2 and 7-3), but the 5 ppm treatment group consumed more feed than the control ($p < 0.05$, Table 7-3).

TABLE 7-1. Body Weight of Male Mice Treated with Mancozeb for 43 Weeks[a]

Time	Mancozeb (ppm)				
	0	5	30	300	3000
(Week)	BW±SE (g)	BW±SE (g)	BW±SE (g)	BW±SE (g)	BW±SE (g)
1	13.0±0.6	12.8±0.6	12.9±0.6	12.8±0.6	12.6±0.5
2	18.4±0.4	18.4±0.5	18.5±0.5	17.9±0.5	16.2±0.6[b]
3	21.4±0.2	22.0±0.3	21.3±0.3	20.8±0.3	19.3±0.4[b]
4	23.3±0.2	23.5±0.3	23.3±0.3	22.9±0.3	21.1±0.3[b]
6	25.7±0.3	26.1±0.3	25.6±0.3	25.0±0.3	23.5±0.3[b]
8	27.2±0.3	27.5±0.3	27.4±0.2	26.7±0.3	25.4±0.3[b]
12	29.1±0.3	29.8±0.3	29.2±0.3	28.4±0.4	27.2±0.3[b]
16	30.3±0.3	30.1±0.7	29.8±0.3	30.9±1.1	29.3±0.5
20	32.2±0.3	32.2±0.7	31.7±0.4	33.4±1.3	30.2±0.4
24	33.1±0.8	33.1±0.6	32.8±0.5	34.4±1.3	30.9±0.4
28	32.1±0.4	37.0±2.1	34.4±0.8	34.7±0.2	31.7±0.5
32	34.3±0.8	35.2±0.6	35.0±0.8	36.2±1.4	32.6±1.4
36	35.9±1.1	38.1±0.7	36.0±1.1	39.0±1.9	32.0±0.5[b]
40	37.5±1.1	39.0±0.8	38.7±1.5	40.2±2.1	32.5±0.6[b]
44	39.3±1.7	41.4±0.9	40.2±1.7	40.4±2.0	32.7±0.6[b]

[a] Values represent means ± SE. From week 1-12, n = 27 to 28. From week 16-44, n = 5.

[b] Denotes statistically significant difference from the control ($p < 0.05$).

TABLE 7-2. Daily Feed Intake of Male Mice Treated with Mancozeb for 43 weeks[a], by weeks

Time (Week)	Dose (ppm) 0 DI±SE (g)	5 DI±SE (g)	30 DI±SE (g)	300 DI±SE (g)	3000 DI±SE (g)
1	3.8±0.3	4.1±0.3	3.9±0.3	3.9±0.3	3.4±0.5
2	4.8±0.4	5.6±0.4	5.5±0.3	5.3±0.4	4.7±0.3
3	5.0±0.3	5.4±0.4	5.9±0.5	5.6±0.3	5.3±0.3
4	5.5±0.4	5.9±0.4	5.9±0.3	5.8±0.4	5.7±0.4
5	5.7±0.5	6.0±0.3	6.0±0.4	5.8±0.4	5.9±0.4
6	5.6±0.4	5.8±0.4	5.7±0.3	5.5±0.5	5.7±0.5
7	5.7±0.4	5.8±0.3	5.6±0.2	5.9±0.3	5.9±0.3
8	5.0±0.5	5.3±0.4	5.5±0.3	5.5±0.4	5.4±0.4
9	5.5±0.4	5.4±0.3	5.3±0.2	5.3±0.3	5.2±0.3
10	4.2±0.9	3.7±0.7	4.0±0.8	3.8±0.8	4.0±0.9
11	5.0±0.2	5.3±0.3	5.1±0.3	5.4±0.4	5.3±0.3
12	4.6±0.2	5.3±0.3	4.9±0.2	4.9±0.3	4.7±0.2
13	5.0±0.5	5.4±0.3	5.5±0.4	5.2±0.4	4.9±0.4
15	5.2±0.1	5.0±0.0	5.2±0.8	5.2±0.3	6.6±0.9
16	4.6±0.2	5.7±0.5	4.9±0.4	5.2±0.1	5.2±0.6
17	4.0±0.1	5.4±0.6	4.4±0.4	5.2±0.3	4.9±0.6
18	3.9±0.1	5.2±0.4	4.0±0.0	4.6±0.2	4.5±1.2
19	3.7±1.1	5.3±0.6	4.7±0.5	4.9±0.9	4.4±0.2
20	3.9±0.4	5.5±1.3	3.8±0.4	5.1±1.0	4.7±1.5
21	2.9±1.2	4.5±0.1	3.1±1.2	3.8±0.7	4.3±0.1
22	4.5±0.1	5.3±0.2	4.0±0.3	4.5±0.1	4.8±0.5
23	4.1±0.2	4.8±0.5	4.1±0.4	5.0±0.4	5.0±0.4
24	4.0±0.1	5.2±0.7	3.9±0.3	4.5±0.1	4.9±0.5
25	5.0±0.4	4.3±0.1	3.9±0.5	4.7±0.4	4.5±0.0
26	5.3±0.3	4.9±0.1	4.1±0.7	4.9±0.1	4.0±0.5

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Time (Week)	Dose (ppm) 0 DI±SE (g)	5 DI±SE (g)	30 DI±SE (g)	300 DI±SE (g)	3000 DI±SE (g)
27	4.8±0.6	6.1±0.0	5.2±0.7	4.8±0.7	6.3±0.2
28	3.0±1.9	5.2±1.6	3.6±1.3	4.3±1.6	6.1±0.7
29	3.8±0.5	5.4±0.3	4.1±0.8	2.7±1.3	3.1±1.9
31	5.1±0.2	4.1±0.4	5.9±0.8	4.6±0.2	5.1±0.2
32	4.2±0.3	4.5±0.5	5.7±0.3	4.8±0.4	4.6±0.1
33	4.6±0.2	4.3±0.6	5.6±0.2	4.8±0.1	4.4±0.3
34	4.7±0.2	4.6±0.2	5.2±0.3	5.1±0.1	3.6±0.0
35	3.4±0.4	4.1±0.7	2.8±0.5	3.6±0.3	3.5±0.7
36	3.2±0.9	2.8±0.7	3.0±1.0	3.2±1.9	3.2±1.4
37	4.2±0.7	4.2±0.1	3.8±0.3	4.0±0.2	4.3±0.6
38	4.5±0.2	4.9±0.4	3.7±0.3	3.8±0.2	3.9±0.1
39	4.3±0.4	4.9±0.5	4.1±0.4	4.6±0.5	4.4±0.2
40	4.0±0.4	4.8±0.6	4.0±0.4	3.4±0.0	3.2±0.2
41	3.7±0.5	4.3±0.1	4.0±0.8	3.9±0.8	3.6±0.6
42	3.5±0.1	4.2±0.7	3.2±0.1	2.8±0.3	3.5±0.1
43	3.7±0.4	4.6±0.3	3.5±0.6	3.9±0.2	3.6±0.7

[a] Values represent means ± SE. No significant difference was observed among the treatments.

TABLE 7-3. Average of Daily Feed Intake of Male Mice Receiving Mancozeb for 43 Weeks

Treatment (ppm)	Daily feed intake[a]	*p*
0	4.86 ± 0.10	
5	5.24 ± 0.09	< 0.05
30	5.07 ± 0.09	
300	5.06 ± 0.09	
3000	4.98 ± 0.10	

[a] Values are mean ± SE of average daily feed intake recorded from 12 cages from week 1 to week 13 and 2 cages from week 14 to week 28. Each cage housed 2 to 4 males.

Fecundity and litter size

In all three breeding studies, at week 9, 13 and 29, the percentages of females giving birth were similar among all the treatment groups regardless of age and the length of treatment of the males (Table 7-4). Maternal exposure to mancozeb during breeding and gestation did not affect the fecundity (Table 7-4). There was no difference in litter sizes of offspring from males treated with mancozeb for 9 weeks before breeding (Table 7-5). A trend for decreased in litter size was observed when females were paired with males treated with 3000 ppm mancozeb for 13 weeks (Table 7-6). After 29 weeks of treatment, males from the 300 ppm- and 3000 ppm-treatment groups produced smaller litters than the control group ($p < 0.05$, Table 7-7).

TABLE 7-4. Fecundity of Male Mice Treated with Mancozeb for 9, 13, and 29 Weeks[e]

Treatment (ppm)	Fecundity (%)			Pooled data[c]	
	week 9[a]	week 13[b]	week 29[b]		week 13[d]
0	83.3	62.5	80.0	75.3	63.6
5	57.1	62.5	80.0	66.5	54.5
30	71.4	62.5	100.0	78.0	63.6
300	85.7	62.5	60.0	69.4	63.6
3000	100.0	50.0	100.0	83.3	72.7

[a] Each male was paired individually with a previously non-treated female for 5 days. The treatment diets were provided to both males and females during breeding. Females received corresponding treatment diets throughout gestation.

[b] Each male was paired with a previously non-treated female for 7 days. The treatment diets were provide to both males and females during breeding. Females received corresponding treatment diets throughout gestation.

[c] Pooled fecundity data of week 9, 13, and 29. The females exposed to the treatment diets during breeding and throughout gestation.

[d] Each male was paired with a previously non-treated female for 5 nights for 14 h without feed. Animals were separated during the day, when males were provided with treatment diets and females were provided with Mouse Chow #5015. Females were never exposed to mancozeb diets.

[e] No statistically significant difference from the control was observed.

TABLE 7-5. Litter Sizes of Offspring from Mancozeb-Treated Male for 9 weeks and Previously Non-Treated Females[b]

Treatment (ppm)	No. of litters observed	Litter size[a]
0	5	8.4 ± 0.8
5	4	8.0 ± 0.6
30	5	8.6 ± 0.8
300	6	8.0 ± 0.4
3000	7	8.3 ± 1.2

[a] Values are mean ± SE of observed litters.

[b] Each male was paired individually with a previously non-treated female for 5 days. The treatment diets were provided to both males and females during breeding. Females received corresponding treatment diets throughout gestation. No statistically significant difference from the control was observed.

TABLE 7-6. Litter Sizes of Offspring from Mancozeb-Treated Males for 13 Weeks and Previously Non-Treated Female

Treatment (ppm)	No. of litters	Litter size[a]	No. of litters	Litter size[b]
0	5	8.0 ± 0.5	7	7.9 ± 0.5
5	4	9.3 ± 0.8	7	7.9 ± 0.5
30	6	9.8 ± 0.2	6	9.0 ± 0.5
300	6	7.2 ± 0.8	6	7.8 ± 0.7
3000	6	6.7 ± 1.3	6	7.0 ± 1.1

[a] Values are means ± SE of observed litters. Each male was paired with a previously non-treated female for 5 nights for 14 h without feed. Animals were separated during the day, when males were provided with the treatment diets and females were provided with Mouse Chow #5015. Females were never exposed to mancozeb diets.

[b] Each male was paired with a previously non-treated female for 7 days. Treatment diets were provided to both males and females during breeding. Females received corresponding treatment diets throughout gestation.

TABLE 7-7. Litter Sizes of Offspring from Males treated with Mancozeb for 29 Weeks and Previously Non-Treated Females

Treatment (ppm)	No. of litters observed	Litter size[a]
0	4	8.5 ± 0.8
5	4	7.0 ± 0.4
30	5	7.6 ± 0.5
300	3	6.0 ± 0.5[b]
3000	5	6.0 ± 0.7[b]

[a] Values are means ± SE of observed litters. Each male was paired with a previously non-treated female for 7 days. Treatment diets were provided to both males and females during breeding. Females received corresponding treatment diets throughout gestation.

[b] Denotes statistically significant difference from the control ($p < 0.05$).

Body weight and sex ratio

No significant difference in birth weights was observed among offspring produced by males after 13 weeks of treatment (Table 7-8). When females were paired with males treated with mancozeb for 29 weeks, the birth weight of the offspring from the 30 ppm group was lower than that the control group ($p < 0.05$, Table 7-8), while the birth weight of the offspring from the 3000 ppm group was higher than that of the control group ($p < 0.05$).

No significant changes in sex ratios (No. of males : No. of females) of the offspring among treatment groups may be concluded from this study (Table 7-9). No external gross anomalies was observed in the offspring.

Sperm analysis and fertilizing ability of sperm in vitro

After 29 and 43 weeks of the treatment, epididymal sperm were collected and sperm concentrations, sperm motilities and in vitro fertilizing ability were examined. No differences in sperm concentration among treatment groups were observed at 29 or 43 weeks of the treatment (Table 7-10). Sperm motility in the 5 ppm- and 3000 ppm-treatment groups was higher than that in the control ($p < 0.05$) at 29 weeks (Table 7-11). The in vitro fertilizing ability of epididymal sperm from 300 and 3000 ppm-treated mice was 31% lower than control ($p < 0.05$)

TABLE 7-8. Birth Weights (BW) of Offspring from Male Mice Treated with Mancozeb for 13 and 29 Weeks

Treatment (ppm)	13 weeks[a]		29 weeks[b]	
	No. of pups	Mean of BW (g) ± SE	No of pups	Mean of BW (g) ± SE
0	56	1.30±0.02	34	1.34±0.02
5	54	1.26±0.01	28	1.29±0.01
30	68	1.29±0.01	38	1.27±0.01[c]
300	50	1.35±0.02	18	1.33±0.02
3000	58	1.35±0.03	30	1.40±0.03[c]

[a] Each male was paired with a previously non-treated female for 5 nights for 14 hrs without feed. Animals were separated during the day, when males were provided with treatment diets and females were provided with Mouse Chow #5015. Females were never exposed to mancozeb diets.

[b] Values are means ± SE of observed litters. Each male was paired with a previously non-treated female for 7 days. Treatment diets were provided to both males and females during breeding. Females received corresponding treatment diets throughout gestation.

[c] Denotes statistically significant difference from the control ($p < 0.05$).

TABLE 7-9. Sex Ratios of Offspring from Male Mice Treated with Mancozeb for 13 and 29 Weeks[a]

Treatment (ppm)	13 weeks		29 weeks	
	No. of litters	Mean of sex ratio ± SE[b]	No. of litters	Mean of sex ratio ± SE[b]
0	7	1.50±0.30	4	1.20±0.27
5	6	1.48±0.39	4	1.48±0.41
30	7	1.05±0.23	5	1.08±0.24
300	7	1.58±0.70	3	0.69±0.14
3000	8	2.40±0.55	5	1.63±0.41

[a] Each male was paired with a previously non-treated female for 7 days. Treatment diets were provided to both males and females during breeding and throughout gestation. No statistically significant differences from the control ($p < 0.05$) were observed.

[b] Values are means of number of males/number of females of each litters.

TABLE 7-10. Concentration of Epididymal Sperm from Male Mice Treated with Mancozeb for 29 and 43 Weeks[a]

Treatment (ppm)	Sperm concentration (million/ml)	
	29 weeks	43 weeks
0	20.81 ± 0.58	23.00 ± 3.09
5	20.75 ± 1.42	26.67 ± 1.36
30	22.84 ± 1.39	24.33 ± 1.44
300	18.88 ± 2.63	23.00 ± 1.70
3000	24.76 ± 2.27	23.00 ± 1.25

[a] Values represent means ± standard error, $n = 3$. No statistically significant differences from control ($p < 0.05$) were observed.

TABLE 7-11. Epididymal Sperm Motility of Male Mice Treated with Mancozeb for 29 Weeks

Treatment (ppm)	No. of experiment	No. of motile sperm /total sperm observed	Motility[a] (%)
0	3	245 / 328	74.5 ± 1.1
5	3	251 / 321	78.3 ± 0.7[b]
30	3	261 / 324	80.5 ± 1.1
300	3	259 / 337	76.6 ± 2.1
3000	3	304 / 372	81.7 ± 0.6[b]

[a] Values are means and ± SE of 3 experiments.
[b] Denotes statistically significant difference from the control ($p < 0.05$).

at 29, and 53% lower at 43 weeks (Tables 7-12 and 7-13).

Sperm intracellular free calcium concentration

After 13 weeks of the mancozeb treatment, dose-related increases in the intracellular free calcium concentration of epididymal spermatozoa were observed in all treatment groups. The increases were 21%, 35%, 64%, and 70% above that in the control for the 5, 30, 300, and 3000 ppm-treated mice, respectively. All concentrations except that from the 5 ppm group were significantly different from that of the control ($p < 0.05$, Table 7-14).

TABLE 7-12. In Vitro Fertilizing Ability of Epididymal Sperm from Male Mice Treated with Mancozeb for 29 Weeks

Treatment (ppm)	No. of experiment	No. of eggs fertilized /No. of eggs observed	Fertilization rates (%)[a]
0	6	152 / 188	82.9 ± 1.9
5	6	154 / 186	85.6 ± 4.9
30	6	127 / 165	78.5 ± 4.5
300	6	131 / 243	57.7 ± 5.3[b]
3000	6	62 / 181	39.3 ± 8.3[b]

[a] Values are means ± SE of 6 experiments.
[b] Denotes statistically significant difference from the control ($p < 0.05$).

TABLE 7-13. In Vitro Fertilizing Ability of Epididymal Sperm from Male Mice Treated with Mancozeb for 43 Weeks

Treatment (ppm)	No. of experiment	No. of eggs fertilized /No. of eggs observed	Fertilization rates (%)[a]
0	6	161 / 169	96.1 ± 1.9
5	6	127 / 138	90.2 ± 2.8
30	6	161 / 174	91.7 ± 2.6
300	6	129 / 151	86.4 ± 3.2[b]
3000	6	123 / 151	79.1 ± 2.3[b]

[a] Values are means ± SE of 6 experiments.
[b] Denotes statistically significant difference from the control ($p < 0.05$).

TABLE 7-14. Intracellular Free-Calcium Concentration ($[Ca^{2+}]_i$) of Epididymal Sperm from Male Mice Treated with Mancozeb for 13 Weeks[a]

Treatment (ppm)	No. of experiment	Mean of $[Ca^{2+}]_i$ (nM) ± SE	p^b
0	6	112.5 ± 7.3	
5	5	136.0 ± 12.1	
30	6	151.8 ± 10.5	< 0.05
300	5	185.0 ± 14.2	< 0.01
3000	5	191.6 ± 13.9	< 0.01

[a] Values are means ± SE of 5 or 6 experiments.
[b] Denotes statistically significant difference from the control ($p < 0.05$).

Production of hydrogen peroxide by spermatozoa

After 13 weeks of the treatment, epididymal spermatozoa were collected and incubated for 1 hr in phosphate buffer before H_2O_2 production was measured. Sperm H_2O_2 concentration from 5 ppm, 30 ppm, and 3000 ppm-treated mice was 23-25% lower than that of the controls ($p < 0.05$, Table 7-15).

Production of free radicals in the epididymal sperm

At 13 and 44 weeks of the study, epididymal sperm were collected and examined for free-radical production using the spin trap 4-POBN and the ESR method. Newly collected sperm

TABLE 7-15. Hydrogen Peroxide Concentration ($[H_2O_2]$) of Epididymal Sperm from Male Mice Treated with Mancozeb for 13 Weeks[a]

Treatment (ppm)	No. of experiment	Mean of $[H_2O_2]$ (μM) ± SE
0	4	9.3 ± 0.7
5	4	7.1 ± 0.1[b]
30	4	7.0 ± 0.4[b]
300	4	8.7 ± 0.7
3000	4	7.2 ± 0.4[b]

[a] Values are means ± SE of 4 experiments.
[b] Denotes statistically significant difference from the control ($p < 0.05$).

were incubated in BMOC-3 for 5 hrs and sampled at 0.5, 1, 2, 3, 4, and 5 hrs.

The ESR spectra obtained from the whole sperm consisted of a triplet of doublets (Figure 7-1). The triplet represents interactions of unpaired electrons with a N nucleus of 4-POBN and the doublet represents splits by interaction with a single H of 4-POBN. The observed hyperfine couplings (a^N=15.8 G, a_{HB}=2.6 G) may be attributed to the 4-POBN radical adduct of lipid peroxyl radicals (Conner *et al.*, 1986, Rosen *et al.*, 1981).

The intensity of the lipid peroxyl radicals-4-POBN adducts varied with the time course of sperm incubation. In the 13-week samples of the treatment, the peroxyl-radical

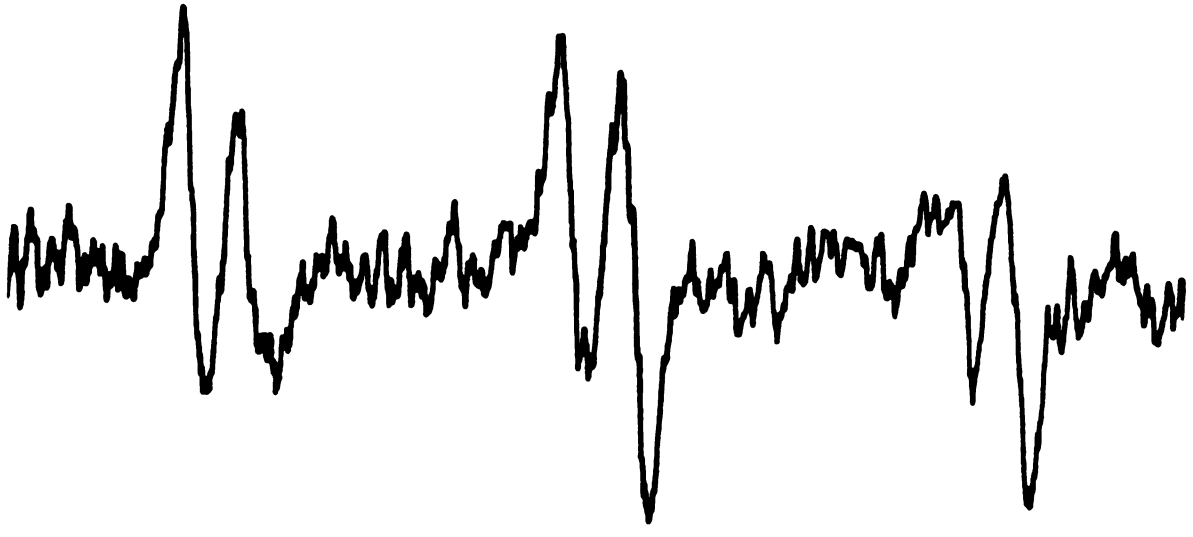

Figure 7-1. ESR spectrum obtained with intact mouse spermatozoa and the spin trap 4-POBN after one hour incubation in the BMOC-3 medium.

production from the control spermatozoa remained at a plateau during the first 2 hrs, declined from 2 to 4 hrs, then increased again from 4 to 5 hrs; spermatozoa from 3000 ppm-treated mice had lower peroxyl-radical production than the controls in all samples taken from 0.5 to 5 hrs (Figure 7-2). Of the 44-week samples, the peroxyl-radical production from the control was the highest at the first hr. It decreased continually at 2, 3, and 4 hrs, then increased again at 5 hrs. Similar to that observed at 13 weeks, the overall sperm peroxyl-radical concentrations of the 3000 ppm group were lower than that of the control, except at 2 and 5 hrs (Figure 7-3).

Progress of spermatogenesis measured by relative distribution of DNA content in testicular germ cells

Using DNA flow cytometry, 3 major populations of germ cells were identified in the testis of mature mice. The germ-cell populations sorted by the flow cytogram based on the fluorescent intensity of DNA-bound PI in individual cells. Different cell populations were obtained based on their DNA content (as opposed to ploidy expression, N). After locating the diploid (2N spermatogonia) peak on the cytogram using mouse peripheral blood lymphocytes as the 2N standard, the DNA content of the other two major populations was identified according to relative fluorescence intensities: 4N represents

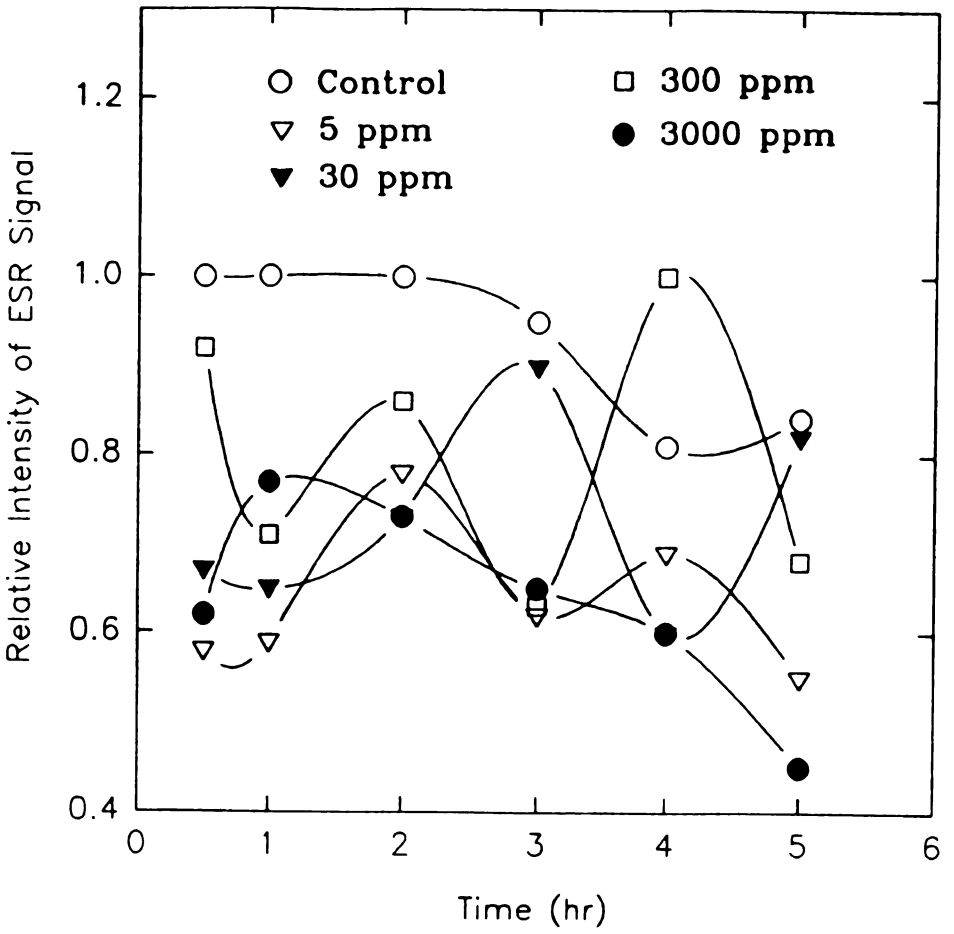

Figure 7-2. **Time cource changes of relative intensity of ESR signals recorded with spermatozoa collected from control, 5, 30, 300, and 3000 ppm mancozeb treated mice for 13 weeks. The ESR signal intensity was expressed as signal heights relative to the signal heights of the controls at 0.5 hour. Sample were taken at 0.5, 1, 2, 3, 4, or 5 hours after the collection from epididymides. Sperm were incubated in BMOC-3 medium at 37°C and 5% CO_2 in air.**

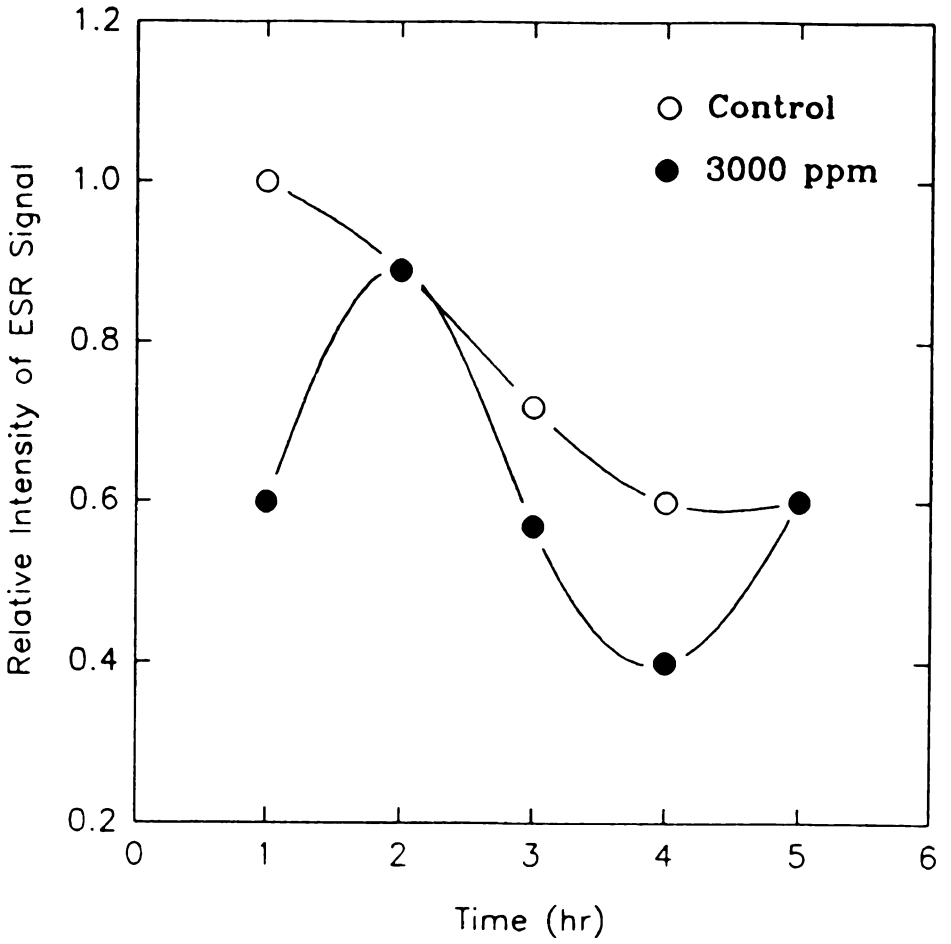

Figure 7-3. Time cource changes of relative intensity of ESR signals recorded with spermatozoa collected from control and 3000 ppm mancozeb treated mice for 44 weeks. The ESR signal intensity was expressed as signal heights relative to the signal heights of the controls at 1 hour. Sample were taken at 1, 2, 3, 4, or 5 hours after the collection from epididymides. Sperm were incubated in modified Tyrode's medium at 37°C and 5% CO_2 in air.

tetraploid or diploid primary spermatocytes; 1N represents haploid round spermatids and haploid elongate spermatid. The DNA-synthesizing cells, which fluorescence intensity falls between the intensity of 2N and 4N cells, account for a small portion of the total population.

After 70 weeks of treatment, testicular germ cells were isolated and the percentages of 1N, 2N, and cells in the 4N total population were analyzed. In the control, 31.1% ± 1.8 of the germ cells were at the 1N stage; 39.8% ± 1.7 at the 2N stage; 12.7% ± 0.8 at the 4N stages. Germ cells isolated from mancozeb-treated males had DNA content distributions similar to that seen in the controls ($p > 0.05$, Table 7-16), suggesting that the progress of spermatogenesis was not altered by mancozeb exposure.

Body weight, sperm concentration and sperm motility of male mice treated with mancozeb for 70 weeks

At 70 weeks of the study, the body weight of 3000 ppm-treated males remained lower than that of the control. This difference was not statistically significant, probably due to the limited number of mice remaining in the study (Table 7-17). Epididymal sperm motility and concentration were not different among all treatment groups ($p > 0.05$, Table 7-17). The mean sperm concentration in these aging mice, however, was only 25% and 22% of that observed at 29 and 43 weeks,

TABLE 7-16. Relative DNA Distribution Patterns of Testicular Germ Cells Isolated from Mancozeb-Treated Males[a]

Treatment (ppm)	No. of sample	Relative DNA content (%)[b]		
		1N	2N	4N
0	4	31.1 ± 1.8	39.8 ± 1.7	12.7 ± 0.8
5	6	32.7 ± 0.8	41.3 ± 1.1	10.5 ± 0.5
30	2	28.5 ± 0.9	41.0 ± 0.7	13.0 ± 1.4
300	6	30.3 ± 0.9	39.6 ± 1.6	13.2 ± 0.8
3000	8	32.0 ± 1.3	39.3 ± 1.1	11.0 ± 0.7

[a] The males were treated with mancozeb diets for 70 weeks.

[b] Values represent means ± SE of observed samples. No statistically significant differences from control ($p < 0.05$) were observed.

TABLE 7-17 Body Weight and Concentration and Motility of Epididymal Spermatozoa from Male Mice Treated with Mancozeb for 70 Weeks[a]

Treatment (ppm)	No. of experiment	Body weight (g)	Sperm concentration (million/ml)	Sperm motility (%)
0	2	44.5±2.1	6.0±0.7	58.6±1.0
5	3	44.2±5.0	6.0±0.8	58.3±8.3
30	1	53.6	4.0	50.0
300	3	52.8±1.8	5.3±0.5	44.4±4.5
3000	4	39.1±1.3	5.5±0.3	45.8±5.1

[a] No statistically significant differences from the control ($p < 0.05$) were observed.

respectively. The overall sperm motility of aging mice also decreased to 34% of that at 29 weeks.

DISCUSSION

Based on body weight and feed intake, the dietary exposures of 5, 30, 300, and 3000 ppm mancozeb are equivalent to the doses of 0.4 to 1.6, 2.5 to 9.1, 25 to 91, and 295 to 870 mg/kg BW/day, respectively. On a body weight basis, young mice at 4 to 5 weeks of age consumed 4 to 5 times the amount consumed after the fast growing stage, at about 39 to 43 weeks of age.

Within a week of mancozeb-treatment, body-weight gain of the males in the 3000 ppm group decreased (Table 7-1), and remained lower than that of the control through the 70-week treatment (Tables 7-1 and 7-17) No decreases in feed intake among treatment groups were observed.

The mancozeb treatment at any dose did not affect sperm concentration or motility, up to 70 week of daily exposure (Tables 7-10 and 7-17). The mancozeb treatment did not alter the progress of spermatogenesis, which was examined in mice at 73 weeks of age by the relative DNA content of testicular germ cells (Table 7-16). The overall sperm concentration in aging males, including the non-treated ones, decreases to about 22% to 25% (Table 7-10 and 7-17), the motility decreased to 34% when compared with young mature males (Table 7-11 and 7-17).

In the past decade, oxygen-free radicals have been shown to be involved in many degenerative diseases, particularly during the aging process (Armstrong *et al.*, 1984). Reactive oxygen species in excessive amounts have been implicated in sperm motility decreases and low fertility. However, other evidence suggest essential biological functions of reactive oxygen species in spermatozoa (Kumar *et al.*, 1989; Bize *et al.*, 1991; and Aitken *et al.*, 1989).

Our time-course studies of non-treated spermatozoa indicated that free-radical generation exhibited spontaneous changes in the capacitation-supporting medium (Figure 7-2 and 7-3). The highest production of peroxyl radicals taking place during the first two hrs of incubation was followed by a gradual decline. In normal sperm, the high production of free-radicals through peroxidation of unsaturated fatty acid in the first 2 hours appears to be the initiation or a part of sperm capacitation. The increase in radical activity at the end of the incubation was probably associated with membrane dysfunction in the dying spermatozoa. Aitken and Clarkson (1987) also observed increases in the production of reactive oxygen species in capacitating spermatozoa within the first two hrs of incubation, which was then followed by a progressive decline.

After 13 weeks of treatment with mancozeb, the lipid peroxyl radical production in the spermatozoa treated with 3000 ppm were lower than those of the controls. After 44 weeks

of treatment, a general trend of lower free-radical production from the 3000 ppm group was observed relative to the control group (Table 7-2 and 7-3).

This observation, on the surface, contradicts the results described in Chapter VI, where peroxyl-radical production increased in mouse spermatozoa incubated with ETU in vitro. The differences in lipid-peroxyl-radical production between in vivo and in vitro studies may be due to the length of the treatments.

Other in vivo studies have demonstrated that aerobic living organisms are in general well protected by antioxidative mechanisms in cells such as glutathione redox reactions. An animal will die if the antioxidative mechanisms are overwhelmed by oxidative challenges. If an animal survives any oxidative challenge, its glutathione contents in cells are most likely to increase to exceed that in the non-challenged animals, and the free radical contents would be lower than that in the non-challenged animals -- a result due to over-compensation by the antioxidative mechanisms.

We expected that after long term dietary EBDC treatment, animals may develop their protective function against oxidative stress. Therefore, instead of seeing increases in free radical activity, we could see a decrease in free radical production in the sperm from mancozeb treated mice.

Similar to the lipid peroxyl-radical production, H_2O_2 production in the sperm from treated mice were also decrease.

The decrease is unlikely due to the variations of the sperm concentration, which is higher in the treatment samples than the control. In a separate study, our laboratory has observed a positive correlation between sperm concentration and H_2O_2 production.

Although the mancozeb treatment caused biochemical changes in intracellular free calcium concentration, hydrogen peroxide production and lipid peroxyl radical generation in spermatozoa, the mouse fertility from natural breeding appears to be unaffected. This may be due to the biochemical changes occurring usually early or before the functional changes. Another reason is that since we studied the entire sperm population, the chemical toxicity may not affect each individual spermatozoa. Natural breeding may apparently be affected only when those biochemical changes reach a certain level or involve large enough sperm population.

Despite the decrease in H_2O_2 and peroxyl radical production in the mancozeb treatment groups and the increase in the intracellular Ca^{2+} concentration in the spermatozoa from the treated mice, the number of females giving birth remained about the same among treatment groups (Table 7-4). Nevertheless, the litter size of the offspring from females bred with 300 ppm- or 3000 ppm-treated males for 29 weeks demonstrated significant decreases (Table 7-7).

The ability of sperm to fertilize eggs decreased in the 300 ppm and 3000 ppm groups after 29 and 43 weeks of the

treatment (Tables 7-12 and 7-13). Similar to the findings in the previous study of dietary maneb and zineb in B_6D_2-F_1 mice (Chapter V), in vitro fertilizing ability is a sensitive parameter to predict subtle decreases in the fertilizing ability of spermatozoa in vivo. A breeding study with small sample sizes is unlikely to reflect this kind of subtle change. Large sample sizes may reflect the decreases in fertility, but large sample sizes will also increase the cost.

In a breeding study, only a fraction of the total number of fertile spermatozoa is necessary to fertilize all of the ovulated eggs. Biochemical changes in sperm cells, such as increases in intracellular Ca^{2+} concentration and decreases in H_2O_2 and peroxyl-radical production, may not reflect an immediate decrease in sperm fertilizing ability, are sensitive markers for perturbed cellular homeostasis. These changes serve as early warning signs for sperm fertilizing ability and point to the toxicity mechanism of the test chemicals. The fecundity, a reproduction index, although the most frequently relied upon parameter in reproductive toxicology for sperm quality, is apparently an unreliable index when sub-fertility, instead of infertility, is the subject of concern.

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CHAPTER VIII

EFFECTS OF LONG-TERM MANCOZEB EXPOSURE ON SPERM VARIABLES IN THE SECOND GENERATION

INTRODUCTION

Mancozeb, maneb, metiram, nabam and zineb are the five EBDC fungicides registered in the United States for the treatment of seeds, cereals, fruits, and vegetables (Federal Register, 1989). EBDC residues in agricultural products became a focal concern of consumers and regulatory agents when laboratory studies showed that their common metabolite ETU was mutagenic. The EPA reported, in a preliminary risk assessment, that no adequate multigenerational studies were available for assessing the reproductive effects of mancozeb. In a chronic feeding study, young Wistar rats treated with 0, 25, 100, and 1000 ppm of mancozeb were mated twice to produce 2 litters for a projected three-generation reproduction study (EPA, 1989). The rats showed a reduction of fertility at the highest dose tested.

Our previous studies showed a decreased in vitro fertilizing ability of spermatozoa from C57BL/6J male mice treated with 300 and 3000 ppm mancozeb in their diets. Sperm intracellular free calcium concentrations increased in mice treated with 30, 300, and 3000 ppm mancozeb in their diets for 13 weeks. The H_2O_2 and lipid-peroxyl radical production decreased in the sperm from mancozeb-treated mice.

In this study, the postnatal development and sperm

quality of the offspring from mancozeb-treated, CBA/CAJ mice were examined. The experiments were based on the hypothesis that mancozeb may alter sperm production and quality from mancozeb treated second generation.

MATERIALS AND METHODS

Chemicals, media, and methods used in this study were the same as those described in Chapter VII; with the exception of animals, intracellular free calcium concentration determination and the chlortetracycline fluorescence assay which will be described here.

Animals and treatments. Twenty-five male CBA/CAJ mice were randomly divided into five groups at 3 weeks of age. Treatment diets contained 0, 5, 30, 300 and 3000 ppm of mancozeb in ground Mouse Chow #5015 (Purina Mills Inc., St Louis, MO). The animal room was maintained at 21°C, 55 ± 5 % humidity, and 14h/10h light/dark cycle.

Breeding study. The reproductive performance of mancozeb treated males was tested by mating them with previously non-treated CBA/CAJ females at 14 and 21 weeks to produced 2 litters. Females received the corresponding treatment diets during breeding and throughout gestation and lactation.

Sperm concentration, motility, in vitro fertilizing

ability, the progress of sperm capacitation and acrosome reaction, and fecundity were measured in the parental generation, while the litter size, birth weight, and postnatal growth were measured in the offspring. The offspring were provided with corresponding treatment diets. Their sperm concentration, motility, intracellular free calcium concentration, and progress of spermatogenesis were examined during puberty.

Chlortetracycline (CTC) fluorescence assay for capacitation/acrosome reaction. The chlortetracycline fluorescence assay described by Ward and Storey (1984) was used to assess the progress of sperm capacitation and the acrosome reaction. Mouse sperm suspension was collected in Tyrode's medium with 0.5% BSA. At sampling time, 0, 1, 2, 3, 4, and 5 hr after the collection, 20 ul sperm suspension was mixed on a clean, warm slide with 20 ul 500 mM-chlortetracycline hydrochloride in 50 mM-NaCl, 5 mM-cysteine, 20 mM-tris-base buffer (pH 7.8). After 30 sec, 15 ul of 5 % glutaraldehyde (v/v) in phosphate buffer (final pH 7.4) was added for fixation. A coverslip was applied and the slide was compressed between two sheets of tissue to remove excess liquid. Sperm were observed on a Nikon-OPTIPHOT microscope equipped with epifluorescence optics with 100x objective lens, 510 Dichroic Mirror, 425 excitation filter and 520 barrier filter. At least 100 sperm were scored in each sample. Sperm

concentration and motility were measured using the Makler chamber at the same time.

Measurement of intracellular free calcium concentration. An estimation of intracellular free calcium by using flow cytometry and the calcium sensitive fluorophore Fluo3 was described by Nolan et al (1992). Epididymal sperm were collected into 1.0 ml calcium buffer containing 0.1 % BSA and held for 15 min. The sperm suspension was then transferred into another culture dish in order to remove epididymal tissue. Acetoxymethylester of Fluo3 10 μl (1.5 mg/ml) was added into 1.0 ml sperm suspension, with a final concentration of 15 μl /ml. Sperm cells were incubated with Fluo3 at 37°C, in 5% CO_2 for 30 min. Cells were washed twice by centrifugation 300 Xg for 2 min and resuspended in 1.0 ml calcium buffer. $[Ca^{2+}]_i$ fluorescence was then determined by loading the cells onto the Ortho Cytofluorograph (Ortho Diagnostics/B-D, San Jose, Ca) with computer-assisted data acquisition (Acqcyte Data Acquisition Software, Phoenix Flow Systems, San Diego, CA). Forward and right-angle scatters were used to selectively gate on the sperm population. Fluorescence was excited at 488 nm and emission was measured at 525 nm on a linear scale. The sample temperature was maintained at 37°C. The intracellular free Ca^{2+} concentration was calculated from the relation: $[Ca^{2+}]_i = K_d\ (F - F_{min})/(F_{max} - F)$, using a K_d of 450 nM. The F_{max} was determined by the addition of 17 μM calcium

ionophore 4-bromo-A23187 and the F_{min} calculated as $F_{max}/40$. Sperm concentration and motility were measured using the Makler chamber at the same time.

Statistical analyses. Statistical analyses of litter size, sperm concentration, sperm intracellular free calcium concentration, and body weight of offspring were made using the General Linear Models (GLM) procedure (SAS institute, Inc., Cary, NC) at the 0.05 significance level. Where significant differences were noted between treatment responses, multiple comparison tests were conducted using the Least Significant Differences (LSD) test. Statistical analyses of fertility, in vitro fertilizing ability and sperm motility were made using the Chisquare procedure (MINITAB Inc., State College, PA) at the 0.05 significance level. Arcsin root transformations were made for the sperm CTC pattern and the relative DNA content and statistical analyses of the transformed values were made using the GLM procedure (SAS institute, Inc., Cary, NC) at the 0.05 significance level.

RESULTS

Breeding study

Results from this study did not reflect any changes in the fecundity of females bred with pre-treated males (Table 8-

1). No significant differences in litter sizes of the offspring were observed (Table 8-2). The offspring' mortalities from the 300 and 3000 ppm treatment groups were 66% and 52% higher than from the control ($p < 0.05$, Table 8-2). Most offspring mortality occurred within 3 days after birth.

TABLE 8-1. Fecundity of Male Mice Treated with Mancozeb for 14 and 21 Weeks

Treatment (ppm)	14 Weeks[a]		21 Weeks[b]	
	No. of females gave birth /No. of females bred	Fecundity (%)	No. of females gave birth /No. of females bred	Fecundity (%)
0	4 / 5	63.6	3 / 6	50.0
5	5 / 5	100.0	5 / 6	83.3
30	4 / 5	80.0	2 / 6	33.3
300	5 / 5	100.0	2 / 6	33.3
3000	5 / 5	100.0	4 / 6	66.7

[a] Each male was paired with a previously non-treated female for 7 days. The treatment diets were provide to both males and females during breeding. Females received corresponding treatment diets throughout gestation and lactation.

[b] Each male was paired with two previously non-treated females for 7 days. The treatment diets were provide to both males and females during breeding. Females received corresponding treatment diets throughout gestation and lactation.

No statistically significant differences from the control ($p < 0.05$) were observed.

TABLE 8-2. Litter Sizes and Mortality of Offspring from Mancozeb-Treated Males for 14 and 21 Weeks and Previously Non-Treated Females

Treatment (ppm)	No. of litters observed	Litter size[a]	Mortality (%)[a]
0	7	6.0 ± 0.6	46.1 ± 14.8
5	10	6.7 ± 0.5	60.1 ± 14.0
30	6	7.5 ± 0.8	41.6 ± 13.9
300	7	7.0 ± 0.6	76.7 ± 11.0[b]
3000	9	6.4 ± 0.5	70.0 ± 13.3[b]

[a] Values are mean ± SE of observed litters.
[b] Denotes statistically significant difference from the control ($p < 0.05$).

Sperm concentration and motility, the progress of sperm capacitation and acrosome reaction, and fertilizing ability of spermatozoa

At 14 weeks of the study, epididymal sperm were collected and examined for sperm concentrations, motilities and in vitro fertilizing ability. No significant differences in sperm concentration between treated mice and the controls were observed (Table 8-3). Time course changes of sperm motilities were measured at 0 time, when sperm were first collected and hourly afterwards, while incubated in a modified Tyrode's medium. The 3-hr samples showed a trend of higher motility in the mancozeb treated groups, although only the 30 ppm groups

were statistically higher than the control group ($p < 0.05$, Table 8-4).

Observed with the CTC fluorescence assay, mancozeb did not alter the progress of sperm capacitation in any of the treatment groups (Tables 8-5 and 8-6). The percentage of acrosome reacted populations at 3 hr were lower in the 30 and 3000 ppm groups than the control group ($p < 0.05$, Table 8-7). Unlike the inhibitory effect of mancozeb on the sperm fertilizing ability in C57BL/6J males (Chapter VII), the in vitro fertilizing ability of epididymal spermatozoa from the treated CBA/CAJ mice did not differ from the control group (Table 8-8).

Peroxyl radical production in the epididymal spermatozoa of mancozeb-treated males

The intensity of the lipid peroxyl radicals-4-POBN adducts varied with the time course of sperm incubation. The peroxyl-radical production from the control spermatozoa was high at 1 hr and declined from 1 to 3 hrs. The same results were observed in C57BL/6J mice (Chapter VII) -- sperm peroxyl radical production in the CBA/CAJ mice, after 21 weeks of the treatment, was lower in the spermatozoa from 3000 ppm-treated mice than in the control group during the first three hrs of the time-course study (Figure 8-1).

TABLE 8-3. Epididymal Sperm Concentrations of Male Mice Treated with Mancozeb for 14 Weeks

Treatment (ppm)	No. of experiments	Sperm concentration (million/ml)[a]
0	3	9.00 ± 1.70
5	3	9.33 ± 1.72
30	3	12.33 ± 1.52
300	3	11.00 ± 1.70
3000	3	10.33 ± 2.13

[a] Values represent means ± SE of 3 experiments. No statistically significant differences from the control ($p < 0.05$) were observed.

TABLE 8-4. Time Course Changes of Epididymal Sperm Motility of Male Mice Treated with Mancozeb for 14 Weeks[a]

Time	Treatment (ppm)				
(hr)	0	5	30	300	3000
0	87.7±2.1	89.5±0.8	87.5±1.2	88.3±0.8	81.1±0.9
1	64.9±4.3	69.4±1.2	75.6±2.4	72.3±4.1	63.8±5.7
2	55.2±9.9	51.3±10.1	47.9±5.0	54.8±5.6	51.7±8.3
3	28.5±9.0	42.1±1.4	49.3±5.2[b]	46.2±3.1	40.1±4.1
4	16.1±3.8	21.3±2.0	24.8±4.6	20.5±5.6	26.5±5.7
5	13.7±0.5	12.6±1.4	12.3±1.0	12.1±2.9	15.5±2.9

[a] Values are means and ± SE of 3 experiments.
[b] Denotes statistically significant difference from the control ($p < 0.05$).

TABLE 8-5. Time Course Changes of Epididymal Sperm of Male Mice Treated with Mancozeb for 14 Weeks Showing the CTC Pattern of Fresh Spermatozoa[a]

Time	Treatment (ppm)				
(ppm)	0	5	30	300	3000
0	41.3±7.1	32.3±1.4	32.3±4.5	41.7±1.5	35.0±3.3
1	31.3±2.4	31.0±3.3	31.0±2.9	26.7±3.3	31.0±1.3
2	27.0±3.7	27.3±3.1	19.0±2.5	22.0±5.0	22.3±4.1
3	19.7±1.7	13.3±3.2	15.3±5.7	17.7±4.0	21.7±3.5
4	11.7±4.4	19.3±4.9	14.7±3.9	15.0±5.0	16.0±4.9
5	12.7±3.9	14.7±4.1	9.7±1.7	13.7±1.5	16.0±4.8

[a] Values are percentage means ± SE of 3 experiments. A minimum of 100 sperm were scored at each time point. No statistically significant difference from the control was observed.

TABLE 8-6. Time Course Changes of Epididymal Sperm from Male Mice Treated with Mancozeb for 14 Weeks Showing the Capacitation-Related CTC Pattern[a]

Time	Treatment (ppm)				
(hr)	0	5	30	300	3000
0	56.3±8.3	65.0±2.4	66.7±4.8	58.0±1.3	63.0±4.5
1	67.7±1.4	68.3±3.5	67.3±2.7	73.0±3.3	67.3±1.2
2	68.0±4.2	69.7±4.8	75.3±1.2	76.0±6.4	72.0±6.4
3	71.3±2.3	79.0±3.9	79.3±5.5	75.0±4.2	73.7±3.1
4	73.0±2.1	66.7±6.4	68.0±6.5	71.3±9.4	68.7±2.6
5	58.0±3.3	69.0±8.2	71.7±6.7	68.3±8.0	63.3±4.0

[a] Values are percentage means ± SE of 3 experiments. A minimum of 100 sperm were scored at each time point. No statistically significant difference from the control was observed.

TABLE 8-7. Time Course Changes of Epididymal Sperm from Male Mice Treated with Mancozeb for 14 Weeks Showing Acrosome Reaction-Related CTC Pattern[a]

Time (hr)	Treatment (ppm)				
	0	5	30	300	3000
0	2.3±1.9	0.3±0.3	0.3±0.3	0.3±0.3	5.3±3.4
1	1.7±0.7	0.7±0.3	1.7±0.7	0.3±0.3	1.3±1.0
2	5.0±2.9	3.0±1.7	5.7±1.4	2.0±1.6	5.7±2.6
3	9.3±1.0	7.7±0.7	5.0±0.5[b]	7.7±0.5	4.7±1.0[b]
4	15.3±3.5	14.3±2.2	17.3±4.8	13.7±4.5	15.3±2.8
5	29.3±6.1	16.3±4.4	12.0±5.3	18.0±6.7	20.7±3.8

[a] Values are percentage means ± SE of 3 experiments. A minimum of 100 sperm were scored at each time point.

[b] Denotes statistically significant difference from the control ($p < 0.01$).

TABLE 8-8. In Vitro Fertilizing Ability of Epididymal Sperm from Male Mice Treated with Mancozeb for 14 Weeks

Treatment (ppm)	No. of experiment	No. of eggs fertilized /No. of eggs observed	Eggs degenerated (%)	Fertilization (%)[a]
0	6	180 / 204	0.9	85.5 ± 6.3
5	6	169 / 193	2.1	89.0 ± 4.8
30	6	216 / 253	2.0	85.2 ± 1.6
300	6	205 / 229	1.7	87.9 ± 3.4
3000	6	199 / 234	2.1	85.1 ± 3.4

[a] Values are means ± SE of 6 experiments. No statistically significant difference from the control was observed.

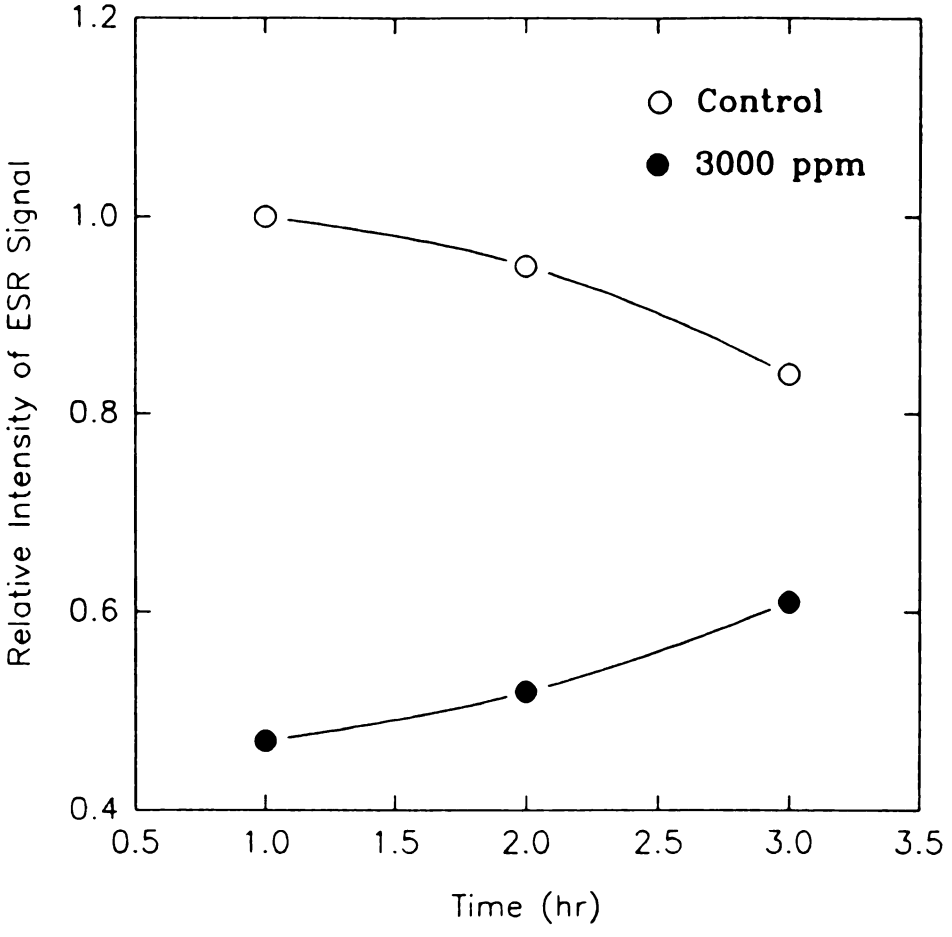

Figure 8-1. **Intensity of ESR signal recorded with sperm collected from control and 3000 ppm mancozeb treated CBA/CAJ male mice for 21 weeks. Sample were taken at 1, 2 and 3 hours after the collection from epididymides. Sperm were incubated in Tyrode's medium at 37°C and 5% CO_2 in air.**

Offspring body weights

The birth weight of the offspring from the 30, 300 and 3000 ppm treatment groups was lower than in the control group. ($p < 0.05$, Table 8-9). From day 4 to week 6, offspring body weights of the 30 and 3000 ppm groups continued to be lower than that of the control group. At 7 weeks of age, only the body weights of the offspring in 5 ppm group was lower than that of the control (Table 8-9).

Offspring sperm concentration and motility

When the offspring were at 6 and 8 weeks of age, epididymal sperm concentration and motility were measured (Tables 8-10 and 8-11). At 6 weeks of age, male offspring of the 30, 300 and 3000 ppm treatments had sperm concentrations 65%, 53%, and 25% lower, respectively, than the control groups. Motility, however, was the same among all treatment groups (Table 8-10). At 8 weeks of age, the sperm concentration from the 5 ppm treatment group was 65% higher, while the concentration of the 300 and 3000 ppm groups was 100% higher, than that of the control group ($p < 0.05$). Sperm motility from the 30 and 300 ppm treatment groups were 46% and 50%, respectively, higher than in the control group ($p < 0.05$, Table 8-11).

TABLE 8-9. Body Weight of Offspring From Male Mice Treated with Mancozeb for 14 Weeks[a]

	Treatment (ppm)				
	0	5	30	300	3000
	(No. of offspring)				
At birth	1.4±0.03 (41)	1.4±0.02 (62)	1.2±0.02[b] (41)	1.3±0.02[b] (48)	1.3±0.03[b] (40)
Day 4	1.7±0.12 (24)	1.6±0.04 (29)	1.4±0.04[b] (26)	1.7±0.07 (13)	1.5±0.03 (15)
Week 1	3.8±0.16 (24)	3.4±0.11[b] (29)	3.0±0.07[b] (24)	3.5±0.12 (13)	3.2±0.12[b] (15)
Week 2	6.8±0.26 (24)	6.5±0.23 (28)	6.3±0.17 (24)	6.9±0.14 (13)	6.3±0.17 (15)
Week 3	9.6±0.22 (24)	10.0±0.2 (28)	8.0±0.24[b] (24)	9.4±0.16 (13)	8.5±0.10[b] (15)
Week 4	14.6±0.4 (12)	14.8±0.4 (18)	12.0±1.2[b] (6)	14.4±0.4 (7)	13.0±0.2 (7)
Week 5	17.8±0.5 (12)	17.7±0.3 (18)	16.0±1.1[b] (6)	17.6±0.2 (7)	16.2±0.2[b] (7)
Week 6	19.7±0.4 (12)	18.4±0.3[b] (18)	18.8±0.4 (6)	18.5±0.4 (7)	18.3±0.4[b] (6)
Week 7	21.7±0.5 (8)	20.4±0.3[b] (17)	21.1 (1)	20.5±0.2 (3)	19.8±0.2 (2)
Week 8	22.6±0.6 (8)	23.1±0.4 (17)	21.6 (1)	21.7±0.2 (3)	21.0±0.02 (2)

[a] Values are means (g) ± SE of observed number of offspring. The average body weights from birthday to week 3 came from both male and female pups. The average body weights from week 4 to 8 came only from male pups.

[b] Denotes statistically significant difference from the control ($p < 0.05$).

TABLE 8-10. Epididymal Sperm Concentration and Motility from Offspring at 6 Weeks of Age

Treatment (ppm)	No. of samples	Sperm concentration (million/ml)[a]	Sperm motility (%)[a]
0	4	4.36 ± 0.71	71.85 ± 7.72
5	1	6.76	76.33
30	5	1.54 ± 0.57[b]	86.72 ± 5.03
300	4	2.07 ± 0.28[b]	72.37 ± 0.90
3000	4	3.27 ± 1.11	72.27 ± 4.74

[a] Values are means ± SE of observed samples.
[b] Denotes statistically significant difference from the control ($p < 0.05$).

TABLE 8-11. Epididymal Sperm Concentration and Sperm Motility from Offspring at 8 Weeks of Age

Treatment (ppm)	No. of samples	Sperm concentration (million/ml)[a]	Sperm motility (%)[a]
0	3	4.03±1.11	31.51±14.96
5	3	6.63±0.97[b]	43.99±15.24
30	1	10.30	77.67
300	3	8.07±0.33[b]	81.89±3.44[b]
3000	2	8.05±3.08[b]	39.72±8.98

[a] Values are means ± SE of observed samples.
[b] Denotes statistically significant difference from the control ($p < 0.05$).

Offspring sperm intracellular free calcium concentration

At 8 weeks of age, epididymal sperm were collected and sperm intracellular free calcium concentrations were measured. Similar to the previous study (Chapter VII), increases of 26, 23, and 64% of the intracellular free calcium concentration was observed in sperm from the offspring treated with 30, 300, and 3000 ppm mancozeb in their diets (p <0.05, Table 8-12).

TABLE 8-12. Intracellular Free Calcium Concentration ($[Ca^{2+}]_i$) of Epididymal Sperm from Offspring at 8 Weeks of Age

Treatment (ppm)	No. of experiments	Mean of $[Ca^{2+}]_i$ (nM) ± SE	Relative change (%)
0	6	577.8±39.3	100
5	5	593.3±61.3	103
30	2	728.8±131.7	126
300	6	713.2±55.5	123
3000	4	845.8±102.0[a]	146

[a] Denotes statistically significant difference from the control (p < 0.05).

Relative distribution of DNA content of testicular germ cells

At 6 weeks of age, testes were removed from the offspring

of the 0, 30 and 3000 ppm treatment groups and were analyzed for relative distribution of DNA content in the testicular germ cells. The percentage of cell in the 1N population from the 30 ppm group was lower than that of the control group (p <0.05, Table 8-13). The percentages of cells in 2N and 4N populations from the 30 and 3000 ppm groups were significantly higher than that of control group ($p < 0.05$). At 8 weeks of age, the relative DNA distribution patterns of the testicular germ cells were analyzed in the 0, 5 and 3000 ppm treatment groups. No significant differences were observed in mean relative percentages of cells in the 1N and 4N populations of the testicular germ cells among all the treatment groups (Table 8-14). The percentage of cells in 2N population from the 5 ppm group, however, was lower than that of control group ($p < 0.05$).

TABLE 8-13. Relative DNA Distribution Patterns of The Testicular Germ Cell from Mancozeb Treated Offspring at 6 Weeks of Age

Treatment (ppm)	No. of samples	Relative DNA content (%)[a]		
		1N	2N	4N
0	4	48.4 ± 2.2	21.7 ± 3.6	6.8 ± 2.1
30	3	45.8 ± 1.9[b]	26.0 ± 1.2[b]	10.2 ± 0.6[b]
3000	3	49.4 ± 2.0	27.7 ± 1.5[b]	9.4 ± 0.4[b]

[a] Values represent means ± SE of observed Samples.
[b] Denotes statistically significant difference from the control ($p < 0.05$).

TABLE 8-14. Relative DNA Distribution Patterns of the Testicular Germ Cell from Mancozeb Treated Offspring at 8 Weeks of Age

Treatment (ppm)	No.of samples	Relative DNA content (%)[a]		
		1N	2N	4N
0	6	53.6 ± 3.2	24.2 ± 2.5	10.5 ± 1.1
5	6	58.2 ± 2.1	19.2 ± 0.9[b]	10.6 ± 1.2
3000	4	53.8 ± 4.3	21.5 ± 2.5	9.7 ± 2.5

[a] Values represent means ± SE of observed samples.
[b] Denotes statistically significant difference from the control ($p < 0.05$).

DISCUSSION

This study demonstrated that dietary mancozeb exposure to males before and during breeding and to females during breeding and gestation decreased the offspring survival at dosage of 300 ppm and above without affecting paternal fecundity or the litter size.

After 3-hr incubation in a capacitation-supporting medium, sperm motility appears to be higher in the 30 ppm-treated mice than in the control group. This trend of long-lasting motility, although appearing in all mancozeb-treated males, lacked a dose-response relationship (Table 8-4).

The decrease in peroxyl radical production in mancozeb-treated paternal CBA/CAJ mice was consistent with that

observed in mancozeb-treated C57BL/6J males described in the previous study (Chapter VII). In this study, the peroxyl-radical production was up to the maximum during the first hr, then declined afterward (Figure 8-1). Like the results discussed in Chapter VII, the hyperactive production of free radical and membrane changes in the first 2 hours may involve sperm function, and may be necessary for sperm capacitation through the peroxidation of unsaturated fatty acids.

Our study on the progress of sperm capacitation and acrosome reaction appeared to approve the biological role of free radicals in sperm capacitation and acrosome reaction. Observed with CTC fluorescence assay, mancozeb caused a subtle delay of the acrosome reaction in spermatozoa from the 30 ppm and 3000 ppm groups (Table 8-7). The delayed acrosome reaction may be due to the low free-radical activity in sperm membrane resulting in a insufficient peroxidation of membrane. However, the alteration of the progress of capacitation and acrosome reaction is subtle in the spermatozoa of CBA/CAJ males, the sperm fertilizing ability in vitro did not show a significant difference in all treatment groups (Table 8-8), unlike the results observed in Chapter VII.

A consistent decrease in the body weight of the offspring was apparent in all mancozeb-treated offspring up to 8 weeks of age; the difference between the treated and control offspring, however, varied at different ages.

Long-term exposure to mancozeb from conception through

gestation and lactation could affect sperm production in the male offspring during the onset of puberty. A delayed sperm production was demonstrated in the pubertal, 6-week-old, offspring. At 8 weeks of age, an enormous increase in sperm production in the 30, 300, and 3000 ppm treated offspring was observed, presumably due to over-compensation of the testicular function in spermatogenesis.

At an early stage of puberty (6 weeks of age), the percentages of testicular germ cells in the 1N population from the 30 ppm treatment groups were significantly lower than that of control group, while the percentages of cells in the 2N and 4N populations from the 30 and 3000 ppm groups were significantly higher than that of control (Table 8-13). The meiosis during spermatogenesis may be partially blocked resulting an accumulation of 2N-spermatocytes and relatively less 1N-sperm population. At 8 weeks of age, the percentages of the cells in the 1N population were the same among all the treatment groups, but, the 2N-cell population was lower than that of the control. Again, this observation may be due to a compensation of the testicular functions in spermatogenesis.

Similar to that observed in the previous study, sperm intracellular free calcium concentrations in the offspring of the 30, 300 and 3000 ppm treatment groups were 26, 23 and 46% higher, respectively, than the control value. Although the mechanisms of increased intracellular free calcium concentration are not fully understood, there are at least

three mechanisms that could accomplish this result: first, direct chemical plasma membrane injury may perturb intracellular calcium homeostasis, resulting in free calcium entry from outside the cells or the release from endoplasmic reticulum, mitochondria, and calcium-binding molecules; second, reactive sulfur, generated from EBDC degradation, may bind to proteins involved in calcium regulation; and third, impairment of energy-dependent, membrane-associated calcium transport systems diminishes the calcium efflux (Shi *et al.*, 1992).

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CHAPTER IX

SUMMARY AND PERSPECTIVES

The objectives of this dissertation were to examine the reproductive performance of mice fed maneb/zineb or mancozeb containing diets, and to explore the role of free radicals in the fertilizing ability of spermatozoa treated with ETU in vitro or collected from EBDC-treated mice. The studies indicated that at high concentrations, mancozeb decreased the body weight of treated males. EBDC exposure decreased the fertilizing ability of spermatozoa in males and decreased neonatal survival of the offspring. Intracellular free calcium concentration was a sensitive marker for EBDC exposure in the first generation. In the presence of ETU, lipid-peroxyl-radical production by spermatozoa increased. After sub-chronic exposure to mancozeb in vivo, sperm H_2O_2 production and lipid peroxidation activity decreased. Continuous exposure to mancozeb, beginning before conception, decreased the relative amount of spermatids and epididymal sperm concentration in pubertal males. After puberty, sperm production in the treated males increased and was more than twice the amount seen in the control.

This study also showed that sperm capacitation and acrosome reaction are under fine regulatory controls. Sperm fertilizing ability relies on a delicate interaction between free radical activity and the redox-defense systems. These regulatory controls, however, can be overwhelmed by chemical or physical perturbation.

Many questions remain unanswered concerning the control of free radical production in spermatozoa. Further investigations are needed to generate more knowledge in understanding cellular mechanisms of EBDC and ETU toxicity.

To explore the detailed process of free radical production, other spin-trap chemicals such as 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 2,2,6,6-tetramethyl-piperidinoxyl (TEMPO), or 2,2,6,6-tetramethyl-piperdinol-1-oxyl (TMPN) can be used to study superoxide and hydroxyl radical production from intact spermatozoa and sub-cellular components.

In order to study the role of free radicals in fertilizing ability, antioxidative mechanisms in spermatozoa should be further investigated. Several antioxidants, such as vitamin E and vitamin C, may protect spermatozoa against oxidative stress. Further studies could also explore the control mechanism of free radical production by comparing species variations in glutathione content and glutathione peroxidase/glutathione reductase activities in spermatozoa.