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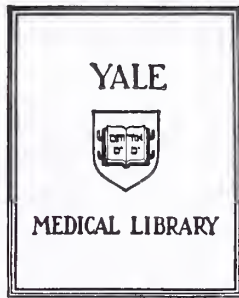
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MALONDIALDEHYDE LEVELS IN UTERINE WASHINGS
IN FERTILE AND INFERTILE WOMEN THROUGHOUT
THE MENSTRUAL CYCLE

Laura Michelle Drabkin

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**MALONDIALDEHYDE LEVELS IN UTERINE WASHINGS IN
FERTILE AND INFERTILE WOMEN THROUGHOUT THE
MENSTRUAL CYCLE**

**A Thesis Submitted to the Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine**

**by
Laura Michelle Drabkin
1992**

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ABSTRACT

MALONDIALDEHYDE LEVELS IN UTERINE WASHINGS IN FERTILE AND INFERTILE WOMEN THROUGHOUT THE MENSTRUAL CYCLE . Laura M. Drabkin, Ray Aten, Maria L Carcangiu, Gad Lavy, Dept. of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, CT 06510.

Partially reduced reactive oxygen species are important mediators of cell injury and cause lipid peroxidation. Malondialdehyde (MLD), a product of lipid peroxidation, can be used to determine indirectly the production of free oxygen radicals. This study was designed to examine lipid peroxidation in uterine fluids throughout the menstrual cycle in normal women and to establish a normogram to be used to evaluate the potential significance of lipid peroxidation in infertility.

Fifty-seven ovulatory women and 18 infertile women comprised the study population. Uterine washings were collected and MLD levels were measured with a fluorimetric assay and normalized for protein content. A pattern consistent with low levels of MLD during the late follicular phase ($LF=0.169\pm 0.035$), a nadir in the early luteal phase ($EL=0.093\pm 0.037$), and high levels in the early follicular phase ($EF=0.303\pm 0.046$) emerged in normal ovulatory women (one way ANOVA, $p=0.0475$). The MLD levels in the infertile group showed no discernible pattern.

Oxygen free radicals are present in both the endometrium of normal cycling women and infertile women, and may significantly affect the microenvironment of embryo implantation. This study indicates marked differences in lipid peroxidation in the various phases of the normal

menstrual cycle. It is possible that abnormal patterns of lipid peroxidation may contribute to infertility.

INTRODUCTION

Knowledge in the field of infertility is rapidly expanding and has recently included many new diagnostic and treatment modalities. However, despite new techniques such as ovulation induction and *in vitro* fertilization, pregnancy rates are still low, perhaps in part as a result of low endometrial receptivity. Unfortunately, knowledge of endometrial function is limited and methods used to evaluate endometrial receptivity are inaccurate. A new method of evaluating the state of the endometrium would be an important advance in the field of infertility.

The interaction between the endometrium and noxious environmental agents, such as oxygen free radicals, is not completely understood. Free radical species induce changes in normal cellular function through the process of lipid peroxidation. Membrane alterations and damage to DNA may be produced. Evidence now exists for the role of oxidant damage to tissues in the pathology of several human diseases, as well as in the process of normal aging.

The endometrium is highly dynamic and undergoes cyclic changes of tissue growth and destruction, including a period in which receptivity for embryo implantation is highest. The role of lipid peroxidation in these different processes is not known. Abnormalities in the equilibrium between oxidants and antioxidants may be a factor in infertility.

Malondialdehyde (MLD), a reactive species of peroxidized lipids that has been found in uterine washings, can be measured to determine levels of free O₂ radicals. MLD has been shown to correlate with tissue destruction in many organs of the body. By using fluorometry to measure the fluorescent reaction product of malondialdehyde and thiobarbituric acid

(TBA), even small differences between the lipid peroxide content of two samples can be measured.

Purpose

The purpose of this study is to examine lipid peroxidation in uterine fluids throughout the menstrual cycle in normal women and establish a normogram, which can then be used to evaluate the potential significance of lipid peroxidation in infertility.

LITERATURE REVIEW

Free Oxygen Radicals

A free radical is a conglomeration of atoms which contains one or more unpaired electrons and behaves as a discrete unit. An unpaired electron is one which occupies an atomic or molecular orbit by itself. This unpaired electron (or electrons) causes the free radical to be slightly attracted to a magnetic field and often causes it to behave in a highly reactive manner. Free radicals may be formed when covalent bonds are dissociated by energy; this energy can be provided by a variety of sources such as heat or electromagnetic radiation (1).

Many reactive species of oxygen can be generated by an input of energy. All respiring cells are capable of reducing molecular oxygen by four electrons (via cytochrome *c* oxidase) to form water. Although this tetravalent reduction may be completed without forming any free intermediary forms of reduced oxygen, most cells have additional mechanisms by which oxygen may be reduced by one, two, or three

electrons to yield free radicals (2). The superoxide radical O_2^- is formed by adding a single electron to the ground state oxygen molecule. The addition of one more electron produces the peroxide ion (O_2^{2-}), which is represented by hydrogen peroxide (H_2O_2) in most biological systems. Augmenting oxygen with three electrons will yield the hydroxyl radical, ($OH\bullet$). Addition of another electron eliminates the free radical state as the molecular orbits become completely filled and water is produced (1). There is evidence that these oxygen-derived free radicals may have a causative role in a variety of pathological conditions including ischemic-reoxygenation injury (2).

Lipid Peroxidation

Lipid peroxidation is a chain reaction made up of three steps: initiation, propagation and termination. In a biological system, LH denotes a membrane phospholipid containing a polyunsaturated fatty acid. Initiation of peroxidation can be accomplished by the attack of any species that has sufficient reactivity to abstract a hydrogen atom from LH to form the free radical $L\bullet$, with an unpaired electron. This readily reacts with oxygen to give a peroxy radical $LOO\bullet$. Following initiation, the propagation stage of the peroxidation sequence occurs in which the peroxy radical abstracts a hydrogen atom from another lipid molecule to form the lipid peroxide $LOOH$. Theoretically, once lipid peroxidation is initiated it can propagate as long as adjacent lipid molecules are accessible. In general, however, propagation of the peroxidation chain reaction will not proceed very far before a protein molecule is encountered, terminating the reaction sequence. The protein molecule itself is attacked and damaged in the process (2,3).

Termination also occurs if two peroxy radicals react to form non-radical inactive products. The length of the propagation phase varies with the substrates involved in the reaction. It is increased by an increase in the concentration of peroxidizable substrate and by an increase in the reactivity of the peroxy radical towards the substrate. It is decreased by an increase in the concentration of radicals and an increase in the reactivity of the peroxy radicals towards each other in the termination reaction (4).

Lipid peroxidation involves the direct reaction of oxygen species with unsaturated lipids and the resultant production of semi-stable peroxides. It is widely believed that oxygen species initiate the peroxidation process in the form of the perferryl radical ($\text{Fe}^{3+}\text{-O}_2\bullet^-$) where the Fe^{3+} is bound to a chelator (2, 5). Other possible pathways of initiation include $\text{O}_2\bullet^-$ generators (hypoxanthine and xanthine) in conjunction with chelators or preformed lipid peroxides bound to chelators (2). In this capacity, the transition metal iron converts a poorly reactive oxygen species into a highly reactive one capable of initiating lipid peroxidation (6). Lipid peroxidation cannot occur in the absence of a transition metal. Furthermore, lipid peroxidation can be inhibited, even after the process has been initiated, by chelating the transition metal (7).

Transition-metal complexes serve another catalytic function in the peroxidation process by aiding in the decomposition of lipid peroxides which are relatively stable molecules at physiologic temperatures. Both iron in its various forms, [Fe(II) and Fe(III) salts, Fe salts with phosphate compounds, non-heme-iron proteins, free heme, hemoglobin, myoglobin and cytochromes] and copper serve as effective catalytic metal compounds, although iron is much more commonly involved. Reduced iron reacts with a lipid peroxide causing fission of the O-O bond to produce an alkoxy

radical (LO•). Oxidized iron combines with lipid peroxides to form a peroxy radical (LOO•). Although peroxy radicals are less reactive than alkoxy radicals, both are able to further stimulate lipid peroxidation by initiation of the free radical chain reaction.

In addition to alkoxy and peroxy radicals, these reactions involving iron produce a variety of hydrocarbons and aldehydes. For example, when reduced iron reacts with a hydroperoxide on the fifth carbon from the methyl end of a fatty acid, pentane gas is the product (3). In a reaction with a polyunsaturated fatty acid with at least three double bonds, after a complex series of breakdown reactions of spontaneous fragmentation (*β-scission*) of peroxides, malondialdehyde is produced (8).

Measuring Peroxidation

Malondialdehyde (MLD) is important in that it enables measurement of the extent of lipid peroxidation through the thiobarbituric acid (TBA) test. In this test the material to be measured is heated with thiobarbituric acid under acidic conditions and the formation of a pink color is measured at 565 nm (9,10). Malondialdehyde proves useful as a marker for lipid peroxidation for two reasons: the rate of MLD formation and disappearance are balanced, providing a constant amount of product to measure (8); MLD readily combines with TBA to generate a fluorescent product which is easily measurable (8,9). Although MLD is unstable, the ability to use tetramethoxypropane (which converts quantitatively to malondialdehyde during the reaction procedure) as a standard eliminates this instability as a problem (10). Unfortunately, the specificity of the TBA reaction is low. Compensation for this low specificity is accomplished by separating the lipid peroxides from interfering substances

which can give false positives and by measuring the fluorescent product at its specific excitation spectrum (565 nm) (10).

Electron spin resonance (ESR) is another method of detecting lipid peroxidation (11). ESR detects the presence of unpaired electrons and therefore can be used to detect free radicals. An electron behaves as a small magnet with a charge of either $+1/2$ or $-1/2$. When exposed to an external magnetic field, an electron can align itself either parallel to or anti-parallel to the field, thereby taking on one of two possible energy levels. If the electron absorbs applied electromagnetic radiation, it can move from a lower energy level to a higher one. In this way an absorption spectrum is obtained. The equation $\Delta E = g\beta H$ [ΔE =the energy gap between two energy levels of the electron, H =applied magnetic field, β =a constant (the *Bohr magneton*) and g =the splitting factor (2.00232 for a free electron)] may be used to describe the absorption spectrum (11).

ESR can be used on any relatively stable radical and is sensitive enough to detect radicals at concentrations as low as 10^{-10} per mole provided they exist long enough to be measured. When dealing with very unstable radicals, a useful technique is spin trapping. In spin trapping, a highly reactive radical is allowed to react with a compound (such as a nitroso compound RNO) to produce a more stable radical with a longer lifetime. Spin trapping has often been used to detect the presence of superoxide and hydroxyl radicals in biological systems and also to measure the formation of organic radicals produced during the process of lipid peroxidation (11).

Lipid Peroxide-Induced Cellular Damage

Cell membranes have fundamental tasks in the proper functioning of cells. The membrane is made up of lipids and proteins, the protein content

varying with the function of the individual cell. Lipid molecules are arranged in a bilayer with a hydrophobic interior and hydrophilic regions facing the exterior. Membranes provide the controlled environment where most of the biochemical and biophysical events of the cell occur. This is accomplished by both active and passive processes. The active processes operate predominantly by metabolic energy. The passive regulation of membrane function is determined by membrane fluidity and the protein-lipid interplay, where lipid dynamics play the dominant role (12).

Membrane fluidity is largely due to the presence of unsaturated and polyunsaturated fatty acids in the lipid bilayer which decrease the melting point and the microviscosity of the membrane interior. The molecules of the bilayer diffuse almost freely in the plane of the membrane. In each half of the bilayer the protein and lipid elements can diffuse very quickly. However, exchange between the two halves of the bilayer is extremely rare (13).

Functionally, membrane fluidity is of vital importance to the cell. Essential cellular functions such as division and differentiation are affected by changing the dynamics of passive diffusion within the membrane. This effect is believed to be due to decreasing the mobility of the functional units (proteins) imbedded in the lipid bilayer. In general, the growth and vitality of cells are suppressed as the lipids of the cell membrane become more viscous and membrane fluidity decreases (12).

Damage to polyunsaturated fats reduces membrane fluidity by increasing the melting point and microviscosity of the cell membrane (14). Because lipid peroxidation specifically attacks fatty acids with polyunsaturated side chains, it would be anticipated that this process would alter membrane function. In fact, the best described mechanism of

membrane damage induced by free radicals is through lipid peroxidation. Dobretsov et al have shown that both translational and rotational diffusion decreases concomitant with an increase in the products of lipid peroxidation (15). The result is a decrease in the membrane's fluidity throughout its hydrophobic space and the subsequent loss of normal membrane function. This physical/functional correlation was demonstrated by a decrease in mitochondrial respiratory control from 6.7 to 2.0 units after 10 nmol of MLD had been accumulated per milligram of lipid; this was the same degree of peroxidation at which changes in membrane viscosity were observed. It has also been shown that lipid peroxidation interferes with such basic functions of the cell membrane as the regulation of ionic permeability. Uncontrolled flow of ions across the membrane can quickly result in a dysfunctional cell (16).

The membranes of intracellular organelles are also subject to lipid peroxidation. Repeated fragmentation of fatty acid side chains in the lipid bilayer may lead to complete loss of membrane integrity and possibly to rupture of the organelle. The integrity of cell structure is largely dependent on the stability of lysosomal membranes. Rupture of lysosomal membranes enables hydrolytic enzymes to spill into the cytoplasm and cause degradation of most of the macromolecules within the cell. Fortunately, the lysosome has fewer polyunsaturated lipids and lacks some of the catalytic agents of peroxidative reactions. These characteristics combine to give lysosomes a considerably slower rate of peroxidation than either mitochondria or microsomes (3,17).

Other subcellular membranes are more susceptible to the damaging effects of lipid peroxidation. This is likely due to the fact that organelles such as mitochondria and microsomes have a higher content of

polyunsaturated fatty acids than the lysosome membrane. The membranous organization of the microsome renders it very labile to peroxidation, especially in the presence of iron and a reducing agent.

The mitochondria and endoplasmic reticulum are examples of organelles whose functions are integrally related to their structure. Disruption of the structure of the mitochondria by lipid peroxidation can lead to an uncoupling of oxidative phosphorylation. Damage to this energy-producing organelle can profoundly affect cellular function. Similarly, disaggregation of polyribosomes on the endoplasmic reticulum caused by free radical attack can disrupt protein synthesis (17).

Peroxidation of membrane-bound proteins often has more significant consequences than its effects on the membranes themselves. Based on measurements of moles of enzymes inactivated per mole of free radical, lipid peroxidation has been shown to be one tenth as damaging to enzymes as ionizing radiation (18). When subject to lipid peroxidation, enzymes and other proteins undergo polymerization, polypeptide chain scission and chemical changes to individual amino acids. Although all three reactions are important in generating cellular damage, emphasis has been placed on polymerization as a result of cross-linking of proteins. When enzymes are exposed to either peroxidizing polyunsaturated lipids or to the products of peroxidation, they undergo cross-linking with resultant molecular weight increases to many multiples of the monomolecular form. The precise organization of proteins and enzymes required for their biological activity is disrupted resulting in impairment or loss of function.

In an experimental model described by Tappel (17), ribonuclease A was reacted with peroxidizing lipid. As the formation of fluorescent product in the reaction mixture increased, the activity of the ribonuclease decreased

through inactivation. The products of the peroxidizing system were shown to include fluorescent monomer, dimer and higher molecular weight species through fractionation. It has also been shown that intramolecular and intermolecular cross-linking of enzymes can cause loss of function by producing structural abnormalities in the membrane in which the enzymes are imbedded (17).

Vaca et al have demonstrated that chemical alterations and damage to DNA may be produced in the various steps of lipid peroxidation (19). DNA destruction would lead to impairment of the functional and metabolic activity of the cell and, ultimately, to cell division. They conclude that there may be two main types of DNA damage caused by peroxidizing lipids, one affecting DNA bases which is sequence specific, and the other affecting the DNA backbone which is not sequence specific. These forms of DNA modification are accompanied by physio-chemical changes such as decreased melting point, decreased template activity, and resistance to enzymatic hydrolysis. Malondialdehyde, the most widely studied product of lipid peroxidation, has been shown to induce inter-strand cross-linking of DNA between the base pairs adenine and guanine *in vitro*, thereby causing the loss of template activity (19).

The research regarding DNA and lipid peroxidation product interaction has been inconclusive to date. The majority of the studies conducted have consisted of oversimplified model systems of pure DNA and lipid peroxidation products. These models fail to account for the actual conditions in the cell; cellular defense mechanisms against oxidative damage and the fact that nuclear genetic material is virtually embedded in protein (histone and non-histone proteins) which is protective against reactive species. Additional *in vivo* studies with more sensitive

methodologies are necessary to determine the role lipid peroxidation plays in mutagenesis (19, 20).

Antioxidants

An endogenous balance exists between the production of free radicals and antioxidant defense mechanisms. Antioxidants can be defined as any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate (21). Damage occurs if the capabilities of these protective agents are overwhelmed either by excessive generation of free radicals or by inadequate antioxidants or free radical scavengers. Each of these conditions are known to occur in various disease states.

There are six major pathways in which antioxidants may function to inhibit lipid peroxidation:

1. By decreasing local O_2 (by combining with O_2 or displacing it).
2. Scavenging species which are capable of abstracting hydrogen atoms.
3. Quenching singlet O_2 (which can react directly with membrane lipids to produce peroxides).
4. Binding metal ions in forms that will not generate reactive species and/or will not decompose lipid peroxides to peroxy and alkoxy radicals.
5. Removing peroxides by converting them into non-radical products, such as alcohols.
6. Reacting with the chain propagating radicals (peroxy, alkoxy) to prevent continued hydrogen abstraction from fatty acid side chains.

Antioxidants that function by mechanisms described in 1-5 above are termed *preventative* antioxidants. Preventative antioxidants function by reducing the rate of chain initiation. *Chain-breaking* antioxidants act by interfering with one or more of the propagation steps, as described in mechanism number 6 above (21).

The primary mechanism for clearance of superoxide anions is superoxide dismutase (SOD). Superoxide dismutases are protective enzymes that specifically scavenge the superoxide radical by catalyzing its dismutation to hydrogen peroxide and oxygen. The enzyme exerts its cytoprotective effect by keeping the intracellular superoxide concentrations very low (22, 23).

Catalase and various peroxidases or glutathione peroxidase function to remove hydrogen peroxide. Catalase and the peroxidases effectively detoxify hydrogen peroxide by catalyzing its reduction to water. Glutathione peroxidase is located strategically in the same cellular compartment as SOD, in a position to remove the hydrogen peroxide produced by that enzyme (2).

The extreme reactivity of the hydroxyl radical precludes its enzymatic removal. Protection against this radical is dependent on either the prevention of its formation or on scavenging by antioxidants (23). N-2-mercaptopropionyl glycine and mannitol scavenge the hydroxyl radical (24).

Xanthine oxidase has been shown to be a source of a large amount of superoxide radical (24). Allopurinol, a competitive inhibitor of xanthine oxidase, protects tissues from the oxidants produced by that enzyme. Oxypurinol, a more potent and noncompetitive inhibitor of xanthine

oxidase, may be a better antioxidant because it not only functions as an inhibitor but also as a radical scavenger. (6)

As stated previously, metal ions, particularly iron, play a large role in generating reactive species. Therefore maintaining low free iron concentrations is important in preventing free radical damage. This can be accomplished through chelation (i.e. with deferoxamine) or by binding iron to proteins. This is accomplished intracellularly by ferritin and extracellularly by transferrin and neutrophil derived lactoferrin (23).

Vitamin E effectively serves as the major lipid-soluble chain-breaking antioxidant, preventing lipid peroxidation in human tissue by inhibiting propagation of chain reactions. It functions synergistically with ascorbic acid (vitamin C), which can react with vitamin E radicals to regenerate vitamin E. Vitamin C radicals can, in turn, be reduced by NADH reductase. Because of its lipophilic nature, vitamin E is likely to function as an antioxidant within membranes. Water soluble vitamin C likely works in the cytosol or extracellular fluid (22).

α -Tocopherol, the most active form of vitamin E, has optimal properties for trapping peroxy radicals. Because it can compete very effectively with substrate for peroxy radicals, small amounts are able to protect large amounts of peroxidizable lipid. The reaction of α -tocopherol with peroxy radical has two important characteristics. First, it produces α -tocopherol radicals with near-maximal stability which ensures that it is unable to continue the propagation reaction by attacking other species. Second, the rate-limiting reaction occurs rapidly. This means that it is better able to compete with oxidizable substrate for the radicals and therefore the substrate is subject to less peroxidative damage (4).

Vitamin E deficient states have been shown to result in free radical related diseases (i.e. in low birth weight infants, which can be exacerbated by the necessity of using 100% oxygen inhalation therapy). Research evidence may be significant in proving a protective role for vitamin E in controlling free-radical related damage in peroxidation in body tissues (25). Therapies based on supplementation with vitamin E and other antioxidants are being investigated .

The Ischemic-Reperfused Myocardium As A Model

As new interventions such as thrombolytic therapy and percutaneous transluminal coronary angioplasty have come into use to reestablish coronary flow in patients with myocardial infarction, experience with reperfusion of anoxic myocardium has broadened. Although it has long been known that reestablishing adequate coronary flow is necessary for the recovery of ischemic myocardium, it has recently been recognized that reperfusion of previously anoxic myocardial tissue has negative consequences of its own (26). During reperfusion, molecular oxygen is converted to oxygen metabolites, which are toxic and promote tissue injury.

Studies have shown that reperfusion of heart muscle after 60 minutes of ischemia is associated with influx of calcium, the release of intracellular enzymes, mitochondrial dysfunction, disruption of cellular membranes, persistent decrease in contractility, transient rise of diastolic blood pressure and eventual necrosis of at least a portion of the tissue. As much of this damage is believed to occur at the time of reperfusion with reintroduction of molecular oxygen, rather than during the period of ischemia, this phenomenon is termed reperfusion damage (22).

Reactive oxygen species have been implicated in the cellular injury that occurs during the reperfusion of ischemic myocardium. Normally, the tissue concentration of reactive free oxygen radicals is small and the production of new oxidants is balanced with antioxidant defenses. It has been shown that in previously ischemic reperfused myocardium there is a large production of active oxygen species (27, 28). Since reperfusion damage has been mimicked by peroxides and several antioxidants have proven to be protective against this type of damage, it is probable that this phenomenon is caused by an overwhelming of protective mechanisms by active oxygen species. This phenomenon has sometimes been called "oxidative stress" (20).

Several *in vitro* studies with isolated hearts subjected to global ischemia and reperfusion have described an oxygen free radical burst occurring within the first few minutes after reperfusion (29, 30, 31). Arroyo et al used electron spin resonance and spin trapping techniques to show that oxygen radicals were present in myocardial tissue of canine hearts following regional ischemia (32). Using electron paramagnetic resonance spectroscopy and a spin trapping agent, Bolli et al demonstrated a burst of free radical formation within the coronary venous outflow of dogs subjected to 15 minutes of coronary occlusion and reperfusion (28).

Due to this oxidative stress, the cellular membranes develop an inability to control calcium gradients causing an increase in cytosolic Ca^{2+} (33). The calcium is believed to originate from three sources: the endoplasmic reticulum, the mitochondria and the extracellular space. These leaks are due to the decrease in cellular ATP which is a consequence of the switch to the less efficient anaerobic metabolic pathways after the decline in tissue oxygen tension. The increase in cytosolic Ca^{2+} has two major effects, both

which cause breakdown of the cytoskeleton: activation of Ca^{2+} - dependent proteases and an activation of phospholipase A_2 (2).

One Ca^{2+} -dependent protease which is activated by this mechanism is calpain. Calpain causes the conversion of xanthine dehydrogenase to xanthine oxidase. Hypoxanthine reacts with molecular oxygen in the presence of xanthine oxidase to form xanthine. Xanthine is then converted into uric acid by xanthine oxidase, and superoxide anions are formed. While one substrate involved in this reaction, hypoxanthine, accumulates during ischemia as a result of ATP degradation, the other, molecular oxygen, is provided by reperfusion. Xanthine oxidase has been shown to be responsible for the production of large amounts of superoxide radical in post-ischemic tissue by participating in this series of reactions (34, 35).

The other major consequence of the influx of Ca^{2+} , the activation of phospholipase A_2 , causes the release of arachidonic acid from phospholipids. This sets the arachidonic acid cascade into motion, which promotes the synthesis of prostaglandins, leukotrienes and thromboxanes. The leukotrienes which are produced stimulate adhesion of leukocytes to the endothelial wall. If the endothelium is damaged, as it usually is in previously ischemic tissue, this can cause activation of the NADPH oxidase which produces $\text{O}_2^{\bullet-}$. Therefore, the influx of Ca^{2+} sets two pathways into motion, both resulting in enhanced enzymatic production of free oxygen radicals with the potential to initiate lipid peroxidation (2).

Other possible intracellular sources of oxygen free radicals are activated neutrophils and mitochondria. The enzyme NADPH oxidase is present within neutrophils as part of their antibacterial armament. Neutrophil activation occurs early in ischemia and reperfusion markedly enhances the infiltration of neutrophils into the ischemic region. When neutrophils are

activated, NADPH oxidase reduces molecular oxygen to form superoxide anion (35).

Superoxide radicals may also be produced from leakage of electrons from the electron transport system within mitochondria. As the adenine nucleotide pool is partially degraded during ischemia, the mitochondria take on a more reduced state than usual. This leads to a higher degree of electron leakage from the respiratory chain that will subsequently react with the molecular oxygen and form superoxide radicals (22, 35). The most crucial source of free radicals for these processes is still unresolved.

Davies et al investigated the role of lipid peroxidation in reperfusion injury by measuring thiobarbituric acid reactive material (TBA-RM) in patients receiving thrombolytic therapy for acute myocardial infarction (36). The study showed that TBA-RM rose in the arteries which regained patency but fell in the arteries which remained occluded. This demonstrates that lipid peroxidation is associated with reperfusion injury and provides indirect evidence that free radical generation occurs at the time of reperfusion .

Further evidence in support of the free radical cause of reperfusion injury is provided by many studies that have reported a decrease in myocardial damage with the use of antioxidants, free radical scavengers and iron chelators. Reduction of infarct size and left ventricular dysfunction was reported in several laboratories when the free radical scavengers superoxide dismutase and catalase were administered before ischemia and reperfusion occurred (37, 38, 39). Przyklenk and Kloner observed that dogs treated with these agents had improved myocardial contractility during the reperfusion phase (38). Gross et al found similar results when using these free radical scavengers with their canine models

(39). Jolly et al found that these agents decreased total infarct size when administered before the ischemic event (40). A study by Myers et al demonstrated enhanced post-ischemic contractile function following pretreatment with the hydroxyl radical scavenging agent N-2-mercaptopropionylglycine (41). Similarly, Gardner and colleagues showed that the administration of the scavenging agents superoxide dismutase, catalase and mannitol resulted in significantly better recovery of left ventricular function after ischemia and reperfusion (42).

Gardner et al also demonstrated that both superoxide dismutase and allopurinol significantly decreased the extent of myocardial necrosis which developed following coronary artery occlusion and reflow (42). This was reinforced by Werns et al, who demonstrated the efficacy of allopurinol in decreasing infarct size when used to pretreat dogs undergoing occlusion of the left coronary artery (43). Studies by Bolli et al and Farber et al using the *in vivo* canine model reported enhanced post-ischemic contractile function of myocardium with the iron chelator deferoxamine (44, 45). The fact that agents which either decrease the amount or the functioning of free radicals consistently prove effective in decreasing the extent of myocardial damage and dysfunction strongly suggests that free radicals play a significant role in reperfusion injury of the myocardium.

The increased use of thrombolytic therapies and transluminal angioplasty has also raised the relative importance of investigating new therapeutic approaches for limiting the free radical-mediated damage which occurs during reperfusion injury. Approaches with the agents mentioned above are being explored to minimize the tissue necrosis which occurs both during arterial occlusion and reperfusion. In addition, investigation is concentrating on inhibitors of activated neutrophils,

leukocyte depletion and substances that reduce endothelial cell damage of capillaries. Each of these methods would interfere with one potential source of free oxygen radical - the neutrophil (24).

Other Examples Of Reperfusion Injury

Free radical damage has been implicated in other types of post-ischemic tissue injury such as brain cell death, small intestinal ischemia and circulatory shock. Post-ischemic brain death secondary to cardiac arrest is a common complication of cardiovascular disease. With the onset of cardiac arrest, decreased cerebral blood flow causes cessation of the supplies of oxygen and glucose to the brain. In three to four minutes high-energy phosphate levels are depleted. The energy-dependent ion pumps then fail and ionic homeostasis is lost. Intracellular calcium levels increase greatly and this sets into motion the set of events previously described. With the resumption of perfusion, abundant molecular oxygen becomes available as substrate for the various reactions. Xanthine oxidase metabolizes the accumulated hypoxanthine to produce superoxide radical and the burst of superoxide delocalizes iron from ferritin. This iron is then available to catalyze and set the chain reaction of lipid peroxidation into motion. Lipid peroxidation eventually destroys the integrity of cellular membranes and causes degradation of DNA and ultrastructural nuclear disorganization (46).

Additional evidence for the importance of oxygen radicals in the etiology of cerebral reperfusion damage has been provided by Cerchiari et al (47). They used cerebral blood flow, oxygen consumption and somatosensory evoked potentials to measure cerebral recovery after cardiac arrest in patients treated with superoxide dismutase and

deferoxamine prior to their arrest. Mitigation of cerebral damage in subjects who received this anti-free radical combination therapy supports the conclusion that oxygen free radicals and lipid peroxidation play a role in the pathogenesis of arrest-related derangements of cerebral blood flow and function. Further research based on this hypothesis may lead to the development of an intervention that will be effective in preventing major neurologic injury after resuscitation from cardiac arrest (47).

The intestinal mucosa is a tissue which is very vulnerable to oxygen free radical injury during post-ischemic reperfusion. This is true not only because the intestinal mucosa is the tissue most sensitive to ischemic injury, but also because the villi of the intestine contain the highest concentrations of xanthine dehydrogenase of any tissue in the body (48). In cats, one hour of local arterial hypotension followed by re-establishment of normal blood flow greatly increases intestinal vascular permeability (49). This effect was mimicked by intraluminal perfusion of animal bowel with a superoxide-generating system consisting of hypoxanthine and xanthine oxidase (50). After three hours of ischemia, the injury is characterized by the development of mucosal lesions, necrosis, hemorrhage and ulceration (51). Subsequent studies have found that damage could be decreased or eliminated by pretreatment with either superoxide dismutase or allopurinol (52, 53).

Hemorrhagic shock can be viewed as a type of 'whole body ischemia,' with insufficient perfusion of all body tissues. ATP levels are severely decreased in the pancreas, liver and intestine, leading to elevated plasma levels of hypoxanthine. Reestablishing blood flow restores tissue perfusion and leads to free radical-induced reperfusion injury. Strong support for this hypothesis is provided by Crowell et al who showed that administration



of allopurinol substantially increased the survival rate of dogs subjected to hemorrhagic shock (54). Another study demonstrated that superoxide dismutase afforded protection to dogs subjected to hypovolemic shock (55). A single intravenous dose of SOD eliminated the sequestration of blood into the gut, persistent vasoconstriction in the kidneys and the development of hemorrhagic lesions in the intestinal mucosa.

The Endometrium

The endometrium is an extremely dynamic tissue, fluctuating with the levels of circulating estrogens, progesterones and androgens. This responsiveness allows the tissue to cycle each month and ready itself for implantation and pregnancy. The endometrium can be divided into two functional components, the functionalis and basalis. The functionalis is the upper two-third portion of the endometrium that is sloughed during menstruation. It is the portion which undergoes morphologic and functional cyclic changes throughout the menstrual cycle and which accepts the blastocyst for implantation. The basalis is the lower one-third portion which remains relatively unchanged throughout the cycle. After the upper portion of the endometrium is shed during menstruation, the basalis provides stem cells for its regeneration each month. Basal arteries are the blood vessels found in the basalis. Spiral arteries are specially coiled blood vessels found in the functionalis (56).

The Menstrual Cycle

The menstrual cycle is a complex endocrinologic event which causes repetitive monthly changes in the functional portion of the endometrium. The process reflects the functional integration of ovary, pituitary gland and

central nervous system. On average, the cycle lasts for a total of 28 days, which can be divided into a follicular phase (approximately 11 days), mid-cycle ovulation, a luteal phase (approximately 14 days) and menstruation which lasts approximately 3 days. Structural changes in the composition of the endometrium reflect each stage of the menstrual cycle.

Ovarian Hormones: During the follicular phase, gonadotropins initiate new follicular development and growth and prepares one follicle for ovulation. Estrogen levels start low in this stage and then begin to rise approximately one week before ovulation to reach an eventual maximum one day before the surge in luteinizing hormone (LH). Following the LH peak, directly before ovulation, a precipitous drop in estrogen levels occur. Progesterone and 17 α -hydroxyprogesterone levels remain low throughout most of this phase. However, just before ovulation, the unruptured but luteinizing graafian follicle begins to produce some progesterone. At the same time there is an increase in 17 α -hydroxyprogesterone production.

At mid-cycle, the final maturation of the follicle follows the preovulatory surge of gonadotropins. This results in the induction of ovulation. At the time of ovulation estrogen levels decline and progesterone rises.

In the luteal phase, the endometrium is prepared by the neuroendocrine system for implantation of the blastocyst by the activation of corpus luteal function. During this phase, estradiol levels rise again and reach a new peak approximately five to seven days after ovulation. Then estradiol levels decrease to baseline shortly before menstruation. Progesterone levels, which were previously low, increase as a result of progesterone and 17 α -hydroxyprogesterone secretion by the newly formed corpus luteum. Like estrogen, progesterone levels peak five to seven days after ovulation

and return to baseline directly before menstruation. Menstruation occurs after the cessation of corpus luteal functioning.

Gonadotropins, Hypothalamic Releasing Hormones and Feedback

Mechanisms: The hypothalamus and pituitary gland play important roles in the cycle and participate in a feedback mechanism which ensures correct functioning of the process. The anterior pituitary produces follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotropins are synthesized and stored in gonadotrophs in the anterior pituitary.

Gonadotropin-releasing hormone (GnRH), is a peptide secreted by the hypothalamus which is responsible for both the synthesis and release of both LH and FSH. GnRH is released by the hypothalamus and travels down the hypophyseal portal vessels to the anterior pituitary where it stimulates the secretion of the gonadotropins. GnRH is released in a pulsatile pattern which is fundamental to normal gonadotropin secretion and ovulation.

With the regression of the corpus luteum, circulating estradiol and progesterone levels decrease which results in a small rise in FSH output. This begins the follicular phase of the menstrual cycle with FSH stimulating follicular growth. Subsequently, LH levels begin to increase. Approximately seven days before mid-cycle ovulation, estradiol levels begin to increase, reaching the maximum shortly before the mid-cycle LH surge. Increasing estradiol levels produce a negative feedback effect on FSH and a positive feedback effect on LH.

Late in the follicular phase, rising estradiol levels initiate the mid-cycle FSH and LH surges. Estradiol increases hypothalamic secretion of GnRH which enhances pituitary secretion of gonadotropins. A small rise in progesterone secretion enhances these effects of estradiol.

The mid-cycle LH surge induces final maturation of the follicle and subsequently, ovulation. The FSH surge is similar to, but smaller than the LH surge. High mid-cycle FSH levels may play a role in inducing LH receptors in the preovulatory follicle, thereby increasing its sensitivity to LH. This facilitates LH-mediated follicular growth, ovulation and luteinization. Following the LH surge, both parts of the mature follicle, the granulosa cells and the theca interna cells undergo luteinization to become a corpus luteum made up of granulosa lutein cells, theca lutein cells, capillaries and connective tissue. This new structure secretes large amounts of progesterone and estradiol.

During most of the luteal phase, high levels of estradiol and progesterone exert potent negative feedback on the gonadotropins. LH and FSH secretion remain low until the corpus luteum begins to regress towards the end of the luteal phase and its secretion of hormones diminishes. This results in removal of feedback inhibition and serum FSH levels again rise to prepare the follicle for the next cycle.

Ovarian steroids can exert either negative or positive feedback on gonadotropin secretion. Usually estradiol inhibits the release of the gonadotropins, especially when combined with high levels of progesterone. However, under specific conditions, estradiol can stimulate gonadotropin secretion through positive feedback.

Endometrial Changes With Menstruation

An important end point of the hypothalamic-pituitary-ovarian interaction is to prepare the endometrial tissue for implantation. Thus, while hormone levels fluctuate, changes occur in the endometrial tissue. Growth (proliferative phase), differentiation (secretory phase) and

regression (menstruation) proceed under the control of the cyclic pattern of hormone secretion.

Estradiol and progesterone exert their effects on endometrial cells by affecting RNA and protein synthesis. These effects are mediated by specific intranuclear receptor sites which undergo cyclic change. The nuclear estradiol receptor concentration reaches its peak during the mid to late proliferative phase, correlating with endometrial growth and cell division. Progesterone receptor concentration experiences a preovulatory rise and is highest directly after ovulation (57).

The proliferative phase of the endometrium, which is concurrent with the follicular phase of the menstrual cycle, is characterized by endometrial proliferation secondary to estrogen stimulation. By day five of the cycle most of the epithelial surface which was shed during menstruation has been reconstituted. Increased estrogen secretion from the ovaries stimulates marked cellular proliferation of the epithelial lining, the endometrial glands and the stromal connective tissue. Evidence of tissue proliferation at this stage of the cycle includes an increase in free and bound ribosomes, mitochondria, Golgi apparatus, and primary lysosomes in gland cells and stroma. These organelles provide the protein matrix and energy necessary for the synthesis of enzymes.

Features typical of the proliferative endometrium are increased ciliogenesis and microvillogenesis. Both seem to be important in facilitating mobilization and distribution of endometrial secretions during the luteal phase. By the end of this phase, cellular proliferation and endometrial growth reach a peak, there is pseudostratification of nuclei in the epithelium, the spiral arteries become long and convoluted, and the glands become tortuous. True secretory function does not occur until

triggered by progesterone secretion by the corpus luteum in the secretory phase (56, 57, 58).

After ovulation, the corpus luteum secretes progesterone which stimulates differentiation in the estrogen-primed endometrium. The glandular cells begin to secrete glycogen, mucus and other substances. Secretion begins as vacuolization in the glandular epithelium beneath the nuclei. The nuclei are eventually displaced to the middle of the cell by a row of glycogen-rich vacuoles at their base and finally appear at the glandular surface. Ultrastructurally, giant mitochondria and the "nucleolar channel system," a substructure which presumably facilitates transport of nuclear RNA to the cytoplasmic substance, appear in gland cells. The glands become tortuous with dilated lumens that are filled with secretions. In addition to glycogen and mucus, the glands also secrete uteroglobin-like protein, progesterone-associated endometrial protein (PEP), and pregnancy-associated endometrial α_2 -globin (α_2 -PEG). These proteins are probably related to implantation and corpus luteum function. Secretions reach a maximum level in the mid-to-late luteal phase and coincide with the time of blastocyst implantation. Glands become so tortuous that they are referred to as having serrated margins. The stroma becomes maximally edematous and the spiral arteries become increasingly convoluted and extend into the superficial layer of the endometrium.

Predecidualization occurs around cycle days 21-23 to prepare the endometrium for pregnancy. This process consists of cytonuclear enlargement with increased nuclear DNA synthesis, mitotic activity, and the formation of a peri-cellular laminin-rich basement membrane and is accompanied by lymphocytic infiltration. The cytoplasm of the cell becomes increasingly eosinophilic and compact. If fertilization occurs,

endometrial stromal fibroblasts are transformed into gestational decidual cells following blastocyst implantation. Decidual cells are large, rich in both glycogen and lipids, and control the invasive nature of the implanting trophoblasts (58).

If fertilization does not occur by day twenty-three of the cycle, without sustaining quantities of human chorionic gonadotropin from the trophoblast, the corpus luteum begins to regress. All of the structural changes stimulated and supported by the corpus luteum begin to decline as well. Secretion of progesterone and estradiol decrease and the endometrium undergoes involution. The lysosomal membranes, previously stabilized by progesterone, lose their integrity and its destructive enzymes are released. As a result, all cellular elements are digested, and in the vascular endothelium this leads to vascular thrombosis, platelet deposition, release of prostaglandins, extravasation of red blood cells and tissue necrosis.

Approximately one day before the onset of menstruation, many vasomotor changes occur. The cause of this is unknown but it is likely that several factors combine to cause constriction and dilation of the spiral arterioles. The hormonal withdrawal which accompanies the regression of the corpus luteum causes shrinkage of tissue height, leading to diminished blood flow within the spiral vessels, decreased venous drainage and eventually to vasodilatation. Thereafter, the spiral arteries undergo successive bouts of vasoconstriction and dilation (59). Results of several studies indicate that prostaglandins, which cause both constriction of the spiral arterioles and myometrial contraction, and are abundant in the premenstrual endometrium and in menstrual blood, may be involved (60, 61, 62). Increased prostaglandin synthesis or an imbalance between

vasoconstricting prostaglandin $F_{2\alpha}$ and vasodilating prostaglandins E_2 and I_2 have been suggested as possible sources of increased menstrual blood loss (60, 61, 62). This rhythmic vasospasm and dilatation coupled with contraction of the endometrium causes ischemia of the functionalis and stasis, followed by leukocyte infiltration and red blood cell extravasation. The combination of these factors results in tissue necrosis and subsequent sloughing of the endometrium and menstrual bleeding (33, 34).

During menstruation, there is dissociation and disintegration of the endometrial glands and stroma. Additional red blood cell extravasation occurs and diffuse interstitial hemorrhage occurs throughout the entire functionalis as a result of breaks in superficial arterioles and capillaries. With the influx of interstitial blood, numerous leukocytes appear in the stroma. The functionalis is completely shed and the basalis becomes compressed due to loss of ground substance. Therefore, the menstrual endometrium is a relatively thin but dense tissue. In the midst of these degenerative changes, new tissue begins to grow in the basalis to replace the functional tissue lost. The endometrium prepares for a new cycle.

The process of menstruation is comparable to that of ischemia and reperfusion injury. Endometrial ischemia results from the constriction of the spiral arterioles rather than thrombotic occlusion of coronary arteries; reoxygenation is the result of vasodilatation and the influx of interstitial blood rather than thrombolysis (56, 57, 58).

Dating The Menstrual Cycle By Endometrial Biopsy

The morphological changes in the human endometrium which occur as the result of endocrine stimulation are highly correlated with the ovarian cycle. Thus, biopsies of endometrial tissue may be used to precisely date

the ovarian cycle. Biopsy is done by suction and has proven to be minimally destructive. Eight biopsy criteria are most useful in dating endometrial tissue are: gland mitoses, pseudostratification of nuclei, basal vacuolation, secretion, stromal edema, pseudodecidual reaction, stromal mitoses and leukocyte infiltration.

Dating is most reliable if biopsies are performed between days seventeen and twenty-four of the cycle. Exact dating of specimens in the proliferative phase is often difficult because this phase is variable in length and daily changes are not sufficiently distinct to allow a high level of precision. Therefore, usually the sub-phases early and late proliferative phase are used. In contrast, endometrial changes during the secretory phase are rapid enough to allow for more exact dating (63).

Implantation

The primary function of the endometrium is to create a suitable environment for embryo implantation and pregnancy sustenance. Implantation is the process by which the fertilized ovum adheres to the endometrial wall and penetrates first the epithelial layer and then the circulatory system of the mother. The embryo reaches the uterine cavity approximately three days after ovulation, undergoes further development for two to three days, loses its zona pellucida and then attaches to the endometrium.

Three components of the endometrium act in concert throughout the process of implantation: the secretory component, the luminal epithelium, and the stroma. The secretory component provides nourishment to the developing embryo, while the stroma is crucial for sustained implantation. The luminal epithelium is involved in uterine receptivity. Hormonal milieu

and protein composition of uterine fluid must also be favorable for implantation to occur. However, even if all of these criteria are met, implantation will not occur unless a close synchrony exists between the stage of development of the embryo and the recipient endometrium. The temporal window of human endometrial receptivity spans days 16 through 19 of a 28-day cycle (56, 59, 64).

The adherence of the blastocyst to the epithelium is probably dependent on changes in the surface characteristics of the embryo and the endometrium. When the embryo comes into close contact with the endometrium, the microvilli on its surface flatten and interdigitate with the microvilli on the luminal surface of the epithelial cells. Junctional complexes form. The embryo becomes imbedded in the endometrium and can no longer be dislodged by flushing the uterus. This marks the completion of the first stage of implantation - adhesion. (56, 59).

The second stage of implantation is penetration. Schlafke and Enders describe three types of interactions which take place between the implanting trophoblast and the uterine epithelium during penetration (65). First, cells of the trophoblast invade between epithelial cells on their way to the basement membrane. Second, epithelial cells lift off the basement membrane and the trophoblast cells are able to squeeze underneath the epithelium. Third, fusion occurs between the cells of the trophoblast and those of the uterine epithelium (65, 66).

The final stage of implantation is invasion. The embryo penetrates the basement membrane, invades the endometrial stroma and taps into the maternal blood supply. The ability to invade the stroma may be derived from the proteolytic capabilities of the embryo (67).

The implantation process involves fusion of the blastocyst membrane with that of the luminal epithelium of the endometrium. Laloraya attempted to decipher the membrane lipid organization of the endometrial cells during implantation using spin labeling technique and ESR spectroscopy (68). The investigation revealed a significant increase in membrane fluidity at the time of implantation with a highly immobilized and viscous phospholipid bilayer on the days immediately before and after implantation. These results, along with other studies (69, 70, 71) showing membrane fusion to be dependent on membrane fluidity, indicate that a high degree of phospholipid bilayer mobility is essential for the process of implantation.

The implantation of the embryo into the luminal epithelium in the uterine cavity represents the limiting factor for the pregnancy rate in both *in vivo* births and in *in vitro* fertilization (IVF) programs. Only three to five out of 100 embryos transferred after *in vitro* fertilization complete implantation and pregnancy. Data indicate that most IVF centers report a high frequency of fertilization (70-90%), but that the pregnancy rate after embryo transfer ranges from only 15 to 25%. Therefore, lack of endometrial receptivity may be an important cause of infertility (67, 72).

Free Radicals And Reproduction

Peroxidase in the Uterus: Peroxidase activity can be found in the uterus of both rats and humans (73, 74, 75). In the rat, uterine peroxidase activity has been shown to increase dramatically following treatment with estrogen (76). In 1955, Lucas et al showed that in ovariectomized rats, the level of uterine peroxidase activity was proportional to the amount of estrogen injected intramuscularly (77). Lyttle and DeSombre found

similar results, and also found that the anti-estrogen CI628 could inhibit this estrogen-stimulated induction (76). In 1964, Lucas et al found that uterine peroxidase activity in the human endometrium varied cyclically with the menstrual cycle (74).

Studies have shown that eosinophils, a component of the leukocytic infiltrate of the uterus, are the major source of peroxidase (73). This was determined by proving that the total eosinophilic peroxidase contained in one horn of the uterus of the rat was equivalent to the total biochemically assayed enzyme in the contralateral horn. Soon after estradiol treatment, the entire circulating quantity of eosinophils will accumulate in the rat uterus. This may result from an estradiol-induced chemotactic factor for eosinophils (78).

Two possible functions for this eosinophil-derived peroxidase are as a bactericidal agent and as a regulator of estrogen levels. Klebanoff and Smith reported an antimicrobial action of uterine peroxidase found in uterine fluid when in the presence of hydrogen peroxide and a halide electron donor (79). The estrogen regulating effect may be mediated by negative feedback mechanisms (80, 81).

Peroxidase levels are highest during the peri-implantation period (74). This is not inconsistent with the need for maximal membrane bilayer fluidity at the time of implantation since the peroxidase reaction controls the extent of lipid peroxidation.

Ishikawa and colleagues have identified the origin of the hydrogen peroxide which is used as substrate for the peroxidase enzyme (82). They have localized it cytochemically to the apical plasma membrane of the rat endometrial endothelium. In this apical location, the readily diffusible hydrogen peroxide would be easily obtainable by the peroxidase contained

in infiltrating eosinophils. Other studies have suggested that hydrogen peroxide production is inducible by both endogenous and exogenous estrogen (83).

Lipid Peroxidation And Sperm: For successful fertilization to occur, sperm must have good motility and have the ability to undergo the processes of capacitation, activation and sperm-zona binding. Motility is essential for transport of the sperm from the hostile environment of the vagina into the safety of the cervical mucus and then into the uterus. Capacitation refers to the surface membrane changes which occur in sperm in preparation for penetration of the zona. Activation, the process in which the acrosome reaction occurs, leads to the fusion of the acrosomal membrane with the plasma membrane and the resultant discharge of the acrosomal contents. This is necessary for the facilitation of the rest of fertilization, zona-pellucida sperm binding (59).

Sperm are complex cells with highly specialized membranous regions which are unusually susceptible to lipid peroxidation. Because of this vulnerability, lipid peroxidation has been implicated in the impairment of sperm function in the process of fertilization. It is interesting, however, that recent evidence has shown a constructive role for reactive oxygen species in capacitation and sperm-zona pellucida binding (84, 85).

Reactive oxygen species have been shown to have destructive effects on spermatic membrane, resulting in defects in impaired motility and consequent infertility (86). Exogenous fatty acid peroxides are highly spermicidal, causing complete loss of sperm motility within minutes of application. Presumably, they act as peroxidation chain initiators in this reaction. Alvarez et al determined that aerobic incubation of washed sperm causes spontaneous auto-oxidation (87). These effects are mediated

by both superoxide and hydrogen peroxide. Superoxide dismutase activity was correlated with sperm survival, whereas the activity of the glutathione system was not. It has also been demonstrated that elevated reactive oxygen species were present in three different groups of infertile men when compared to a control group (88). Defective sperm have been found to have forty times the normal amount of reactive oxygen species production, with superoxide anion making up a substantial portion (89).

Despite the presence of a NADPH-mediated superoxide generator, sperm exhibit an extreme susceptibility to lipid peroxidation. This is partially due to a paucity of catalase activity to detoxify the hydrogen peroxide formed by the dismutation of superoxide by SOD (86, 87, 89). An additional reason for their vulnerability is the large amount of polyunsaturated fatty acids contained in their membranes. The relative lack of free radical scavenging agents may be due to a functional role for free radicals in capacitation and sperm-zona binding.

A recent study suggests a role for hydrogen peroxide during capacitation (84). When sperm are aerobically induced they generate hydrogen peroxide from the conversion of superoxide anion by SOD. Incubating sperm with exogenous catalase, to reduce the generation of hydrogen peroxide, resulted in a significant delay or inhibition of capacitation. It was also found that when hydrogen peroxide levels were enhanced, the capacitation period was reduced.

Evidence is also available for a role for lipid peroxidation in facilitating the binding of sperm to the oocyte zona pellucida (85). Incubating the sperm and oocyte with a superoxide generating system enhanced their binding to each other. This effect was reversible with vitamin E, a chain-breaking antioxidant. It was also shown that peroxidation did not increase

the agglutination of sperm in the absence of the oocyte, suggesting that this was not the result of non-specific adhesiveness of the sperm.

Due to the delicate balance between the positive and negative effects of the oxygen free radical system on endometrial and sperm function, it is important to establish normal values for lipid peroxidation during a normal ovulatory cycle. This may enable further investigation into the role of lipid peroxidation in infertility.

MATERIALS AND METHODS

Patient Population

I. Fertile Group - Fifty-seven ovulatory women ages 27-49 (mean age = 40.3) who underwent hysterectomies for benign disease were entered into the study. Hysterectomy patients were chosen due to the relative ease with which uterine washings could be obtained. Patients without proven fertility, with abnormal bleeding or with irregular menses were excluded from analysis.

II. Infertile Group - Eighteen ovulatory women ages 22-45 (mean age = 34.3) who were being treated at the Reproductive Endocrinology Clinic at Yale New Haven Hospital for their inability to conceive or to continue a pregnancy to full term. Samples from patients who were undergoing procedures to diagnose or treat their infertility were obtained to avoid instrumenting patients unnecessarily. Seven patients had hysterosalpingograms, ten had diagnostic laparoscopies and/or hysteroscopies and one had a tuboplasty. These patients were then divided into six groups based on etiology of infertility: uterine (anatomic abnormality), tubal (fallopian tube blockage or damage), endometriosis, anovulatory, hormonal (endocrinologic dysfunction), and idiopathic.

Experimental Design

Fifty-seven women from the fertile group and eighteen from the infertile group were assigned to one of four groups based on their menstrual phase at the time the samples were obtained.

I. For the fertile group, date in the menstrual cycle was determined by staging tissue obtained from the endometrial biopsy according to the

method described by Noyes (63). Biopsies were labeled as one of the following; early follicular (cycle days 1-7), late follicular (cycle days 8-14), early luteal (days 15-21), or late luteal (for day 22 or later in the cycle). To date the follicular phase specimens, the criteria gland mitoses, pseudostratification of nuclei in the epithelium and tortuosity of glands were mainly used. The behavior of the glandular epithelium, (especially mitosis, vacuolization and secretion) was the key to dating specimens in the first half of the luteal phase. Dating in the later part of this phase depends heavily on stromal characteristics (i.e. edema, predecidual reaction, mitosis and leukocytic infiltration) (63). Distribution of specimens in the different phases of the cycle is shown in Table 1.

Table 1

EXPERIMENTAL DESIGN - FERTILE GROUP

Ovulation
↓

Phase of cycle	Early follicular	Late follicular	Early luteal	Late luteal
Cycle day	1-7	8-14	15-21	≥ 22
Patients (N)	12	18	10	17

II. Stage of the menstrual cycle for the infertile patients was determined by using the last menstrual period as reported by the patient and calculating

based on a 28-day cycle. As in the fertile group samples were labeled early follicular, late follicular, early luteal and late luteal.

Method For Collection

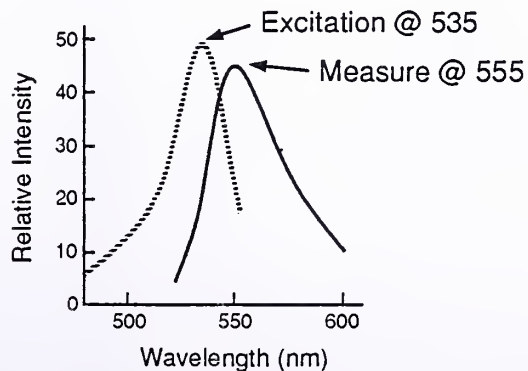
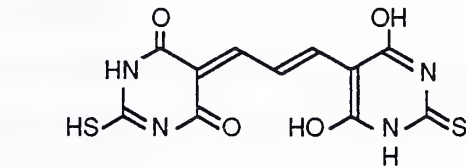
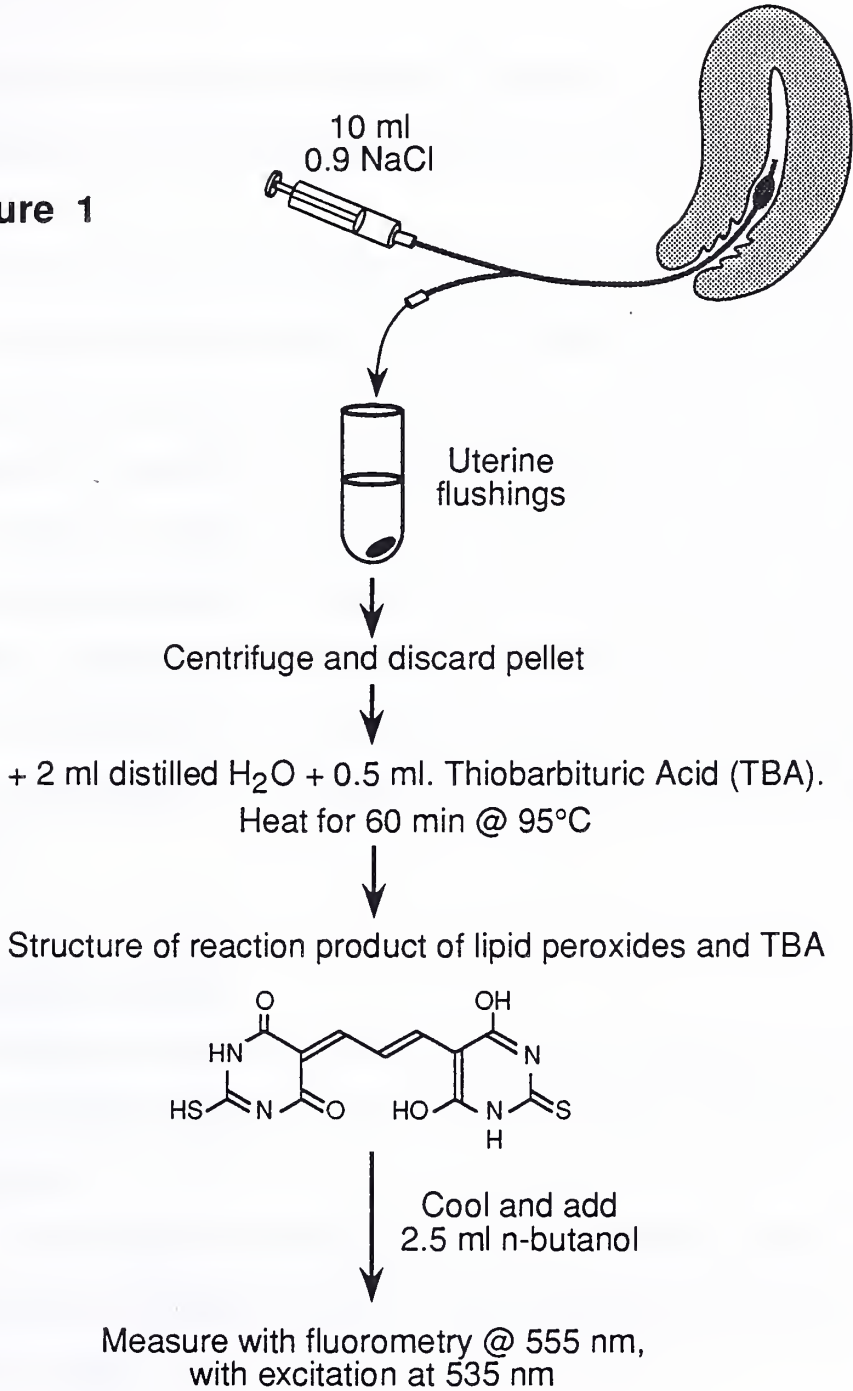
I. Uterine washings from the fertile group were obtained within 30 minutes of surgery by placing a Foley[®] catheter through the cervix of the resected uterus, after clamping the fallopian tubes to avoid leakage of saline, injecting 10 ml of normal saline and aspirating through the same catheter. (Figure 1).

II. Washings from the infertile group were obtained after the patient was prepared for the surgical procedure but before the actual procedure began. A syringe was attached to a cervical cannula already in place. Ten ml of normal saline was injected into the uterine cavity and aspirated through the same instrument.

Assay

Malondialdehyde (MLD) levels of uterine washings were obtained by a simple fluorometric assay (10). This assay measures the fluorescent reaction product of MLD (released from lipid peroxides) and thiobarbituric acid. The reaction was produced by combining different dilutions of the supernatant of the uterine flushing with .5ml thiobarbituric acid and 2 ml of distilled H₂O and heating the mixture in a 95° C water bath for 60 minutes. The addition of 2.5 ml of *n*-butanol separates out the reacted substance. Levels are measured by exciting the product at a wavelength of 535nm and measuring the

Figure 1



emission at a wavelength of 555nm. Tetramethoxypropane which converts quantitatively to MLD during the reaction procedure is used as the standard.

MLD levels were corrected for protein content using the Pierce BCA protein assay (90). This assay is a very sensitive indicator of proteins , with the ability to determine concentrations in the range of 10µg/ml to 2000µg/ml. It consists of a bicinchoninic acid (BCA) solution and a copper sulfate solution. Copper II ions in the reagent are reduced to Copper I ions in the presence of protein. The Copper I ions then chelate with two BCA molecules, effecting a color change in the solution. After being incubated at 60° C for 60 minutes, the samples were read in a spectrophotometer at 562nm and compared to a standard curve made up of Bovine Serum Albumin.

Analysis Of Results

I. Statistical analyses were done by a one way analysis of variance and Duncan multiple range test. $P < 0.05$ was considered statistically significant.

II. The results for infertile patients were compared against the normogram established for fertile women, and were categorized as low, average or high.

RESULTS

Demographics (Table 2)

I. Fertile group: the patients in the four groups (different phases of the menstrual cycle) did not differ in age (ANOVA, $p=0.55$) or in the indications for hysterectomy.

II. Infertile group: the patients in the different diagnostic categories did not differ in age or procedure performed. The mean age was 34, with a range of 22 to 45 years of age. Ten patients had diagnostic laparoscopies, seven hysterosalpingograms and one, a tubal catheterization.

III. Fertile vs. infertile: the patients in the infertile group were significantly younger than the patients in the fertile group (ANOVA, $p=0.0001$).

MLD Levels

I. Fertile group: MLD levels corrected for protein content demonstrated a pattern consistent with low levels during the late follicular phase ($LF=0.169 \pm 0.035$ [mean \pm standard error]), reaching minimum levels in the early luteal phase around implantation ($EL=0.093 \pm 0.037$). Levels were in the mid-range in the late luteal phase ($LL=0.201 \pm 0.05$) and were highest in the early follicular phase ($EF=0.303 \pm 0.046$) (one way ANOVA, $p=0.0475$).

MLD levels in the early follicular phase were significantly higher than levels in both late follicular and early luteal phases (Duncan multiple range, $p=0.05$).

Table 2**PATIENT DEMOGRAPHICS**

	Fertile	Infertile
Characteristics		
No. of patients	57	18
Mean age of patients	40.3 (27-49)	34.3 (22-45)
Menstrual phase		
	no. (mean age)	no. (mean age)
EF	12 (39.2)	9 (34)
LF	18 (39.6)	7 (34)
EL	10 (41.4)	2 (38)
LL	17 (41.2)	0
Diagnosis		
	no.	no.
Fibroids	35	0
Prolapse/USI	6	0
DUB	8	0
Endometriosis	8	6
Uterine	0	1
Hormonal	0	3
Tubal	0	6
Anovulatory	0	1
Idiopathic	0	1

USI=urinary stress incontinence, DUB=dysfunctional uterine bleeding

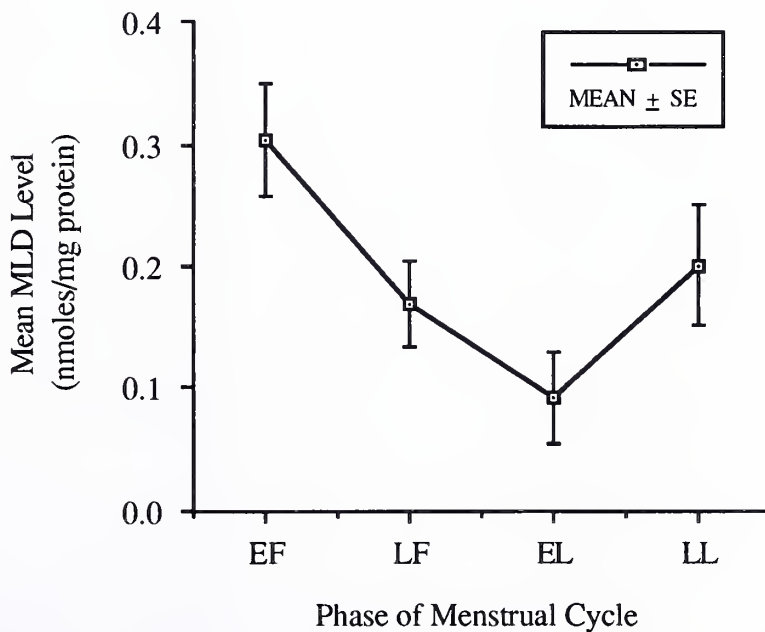
Table 3

MLD LEVELS IN FERTILE PATIENTS
(nmoles/mg protein)

Phase	EF	LF	EL	LL
Patients (N)	12	18	10	17
Mean MLD Level	0.303	0.169	0.093	0.201
Standard Error	0.046	0.035	0.037	0.050
	ANOVA $p = 0.0475$ $F = 3.0$			

Figure 2

CYCLIC VARIATIONS IN MLD LEVELS IN FERTILE WOMEN



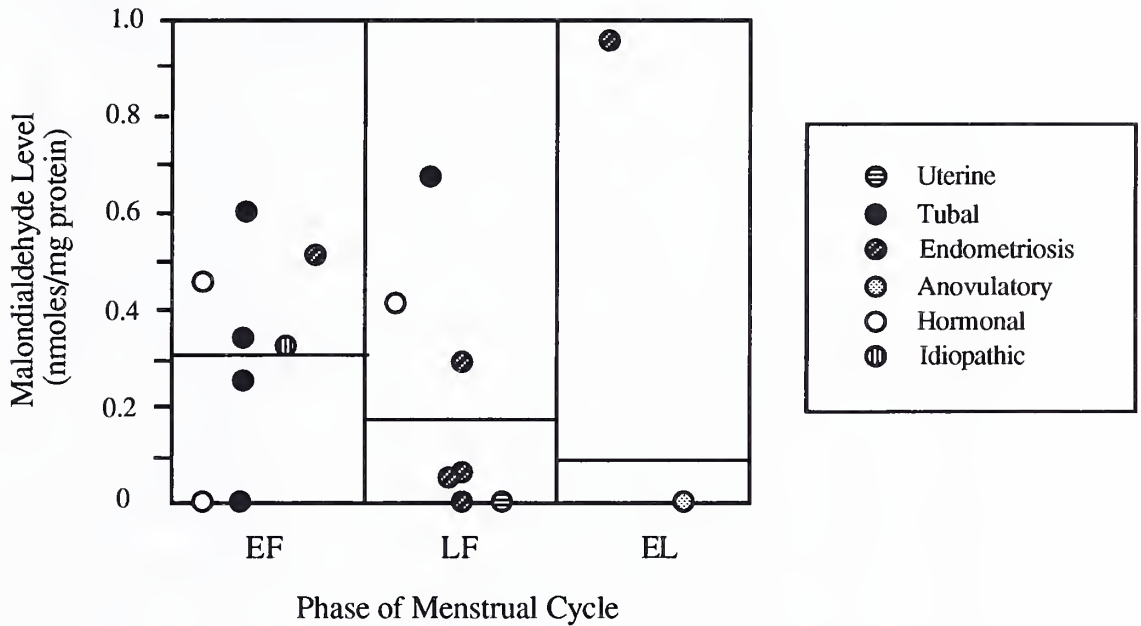
* EF > LF, EL $p=0.05$

II. Infertile group: the results of the infertile group can be seen in table 4 and figure 3. In each phase of the menstrual cycle, the results of the infertile group in different diagnostic categories were arranged from low to high MLD levels as shown in figure 3. No particular pattern was obvious upon analysis.

Comparison of MLD levels in fertile and infertile patients with endometriosis was performed using unpaired t-test. MLD levels measured during the late follicular phase were not statistically significant ($p=0.071$) between the two groups. Statistical comparisons in the other phases of the menstrual cycle was not possible due to the small number of patients with endometriosis in these groups.

Figure 3

MLD LEVELS IN INFERTILE WOMEN BY DIAGNOSIS*



* Circles denote MLD level for an individual infertile patient; horizontal lines represent mean MLD level of fertile patients in each phase of menstrual cycle.

Table 4
MLD LEVELS IN INFERTILE PATIENTS

Patient number	Diagnostic group*	Phase	MLD level	Comparison**
1	E	LF	0.0	low
2	U	LF	0.0	low
3	H	EF	0.0	low
4	E	LF	0.088	low
5	T	EF	0.269	avg
6	A	EL	0.0	low
7	T	EF	0.0	low
8	T	EF	0.344	avg
9	E	LF	0.084	low
10	H	EF	0.471	high
11	H	LF	0.405	high
12	T	LF	0.682	high
13	T	EF	0.603	high
14	I	EF	0.315	avg
15	E	EF	0.052	low
16	E	LF	0.294	high
17	E	EL	0.958	high
18	T	EF	0.0	low

* U = Uterine
T = Tubal
E = Endometriosis
A = Anovulatory
H = Hormonal
I = Idiopathic

** Comparison = subject's MLD level compared to the normal standard curve for fertile women at that menstrual phase.

DISCUSSION

This paper was designed to explore the hypothesis that lipid peroxidation plays a role in infertility. The approach taken was to study the lipid peroxidation patterns of fertile and infertile women throughout the menstrual cycle and compare these results.

For the group of fertile women studied, the highest MLD levels were in the early follicular phase, with mid-range values in the late luteal phases. The lowest MLD levels were in the peri-ovulatory late follicular and early luteal phases, with the nadir in the early luteal phase around the time of blastocyst implantation. By contrast, no pattern was seen in the MLD levels of the infertile group.

The high levels of MLD found in the period following menstruation are indicative of ischemic/reperfusion injury occurring in the endometrium. The menstruation-associated vascular changes which occur and the subsequent ischemic tissue necrosis are reminiscent of the reperfusion damage seen in the heart model. A burst of oxygen free radical production ensues, initiating lipid peroxidation, resulting in the high levels of MLD that were observed in this period of the cycle.

Role Of Lipid Peroxidation In Infertility - A Hypothesis

Based on these results, it is possible to hypothesize a role for lipid peroxidation in the normal menstrual cycle and the possibility that deviations from the normal pattern of peroxidation could lead to infertility. In all women, approximately one day before menstruation, endometrial vasomotor changes occur which cause tissue ischemia and reperfusion, eventually resulting in the onset of menses. The same rhythmic

alternations of vasoconstriction and dilation which cause ischemia and reoxygenation injury leading to menstruation at the end of the cycle could also promote smaller ischemic/reperfusion events during the rest of the cycle. A premature withdrawal of hormones (a weak corpus luteum), an early prostaglandin surge or imbalance, or some other unknown event could be sufficient. These limited vascular injuries, which by themselves may be insufficient to cause menstrual bleeding, still have the potential to promote reperfusion injury resulting in oxygen free radical-induced lipid peroxidation. Consequently, the endometrial tissue is vulnerable to the cellular changes described above, particularly membrane alterations which lead to increased membrane rigidity. It has been demonstrated that membrane fluidity at the time of implantation is crucial (68). A decrease in membrane fluidity will decrease endometrial receptivity. The endometrium is transformed into an unsuitable environment for implantation of the blastocyst. The sequence of events proposed above weakens an already tenuous link (implantation) in the process of pregnancy and could possibly result in infertility. An important implication of this hypothesis, then, is that lipid peroxidation should be kept to a minimal level around the time of implantation.

Alternatively, there are other structures which lipid peroxidation could damage. It is well known that lipid peroxidation affects structures imbedded in the membrane as well as the membranes themselves. Therefore, hormone receptors and cilia, both of which are imbedded in the endometrial epithelium, are vulnerable to destruction by lipid peroxidation. Damage to membrane receptors could lead to infertility on the basis of decreased hormonal responsiveness. Likewise, inadequate distribution of endometrial secretions along the endometrial surface secondary to damaged

cilia could result in an unsuitable environment for the embryo. Proteins are also attacked and destroyed by lipid peroxidation. Enzymes are another essential component in the process of implantation and pregnancy. They provide nutritional support to the embryo and function in lytic processes at the time of implantation. Enzyme dysfunction through interaction with lipid peroxidation represents another possible pathway for infertility.

Due to their relative lack of antioxidant defenses and high polyunsaturated fatty acid content, spermatozoa are extremely vulnerable to damage by lipid peroxidation. The hypothesis that lipid peroxidation activity is high in infertile women in the peri-implantation period has important consequences for sperm, whose longevity and function during this period is essential for proper fertilization. Lipid peroxidation of sperm in the uterus may cause loss of motility and even sperm cell death leading to infertility.

Contradictory evidence is provided by Laloraya et al, who report an upsurge of the superoxide anion radical level at the time of implantation (68). They suggest that the superoxide radical may play a role in implantation and in the synthesis of progesterone during early pregnancy. An increase in superoxide radical concentration and its possible constructive role at implantation is inconsistent with the hypothesis of this study. The increased superoxide radical concentration at the time of implantation, which would predict a high rate of lipid peroxidation, is not supported by the results of this study.

Comments On Study

There are a number of considerations which make it difficult to draw general conclusions based on this study. Limited time and patient availability made it possible to use only a small number of infertile patients. This made it difficult to do either intra-group or comparative statistical analyses with this population, especially since these patients were subdivided into smaller groups based on both phase of menstrual cycle and diagnosis.

Another problem with this population was the lack of subjects studied at different menstrual phases. While hysterectomies are done throughout the menstrual cycle, most routine infertility procedures are done in the early and late follicular phases, to avoid interrupting a new pregnancy. This caused the data to be heavily weighted towards follicular specimens with almost no data from secretory specimens.

Estimating the day of the menstrual cycle introduces inaccuracy into the study, particularly in the infertile population. In these women, dating was based on a 28-day cycle using the date of onset of the last menstrual period, as reported by the patient. This may result in a large degree of error, especially in anovulatory women. A better system of dating is by endometrial biopsy, the method used for the fertile population, however, this technique has its limitations as well.

Although dating by endometrial biopsy is reproducible when highly experienced morphologists examine a specimen taken from a uterus at the optimal time (between the seventeenth and twenty-fourth day), several studies have shown that when these conditions are altered, considerable variability results. Several studies have found histological dating of endometrium to be imprecise. Li and colleagues found that when the same

observer dated the same endometrial biopsy on two separate occasions the two readings were in agreement only 24% of the time, with disagreement of more than two days occurring 10% of the time (91). Gibson et al found that 5% of duplicate readings (the same slide by the same evaluator at a different time) were discordant by ≥ 3 days and 22% of readings of the same slide by different evaluators were different by > 2 days (92). In the original paper defining the criteria to use in dating endometrial biopsies, Noyes and colleagues concluded that error ranged from 2 days early to 2 days late (in predicting ovulation), and that dating specimens in the secretory phase of the cycle maximized precision (63). Since the specimens for this study were collected from women at all times of the menstrual cycle, minimal error in dating cannot be assumed. Therefore, the dating of endometrial biopsies adds a moderate degree of inaccuracy to this investigation.

An additional source of imprecision in this study is this grouping of subjects in both the fertile and infertile populations. The design places patients in the first day of the cycle (who are immediately post-menstrual) in the same category as patients in the seventh day of the cycle (mid-follicular). Inability to stratify specimens more narrowly may skew results in a unpredictable way.

In the infertile population, patients were broken up into smaller groups based on etiology of infertility. These groups, however, were not comprised of patients with one pure diagnosis. Many of the patients carried two diagnoses, and the possibility exists that a more obvious problem could be masking another, yet undiagnosed cause of infertility in the patients who still had been unable to conceive. These impure diagnostic groups are both difficult to analyze and compare to the normal standard

values of the fertile population and various combinations of diagnoses may confound results.

Finally, age differences create a bias in the study design. Hysterectomy patients are generally older than patients being seen in an infertility clinic. The difference between these groups, inherent in the populations from which they are drawn, is significant.

It is well documented that serum lipid peroxide levels increase with age (93). Hagihara et al studied healthy individuals in two age groups: individuals under 40 and those over 40 years of age. They found that the older group had a significantly elevated lipid peroxide level. The use of MLD as a marker for fertility in these two groups may be confounded by age. This is a difficult problem to overcome as fertile women usually do not present in a situation which would facilitate collection of uterine washings earlier in life. Perhaps, in the future fertile women coming in for routine gynecological exams could be studied.

Future Research/ Possible Treatment Approaches

Future research in this area might include a study of an infertile population of women with more subjects, more evenly distributed throughout the menstrual cycle. Evaluation of these patients would be more accurate if it were possible to obtain endometrial biopsies to determine their stage in the menstrual cycle. In addition, investigations into treating endometrial tissue with antioxidants and/or free-radical scavenging agents and measuring lipid peroxidation levels in the endometrium would provide useful information. The efficacy of measuring lipid peroxidation by assays for malondialdehyde in uterine washings must be addressed. Studies quantifying implantation events (after

multiple fertilizations) with antioxidant pretreatment, is a possible next step. Finally, a controlled study using antioxidant therapy in infertile women with high lipid peroxidation levels, and measuring pregnancy rate, would be invaluable in the progress towards the treatment of infertility. Therapies to investigate include all of the antioxidants and free-radical scavengers discussed above: superoxide dismutase, catalase, allopurinol and oxypurinol, iron chelators and vitamin E.

Despite its limitations, this study reveals some important trends. The pattern of low MLD levels around the time of ovulation in normal ovulatory women is significant. In addition, there are several infertile patients in the late follicular and early luteal phases with MLD levels which are substantially higher than the mean MLD levels determined in the normogram for normal fertile women and perhaps in a larger study a pattern may emerge in the infertile population. These findings indicate that lipid peroxidation may play a role in infertility. The development of MLD levels as a marker for infertility would be of great clinical benefit to the field of in vitro fertilization. Future investigations into lipid peroxide testing and antioxidant/free-radical scavenging treatment for infertile women may make it increasingly possible to treat a previously unrecognized cause of infertility. The effect would be to maximize the chance for a successful pregnancy for many infertile couples.

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