# MINIREVIEW

# Oxygen Radicals and Reactive Oxygen Species in Reproduction (43321C)

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*Abstract.* **Free radicals and reactive oxygen species play a number of significant and diverse roles in reproductive biology. In common with other biological systems, mechanisms have evolved to minimize the damaging effects that these highly reactive molecules can have on reproductive integrity. Conversely, however, recent findings illustrate the constructive roles that oxygen radicals and reactive oxygen species play in a number of important junctures in the development of germ cells and the obligate endocrine support they receive for the successful propagation of the species. Specifically addressed in this review are some aspects of sperm development and action, the uterine environment, oocyte maturation and ovulation, and corpus luteum function and regression.** [P.S.E.B.M. 1991, Vol 198]

In this review a relatively new group of cell and tissue regulators in the reproductive system is addressed, namely, oxygen radicals and other reactive oxygen species such as hydrogen peroxide. Beyond their role in the thyroid and the immune system, much of what is known about free radical chemistry in biology concerns damaging or pathological processes, including aging, cancer, radiation damage, various diseases, and toxicity of xenobiotics. Here, however, a different perspective will be brought to bear, where potentially important functional roles of oxygen radicals and hydrogen peroxide are discussed with respect to cells and tissues of the reproductive system. Most tissues of the reproductive system have an intrinsic plasticity with a host of developmental and regressive states. It may not seem unnatural, therefore, that oxygen radicals and associated agents may serve as important mediators in tissue remodeling, hormone signaling and steroidogenesis, and germ cell function. While this field of investigation is just emerging, an overview of the early information in this area is presented, as well as a brief review of the general nature and actions of oxygen radicals and reactive oxygen species.

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## Free Radicals and Reactive Oxygen Species

Free radicals have been described as molecular entities that contain at least one unpaired electron in a given atomic or molecular orbital (1). The generally enhanced reactivity of free radicals over more stable molecules results from the fact that more energy is required, for example, to maintain two separate species each with an unpaired electron than to allow them to come together and share electrons such that a filled molecular orbital is formed, with the attendant formation of a covalent bond. The reactivity of a free radical is inversely related to its stability.

Oxygen radicals are intermediate, short-lived species produced by the reduction of oxygen (addition of electrons), ultimately forming water. The addition of a single electron to oxygen leads to the formation of the superoxide anion radical, gaining another electron produces hydrogen peroxide, and trivalent reduction generates the hydroxyl radical. Some enzymes catalyze either single (NADPH oxidase) or double (glucose oxidase) electron additions that form superoxide or hydro-

gen peroxide, respectively. The hydroxyl radical can be generated from hydrogen peroxide in the presence of ferrous or cuprous ions and is one of the most reactive radicals known (2). So quickly does the hydroxyl radical react with neighboring molecules that it rarely strays from its site of production (1). Other agents that play a role in free radical chemistry are not free radicals themselves, but become such in the appropriate environment. These reactive oxygen species include hydrogen peroxide, singlet oxygen, hypochlorous ion, and lipid hydroperoxides. Transition elements, notably iron and copper, can propagate reactions via redox cycling because of their multivalent capability (1).

### Actions of Reactive Oxygen Species

Generally, direct and indirect effects of reactive oxygen species on cells can be summarized in three distinct categories. These include damage of DNA and RNA, lipid peroxidation, which principally affects membrane structure and function, and protein damage.

It has long been thought that the toxic effect of ionizing radiation on cells is largely the result of genomic derangement. Since DNA resides within a hydrophilic compartment, ionizing radiation can generate hydroxyl radicals from nearby water that alter the sugar residues or bases and which may induce either single- or double-strand breaks. DNA repair processes play a vital role in protection against oxygen radicals. Notable among such repair mechanisms is poly(ADP-ribose) polymerase. The marked depletion of cell levels of ATP caused by exposure to reactive oxygen species is linked to DNA strand scission, which causes activation of poly(ADP-ribose) polymerase. The depletion of NAD, which is utilized as substrate by this repair enzyme complex, can be so severe that the ability of the cells to generate ATP becomes compromised (3, 4).

Biological membranes are subject to attack by oxidants because phospholipids contain a significant proportion of esterified polyunsaturated fatty acids, which are particularly sensitive to oxidative reactions (5). Lipid peroxidation has been described as a three-step process: initiation, propagation, and termination (1, 5). Initiation begins with the abstraction of a hydrogen atom from a methylene group, a process made easier if there is an adjacent carbon double bond as in polyunsaturated fatty acids. The hydroxyl radical is a typical initiator; neither superoxide anion nor hydrogen peroxide is sufficiently energetic to act directly as initiators (1), although their conversion to hydroxyl radical by ferrous or cuprous ion can result in initiation of lipid peroxidation. Since oxygen is abundant in membranes due to its hydrophobic character, rapid addition of oxygen occurs, leading to the formation of a peroxy radical. Propagation ensues because the peroxy radical can abstract the hydrogen from the methylene group of another polyunsaturated fatty acid, leading to a chain reaction culminating in the generation of lipid hydroperoxides. This chain reaction is terminated by the lipid-soluble antioxidant vitamin E, and this appears to be the major role of this vitamin (5). Lipid hydroperoxides and alkoxy radicals are prone to scission, a process of fatty acid decomposition in which small hydrocarbons (such as ethylene and pentane) and aldehydes are produced. Of notable importance in lipid peroxidation is malondialdehyde (MDA), a toxic compound with the ability to cross-link lipids and proteins; the detection of MDA and related compounds by the thiobarbituric acid test (1) has been used by many investigators as a indicator of lipid peroxidation in a variety of systems. Many aspects of membrane function are changed by lipid peroxidation, including reduction of membrane fluidity, aggregation and rearrangement of phospholipid organization, "blebbing," and increased permeability leading to leakiness and loss of compartmentalization. Extensive peroxidation can lead to a complete breakdown of membrane architecture.

Products of lipid peroxidation are damaging to many membrane-associated proteins. Particularly susceptible are those proteins with exposed tryptophan or cysteine residues. Sulfydryl groups will react with aldehydes and MDA, causing the development of both intra- and intermolecular cross-links. MDA will also attack amino groups with the same result. Additionally, reduced membrane fluidity caused by lipid peroxidation can alter the function of intrinsic proteins. Proteins can also be damaged by direct attack of the hydroxyl radical. One cellular defense scheme that has been interpreted as an antioxidant mechanism is accelerated activity of proteolytic enzymes which degrade and remove oxidized proteins (6).

### Defenses Against Oxygen Radicals

Oxygen radicals and reactive oxygen species are ubiquitous in aerobic organisms, and the reproductive system must be protected from their potentially damaging effects. One avenue of defense against reactive oxygen species is to provide a low oxygen environment such as that seen with developing germ cells within their barrier of protective nurse cells. More common defenses include scavenging by antioxidants, inhibition of production, enzyme-catalyzed degradation, and repair of damaged cell products.

A major line of defense is detoxification of superoxide anion and hydrogen peroxide, catalyzed by superoxide dismutase, catalase, and glutathione peroxidase (7–9). Both mitochondria and cytosol contain unique enzymes that catalyze the dismutation of superoxide into hydrogen peroxide and oxygen. Cytosolic superoxide dismutase is a copper-zinc metalloenzyme (also found in the extracellular fluid), and the mitochondrial form is a manganese metalloenzyme (7). Hydrogen peroxide is detoxified by two major enzymes:

catalase and glutathione peroxidase. Catalase is found primarily within peroxisomes of most cells; this iron metalloenzyme catalyzes the conversion of hydrogen peroxide into water and oxygen (8). Glutathione peroxidase, in contrast to catalase, is a selenium-containing enzyme and catalyzes the degradation of lipid peroxides as well as hydrogen peroxide (8).

A major source of protection against the damaging effects of oxygen radicals is provided by antioxidant vitamins, with E serving such a role in membranes and C in aqueous compartments; carotenoids also play a role. membranes are major sites of damage by radicals, and the protective role of vitamin E is of paramount importance in terminating peroxidative chain reactions of unsaturated lipids. Vitamin C recycles oxidized E back to the reduced state, and the vitamin C radical can be regenerated by transhydrogenases or replaced from extracellular sources. Thus, depletion of vitamin C can reflect the production of oxygen radicals. Little is known about the levels of vitamins E, A, and carotenoids in the tissues of the reproductive system in different functional states, or about the mechanisms that govern accumulation and depletion of these antioxidants.

The final cellular approach to counter deleterious effects of free radicals involves repair processes invoked to remove damaged cellular components. The replacement of peroxidized fatty acids in membrane phospholipids by activation of phospholipase $A_2$ and acyltransferases is one example. Another is the activation of poly(ADP-ribose) polymerase produced by damage of DNA, as described earlier. There appears to be less information on the role of proteases in removal, or repair, of proteins subjected to oxidative damage, but such phenomena would be expected.

**Reactive Oxygen Species and Sperm**

Spermatogenesis, the period during which dormant spermatogonia differentiate into mature spermatozoa, occurs over a period of about 64 days in humans; after deposition in the female tract, survival is limited to 30–45 hr (10). Over this period, three important changes appear in sperm if successful fertilization occurs: capacitation, activation (during which the acrosome reaction takes place), and zona-sperm binding. Spermatozoa are highly differentiated and complex cells, with specialized membranous regions that undergo substantial structural and morphological change in the interval between capacitation and fertilization (11). Reactive oxygen species have been implicated as agents that can impair some of these changes, which result in defective sperm, impaired motility, and consequent infertility. Two recent reports, however, provide evidence for a constructive role for reactive oxygen species in capacitation, and in sperm-zona pellucida binding.

Capacitation is a process occurring over a few hours that is required before sperm are capable of fertilization. Several plasma membrane changes occur; among these are the stripping of extrinsic glycoproteins acquired from the epididymis and seminal fluid, changes in lipid and protein composition, and alterations in membrane fluidity, with the disappearance of organized discrete membranous domains (11). Accompanying these changes are functional alterations in metabolism, ion flux, and motility. Recent work by Bize *et al.* (12) implicates a role for hydrogen peroxide during capacitation. Sperm generate hydrogen peroxide when aerobically incubated, owing to their lack of catalase and the conversion of superoxide anion to hydrogen peroxide by superoxide dismutase (SOD). Incubation of sperm with exogenous catalase, to remove hydrogen peroxide, substantially delayed or inhibited capacitation when the samples were scored for the acrosome reaction (signifying completion of capacitation) 5 hr later. The authors suggest this is not a direct effect on the activation process, since catalase addition 3 hr into the incubation was less effective in delaying the acrosomal reaction. It was also found that the capacitation period was reduced when glucose oxidase (a hydrogen peroxide generator) or exogenous hydrogen peroxide was added. Although relevant mechanisms are unknown, the suggestion was made that the role of hydrogen peroxide during capacitation is to facilitate membrane reorganization.

Evidence for a role of lipid peroxidation in facilitating binding of sperm to the oocyte zona pellucida was provided by Aitken et al. (13). Sperm-zona binding was enhanced during incubation with a superoxide-generating system, and this could be reversed by the presence of the chain-breaking antioxidant vitamin E. Enhanced sperm-zona binding was not a result of increased nonspecific adhesiveness on the part of the sperm, because peroxidation did not alter agglutination of sperm in the absence of a zona pellucida. This finding seems consistent with the next major event that occurs immediately after initial sperm-zona binding, the acrosome reaction. The acrosome is a large, flattened, secretory granule at the anterior end of the sperm head that contains proteolytic and lipolytic enzymes essential for zona penetration and oocyte binding. The acrosome reaction, in which the plasma membrane and acrosomal membrane fuse and discharge the acrosomal contents, is triggered by a calcium ion influx. This can be demonstrated *in vitro* by incubating sperm with the calcium ionophore A23187, which has also been shown to rapidly induce lipid peroxidation (14). By the time sperm-oocyte fusion occurs, the sperm outer membrane has undergone extensive modification, conferring fusogenic properties that may be caused in part by phospholipase $A_2$. In other systems, oxidants can activate phospholipase $A_2$, perhaps by inactivating its inhibitor, lipocortin (15). Lipid peroxidation also enhances the

activity of phospholipase $A_2$ by increasing either substrate availability or affinity (16). In neutrophils, phospholipase $A_2$ activity appears to be required for the generation of superoxide by NADPH oxidase (17).

Spermatozoa are unusually susceptible to lipid peroxidation, partly because they appear to contain an NADPH-mediated superoxide generator and little or no catalase activity to purge hydrogen peroxide after dismutation of superoxide by SOD (18–20). Additionally, sperm are well endowed with polyunsaturated fatty acids, which are central targets of lipid peroxidation. It has been suggested that gossypol, a nonsteroidal compound derived from cottonseed that has contraceptive actions in men, may exert its effects by promoting membrane lipid peroxidation, thereby inhibiting glucose uptake (21). Exogenous fatty acid peroxides, presumably acting as peroxidation chain initiators, are highly spermicidal, causing complete loss of sperm motility in minutes (18). Aerobic incubation of washed sperm causes spontaneous auto-oxidation, as determined by measurement of malondialdehyde and mediated by both superoxide and hydrogen peroxide (19). These workers determined that although present in human sperm, the efficacy of the glutathione system (for scavenging hydrogen peroxide) was not correlated with sperm survival, whereas SOD activity was. They suggest that measurement of SOD activity in fresh sperm samples may be diagnostically useful in semen analysis. In one study, it was found that elevated reactive oxygen species production was evident in the sperm of each of three etiologically distinct groups of infertile men compared with a control group (14). In another, reactive oxygen species production from defective sperm was determined to be elevated 40-fold over normal cells; superoxide anion was found to be a principal component (20).

The relative lack of vigorous cellular defense mechanisms against oxidative agents in sperm, particularly outside the male reproductive tract, may be related to functional aspects of peroxidation in capacitation and in sperm binding to the oocyte zona pellucida, as discussed above. Interestingly, the seminal plasma has been shown to have 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 treatment with seminal plasma (22). The protective action in seminal fluid was found to be heat stable and of a multicomponent nature; fractionation by either dialysis or ultrafiltration abolished antioxidant activity, which could be restored upon reconstitution. Furthermore, the antioxidant activity of seminal plasma is not sperm specific, since it also inhibits spontaneous autoxidation of brain homogenates (22).

**Reactive Oxygen Species and the Uterus**

In species that exhibit a menstrual cycle (primates) and those with an estrous cycle (most other mammals), the uterus undergoes significant hormonally induced morphological and functional changes during the reproductive cycle. The uterus provides the appropriate environment to receive the blastocyst and to ensure successful implantation, which is followed by large-scale tissue remodeling to accommodate the developing fetus. Coordinated contractions of the uterus are required for parturition, which is followed by regression of uterine tissue.

Well established is the increase in uterine weight that occurs in response to estradiol. Another remarkable response of the uterus to estradiol is the more than 200-fold increase in peroxidase activity (23, 24). Eosinophils, a component of leukocytic infiltrate of the uterus, are the major source of peroxidase. Within 24 hr of estradiol treatment, the immature rat uterus will accumulate as many eosinophils as are normally found in the animal's circulation; Lee *et al.* (25) showed that this may result from an estradiol-induced chemotactic factor for eosinophils with a mass of about 20 kDa. Early suggestions for the function of eosinophil-derived uterine peroxidase included that it was a means of regulating uterine levels of estradiol (23) and that it provided an antimicrobial effect (26). These authors reported that peroxidases found in uterine fluid, in combination with hydrogen peroxide and a halide electron donor (iodide or bromide), exerted a pronounced bactericidal effect. It was argued that there is sufficient endogenous iodide in the uterus to support the hypothesis that a function of uterine peroxidase is to prevent infection. The antiestrogen effect of uterine peroxidase has been confirmed by others (27, 28), and it may be a component of a negative feedback system to regulate estrogen levels. The magnitude of peroxidase induction by estrogen is among the highest of all estrogen-induced proteins in the uterus (29).

If uterine peroxidase is of functional importance, there must be a uterine source of its substrate, hydrogen peroxide. Evidence for estrogen-stimulated hydrogen peroxide production in the uterus was provided by Johri and Dasgupta (30). They found that in uterine homogenates, estrogen provoked a marked increase in hydrogen peroxide production, an effect antagonized by progesterone. Moreover, hydrogen peroxide concentrations were elevated in uterine samples obtained from rats in estrus, when native estradiol levels are elevated, causing among other things, induction of peroxidase. Information on the cytochemical localization of hydrogen peroxide production was provided by Ishikawa *et al.* (31), who utilized cerium, which forms an electron-dense perhydroxide precipitate upon reaction with hydrogen peroxide. Electron microscopy of endometrial

sections revealed dense staining on the apical plasma membrane of the endometrial surface epithelium, but little or none at the basolateral side. Staining was dependent upon incubation with NADH or NADPH, and they suggested that an NAD(P)H oxidase, similar to that found in phagocytic cells, was responsible for generating superoxide, which would be dismutated by SOD to hydrogen peroxide. Since hydrogen peroxide readily diffuses through membranes, its production in this location would be consistent with the idea that endometrial generation of hydrogen peroxide would supply neighboring eosinophil peroxidase with substrate.

Eicosanoids are a family of lipid compounds, many with hormone-like properties, derived from unsaturated fatty acids, principally arachidonic acid. A major class are the prostaglandins, produced by the cyclooxygenase system, in which oxygen is inserted into arachidonic acid to yield a ring structure. Prostaglandins are produced throughout the body and have a diversity of effects; uterine-derived eicosanoids are worthy of attention for their roles in chemotaxis, implantation, and invoking uterine contractions at parturition, and as a source, in several species, of prostaglandin (PG) $F_{2a}$, the luteolytic signal that induces regression of the corpus luteum in the ovary. In general, the precursor arachidonic acid is provided by the action of phospholipase $A_2$, a calcium-dependent lipolytic enzyme, on membrane phospholipids. The activity of phospholipase $A_2$ can be enhanced by lipid peroxidation, an effect demonstrated by the addition of lipid hydroperoxides to arterial endothelial cells (32). In this system, *tert*-butyl hydroperoxide displayed a dose-dependent activation of phospholipase $A_2$ activity and consequent generation of prostaglandins. Another study demonstrated inhibition of lipid peroxide-induced phospholipase $A_2$ activity by an antioxidant, showing that active lipid peroxidation is probably needed for this effect (33). These authors suggest that lipid peroxides promote phospholipase activity in two ways: the enzyme has a greater affinity for oxidized phospholipids, and lipid peroxides cause membrane defects that enhance substrate availability.

Classically, free arachidonic acid acid is metabolized by lipoxygenases and cyclooxygenase, widely distributed heme-containing enzymes that utilize molecular oxygen, to form lipid hydroperoxide intermediates, which undergo further nonenzymatic conversion to eicosanoids. The action of cyclooxygenase is retarded, although not eliminated, when an efficient antioxidant system is present to remove trace lipid peroxides, and it is enhanced in the presence of hydrogen peroxide (1, 34). Results from a recent report make the relationship between peroxide and PG synthesis appear to be even more complex. Morrow *et al.* (35) demonstrated *in vivo* generation of $PGF_2$-like compounds that was catalyzed by free radicals independently of cyclooxygenase activity. The radical-catalyzed peroxidation of arachidonic acid caused the formation of a bicyclic endoperoxide that was subsequently reduced to form the F-series prostanoids, and at least one of these displayed potent biological activity. Significantly, in normal human urine, the level of nonenzymatic, peroxide-generated prostaglandins substantially exceeded that produced by cyclooxygenase. These findings may well have an important impact in reproductive biology in which reactive oxygen species may promote both cyclooxygenase-catalyzed and non-enzyme-induced generation of bioreactive prostaglandins.

It has been shown that hydrogen peroxide elicits prostaglandin production and uterine contractions in the pregnant rat uterus (36, 37). This effect could be blocked by indomethacin, a cyclooxygenase inhibitor, apparently precluding the nonenzymatic, direct generation of prostaglandins by reactive oxygen species, as discussed above. The authors found evidence of lipid peroxidation as a consequence of hydrogen peroxide treatment and also showed that the antioxidant, butylated hydroxyanisole, prevented peroxide-stimulated uterine prostaglandin production and contractions. The preventative effect of butylated hydroxyanisole was observed in spontaneously contracting uteruses obtained at parturition as well as those treated with hydrogen peroxide, suggesting that endogenous reactive oxygen species may be involved with parturition. Because butylated hydroxyanisole did not interfere with the contractile response caused by exogenous prostaglandins, it was reasoned that the antioxidant interfered with prostaglandin production rather than with prostaglandin action in the uterus. Finally, uterine and amniotic sepses are known to cause premature delivery (38), which may be due to the release of reactive oxygen species following leukocytic infiltration (39). Cytokine interactions with leukocytes were reviewed recently from the standpoint of uterine function and reactive oxygen species (39).

### Reactive Oxygen Species in Oocyte Maturation and Ovulation

In the follicular phase of the reproductive cycle, a cohort of primordial follicles are recruited for further development and maturation, with the end result being ovulation of one or more dominant mature follicles. Follice-stimulating hormone and estradiol are the principal hormones regulating follicular growth and development, while the midcycle luteinizing hormone (LH) surge is of central importance in ovulation. Within the periovulatory follicle, generation of both $PGE_2$ and $PGF_{2a}$ occurs, and this appears to be obligatory for ovulation. Ovulation has been compared to an inflammatory event (40), with common features such as vascular swelling, the accumulation of immune cells, prostaglandin production, and the presence of cytokines.

Only one or a few (depending on the species) follicles are destined for ovulation; others in the cohort that undergo development during the follicular phase become atretic and regress. Follicular atresia is characterized, in part, by loss of sensitivity to gonadotrophic hormones, particularly follicle-stimulating hormone, and steroidogenic function. Indirect evidence from Margolin *et al.* (41) suggests that reactive oxygen species may be involved in the atretic response. They reported that granulosa cells are extremely sensitive to low concentrations of hydrogen peroxide that rapidly inhibited FSH-sensitive cyclic AMP accumulation and progesterone production. This effect may be due to interference with the G-protein activation of adenylate cyclase in the granulosa cell plasma membrane.

Oocyte maturation, in which the resumption of meiosis is associated with germinal vesicle breakdown, can be demonstrated *in vitro* and is dependent on an appropriate oxygen tension. An atmosphere of 5% oxygen is optimal for *in vitro* maturation of hamster oocytes (42), whereas inhibition of maturation occurs at higher levels. A respiratory increase at the time of maturation may be one reason for the oxygen requirement. Zeilmaker and Verhamme (43) showed that when pyruvate was present, maturation proceeded independently of oxygen tension, presumably because the oocytes could switch to anaerobic metabolism. Selstam and Gafvels (44) showed that low oxygen levels caused the induction of glycolysis in maturing oocytes, which were still able to respond to LH, except in the complete absence of oxygen. The failure of SOD to influence oocyte maturation (45) suggests that superoxide is probably not involved in this process. However, support for a role of other reactive oxygen species in oocyte maturation was recently provided in a preliminary report by Doyle *et al.* (46). The LH analog, chorionic gonadotropin, induced oocyte maturation and this effect was accompanied by an increase in follicular lipid peroxidation, and spontaneous oocyte maturation was blocked by treatment with butylated hydroxyanisole.

Levels of both superoxide anion and SOD have been shown to undergo cyclical variations, in an inverse relationship to each other, throughout the reproductive cycle (47). Notably, superoxide levels were found to increase rapidly during estrus when ovulation occurred, and it was shown that LH caused a transient increase of SOD activity. Recently, a relationship between oxygen free radicals and ovulation was demonstrated by Miyazaki *et al.* (48). With an *in vitro* rabbit ovary preparation, they showed that ovulation was markedly reduced when SOD was added to the incubation medium. Incubation with catalase had no such effect, suggesting that hydrogen peroxide is not involved. These authors propose that free radical-induced membrane damage causes activation of phospholipase and that the resultant arachidonic acid is converted to prostaglandins via the cyclooxygenase system. It can be envisioned that the process could be self-propagating, since in the cyclooxygenase cascade, oxygen free radicals are produced as PGG2 is converted to PGH2 by the inherent peroxidase activity of cyclooxygenase. It is well known that prostaglandins (particularly PGE and $PGF_{2a}$) are a prerequisite for ovulation (49) and that inhibition of cyclooxygenase by indomethacin inhibits ovulation. Ovarian levels of cyclooxygenase increase during the periovulatory period, and this effect was shown to be an LH-stimulated, cyclic AMP-mediated event (50), although the precise role of cyclic AMP is complex (51).

In the sea urchin, there is an obligatory role for hydrogen peroxide in the fertilization process, in which it mediates the formation of the fertilization envelop (reviewed in Ref. 52). A "respiratory burst of fertilization" generates hydrogen peroxide via a NADPH oxidase. A specific ovoperoxidase utilizes hydrogen peroxide to cross-link dityrosyl moities in a protein matrix derived from the cortical granules and deposited on the extraoocyte vitelline layer. Dityrosyl cross-linking occurs within 3 min and forms a hard, impenetrable membrane that surrounds the fertilized egg. The fertilization envelop provides a barrier that prevents additional sperm penetration and a protected environment in which embryo development proceeds until hatching of the blastula.

### Reactive Oxygen Species and the Corpus Luteum

Granulosa and thecal cells, which enclose the oocyte in the mature follicle, differentiate after ovulation to form the corpus luteum, a temporary endocrine gland that plays a vital role in the maintenance of pregnancy. In response to LH, the corpus luteum secretes progesterone, and in primates, estradiol as well. In the absence of conception, luteal regression occurs, an event functionally described as the abrupt decline in progesterone secretion, and is followed by structural regression, culminating ultimately in resorption of the corpus luteum. It is generally agreed that in many species, corpus luteum regression is triggered by $PGF_{2a}$ derived from the uterus and supplied to the ovary via transfer between the uterine vein and the ovarian artery. An important exception are primates, in which the factor(s) initiating luteolysis remains elusive. We suggest that intraluteal generation of hydrogen peroxide may play a luteolytic role in primates, as appears to be the case in the rat.

While LH is known to stimulate steroidogenesis, it is also equally well established that LH provokes a substantial drop in luteal levels of ascorbic acid (53). Luteal depletion of ascorbic acid is also an early effect of *in vivo* $PGF_{2a}$ treatment (54). The time course of $PGF_{2a}$-induced ascorbic acid depletion in rat luteal tissue was found to occur in parallel with functional

luteolysis and increased lipid peroxidation of the corpus luteum (55). In these preliminary studies, both ascorbic acid depletion and lipid peroxidation were evident 2 and 4 hr after treatment with $PGF_{2a}$, but both returned to normal levels 24 hr later. Earlier, Sawada and Carlson (56) reported the occurrence of lipid peroxidation in rat luteal tissue that was evident 24 hr after $PGF_{2a}$ treatment *in vivo*. Since progesterone levels are reduced within 1 hr of $PGF_{2a}$ treatment, these results also point to a late effect of $PGF_{2a}$ on lipid peroxidation in the corpus luteum. In contrast to that seen with $PGF_{2a}$, LH-induced ascorbic acid depletion in rat luteal tissue was not associated with an increase in lipid peroxidation or a decrease in serum progesterone levels (55). Vitamin E levels in the corpus luteum were not changed by $PGF_{2a}$, whereas LH doubled vitamin E levels within 24 hr *in vivo* (55). These studies reveal a novel aspect of the luteotropic action of LH that is related to the maintenance of high levels of vitamin E in the corpus luteum. In other tissues, vitamin E is transported in serum complexed with lipoproteins and taken up by cells via lipoprotein transport processes. These findings, in addition to the abundant levels of antioxidant vitamins in luteal tissue, lend indirect support for the functional involvement of reactive oxygen species in the corpus luteum.

There is direct evidence for the generation of hydrogen peroxide in the corpus luteum early in the luteolytic process. $PGF_{2a}$ increases the generation of hydrogen peroxide in rat luteal tissue *in vivo* within 2 hr and decreases serum progesterone levels in the same interval (57). In this study, $PGF_{2a}$-treated animals were injected with the hydrogen peroxide-dependent catalase inhibitor, 3,4-aminotriazole, which was followed by an assay of luteal catalase activity. Significant depression of catalase activity was found when the rats were administered the inhibitor 1 hr after $PGF_{2a}$ treatment, denoting increased hydrogen peroxide generation. An increase in hydrogen peroxide production within luteal tissue was also seen during natural luteal regression, precluding the possibility that the results from the $PGF_{2a}$-treated rats were pharmacological artifacts. Preliminary evidence by Pepperell *et al.* (58) showed that membrane preparations of rat luteal tissue generate hydrogen peroxide when incubated with either NADH or NADPH and both of these responses were heat and catalase-sensitive. In these studies, NADH-dependent hydrogen peroxide production was almost 10-fold greater than that seen with NADPH. Unlike that seen with NADH, NADPH-stimulated hydrogen peroxide production in luteal membranes was calcium sensitive. These results point to the presence of a NAD(P)H oxidase in the corpus luteum that generates superoxide like that in leukocytes (59), or like the thyroid hydrogen peroxide generator responsible for iodide organification (60).

Significantly, low levels of hydrogen peroxide are directly luteolytic in isolated rat luteal cells (61). In this paradigm, hydrogen peroxide inhibits LH-dependent cyclic AMP and progesterone production within minutes, followed by a decline in cellular levels of ATP. The depletion of ATP was shown to be primarily the result of peroxide-induced DNA damage that caused induction of DNA repair mechanisms (61). However, maintenance of ATP levels did not affect the luteolytic actions of hydrogen peroxide and no early impairment of LH receptor binding was seen. Not only does hydrogen peroxide interfere with cyclic AMP production, which appears to be due to uncoupling of the G-protein (61), but it also blocks steroidogenesis at a post-second messenger site (62). Dibutryl cyclic AMP- and 8-bromo-cyclic AMP-stimulated progesterone production are inhibited by hydrogen peroxide, an effect reversed when the cells are supplied with mitochondria-permeant cholesterol analogs as substrates for steroidogenesis. Translocation of cholesterol across the outer mitochondrial membrane, after which oxidation to pregnenolone prior to final conversion to progesterone occurs, is a rate-limiting step in acute stimulation of steroidogenesis, in addition to cholesterol side-chain cleavage. Membrane translocation of cholesterol in the mitochondria, or intracellular transport of cholesterol to the mitochondria, is inhibited by hydrogen peroxide. It is this response, rather than inhibition of side-chain cleavage of cholesterol, that is blocked by hydrogen peroxide, causing inhibition of steroidogenesis. While hydrogen peroxide has luteolytic actions similar to those of $PGF_{2a}$, the action of hydrogen peroxide is not mediated by $PGF_{2a}$ because $PGF_{2a}$ production is unaffected, and treatment with the cyclooxygenase inhibitor indomethacin does not reverse the effects of hydrogen peroxide (61). A preliminary report by Endo *et al.* (63) shows that hydrogen peroxide evoked antigonadotropic effects on cyclic AMP accumulation and inhibited progesterone synthesis in human granulosa luteal cells in culture similar to that seen in rat luteal cells.

While the superoxide anion may be the progenitor of hydrogen peroxide, it is not directly luteolytic. The evidence for this conclusion was derived from studies with xanthine oxidase, an enzyme that generates superoxide when incubated with hypoxanthine or xanthine. Under these conditions, xanthine oxidase mimicked completely the actions of hydrogen peroxide in luteal cells (64). However, these luteolytic actions of superoxide were reversed completely by catalase, showing that the superoxide anion was rapidly dismutated to hydrogen peroxide, which mediated the luteolytic effects of the superoxide radical (64).

The origin of hydrogen peroxide in the ovary is unknown. It could be derived directly by bivalent reduction of oxygen, or from univalent reduction of oxygen to superoxide followed by dismutation into

hydrogen peroxide by superoxide dismutase. The rat ovary has been shown to generate the superoxide anion (47). Recently, more convincing evidence for the early involvement of superoxide production in luteal regression was reported by Sawada and Carlson (65). They showed a rapid and transient increase in superoxide levels in luteal membranes that achieved statistical significance when ovaries were removed after 10 min of *in vivo* $PGF_{2a}$ treatment. Interestingly, the superoxide burst preceded the fall in progesterone levels and the superoxide levels returned to baseline levels in samples prepared from ovaries 40 min after $PGF_{2a}$ administration. In addition, a concomitant decrease in membrane fluidity and activation of phospholipase $A_2$ activity occurred, earlier shown to be components of luteal regression in this paradigm (66, 67). Evidence of lipid peroxidation, following the luteal superoxide burst, was also observed (65).

The mechanism by which hydrogen peroxide blocks hormone-sensitive cyclic AMP accumulation and steroidogenesis in luteal cells is not known. Presumably this could occur by peroxidase-dependent events in which hydrogen peroxide serves as a substrate, or by generation of the highly reactive hydroxyl radical by ferrous or cuprous ions via Fenton-like reactions. Rat luteal peroxidase activity has been reported in crude extracts of luteal homogenates in which activity was inversely related to ascorbic acid levels (68); this is not a surprising finding in view of the ability of ascorbic acid to inhibit many peroxidases. Indirect evidence for the generation of hydroxyl radicals by hydrogen peroxide was shown recently in a preliminary report by Musicki and Behrman (69). Nearly complete reversal of the effects of hydrogen peroxide in luteal cells was achieved by treatment of luteal cells with *o*-phenanthroline, a cell-permeant and tenacious chelator of iron and copper. Complicating the interpretation of this finding, however, was the observation that ethanol and other scavengers of the hydroxyl radical were not effective in reversing the luteolytic actions of hydrogen peroxide (69), suggesting that the hydroxyl radical may either be cryptic, or not involved in the action of hydrogen peroxide.

While it seems clear that reactive oxygen species are implicated in luteal regression, their nature and source are not as yet understood. To date, there has yet to be a convincing demonstration for the production of significant levels of reactive oxygen species exclusively from luteal cells. Luteal cells may be an important source because they have active steroid-metabolizing microsomal and mitochondrial P-450 monoxygenase systems that are known to generate reactive oxygen species. Also, parenchymal luteal cells may contain a generator of hydrogen peroxide like the thyroid-stimulating hormone- and $PGF_{2a}$-sensitive generator of reactive oxygen species of the thyroid (59).

In addition to endothelial cells, other likely candidates for generation of reactive oxygen species in the corpus luteum are leukocytes, which, along with associated cytokines, are increasingly being recognized as mediators for a variety of ovarian and reproductive functions (70). Resident macrophages, lymphocytes, and granulocytes have been identified in the ovary, and macrophages, neutrophils, and eosinophils are well known to generate toxic bursts of superoxide anion and hydrogen peroxide (71). The cytokines interleukin 1 and tumor necrosis factor-$\alpha$, secreted by macrophages and perhaps granulosa/luteal cells, activate neutrophils and have been shown to evoke antigonadotropic effects (72). At the conclusion of pseudopregnancy in the rabbit, a large increase in the number of resident macrophages is evident, and this is associated with a rise in tumor necrosis factor-$\alpha$ (73). In the ovine corpus luteum, $PGF_{2a}$ causes a significant increase in eosinophils that precedes the fall in progesterone, which was shown to be mediated by a specific chemoattractant produced by luteal cells (74). This work, and the evidence linking hydrogen peroxide to luteolysis, has led to the concept of immunoregulation of luteal regression (75, 76). It seems plausible that resident ovarian immune cells, activated by cytokines of luteal origin and stimulated by $PGF_{2a}$, could generate superoxide and other reactive oxygen species. The evidence points to hydrogen peroxide as the principal luteolytic effector of reactive oxygen species, and two sites among several possibilities include signal transduction across the plasma membrane and transport of cholesterol at the mitochondrial level. Involvement of resident ovarian immune cells in luteolysis might, therefore, explain the difficulty in demonstrating all elements of $PGF_{2a}$-orchestrated luteolysis in populations of luteal cells *in vitro*.

Consistent with the luteolytic actions of hydrogen peroxide, it follows that suppressed generation of hydrogen peroxide may be an important avenue for the prevention of corpus luteum regression, a vital response necessary for maintenance of early pregnancy. Transforming growth factor-$\beta$, an ovarian product of which activin is a family member, deactivates macrophages and suppresses the respiratory burst (77). Adenosine, which exerts progonadotropic actions in ovarian cells (78), inhibits neutrophil production of reactive oxygen species in response to activators such as colony-stimulating factor (79). $\alpha$-Interferon has also been shown to antagonize activation of the respiratory burst induced by $\gamma$-interferon in macrophages (80), an interesting finding in view of the similarity between $\alpha$-interferon and trophoblastic proteins that extend luteal function (81, 82). Such interactions between cytokines and other paracrine regulators may play important roles in prevention of luteal regression in early pregnancy by blocking the production of reactive oxygen species such as

hydrogen peroxide and, thereby, preventing corpus luteum regression.

## Summary

Many components of the reproductive system operate under finely tuned regulatory control and, as such, are sensitive to perturbation, whether of an extrinsic or intrinsic nature. Reactive oxygen species and their associated free radicals are a literal fact of aerobic life; consequently, adaptations have evolved in reproductive processes that not only protect against the pernicious aspects of reactive oxygen species, but also exploit their reactive nature for beneficial ends. The latter principle seems natural in light of the frequent and substantial morphological and functional modulation that reproductive tissues experience from the endocrine, nervous, and immune systems. A variety of imaginative investigative techniques have been employed to study interactions between free radicals and biological systems, but due to their ephemeral nature, experimental and interpretive complexities remain. Despite this, it seems likely that future research exploring the relationship between radical chemistry and cellular function will yield fundamental insights, into both pathological and mechanistic features of reproductive biology.

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