CHAPTER 16
Role of oxidants and antioxidants in female reproduction

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THEMATIC SUMMARY BOX
At the end of this chapter, students should be able to:
• Define the term oxidative stress
• Describe the importance of reactive oxygen species and reactive nitrogen species in oxidative stress
• Describe the general physiological roles of reactive oxygen species
• Discuss the significance of antioxidants in our body and their therapeutic role in female infertility
• Describe how endogenous and exogenous free radicals stimulate and initiate disease
• List the different methods used to detect reactive oxygen species
• Discuss how reactive oxygen species play a role in female reproductive physiology
• List the factors that contribute to oxidative stress in the female
• Describe the pathological roles of oxidative stress in the female reproductive tract

Introduction
Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are naturally produced in the human body. In fact, they are key by-products in oxygen-utilizing metabolic processes such as oxidative phosphorylation and play essential roles as secondary messengers in intracellular signaling pathways.¹,² Levels of ROS and RNS are kept within physiologic limits by antioxidants. Thus, normal cell function is a balanced interplay of antioxidant defenses and reactive oxygen and nitrogen species production. As with any concept of balance in the human system, it is assumed that

Edited by Donald Armstrong and Robert D. Stratton.
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a graded response will occur when it is disrupted, with small and immediate changes manageable by homeostatic mechanisms. Major changes, on the other hand, either due to aggressive increases in ROS or RNS or inadequate antioxidants, lead to a condition called oxidative stress. Oxidative stress is highly pathogenic and associated with reproductive diseases such as polycystic ovary syndrome and endometriosis as well as pregnancy complications including spontaneous abortion and preeclampsia – these will be further outlined in this chapter.

**Reactive oxygen species**

**Characteristics of reactive oxygen species**

The production of ROS requires the presence of oxygen. Oxygen is necessary as a final electron acceptor in the production of adenosine-5-triphosphate (ATP) during mitochondrial oxidative phosphorylation to yield water. Nonetheless, what makes oxygen necessary is also what makes it potentially damaging. Oxygen (O₂), by nature, is a highly reactive, diradical species that accepts free electrons readily. When oxygen consumption occurs during electron transfer, radicals or oxygen ions are produced, which are highly reactive due to unpaired electrons in their outermost shell. These short-lived, toxic ROS are produced due to electron leakage from the electron transport chain process toward the end of oxidative phosphorylation. The main type of ROS that is produced includes the superoxide anion – a product of NADPH oxidase reactions and the hydroxyl radical – by spontaneous degeneration of hydrogen peroxide. There are two redox reactions highly implicated in ROS production:

1. **The Haber–Weiss reaction**:

   \[
   \cdot \text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \cdot \text{OH} + \text{OH}^- + \text{O}_2
   \]

   The Haber–Weiss reaction produces the highly reactive hydroxyl radical (•OH), which can also facilitate interactions between the superoxide anion (•O₂⁻) and hydrogen peroxide (H₂O₂). Hydrogen peroxide is not a free radical per se, but is highly implicated in their generation and breakdown. This reaction is thermodynamically unstable and, hence, requires a catalyst to proceed – this is known as the Fenton reaction.

2. **The Fenton reaction**:

   \[
   \text{Fe}^{3+} + \cdot \text{O}_2^- \rightarrow \text{Fe}^{2+} + \text{O}_2
   \]

   \[
   \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \cdot \text{OH}
   \]

   In the Fenton reaction, a metal ion acts as a catalyst and produces a net product of hydroxyl radicals (•OH). Thus, metallic cations such as copper or iron ions contribute to ROS generation by acting as catalysts.

   Other systems contributing to superoxide production include NADPH oxidase, which is a core enzyme in cellular respiration. It is found in the form of NADPH oxidase 1 (NOX1), NADPH oxidase 2 (NOX2), and NADPH oxidase 3 (NOX3) in smooth muscle and vascular endothelium, dual oxidases 1 and 2 (DUOX1 and DUOX2), and NADPH oxidase 4 (NOX4) in epithelial cells.
Furthermore, the short electron chain in the endoplasmic reticulum, cytochrome P450 of the liver, and the enzyme xanthine oxidase also produce ROS.\(^3\)

**General physiological roles**

ROS are produced as a leukocyte defense mechanism. When foreign microbes are detected, phagocytic leukocytes such as neutrophils and macrophages ingest and kill microbes via ROS-releasing, respiratory bursts in the phagolysosome. This is facilitated by a phagosome membrane enzyme called myeloperoxidase, which produces superoxide (\(^{\cdot}\)O\(_2^-\)).\(^5\) Subsequently, hydrogen peroxide (H\(_2\)O\(_2\)), followed by a highly reactive compound called hypochlorite (ClO\(^{-}\)), is generated.\(^5\)

Beyond the immune system, ROS are also vital secondary messengers that activate downstream signal transducing pathways that influence expression and activity of cytokines, ions, and growth factors. As a matter of fact, ROS have been specifically implicated in core apoptotic pathways. The final product of this pathway is the release of cytochrome \(c\) and other proapoptotic cell mediators. This is achieved by activating c-Jun N-terminal kinase (a member of the mitogen-activated protein (MAP) kinases family) in response to stress stimuli, which in turn phosphorylates and releases Bcl-2-related proteins.\(^4\) This initiates the uncoupling of protein Bax and its translocation to oligomerize inside the mitochondria.\(^5\) Bax in the mitochondria catalyzes the production of the final proapoptotic mediators. Both Bax (proapoptotic) and Bcl-2 (antiapoptotic) are proteins implicated in the mitochondrial apoptotic pathway (Figure 16.1).

![C-jun-N terminal kinase pathway and apoptosis](https://example.com/cjun-n-terminal-kinase-pathway.png)

**Figure 16.1** c-Jun N terminal kinase pathway and apoptosis. ROS act as secondary messengers that activate core apoptotic pathways via the activation of the c-Jun N-terminal kinase. (See color plate section for the color representation of this figure.)
ROS and potential means of damage

When the effects of ROS are either exaggerated or left unchecked, cellular injury occurs. The extent of damage depends on rates of production and removal of ROS.\(^5\) Cell injury manifests as (Figure 16.2):

1. **Lipid peroxidation of membranes:** The cell membrane is a compilation of a variety of macromolecules. However, lipids in the form of polyunsaturated fatty acids (PUFAs) make up the majority of the cell membrane. Thus, being unsaturated, exposed double bonds that form kinks (turns) in the fatty acid chain are susceptible to oxidation.\(^5\) Specifically, at these kinks, PUFAs and ROS react to produce peroxide (\(O_2^{2-}\)), which is highly implicated in inducing an autocatalytic chain reaction.\(^5\) Furthermore, excess peroxide may induce apoptosis. It should be noted that lipid peroxidation depends on the enzyme sphingomyelinase and on the subsequent ceramide release.\(^4\)

2. **DNA injury:** Because reactive species are highly diffusible, both nuclear DNA and mitochondrial DNA are susceptible to attack. Highly damaging single-strand breaks occur via thymine reactions.\(^5\) DNA injury is vastly detrimental and produces defects in several downstream processes including future transcription and translation of proteins and even gene expression and cellular adaption. Premature cellular aging, cell death, and malignant transformation could also occur.\(^5\)

![Figure 16.2](image)

Figure 16.2 The consequences of ROS and oxidative stress. Exposure to pathological levels of ROS and oxidative stress causes cellular membrane and DNA damage along with protein manipulation. (See color plate section for the color representation of this figure.)
3 **Protein manipulation:** Proteins, regardless of their degree of structural complexity, pose a viable site of attack for ROS. Free radical interaction may cross-link sulfhydryl molecules, leading to conformational change of the polypeptide and, thus, loss of function or enhanced protein degradation. Direct oxidation or nitrosylation of proteins could also produce carbonyl, nitration, or nitrotyrosine, all of which are capable of disrupting enzyme function as well.

**Characteristics of reactive nitrogen species, physiological roles, and mechanisms of damage**

Reactive nitrogen species are also vital contributors to oxidative stress. These include both nitric oxide (NO) and nitrogen dioxide (NO₂) and other less reactive species such as nitrosamines and peroxynitrite (ONOO⁻). Nitric oxide is physiologically fundamental in human vasoregulation and also plays a role in signaling at the cellular level. Nonetheless, although RNS exist at some level in humans, excess sources of RNS in mammals also lead to oxidative stress. RNS are produced mainly through a reaction of oxygen and L-arginine and their reaction with superoxide anions, which ultimately leads to peroxynitrite formation.

To understand how RNS can cause damage, a general overview of the physiological attributes of RNS must be discussed. Nitric oxide production and function depend on its location within the body and nitric oxide synthase (NOS) enzymes present in tissue. For example, NO acts as a neurotransmitter in the central nervous system and is produced by the neuronal nitric oxide synthase (nNOS) enzyme system. NO is also produced in macrophages by inducible nitric oxide synthase (iNOS) and plays a vital role in the initial vasodilation and immune cell recruitment in inflammation.

In the female reproductive system, the endothelial nitric oxide synthase (eNOS) enzyme produces NO at an elevated level in response to luteinizing hormone (LH) and human chorionic gonadotropin (hCG). This is because eNOS activity is sensitive to intracellular calcium, and in a normal pregnancy, sustained or capacitive calcium entry occurs to maintain eNOS activity. Lack thereof will lead to inadequately vasodilated uterine vessels and hypertensive-related complications. On the other hand, overproduction of RNS may lead to an exaggerated response.

Other effects of high levels of RNS include protein structure anomalies, which in turn compromise enzymatic activity, cytoskeletal organization, and cell signal transduction.

**Antioxidants**

Throughout our evolutionary adaptations, the human body has developed several defense mechanisms to balance levels of reactive species in our bodies. There are repair mechanisms, preventative mechanisms, physical defenses, and antioxidant defenses. The latter can be divided into enzymatic and nonenzymatic molecules.
Nonenzymatic antioxidants

Nonenzymatic antioxidants are generally exogenous and must be ingested in the form of nutrients.\textsuperscript{6} These low molecular weight molecules, nonetheless, are necessary.

The most vital molecules include ascorbic acid (vitamin C) and α-tocopherol (vitamin E). They act as dual cofactors, with vitamin C needed to regenerate vitamin E.\textsuperscript{2} Other molecules include thiol compounds such as thioredoxin, which breaks down H\textsubscript{2}O\textsubscript{2}.\textsuperscript{2} Once oxidized, thioredoxin reductase in the vicinity will reduce and recycle the thiol compounds, maintaining viable thiol levels.\textsuperscript{2} Other nonenzymatic antioxidants include ceruloplasmin and transferrin, which aim to sequester free iron ions – a vital catalyst for the previously mentioned Fenton reaction.\textsuperscript{2}

Although these antioxidants are vital, the glutathione redox system is the most significant and abundant. Arguably the decisive antioxidant in oxidative balance, glutathione is a tripeptide, thiol antioxidant, and redox buffer. Glutathione, in its reduced form and in the presence of ROS, becomes oxidized to glutathione disulphide.\textsuperscript{7} Unlike other antioxidant patterns in the body, this peptide is found in different concentrations within the cell and is a major soluble antioxidant within cell compartments. Its presence in different cellular compartments suggests that glutathione is involved in an intricate, intracellular transport system. Glutathione, a combination of L-glutamate, L-cysteine, and glycine, is synthesized by glutamate–cysteine ligase and glutathione synthetase – enzymes found only in the cytosol.\textsuperscript{7} A concentration gradient drives the transport of glutathione into the mitochondria.\textsuperscript{7}

Moreover, as an example of the interplay of enzymatic and nonenzymatic antioxidants, glutathione can act as a cofactor for several detoxifying enzymes, which include glutathione peroxidase (GPx) and glutathione transferase.\textsuperscript{7} Glutathione also participates in amino acid transport through the plasma membrane and can take up and neutralize hydroxyl radicals (•OH) and singlet oxygen molecules (\textsuperscript{1}O\textsubscript{2}) directly.\textsuperscript{7} Glutathione regenerates vitamins C and E back to their active forms.\textsuperscript{7} More specifically, through the subsequent reduction of semi-dehydroascorbate to ascorbate, glutathione is capable of reducing α-tocopherol radicals to vitamin E.\textsuperscript{7} A lack of any of these antioxidants will leave the body, especially the reproductive system, susceptible to oxidative damage.

Enzymatic antioxidants

On the other hand, enzymatic antioxidants are endogenous and are generally more efficient in their role. The core of these antioxidants is a transition metal capable of different valences, which is necessary for the transfer of electrons during detoxification reactions.\textsuperscript{2} These antioxidants help maintain homeostatic oxidative balance.

They include the following:

1. Superoxide dismutases (SOD): This enzyme catalyzes the dismutation of the superoxide anion – a free radical produced after oxygen reduction and radical chain propagation thereafter.\textsuperscript{6} There are many isoforms but the main isoforms are SOD1, SOD2, and SOD3. SOD1 (Cu–Zn SOD), a mainly cytosolic form, contains a core of two metal cofactors forming a copper–zinc enzyme.\textsuperscript{9} A specific mutation in this enzyme alone can produce a phenotype of female infertility.\textsuperscript{6} SOD2 (Mn-SOD), on the other hand, is the manganese counterpart, encoded by nuclear DNA and restricted in activity to the mitochondria.\textsuperscript{2} SOD2 is inducible under various levels
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of oxidative stress and inflammatory conditions. SOD3 encodes an extracellular form of this enzyme and is structurally similar to SOD1 but has not been linked to fertility or reproduction. Therefore, SOD enzymes vary in type, location, and makeup.

2 Peroxidases: These are a large family of enzymes that preferably use hydrogen peroxide as a substrate. One of the most vital of these is GPx. GPx is a tetrameric selenoprotein that depends on reduced glutathione as a hydrogen molecule donor. With a main role of detoxifying peroxides, the selenocysteine core is critical. In fact, four isoforms exist in mammals, and it is a multisystem antioxidant defense system.

Another vital peroxidase is catalase, which detoxifies hydrogen peroxide but does not require an electron donor. Its role is that of an antiapoptotic and will be detailed in a later section.

3 Thioredoxin (Trx) system: The thioredoxin system regulates various enzymes and transactivating factors for genes and is substantially involved in cell growth, differentiation, and death. It is also a repair system rather than just a protective mechanism. Trx is a protein disulfide isomerase that corrects errors such as disulfide bridges and is a cofactor for the DNA-synthesizing ribonucleotide reductase enzyme. Trx is not only necessary to maintain an oxidative balance and limit oxidative damage but is also critical for continued cell survival. This is highly evident, as Trx-deficient mice are embryonically lethal.

Antioxidant treatment for female infertility

The global vitamin and supplement market is an enormous and growing industry, worth $68 billion. However, it is unregulated and supplements can be purchased at retail stores. This could pose issues of unnecessary supplementation, overdose, and ineffective treatment.

Antioxidants are chemical or biological compounds that include vitamins, minerals, and PUFAs, the latter of which includes omega-3, omega-6, and omega-9. These can be taken as oral supplements. Other antioxidants given as supplements and studied in female infertility include melatonin, folic acid, myo-inositol, zinc, selenium, N-acetyl-cysteine, and vitamins A, C, and E, which can be used individually or as a combination therapy. Other commonly used antioxidant supplements include pentoxifylline, a trisubstituted xanthine derivative, and l-arginine. Apart from synthetic, nonenzymatic antioxidants, antioxidant mimetic molecules are currently being developed, including porphyrinic, peptidylic, and phenolic structures of zinc, copper, and manganese complexes that mimic SOD and its enzymatic role.

In present-day medicine, antioxidants are utilized clinically. For example, women take antioxidant supplements to improve their fertility before undergoing assisted reproductive techniques (ART). Theoretically, replacing deficient antioxidants should be effective as a means of treatment. Nevertheless, evidence of the efficacy of antioxidant supplementation is extremely limited, and the literature further demonstrates the controversy behind this topic (Figure 16.3).
Antioxidant supplementation efficacy as a therapeutic treatment

<table>
<thead>
<tr>
<th>Supporting studies</th>
<th>No effect studies</th>
</tr>
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</table>
| Noda et al. (1994)                  | Torin et al. (2002)  
| Blastulation                                                                     | No effect on fertilization                                                      |
| Lighten et al. (1998)               | Iwata et al. (1998)  
| Enhanced embryo survival             | SOD/catalase/mannitol had no positive effects on embryo                         |
| Blastocyst formation                |                                                                                  |
| Embryo quality                      |                                                                                  |
| Henmi et al. (2003)                 |                                                                                  |
| Progesterone                        |                                                                                  |
| Pregnancy rates                     |                                                                                  |

Adverse effects

<table>
<thead>
<tr>
<th>Westphal et al. (2006)</th>
<th>Reported miscarriage and GI disturbances</th>
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<tbody>
<tr>
<td>Agarwal et al. (2012)</td>
<td>Reported ectopic pregnancy</td>
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</table>

Figure 16.3 Antioxidant supplementation efficacy as a therapeutic intervention. The efficacy of antioxidant supplementation as a therapeutic intervention remains inconclusive at this stage. The outcomes of studies involving intervention with oral antioxidant supplementation either support its use in alleviating the damaging effects of oxidative stress or show no effects on the reproductive parameters studied (selected studies are shown here).

Although controversial and contradictory, this topic has received some support. A 1994 study by Noda et al., incorporating low oxygen tension and low illumination, showed enhanced blastulation. A 1998 study by Lighten et al. used human insulin-like growth factor-I (IGF-1) ligand and reported enhanced embryo survival and blastocyst formation. Other studies, including that of Ali et al. in 2000, reported improved embryo quality with a combination of antioxidant regimens. A study done by Henmi et al. in 2003 assessed vitamin C supplementation in patients with a luteal phase defect and found that the treatment increased pregnancy rates and progesterone levels. Another study found that vitamin C levels in follicular fluid were higher in supplemented individuals than in controls, but the pregnancy rate between the groups was not statistically significant.

These types of studies shine a bright light on the potential of antioxidant treatments. However, not all studies have found supplementation to be beneficial to fertilization, including a 2002 study by Tarin et al., which used 62.5 μmol/l of ascorbate in a human tubal fluid medium and found no correlation with increased probability of fertilization. Moreover, antioxidants can have side effects. Indeed, vitamin C is a prooxidant at high doses and may cause further reproductive damage.
With such an abundant amount of studies with contradicting results, a systematic review with predefined inclusion and exclusion criteria was performed by Showell et al. and published in the Cochrane Library in 2013. This systematic review was significant because it excluded quasi-randomized trials and studies that tested antioxidants alone against fertility drugs as controls, as well as studies that exclusively included fertile women attending fertility clinics due to male factor infertility. Hence, the review included randomized controlled trials and crossover trials, which were limited to only first-phase data usage. Participants in these studies also were either subfertile women who had been referred to a fertility clinic and who may or may not have undergone ART. The participants were randomized to treatment groups taking oral antioxidant supplementation versus control groups (placebo or no treatment/standard treatment). Other interventions that were included are individual or combined oral antioxidants versus any antioxidant (head-to-head trials), pentoxifylline versus control (no or standard treatment), and antioxidants versus fertility drugs such as metformin and clomiphene citrate. The desired primary outcomes included live birth rate per woman, and secondary outcomes included clinical pregnancy rate per women and any adverse effects reported by the trials.

Primary outcomes showed that antioxidants were not associated with an increased live birth rate. In fact, reports on these studies revealed that among subfertile women with a pretreatment live birth rate of 37%, those on antioxidant therapy exhibited live birth rates between 10% and 83%. This indicates the heterogeneity of the results. Even in the two specific trials that reported a live birth, it was shown that the results were overestimated. In trials comparing antioxidants, both the odds ratio and confidence intervals indicated a possibility of not having an effect. This included a study comparing supplementation with myo-inositol plus folic acid and melatonin versus just myo-inositol and folic acid, as well as a study that compared supplementation with myo-inositol versus D-chiro-inositol. Hence, these studies had statistically insignificant results.

Secondary outcomes included clinical pregnancy rates, and even with a random-effects model, the heterogeneity remained high and, thus, no relation was shown. Although none of the single antioxidants, including melatonin, vitamins E and C, L-arginine, and N-acetyl-cysteine, led to clinical pregnancy; combined antioxidant therapy was associated with increased clinical pregnancy rates. Yet, heterogeneity remained high. Only one study assessed the use of vitamin E, ascorbic acid, and L-arginine as combination therapy, which made it impossible to pool results. In that study, the authors found no effect on clinical pregnancy rates.

In regard to another secondary outcome measures, the systematic review found no association between antioxidants (N-acetyl-cysteine, zinc, biotin, selenium, etc.) and adverse events such as multiple pregnancies and miscarriage. A few studies reported gastrointestinal disturbances and ectopic pregnancies, all of which were statistically insignificant.

From the findings of this systematic review, we can conclude that as of now, there is no viable indication or evidence suggesting that antioxidant efficacy in the clinical setting is an effective treatment for subfertility. Nonetheless, as much as there is a lack of evidence for their efficacy, there is also a lack of evidence suggesting that antioxidant intake causes adverse events. Treatment with antioxidants may not be effective, but it is also highly unlikely to be harmful.
Methods of detection of ROS in the female

Oxidative stress biomarkers such as ROS and the end-products of ROS reactions can be measured and used to assess oxidative damage in tissues and biological fluids. Prime locations of these biomarkers in the female reproductive system include the peritoneal, amniotic, and follicular fluids. The major methods used to measure biomarkers of oxidative stress are listed and detailed in Table 16.1.

Physiological roles and sources of ROS

Although ROS are dangerous in excess, their presence in moderate amounts is critical for normal cellular function. They play a physiological role in nearly every human system, including the female reproductive system.

Hence, this section will outline how ROS play a vital role in the menstrual cycle, a process in which a single ovum is released monthly and the uterine endometrium is prepared for implantation. A total of 6–12 primary follicles are formed per cycle. Initial folliculogenesis begins when the ovum and other layers of the granulosa layers grow, with the formation of ovary interstitium-derived spindle cells in the form of theca interna (responsible for steroidogenesis) and the capsule, theca externa. Granulosa cells secrete follicular fluid, and the antrum appears.

Oogenesis and folliculogenesis

In the ovaries, parenchymal steroidogenic cells, endothelial cells, and phagocytic macrophages produce ROS.17 Initially, it was thought that ROS were present in the ovaries and played a significant role there because ovarian tissue expressed markers of oxidative stress. Also, adverse effects were thought to occur in the absence of ROS. An example of oxidative stress biomarkers includes antioxidants such as Cu-SOD, Zn-SOD, and Mn-SOD, as well as GPx glutamyl synthetase and lipid peroxides.17 Decreased antioxidant levels were associated with negative effects on fertilization.17 Further studies have also suggested that GPx and Mn-SOD can be used as markers to detect oocyte maturation, thus implying that ROS are involved in this process as well. Moreover, aldose reductase and aldehyde reductase are found in granulosa cells and epithelia of the genital tract.17 NO also has a physiological significance. High levels of NO have adverse effects on cleavage rates, implantation rates, and overall embryo quality.17 High levels of NO have been positively correlated with peritoneal factor infertility.17 Therefore, it is apparent that ROS/RNS are present in the female reproductive tract and that they play important roles. The rest of the section will discuss the specifics of their physiological roles in folliculogenesis.

Each month, a cohort of oocytes begins to grow and develop in the ovary, but only meiosis I resumes in one of the embryos, which becomes the dominant oocyte. This initial process is promoted via ROS and is inhibited by antioxidants.3 However, ROS inhibits and antioxidants promote this process when progressing into meiosis II.3 During growth, steroid production in the growing follicle causes an increase in P450, which leads to ROS production. ROS is also generated by the post-LH surge inflammatory precursors.3 Thus, physiological levels of ROS regulate oocyte maturation.
### Table 16.1  Overview of the major methods of ROS and RNS detection.

<table>
<thead>
<tr>
<th>Method</th>
<th>Overview</th>
<th>Instrumentation</th>
<th>Means of error</th>
<th>Advantages</th>
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</thead>
<tbody>
<tr>
<td>Chemiluminescence assay and luminometers</td>
<td>This method excites molecules and then measures the amount of light they emit in a selected amount of time. The oxidative end-products produced by an <em>in vitro</em> reaction between a reagent and ROS are detected via light emission as measured by a luminometer. Luminometers measure light intensity using a photomultiplier tube to detect photons on a luminol-stained sample. Intracellular and extracellular radicals can be measured. Nonetheless, it is not possible to differentiate between the types of ROS detected. Other reagents can be used for this task such as lucigenin, which is specific for extracellular superoxide anions.</td>
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<tr>
<td></td>
<td><strong>Instrumentation:</strong></td>
<td>(a) <em>Photon counting luminometer</em> – measures individual photons</td>
<td>(b) <em>Direct current luminometer</em> – measures electric currents that are proportional to the photon flux in the photomultiplier tube.</td>
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<td></td>
<td><strong>Means of error:</strong></td>
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<td></td>
<td><strong>Advantages:</strong></td>
<td>Studies evaluating oxidative stress and damage in sperm found that flow cytometry is more sensitive, accurate, and specific than chemiluminescence.</td>
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<tr>
<td>Flow cytometry</td>
<td>This method can be used to detect changes in cells via fluorescence emitted by ROS-binding probes. Flow cytometry detects individual intracellular reactive oxygen radicals. What follows is oxidation of 2,7-dichlorofluorescein diacetate by these radicals and subsequent fluorescence emission. Using hydroethidine to measure intracellular superoxide is possible, and it will emit a red fluorescein color when oxidized.</td>
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<td>General microscopy and stain</td>
<td>This method is a simple and cost-effective but limiting tool. In order to detect ROS, a simple histochemical method called nitroblue tetrazolium staining is used. This compound is reduced in the presence of ROS to form a bluish-black insoluble compound, dubbed formazan.</td>
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<td><strong>Method:</strong> Microscopy usage is strenuous as the operator must observe 100 consecutive cells under oil immersion. Cells are then graded based on density of formazan granules inside the cells.</td>
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<tr>
<td>Epifluorescence microscopy</td>
<td>This is another method of microscopy where an end-product is detected via fluorescence. A similar method was mentioned in flow cytometry. Hydroethidine is added to react with superoxide, and a red-fluorescence emitting product called ethidium bromide is produced. This technique is more commonly used because the equipment is less complex and expensive.</td>
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<td>Enzymatic antioxidants</td>
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(continued)
Similarly, ROS have a dominant presence in the follicle and act as the main inducers of ovulation in the preovulatory follicle. Oxygen deprivation promotes the angiogenesis that is necessary for adequate growth and development of the ovarian follicle, and ROS may play a role in this process as well. Another physiological role is the induction of apoptosis. Follicular GSH and FSH counterbalance this action in the growing follicle. This is shown in the dominant follicle, which experiences a surge of estrogen due to FSH, which in turn triggers the generation of catalase in the dominant follicle, lowers ROS levels, and thus, avoids apoptosis.

Atresia accounts for up to 99% of germ cell depletion in the female. In folliculogenesis, only one of the 6–12 follicles proceeds to mature. This is not regulated

### Table 16.1 (Continued)

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
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<tbody>
<tr>
<td>Measurement enzymes measured</td>
<td>Superoxide dismutase activity is detected via a tetrazolium salt, and the formed chromophore is then measured at its maximal absorbance of 525 nm. Glutathione peroxidase can be measured via a kinetic colorimetric assay that will measure the indirect glutathione reductase-coupled reaction at its maximal wavelength absorbance. Catalase can be detected via a CAT assay based on the reaction of this enzyme with methanol in the presence of hydrogen peroxide, which produces formaldehyde.</td>
</tr>
<tr>
<td>Immunohistochemistry and Western blotting</td>
<td>Overview: This method can measure oxidative DNA adducts via immunohistochemistry, as exhibited in a study done by Takagi et al. where oxidative stress molecules such as 8-hydroxy-2'-deoxyguanosine (8-OHdG), 4-hydroxynonenal (4-HNE), thioredoxin (Trx), and redox factor-1 (ref-1) were detected via similar methods. Incubation with enzyme-linked secondary antibodies and diaminobenzidine staining could reveal the presence of these molecules. As an alternative, Western blotting may be used to as a means of detecting specific proteins in a sample.</td>
</tr>
<tr>
<td>Total antioxidant capacity (TAC)</td>
<td>Overview: This method assesses the actual antioxidant power present in the sample, as both endogenous and nutrient-derived antioxidants are measured. Total antioxidant assays are similar, in that they all rely on the antioxidants in the sample to prohibit oxidation of 2,2'-azino-di-ethylbenzthiazoline sulfonate by metmyoglobin. This is then compared with a control solution.</td>
</tr>
<tr>
<td>Enzyme-linked immunosorbent assay (ELISA)</td>
<td>Overview: This method can be used to detect certain antigens or antibodies that are produced as by-products of ROS interactions or production. Fluid is analyzed for targeted molecules via a spectrophotometer. Antibodies for certain antioxidants can also be used.</td>
</tr>
<tr>
<td>Nitrate reductase and Griess reaction</td>
<td>Overview: In this method, nitrate and nitrite can be detected via nitrate reductase and the Griess reaction as well as total nitric oxide levels in serum via rapid response chemiluminescence assay.</td>
</tr>
<tr>
<td>Other methods</td>
<td>Other methods include measuring nitric oxide levels, using enzyme-linked immunosorbent assays (ELISA) to detect damaged proteins, and measuring lipid peroxides, protein oxidation, total plasma lipid hydroperoxides, total 8-F2-isoprostane, and fat-soluble antioxidants. Further methods include the Raman analysis, near-infrared spectroscopy, and protein nuclear magnetic resonance.</td>
</tr>
</tbody>
</table>
by hormones but by an intrinsic positive feedback, which studies suggest is ROS related.\textsuperscript{21,22}

Although the apoptosis pathway of follicular atresia can be hormonally influenced, molecular mechanisms regarding the initiator of apoptosis remained vague until not long ago. Recently, key studies have elucidated the specific processes that involve ROS. A study by Tilly’s group used Northern blotting analysis to study equine chorionic gonadotropin (eCG)-induced follicular growth and survival. They reported steady counts of Bcl-2 and Bcl-x expression with lower levels of Bax mRNA.\textsuperscript{21} Bcl-x long was also dominant in granulosa cells in eCG primed ovaries.\textsuperscript{21} With Bcl-2 and Bcl-x both being present and having primary antioxidant properties in the surviving follicle, we can assume that ROS play a role in follicular atresia. Researchers thereafter went on to develop a study with preovulatory follicles isolated from ovaries of immature rats. The follicles were separated into a group with medium alone, FSH and medium, and FSH and buthionine sulfoximine (BSO) – a specific inhibitor of glutathione synthesis.\textsuperscript{22} As FSH is known to inhibit apoptosis, the study’s most significant discovery was that the antiapoptotic effect of FSH was reversed in the group in which glutathione synthesis was inhibited (FSH and BSO group).\textsuperscript{22} In fact, confocal microscopy showed that ROS were present in apoptotic follicles and that FSH significantly suppressed their production.\textsuperscript{22} Thus, the antiapoptotic FSH works by inducing glutathione production, and oxidative stress plays a role in the remaining follicles that undergo apoptosis. In another study, researchers dissected goat ovarian follicles and found that the large follicles exhibited greater catalase activity than other granulosa cells from small- or medium-sized follicles.\textsuperscript{17} Accordingly, ROS and oxidative stress are proven to play a role in follicular atresia.

**Corpus luteum apoptosis and steroidogenesis**

After ovulation, the corpus luteum is formed and secretes progesterone, which is vital to conception and pregnancy. In fact, when pregnancy does not occur, the corpus luteum disintegrates and regresses. During degeneration, ROS are produced via the monooxygenase reaction due to steroid hormone synthesis.\textsuperscript{23} These P450 systems, coupled with normal levels of ROS in electron transport chain leakage reactions, cause inevitable damage.

Here, ROS play a role in progression and regression of the corpus luteum. In progression, progesterone levels decline and levels of Cu-SOD and Zn-SOD antioxidants increase during the early to mid-luteal phase.\textsuperscript{3} Conversely, they decrease if regression occurs.\textsuperscript{3} Lipid peroxide levels increase during luteal regression and decrease during luteal progression.\textsuperscript{3} Levels of Cu-SOD and Zn-SOD decrease due to prostaglandin F2-\(\alpha\) stimulating accumulation of the superoxide anion by luteal cells and nearby phagocytic leukocytes.\textsuperscript{3} Nonetheless, Mn-SOD is directly associated with inflammatory reactions induced by oxidative stress.\textsuperscript{3} Confirmatory to what was previously mentioned, studies have found that ROS concentrations in the rat ovarian corpus luteum increased during the regression phase, and that the prime identified antioxidants are SOD.\textsuperscript{14}
Endometrium and endometrial cycle

The endometrium is a layer of epithelium lining the uterus that experiences cyclical degradation and regeneration. The stages of the endometrial cycle include the proliferative, secretory, and menstrual phase. ROS play a role in these stages and their progression.24

This was first discovered when variations of antioxidant SOD levels in the endometrial cycle were reported, as fluctuating SOD levels and elevated lipid peroxide levels were reported specifically in the late secretory phase, directly before the onset of menstruation.20 Thus, ROS could play a regulatory role in endometrial shedding. The source or reason for heightened ROS production remains unknown. Nonetheless, a study by Serviddio et al. observed how varying hormone levels influenced redox reactions and lipid peroxidation in human endometrial cells.25 They found that hormonal patterns were able to regulate GSH levels and GSH metabolism.25 Thus, hormone changes across the endometrial cycle could mediate antioxidant activity. This was later supported by several literature reports, suggesting that estrogen and progesterone withdrawal could be the prime instigator in ROS elevation. When these hormones were removed from endometrial cells in vitro, antioxidant SOD activity dropped.24,25 Thus, ROS are key players in the endometrial cycle. Their role is based on their ability to activate nuclear factor-kappa B (NF-κB), which leads to increased COX-2 mRNA expression and prostaglandin F2-α synthesis.24 This physiological impact is detrimental to the endometrial cycle.

In other states, ROS play a potentially pathogenic but defensive role. This is indicated as vascular endothelial growth factor (VEGF, proangiogenic factor) and Angiopoietin-2 (Ang-2, angiogenic antagonist) regulate endometrial vasculature formation and are regulated by both hypoxia and ROS.24 When, for instance, long-term progestin contraceptives are used and subsequent hypoxia and ROS production ensue, abnormal angiogenesis occurs as VEGF and Ang-2 are upregulated in an attempt to maintain homeostasis.24

With RNS, NO has also been implicated in the regulation of endometrial microvasculature. This was touched on earlier in the chapter and NO’s vital role in vasomotor functions and regulation was explained. Endothelial NOS mRNA was observed specifically in mid-secretory and late secretory phases, implying its significance in decidualization in the preparation for pregnancy and menstruation.24

Factors contributing to oxidative stress in the female

Several factors that generate oxidative stress play a role in female fertility and pregnancy (Figure 16.4). In general, the first trimester is a vital point in development and carries the highest risk for miscarriage.3 Obesity, malnutrition, drug and/or alcohol use, smoking, and exposure to environmental toxins have their greatest effects in the second trimester and can lead to poor fetal viability and influence outcomes in the third trimester.3
Factors contributing to oxidative stress in female reproduction include obesity, malnutrition, alcohol intake and smoking, misuse of drugs such as marijuana and cocaine, and exposure to environmental toxins.

**Obesity and overnutrition**

Today, obesity has become epidemic-like and its negative effects on human health have been documented extensively. Naturally, the number of obese women of reproductive age has also increased. More specifically, two-thirds of the female population in the United States who are within the reproductive age are considered either obese or overweight. In fact, obesity and overnutrition are vital when it comes to female infertility as obese women take longer to conceive and have higher risks of miscarriage than women with a normal BMI. Therefore, it is important for clinicians to educate their patients about obesity and its link to reproductive diseases and complications.

Linking obesity to oxidative stress has been a sequential process. Visceral fat is associated with disordered metabolism and insulin resistance. Moreover, centrally stored fat deposits are more likely to exhibit fatty acid overflow. Thus, preferential storage of fat in the abdomen and visceral areas leads to lipotoxicity. Lipotoxicity is the main culprit in oxidative stress, which in turn can lead to endothelial dysfunction. In addition, obesity is also linked to ROS generation as intracellular fat deposits disrupt mitochondrial function. This disruption leads to electron accumulation and leakage from the electron transport chain, which is a prime pathway for ROS generation. Lipid peroxides, oxidized low-density lipoproteins (oxLDL), and oxysterols are thus produced as a combined reaction between high lipid levels and oxidative stress. It should be noted that in the ovaries, the mitochondrial energy...
production is vital to the embryonic metabolism of the oocyte.\textsuperscript{3} Thus, mitochondrial dysfunction will directly influence embryonic metabolism. Another means of production of ROS is through increased plasma nonesterified fatty acid levels, which prompt the formation of the nitroxide radical.\textsuperscript{3}

Oxidative stress can also be linked to obesity due to adipose tissue physiology. Adipose tissue has a bilateral relationship with the gonads, as fat secretes adipokines such as leptin, ghrelin, and resistin.\textsuperscript{26} Of these, leptin plays a regulatory role in early embryo cleavage and development; it also stimulates the Hypothalamic-pituitary-adrenal axis and inhibits developing ovarian follicles.\textsuperscript{26} Obese individuals also have impaired lipid and glucose metabolism due to GLUT-4 translocations, insulin–insulin receptor binding, and postreceptor signaling, along with decreased lipoprotein lipase activity.\textsuperscript{27} With these impairments, mitochondrial dysfunction occurs, energy metabolism is impaired, and oxidative stress ensues.

The processing of macronutrients is associated with ROS production as well. When consumed and processed, don’t need that there, but you can keep it. High carbohydrate or fat intake is proportional to an increase in oxidative stress biomarkers. Hence, overnutrition is also an issue. This type of oxidative stress is dubbed postprandial and is directly correlated with both postprandial glycemia and lipemia.\textsuperscript{27} Its severity is immensely increased in diseased individuals such as those with diabetes and heart disease. Obese patients experience higher rates of disease than their healthy cohorts and are thus targets of higher postprandial oxidative stress. In fact, obese individuals have been shown to have higher resting and fasting levels of oxidative stress biomarkers compared to nonobese individuals.\textsuperscript{27} This also is true in regard to exercise-induced oxidative stress.\textsuperscript{27} Regardless, systemic oxidative stress has implications in all systems in the circulation, including the female reproductive system.

As mentioned earlier, endothelial dysfunction can be increased by obesity. Insulin resistance and visceral adiposity increase inflammatory reactants, diminish blood flow to skeletal muscle, and further increase ROS levels.\textsuperscript{28} A vicious circle of endothelial dysfunction and formation of OS ensues.

Obesity also plays a role in pregnancy. Pregnancy entails an increased state of metabolic demand, which is necessary to support both maternal hormonal physiology and normal fetal development. It should be noted that a healthy pregnancy is associated with mobilization of lipids as well as increased lipid peroxides, insulin resistance, and enhanced endothelial function.\textsuperscript{3} Obese women, on the other hand, experience increased lipid peroxide levels and limited progression of endothelial function during their pregnancies, along with an additive innate tendency for central fat storage.\textsuperscript{3} Normally, increases in total body fat peak during the second trimester. Excessive weight gain in pregnancy can cause further complications. It also should be noted that both obese and lean women gain a similar amount of fat during pregnancy. Nonetheless, lean women tend to gain excess fat in their lower extremity or thighs, whereas obese women gain fat in their trunk and visceral areas (central obesity).\textsuperscript{28} Preferential storage of fat in these areas leads to lipotoxicity, and its oxidative effects can be devastating. In pregnancy, oxidized lipids can inhibit trophoblast invasion and influence placental development, lipid metabolism and transport, and fetal developmental pathways.\textsuperscript{28}
Malnutrition

In pregnancy, undernutrition leads to impaired or stunned fetal growth as well as higher rates of low birthweight and potential endothelial dysfunction. In utero, NO levels are diminished and endothelium-dependent vasodilation is impaired. In studies of undernourished dams, the offspring exhibited decreased SOD activity and increased superoxide anions, which increases NO scavenging. Undernutrition during critical periods of fetal or embryonic growth can lead to overall increases in oxidative stress in the female offspring's ovaries. In fact, oxidative stress coupled with mitochondrial antioxidant defense impairment may decrease primordial, secondary, and antral follicles in the offspring. A study by Bernal et al. found that ovarian follicle numbers and mRNA levels of regulatory genes in the offspring of malnourished rats were decreased, and that this was mediated by a mechanism of oxidative stress in the ovaries and a diminished antioxidant system. This is relatable to human maternal undernutrition.

Nutrition is also a time-dependent factor. Pregnant adolescents experience two states of high metabolic demand because both puberty and pregnancy require additional nutrition to sustain growth. Thus, a tug-of-war scenario ensues.

Alcohol

Alcohol (ethanol) and mechanisms of ethanol breakdown are correlated with female fertility. Specifically, alcohol can increase apoptosis, damage tissue, and alter cell structures by intensifying oxidative stress both directly and indirectly.

Hepatic oxidative metabolism plays a prime role in the primary elimination of ethanol. Specifically, ethanol is dehydrogenated to produce acetyl aldehyde and is further metabolized to produce a combination of acetic acid and acetyl and methyl radicals. Naturally, these are ROS, and oxidative stress is thus a component of ethanol metabolism. Nonetheless, occasional or minimal alcohol ingestion is balanced by the endogenous antioxidant systems. Regular alcohol ingestion, on the other hand, leads to an overproduction of ROS, excessive SOD and GSH antioxidant depletion, and excess lipid peroxidation. Further studies have shown that acetyl aldehyde is highly implicated in ROS production, and that its presence may potentially "propagate redox cycling and catalytic generation of OS." More specifically, ethanol metabolism is linked to three metabolic pathways: alcohol dehydrogenase system, microsomal ethanol oxidation system (MEOS), and catalase system. MEOS, specifically, aggravates oxidative stress directly as well as indirectly by impairing defense systems. In fact, this metabolic pathway involves alkylation of hepatic proteins, and hydroxyethyl radicals are produced as coproducts. The primary elimination of ethanol has been shown to potentiate the oxidation seen in the Mallard reaction, leading to a overproduction of advanced glycation end-products (AGE), which can be toxic in high numbers. In fact, a study by Kalousova et al. found that serum levels of AGE are more abundant in people who are chronic alcoholics than in those who are nonalcoholics. This is supported by the fact that AGE production in chronic alcoholics may be high due to a lack of antioxidant systems, with malnutrition, cachexia, and vitamin deficiencies being common in this patient group. The physiology behind AGE products is that they bind with the receptor for advanced glycation end-products (RAGE) responsible for
activating the NF-κB transcription factor and the subsequent cytokine expression. Several studies have also found that antioxidant supplementation in form of vitamin B derivatives and vitamins A, C, or E may decrease production of AGE. Thus, accumulation of AGE has been linked to an inflammatory state and a reflex upregulation of antioxidant systems, which insinuates that ROS are involved in the process. Moreover, weakened or strained antioxidant systems may spur free radical formation in light of minimal AGE elevation.

As explained earlier, NO is vital in homeostasis of the body when it comes to regulation of vascular tone. Nonetheless, it may have cytotoxic effects in excess. Stable metabolites of nitrate and nitrite are increased in alcoholics. High concentrations of NO have also been linked to vascular and endothelial dysfunction. In the female reproductive system, NO in excess will disrupt the normal vascular tone of uterine vessels. Alcoholics also exhibited increased levels of oxidized LDL, which is pathogenic for atherogenesis and can lead to decreases in enzymatic antioxidants such as SOD and GPx, possibly due to their exhaustion.

A Danish study found that alcohol consumption is related to delayed conception. Women older than 30 years who consumed seven or more beverages per week experienced a higher rate of infertility. Thus, alcohol may hasten age-related infertility in women.

Maternal alcohol ingestion undoubtedly negatively affects the fetus in utero. Alcohol’s effects on the fetus are amplified in pregnancy, in part, due to the low levels of alcohol dehydrogenase in the fetal liver, with higher rates of low birthweight babies, congenital anomalies, and intrauterine growth retardation. In fact, spontaneous abortion and early pregnancy loss are also hastened by alcohol consumption in pregnancy. Alcohol metabolism leads to a prooxidant environment and thus, the antioxidant protective systems of the placenta will be depleted, and oxidative placental damage plays a large role in the pathophysiology. Gauthier et al. reported that in pregnant women, drinking more than three alcoholic beverages at a time produces stressful systemic oxidative stress. In fact, women who drank during pregnancy exhibited significant depletion of GSH, with higher rates of oxidized glutathione molecule (GSSG), during the postpartum period. Thus, alcohol plays a significant role in inducing oxidative stress.

Smoking

The negative effects of cigarette smoking are well documented and researched. The most addictive and toxic component of cigarettes is nicotine. One-third of women of reproductive age smoke cigarettes. Nicotine receptors and their actions have been linked with female reproductive pathologies. Only recently has oxidative stress become a prime focus.

Prooxidants and toxic chemicals in cigarette smoke are abundant and can be found in the two phases of smoking. The first phase is known as the tar or particulate phase. Water-soluble constituents of tar, when inhaled, react to form ROS, which include the superoxide anion, hydrogen peroxide, and hydroxyl radicals – the latter of which is notorious for its role in DNA damage. The gas phase, on the other hand, includes free radicals and toxins as well. NO is also produced via smoking, and overproduction of NO will lead to production of the RNS known as peroxynitrite.
This process begins when nicotine is inhaled and is oxidized into nicotine iminium and monoamine iminium, both of which have high reduction potentials. This high free radical state will lead to the depletion of antioxidant systems such as vitamin E, β-carotene, SOD, and catalase.

The effects of cigarette smoking on female fertility have been assessed in a number of studies. A meta-analysis based on 12 studies reported that smokers were 1.6 times more likely to experience infertility and increased time to conception compared to nonsmokers, in a dose-dependent manner with the number of cigarettes smoked.

Assisted reproductive techniques are less successful in smokers as well, perhaps because ROS of exogenous origin have direct effects on the follicular microenvironment and are correlated with decreased β-carotene antioxidant systems. Chelchowska et al. found that cigarette smoking depleted other antioxidants such as vitamin A, while other studies reported high lipid peroxidation in the follicular environment. Passive smoking also had similar effects.

As for smoking during pregnancy, complications and embryo damage are common. In fact, smokers have higher rates of fetal loss, preterm deliveries, decreased fetal growth, and spontaneous abortions because placental transfer of nicotine and carbon monoxide lead to placental hypoxia.

Recreational drugs
Marijuana is the most commonly used recreational drug worldwide and many leaders have called for its legalization. Nonetheless, its effects on female reproduction are evident and in pregnancy, it poses several dangers.

The main active constituents of marijuana are the cannabinoids. Cannabinoids generate free radicals when metabolized, directly affecting the peripheral and central nervous systems. The fundamental component of cannabinoid is delta-9-tetrahydrocannabinol or THC, which is also responsible for producing the psychological effects attributable to this drug. Cannabinoids bind to cannabinoid receptors, which include CB1 and CB2, both of which are a superfamily of G-protein-coupled receptors. The first indication that marijuana may play a role in reproduction occurred when endocannabinoid receptors were localized to female reproductive organs such as the ovaries and the uterine endometrium. Thus, exogenous administration of cannabinoid agonists may alter the reproductive processes within the female. THC, administered acutely, has also exhibited an ability to suppress LH. In fact, this is a time-dependent process, depending on the stage of the menstrual cycle. During the luteal phase, 30% of LH production is suppressed with marijuana exposure. In the follicular phase, however, this effect does not occur.

THC has been shown to disrupt the menstrual cycle by directly inhibiting oogenesis. The mechanism of action is hypothesized to be ROS related as the negative effects of marijuana can be counterbalanced by antioxidant (i.e., vitamin E) supplementation. Also, a study by Sarafian et al. in 1999 found that marijuana’s effects are dose dependent, and that a group of controls did not exhibit increased ROS production. In fact, it was later shown that generation of ROS through THC occurs via epoxidation of the 9, 10 alkene linkages in DNA.
During pregnancy, THC can inhibit initial implantation and disturb embryo development. The placenta is also capable of transporting THC, which accounts for its buildup in reproductive fluids. Moreover, THC-exposed fetuses had low birthweights, prematurity, congenital abnormalities, and intrauterine growth retardation.36

Another drug of importance to fertility is cocaine. Cocaine is a potent stimulant that is highly addictive and highly detrimental to the body both psychologically and physically. When taken, cocaine is immediately oxidized into several metabolites that lead to lipid peroxidation and production superoxide anions and lipid peroxyl radicals.3 Formaldehyde and norcocaine are oxidative metabolites, the latter of which could produce NO or peroxynitrite. Oxidative stress thus leads to GSH depletion and further oxidative damage such as apoptosis. The antioxidants thiol and deferoxamine were found to inhibit apoptosis during cocaine use, suggesting that ROS play a role in this process.3 Cocaine also has an inherent vasoconstrictive nature that can affect the uterine and placental vasculature, leading to hypoxia and further decreased GSH levels and heightened GSSG levels.3 In fact, Lee et al. found a dose-dependent reduction of GSH levels as well as an inflammatory state with heightened expression of TNF-α and NF-κB in cocaine users.38

In pregnancy, cocaine use can cause adverse outcomes such as intrauterine growth retardation, miscarriage, and low birthweight by causing peroxidative damage to underdeveloped fetal membranes.39 Cocaine use and subsequent ROS and RNS production also lead to limb defects, making it teratogenic as well.39

**Chemical compound exposure**

Female infertility can occur due to environmental and occupational exposure to toxins and chemicals. These substances are capable of deregulating a balanced oxidative environment, and our continued exposure to them via air, soil, contaminated food, and water poses a danger. Organochlorine pesticides (OCP), for example, accumulate in the body over time and are toxic to nerve fibers. OCPs are lipophilic and hydrophobic and thus are broken down slowly.3 They also can accumulate in the embryo, fetus, blood, placenta, and other parts of the female reproductive system.3 Hexachlorocyclohexane (HCH), a type of OCP, damages cell membranes via lipid peroxidation and increases superoxide radical and hydrogen peroxide production, leaving mitochondria and microsomes more susceptible to damage.40 In a study by Pathak et al. (2011), a xenobiotic (insecticidal isomer in OCPs known as γ-HCH) was positively correlated with markers of oxidative stress, which included 8-OHdG, MDA, and protein carbonyl levels.40 Moreover, as it has a tendency to conjugate with GSH, this antioxidant system was depleted, and the ferric reducing ability of plasma (FRAP, a measure of total antioxidant power) was low.40

An organochlorine insecticide commonly used in the past was 1,1,1-trichloro-2,2-bis (4-chlorophenyl)-ethane or DDT. Acute or transient DDT exposure is non-toxic, but continuous exposure negatively affects several systems in the body, including the reproductive system. DDT is lipophilic, and so it can remain stored in the body fat and even follicular fluid for up to two decades.3 Its effects were outlined in a study by Jirosova et al. (2010), which linked DDT to decreases in diploid oocyte numbers.41 Other studies reported correlations with miscarriages and spontaneous
abortions, especially in spouses of DDT-exposed agricultural workers. DDT exposure has also been correlated with intrauterine growth retardation. Furthermore, another OCP associated with intrauterine growth retardation is HCH. In fact, a study by Pathak et al. exhibited a statistically significant elevation of gamma-HCH in maternal and cord blood of intrauterine growth retardation cases compared to controls.

Polychlorinated biphenyls (PCBs) are another group of compounds implicated in female reproductive issues. PCBs are commonly found in pesticides and even cosmetics. Like OCPs, they are highly lipophilic and are slow to degrade and therefore accumulate in the body over time. Humans can be exposed to PCB via contaminated occupational environments and air and through the ingestion of meats, dairy, or fish with PCB traces. PCB has been found in follicular fluid, ovaries, uteri, and even in fetuses and embryos.

PCB exposure is associated with several pathologies although several studies contradict each other. Studies on Native American women who consumed fish containing PCBs had shorter than normal menstrual cycles. Other studies reported decreased fecundability. Studies by Meeker et al. (2011) and Toft et al. (2010) found that women exposed to PCBs had a heightened risk of IVF failure. Other studies have assessed the effects of PCBs on pregnancy. High exposure had no effect on the mean number of pregnancies in a study by Taylor, but birthweight and gestational age were decreased with higher PCB blood levels. Furthermore, high PCB blood levels were found in women who had three or more miscarriages. PCBs may induce oxidative stress via endothelial dysfunction and membrane damage with subsequent free radical formation. Regardless, connections with oxidative stress are implied as antioxidant systems such as vitamin E decrease with PCB exposure.

Another highly implicated group of chemicals in female reproductive oxidative stress are the organophosphorus pesticides (OPCs). OPCs have been linked to oxidative stress because GSH levels are depleted in exposed fetuses; other effects occur via lipid peroxidation. In fact, Samarawickrema et al. found that low-grade, long-term exposure to OPCs elevated levels of oxidative markers. These researchers also found elevated MDA in cord blood, DNA fragmentation in the fetus, as well as OPC accumulation in the placental-fetal compartment, suggesting the presence of fetal oxidative stress. Nonetheless, oxidative biomarkers in the mothers or females in general are unaltered, and this could be due to lower maternal metabolic detoxification capacities (with continued accumulation of OPCs) or diminished conversion to toxic metabolites.

Pathological effects and associations of oxidative stress

Menopause and OS

Menopause is a gradual event that occurs in the female and is marked by decline and cessation of reproductive capability due to diminished hormone production. It occurs at a mean age of 51 years in the United States but it can also occur prematurely, as early as 40 years. Symptoms of menopause vary but are generally typical: breast tenderness, vaginal dryness, irregular menses, hot flashes, and osteoporosis. These
symptoms occur due to diminished estrogen levels as well as heightened oxidative stress. This is hypothesized to be a result of a deficient antioxidant system. In fact, as women age, they have higher serum concentrations of TNF-α, IL-4, IL-10, and IL-12 than their younger counterparts.47

In regard to TNF-α, it is a highly proinflammatory mediator and the subsequent increase in IL-4 leads to an inflammatory state in menopause. Reactive oxygen species are highly implicated in inflammation and thus, heightened inflammatory markers also imply heightened ROS levels. Moreover, a study by Signorelli et al. reported elevated oxidative stress markers such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), oxLDL in postmenopausal women.48 GPx, a vital antioxidant, was also found to be diminished.48

Differentiated cells in the female reproductive system are quite susceptible to oxidative damage, including estrogen-producing ovary cells.49 These specific cells already have high cellular respiration levels, which imply that a large amount of ROS are being produced via electron leakage.49 Hence, with ROS already present in a chronic state, further accumulation of ROS during menopause will damage mitochondrial structures and genes, with consequent homeostatic, bioenergetics, and functional decline.49

Other menopause-related research shows that low NO concentrations may lead to vasomotor defects in the uterus and in the body as a whole. Moreover, vitamin C has been shown to decrease oxidized LDL concentrations, leading to improved parameters of cardiovascular and vascular health.47

Reproductive diseases and oxidative stress
Endometriosis
Endometriosis is a chronic inflammatory disease characterized by the invasion of the endometrial layer into extrauterine sites such as the ovaries and other pelvic sites with a few cases found in abdominal viscera, lungs, and the urinary tract.3 Studies disagree on whether oxidative stress plays a role in this condition.

Positive studies found higher concentrations of oxidative stress biomarkers such as MDA, proinflammatory cytokines (IL-6, TNF-α, and IL-β), angiogenic factors (IL-8 and VEGF), monocyte chemoattractant protein 1 (MCP-1), and oxLDL.3 Phagocytic cells are also highly implicated in ROS production in endometriosis. Oxidative stress may occur via the nonenzymatic peroxidation of arachidonic acid, which produces F2-isoprostanes.3 Lipid peroxidation, in fact, leads to 8-iso-prostaglandin F2-α. This molecule is not only a vasoconstrictor and initiator of necrosis in endothelial cells but also plays a role in mediating immune cell adhesion.3

Another class of proteins called heat shock proteins (HSP) is also highly implicated. HSP-70B has been singled out and is a chaperone for protein metabolism and production.3 Under heavy stress and heightened protein misfolding, levels are elevated.3 Hence, HSP-70B is an oxidative stress biomarker. Furthermore, it supports the production of inflammatory cytokines, further worsening oxidative stress.3

The most viable hypothesis at the moment regarding endometriosis and its pathophysiology is retrograde menstruation. This is built on the idea that highly prooxidant factors are carried from the endometrium into the peritoneal cavity and ovaries.50 These factors include iron and heme, as well as apoptotic endometrial cells.50 Iron
has no direct link to ROS *per se* but may be associated with catalyzing reactions of free radicals.  

**Polycystic ovarian syndrome**

Polycystic ovarian syndrome (PCOS) is a relatively common condition in women of reproductive age. In fact, its prevalence is around 18% in the female population. Polycystic ovarian syndrome is a combination of hyperandrogenism, ovulatory dysfunction, and polycystic ovaries. Clinical symptoms include amenorrhea or menorrhagia and dermatological manifestations such as acne and skin darkening. Furthermore, 90% of sufferers are infertile. The true cause of PCOS is hypothesized to be due to insulin resistance, which is accompanied by hypertension, central fat distribution, and general obesity, the latter two being proportional to oxidized LDL serum concentrations. In fact, metabolic syndrome, sleep apnea, and other diseases are intimately related to obesity and, thus, PCOS.

In conditions that predispose women to PCOS, oxidative stress has been found to be abundant. However, oxidative stress also plays a role in the essence of PCOS, as antioxidant levels are diminished. Furthermore, mitochondrial dysfunction could be present as mitochondrial oxygen consumption decreases along with GSH levels, but ROS production remains elevated. Studies also indicate that oxidative stress alters steroidogenesis in the ovaries, disturbs follicular development, and leads to infertility and increased androgen production. Furthermore, this is an inflammatory state. Inflammation is a process by which the body reacts to an unfavorable environment, which in this case is hyperglycemia. Mononuclear cells and C-reactive protein are increased. Hence, ROS produced by these processes are potentiated.

**Unexplained infertility**

Unexplained infertility is diagnosed via a process of exclusion. In other words, when a couple fails to conceive after 1 year of unprotected sex and all other infertility-related conditions are excluded, unexplained infertility is diagnosed. It affects up to 15% of couples in the United States. Unfortunately, unexplained infertility is a vague term. In fact, the pathophysiology behind it is still unclear. Nonetheless, it has been shown that oxidative stress may play a role in the etiology of unexplained infertility. In patients with unexplained infertility, oxidative stress markers such as MDA are somewhat elevated in the peritoneal cavity. Antioxidant defenses and ROS are in an unbalanced state. Levels of vitamin E and GSH are especially low.

Other causes proposed include genetic polymorphism of folate metabolism. Polymorphism in the methylenetetrahydrofolate reductase (MTHFR) enzyme can be detrimental to folate metabolism. A study by Altmae et al. in 2010 found that genetic polymorphisms in this enzyme led to the accumulation of homocysteine, an inducer of apoptosis, and oxidative stress. Other studies found a low concentration of N-acetyl cysteine (NAC) enzyme, leading to heightened levels of homocysteine.

**Pregnancy complications and oxidative stress**

As explained previously, pregnancy is a state of heightened metabolic activity and requirements. Thus, it is a delicate balance that can be easily disrupted by oxidative
Spontaneous abortions

- **Definition:** An unintentional termination of pregnancy either before 20 weeks of gestation or when fetal weight is below 500 g. This condition is attributed most commonly to chromosomal aberrations that inhibit further viable fetal development and, hence, abortion.

- **Pathology:** Oxidative stress has been associated with spontaneous abortions. At the 10th or 12th week of gestation, an oxidative burst occurs. Antioxidant activity usually keeps this in check. However, in certain women, premature maternal intraplacental circulation develops early in the 8th and 9th week. This development overwhelms the body. Early in the pregnancy, antioxidant development is still premature and, thus, is unable to offset the buildup. Placental development is impaired and syncytiotrophoblast degradation is heightened.

Recurrent pregnancy loss

- **Definition:** Three or more consecutive pregnancy losses. It occurs in 1–3% of women in their childbearing years.

- **Pathology:** Although a cause–effect relationship has not been established, oxidative stress seems to play a role. Women with recurrent pregnancy loss (RPL) exhibit heightened uterine natural killer cells in preimplantation angiogenesis leading to premature maternal intraplacental circulation. Thus, oxidative stress in early pregnancy may lead to pregnancy loss. It should be noted that patients with RPL have also been found to have high plasma lipid peroxides and low levels of carotene and vitamin E. Genetic polymorphisms, especially those of antioxidant genes, may also predispose women to this condition.

Preeclampsia

- **Definition:** A disorder occurs during pregnancy and affects multiple systems in the body. This condition can occur in all women, even those who lack a hypertensive history. It is the leading cause of morbidity and mortality during pregnancy in the mother and child, with a prevalence of 3–14%. Preeclampsia usually occurs after 20 weeks of gestation and is diagnosed after two blood pressure measurements, taken 6 h apart, of over 140/90 mm Hg.

- **Pathology:** Focal vasospasm occurs leading to placental ischemia and hypoxia. With ischemia, ROS is heightened and oxidative stress ensues. This is implied as studies show elevated levels of carbonyls, lipid peroxides, and other oxidative markers, both in placental and maternal serum, and decreased levels of antioxidants such as vitamin E.

Table 16.2 briefly discusses pregnancy complications associated with oxidative stress, which are depicted in Figure 16.5.

### Conclusion and key points

Reactive species are necessary and play important physiological roles in the human body, but are pathological when their levels become too high and lead to a state of oxidative stress. Thus, the body attempts to maintain healthy levels of ROS/RNS with the concomitant production of antioxidants. In the female reproductive system, this balance is highly delicate, and any failure of homeostasis will lead to damaging effects. This includes the aforementioned reproductive diseases and complications. Studies of
multiple choice questions

1 In the female, physiological levels of reactive oxygen species
   a. Regulate oocyte maturation
   b. Are the main inducer of ovulation
   c. Suppresses apoptosis
   d. Plays a role in follicular atresia

2 Oxidative stress plays a role in the pathology of
   a. Polycystic ovarian syndrome
   b. Spontaneous abortion
   c. Menopause
   d. Preeclampsia

3 Factors that may induce oxidative stress in the female include
   a. Regular alcohol ingestion
   b. Abdominal fat deposition
   c. Nicotine inhalation
   d. Cocaine use
References

Role of oxidants and antioxidants in female reproduction


