

Chapter 2

Methods for Detection of ROS in the Female Reproductive System

**Rakesh K. Sharma, Nathan Reynolds, Mitali Rakhit
and Ashok Agarwal**

Abstract The role of reactive oxygen species (ROS) within the female reproductive system is complex and can contribute to multiple gynecological diseases including infertility. This chapter will describe the various methods available to measure both ROS and other markers of oxidative stress in female infertility. Methods including chemiluminescence, flow cytometry, ELISA, metabolomics that utilize various markers of oxidative stress will be discussed. The effects of these markers in various female diseases are also briefly described.

Keywords Methods for detection • Reactive oxygen species (ROS) • Female Reproductive System • Chemiluminescence • Flow Cytometry • ELISA

R. K. Sharma (✉) • M. Rakhit
Center For Reproductive Medicine, Cleveland Clinic, Desk A19.1
9500 Euclid Avenue, Cleveland, OH 44195, USA
e-mail: sharmar@ccf.org

M. Rakhit
e-mail: mitali.rakhit@gmail.com

N. Reynolds
Biological Sciences, Western Michigan University, 1903 W. Michigan Avenue,
Kalamazoo, MI 49009, USA
e-mail: nathan.m.reynolds@wmich.edu

A. Agarwal
Center for Reproductive Medicine, Lerner College of Medicine,
Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA
e-mail: agarwaa@ccf.org

2.1 Introduction

Free radicals are molecules that contain one or more unpaired electrons in their outer shell. They are very unstable and reactive, and attempt to acquire an electron from the surrounding molecule and trigger a chain reaction. Oxygen radicals, such as the superoxide anion (O_2^-), the hydroxyl radical (OH^\cdot), hydrogen peroxide (H_2O_2), hypochlorite radical ($OHCl^\cdot$), and the peroxy radical (ROO^\cdot) comprise the highly reactive group of oxygen species called reactive oxygen species (ROS). Also included in this group are the reactive nitrogen radicals, such as nitric oxide (NO^\cdot) and nitric dioxide (NO_2^\cdot), peroxyxynitrite anion, and nitroxyl ion [1–5].

ROS are involved in various signal transduction pathways and act as second messengers. Physiological amounts of ROS are necessary for healthy body function. A delicate balance exists between ROS and antioxidants throughout the female reproductive system. Antioxidants are able to break the cycle by donating an electron to the free radical and stabilizing it without destabilizing itself. When this balance is disrupted due to excessive production of ROS or the inability of the antioxidants to neutralize ROS, it results in oxidative stress (OS). OS can damage fats, lipids, nucleic acids, and proteins which are the fundamental building blocks of life. OS is involved in the pathophysiology of a number of diseases of the female reproductive system such as endometriosis, polycystic ovary syndrome, preeclampsia maternal diabetes, and recurrent pregnancy loss [2, 6–12].

2.2 Measurement of Reactive Oxygen Species and Oxidative Stress Markers

A number of OS biomarkers have been investigated in various fluids such as peritoneal fluid, follicular fluid, amniotic fluid, and hydrosalpingeal fluid. ROS is the initial marker and a number of other markers are available to measure the end product of ROS-induced damage such as lipid peroxidation, oxidation of proteins, and DNA damage. Some of the more common methods are described below.

2.3 Measurement of ROS by Chemiluminescence Assay

In this assay, a probe such as luminol (5-amino-2,3 dihydro-1,4 phthalazinedione) is added to the target fluid that reacts with any ROS present in the biofluids. The byproduct of this reaction is the production of photons of light [3, 12]. Luminol reacts with free radicals within the fluid including O_2^- , OH^\cdot and hydrogen peroxide. Luminol reacts quickly with both intracellular and intercellular free radicals. However, it does not differentiate between the types of free radicals and therefore measures global ROS. The results are expressed in relative light units (RLU), counted photons per minute (cpm), or milliVolts/sec [12].

Herein, we describe the details of measurement of ROS by chemiluminescence assay in unprocessed or neat sample i.e. without any further processing of the sample.

1. Equipment and material

- (a) Disposable polystyrene tubes with caps (12 × 75 mm)
- (b) Eppendorf pipettes (5, 10, 50 and 1000 µL)
- (c) Serological Pipettes (1, 2 mL)
- (d) Desktop centrifuge
- (e) Dimethyl Sulfoxide (DMSO; Catalog # D8779, Sigma Chemical Co., St. Louis, MO)
- (f) Luminol (5-amino-2,3 dihydro-1,4 phthalazinedione; Catalog # A8511, Sigma Chemical Co., St. Louis, MO)
- (g) Dulbecco's Phosphate buffered saline solution 1X (PBS-1X; Catalog # 9235, Irvine Scientific, Santa Ana, CA)
- (h) Luminometer (Model: Berthold Technologies, Autolumat plus LB 953, Oakridge, TN)

2. Preparation of reagents

- (a) *Stock Luminol (100 mM)* Weigh 177.09 mg of luminol and add it to 10 mL of DMSO solution in a polystyrene tube. The tube must be covered in an aluminum foil due to the sensitivity of the luminol to light. It can be stored at room temperature in the dark.
- (b) *Working Luminol (5 mM)* Mix 20 µL luminol stock solution with 380 µL DMSO in a foil-covered polystyrene tube. This must be done prior to every use. Store the solution at room temperature in the dark.
- (c) *DMSO solution* Provided ready to use; store at room temperature until the expiration date.

3. Specimen preparation

Upon arrival of the specimen, complete record of patient's name, allocated identity number, date, and time of collection is noted.

4. ROS measurement by luminometer

The luminometer is attached to the desktop computer and a monitor. The software used is Tube master (Berthold Technologies, Autolumat plus LB 953, Oakridge, TN). This procedure is performed in a dark room.

1. Label 11 tubes (12 × 75 mm) in duplicates and add the following reagents as indicated in Table 2.1. Blank (tubes labeled 1–3), Negative Control (tubes labeled 4–6), Patient Sample (tubes labeled 7–8), and Positive Control (tubes labeled 9–11).

Note: To avoid contamination, change pipette tips after each addition.

2. Gently vortex the tubes to mix the aliquots uniformly.

Table 2.1 Set up for the measurement of ROS

No.	Labeled tubes (no.)	PBS-1 X (μL)	Specimen volume (μL)	Probe luminol (5 mM) (μL)	Hydrogen peroxide (μL)
1	Blank (tubes 1–3)	400	–		–
2	Negative control (tubes 4–6)	400	–	10	–
3	Patient (tubes 7–8)	–	400	10	–
4	Positive Control (tubes 9–11)	400		10	50

- Place all the labeled tubes in the luminometer in the following order: Blank (tubes labeled 1–3), Negative Control (tubes labeled 4–6), Patient Sample (tubes labeled 7–8), and Positive Control (tubes labeled 9–11).

Instrument set up

- Turn on the instrument and the computer. From the desktop, click on ‘Berthold tube’ master icon to start the program.
- From the ‘Setup menu’ select ‘Measurement Definition’ and then ‘New Measurement’. You will be prompted to the following:
 - ‘Measurement Name’ (Initials, Date, Analyte, and Measurement).
 - It will show ‘Measurement Definition’ on the ‘Tool bar’
 - Click ‘Luminometer Measurement’ protocol and from the drop menu click on ‘Rep. assay’.
 - Next define each ‘Parameters’ as follows:
 - Read time 1 s
 - Background read time 0 s.
 - Total time 900 s.
 - Cycle time 30 s
 - Delay ‘Inj M read (s)’ 0 s.
 - ‘Injector M (μL)’ 0 s
 - ‘Temperature ($^{\circ}\text{C}$)’ 37 $^{\circ}\text{C}$
 - ‘Temperature control (0 = OFF) 1 = ON’
 - Press ‘Save’

Note: steps ‘v and vi’ are used for a large number of samples and the reagents can be added by means of injectors.

- From the ‘Setup’ menu select ‘Assay Definition’ and then ‘New Assay’: It will ask for the following:
 - ‘Assay Name’ (Initials, Date, Analyte, Assay). Click ‘OK’
 - Select ‘Measurement Method’ and from the drop down menu select the measurement from Step 2a above.
 - Go to ‘Column Menu’ Hide everything except the following:

- i. Sample ID
 - ii. Status
 - iii. RLU mean
 - iv. Read date
 - v. Read time
 - d. Go to 'Sample Type' menu and select 'Normal'
 - e. Press 'OK'
 - f. Go to file, 'New' click 'Workload' Press 'OK.'
4. Save the 'Work Load' (Date, Initial, Sample or experiment ID) in 'Work Load' file.
 5. Click 'File name'
 6. After saving the 'Work Load' the name of the file will show in the 'Title Bar'
 7. The specimens are ready to be analyzed

VIII. Analyzing the samples

1. Load the tubes into the instrument and click 'Start'. It will start scanning for tubes.
2. After scanning it will show how many tubes are detected by the instrument in each batch, press 'Next'.
3. Select the 'Assay Type' and type file name and then click 'Finish'.
4. The 'Excel spreadsheet' will open measurement of the tubes will start.
5. Do not touch or change the screen, wait (3–5 min) to make sure everything is working fine.
6. After finishing measurements, it will ask for 'Save Excel Spread Sheet', save it in the 'My Document' under 'ExcelSheet' folder.
7. Select 'Excel Sheet' name the file and save type as 'Measurement Files' (*.txr). Save the 'Excel Sheet'.

IX. Printing ROS results

1. Print Excel as well as the 'chart'.
2. Close the 'Excel sheet'.
3. Print the 'Work Load' sheet, save, and close it.

X. Calculating Results and Quality Control

1. Calculate the 'average RLU' for Negative control, Samples, and Positive control.
2. Calculate sample ROS by subtracting its average from negative control average.
3. Sample ROS = Average 'RLU mean' for sample—Average 'RLU mean' for negative control.

Calculated sample ROS = XX (RLU/sec)

Reference values are established for each lab by testing a number of samples both from healthy women and infertile subjects. It is important to perform a regular quality control of the instrument as well as the reagents.

2.4 Factors Affecting Chemiluminescent Reaction

Having a reliable method of detection of ROS is important [13]. The luminol assay is robust; however, there are various factors that can affect ROS detection by the chemiluminescent reactions [3, 12, 14, 15]. Some of these confounding factors are:

1. The luminometer instrumentation, its calibration, determination of sensitivity, dynamic range, and units used.
2. The volume of the sample, use of reagent, and temperature of the luminometer.
3. Repeated centrifugation: Artificial increase in chemiluminescent signal because of the shearing forces generated by centrifugation [16].
4. Medium pH: Luminol is sensitive to pH changes.

2.5 Types of Luminometers

Several types of luminometers, ranging in features, design, and pricing, can be used in measuring the emitted light from the chemiluminescence assay reaction [17]. There are two different kinds of processing designs for luminometers. While direct current luminometers measure electric current, photon counting luminometers count individual photons [12]. Currently, there are three types of luminometers available for commercial uses. Single and double tube luminometers, which measure one or two samples at a time, are inexpensive and are typically used by small research laboratories. Multiple tube luminometers measure several tubes at a time, and as they are more expensive than single and double tube luminometers. Lastly, plate luminometers measure multiple samples (96 or 360) at a given time, and are typically used by commercial enterprises.

2.6 Measurement of ROS by Other Methods

2.6.1 Measurement of ROS by Nitroblue tetrazolium staining

Nitroblue tetrazolium (NBT) is an electron acceptor that becomes reduced in the presence of ROS to form a blue-black compound, formazan. This simple histochemical staining method can help target cells generating ROS. Cells generating ROS are prepared at a concentration of $1-5 \times 10^6/\text{mL}$ in Kreb's buffer and about 10 μL is loaded in a glass slide and placed in an incubator for 20 min at 37 °C. It is gently rinsed with 0.154 M NaCl, and the adherent cells are over-layered with an equal volume of NBT (0.4 %) and phorbol 12-myristate 13-acetate (0.20–1 $\mu\text{g}/\text{mL}$) in Kreb's buffer with glucose (5 mM). After 15 min incubation, the slides are washed with 0.154 M NaCl, fixed for 1 min in absolute methanol, and counter stained with 1 or 2 % Safranin. Total 100 consecutive cells on each slide are observed microscopically under oil

($\times 100$) and scored as: cells filled with formazan granules (+++); intermediate formazan density (++); scattered or few formazan granules (+) or no formazan detectable (–). The data are represented as percentage of NBT-positive cells.

2.7 Epifluorescence Microscopy

This technique detects the presence of ROS using a fluorescent end product of an oxidation reaction. The product is generated by the reaction of hydroethidine and a O_2^- , which yields ethidium bromide. This end product emits a red fluorescent light that can be seen through an epifluorescence microscope. Because this technique requires a less expensive piece of equipment, it is more common and easily available to many labs for ROS detection.

2.8 Measurement of ROS by Flow Cytometry

This method utilizes the use of fluorescent probes to detect ROS within the cells [18]. The single cells are required to be suspended at a density of 10^5 – 10^7 cells/mL and 10,000 events are measured. Individual intracellular ROS radicals can be detected by flow cytometry. Oxidation of 2, 7 dichlorofluorescein diacetate (DCFH-DA) by ROS, which is generated within the cell, makes them highly fluorescent and can be used to measure formation of intracellular levels of hydrogen peroxide. Hydroethidine (HE) can be used for measurement of intracellular levels of superoxide. It is a substance that is oxidized by the O_2^- to become ethidium bromide with red fluorescence emission. Flow cytometry method has a higher specificity, accuracy, sensitivity, and reproducibility as compared to chemiluminescence for intracellular ROS [18].

2.9 Measurement of Enzymatic Antioxidants

Enzymatic activities can be measured using commercially available assay kits (Cayman Chemical, Ann Arbor, MI) following the methodology described by the manufacturer. Superoxide dismutase (SOD) activity is measured using a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The chromophore produced has a maximal absorbance at 525 nm. One unit of SOD is defined as the amount of enzyme needed to exhibit 50 % dismutation of the superoxide radical. Glutathione peroxidase (GPx) activity is measured using a kinetic colorimetric assay that measures activity indirectly by a coupled reaction with glutathione reductase. Glutathione reductase and NADPH reduce oxidized glutathione. NADPH oxidation is accompanied by a decrease in

absorbance at 340 nm, and the decrease is directly proportional to the GPx activity in the sample. Similarly, Catalase (CAT) is involved in the detoxification of H_2O_2 , which is a toxic product of both normal aerobic metabolism and pathogenic ROS production. This enzyme catalyzes the conversion of two molecules of H_2O_2 to molecular oxygen and two molecules of water (catalytic activity). CAT assay kit utilizes the peroxidatic function of CAT for determination of enzyme activity. This method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2 . The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple color [19, 20].

2.10 Measurement of Total Antioxidants

The sum of endogenous and food-derived antioxidants represents the total antioxidant activity of the extracellular fluid. Measuring the overall antioxidant capacity may give more relevant biological information compared to that obtained by the measurement of individual components, as it considers the cumulative effect of all antioxidants present in plasma and body fluids. The total antioxidant assay relies on the ability of antioxidants in the sample to inhibit the oxidation of 2,2'-azino-di-3-ethylbenzthiazoline sulphonate (ABTS) to ABTS^+ by metmyoglobin. Under the reaction conditions used, the antioxidants in the seminal plasma cause suppression of the absorbance at 750 nm to a degree which is proportional to their concentration. The capacity of the antioxidants present in the sample to prevent ABTS oxidation is compared with that of standard—Trolox, a water-soluble tocopherol analog. Results are reported as micromoles of Trolox equivalent. This assay measures the combined antioxidant activities of all its constituents including vitamins, proteins, lipids, glutathione and uric acid.

2.10.1 Measurement of Oxidative DNA Adducts

Immunohistochemistry (IHC) or western blot analysis has been used to study oxidative DNA adducts 8-hydroxy 2-deoxyguanosine [21]. IHC staining can be performed on paraffin sections ($5\ \mu$) using mouse monoclonal antibodies specific against 8-hydroxy-2'-deoxyguanosine clone N45.1 using indirect methods. Briefly, after sections are deparaffinized and rehydrated, tissue sections are incubated for 120 min at $37\ ^\circ\text{C}$ with the primary antibody at 1:50 dilution. Slides are then incubated with a secondary antibody using horse anti-mouse dilutions (1:200) for 30 min at $37\ ^\circ\text{C}$. Finally, sections are stained with diaminobenzidine (DAB).

2.10.2 Measurement of Nitric Oxide

Metabolites of NO such as nitrite and nitrate can be determined by nitrate reductase and the Griess reaction [22, 23]. Total NO (nitrite and nitrate) levels in the serum and NO can also be measured using a rapid response chemiluminescence assay [24].

2.10.3 Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) is based on the ability for antigens to detect proteins that have been damaged by OS. This method can detect the presence of a specific antibody or antigen within a fluid sample making it a useful tool to detect the end products of ROS interactions. This is accomplished by exposing the biofluids to the desired assay and analyzing the fluid for the amount of the targeted molecules using a spectrophotometer [12]. Utilizing appropriate antibodies, activities of various antioxidant enzymes such as total superoxide dismutase, CAT and GPx can be measured within the tissue in order to evaluate the OS [12].

2.10.4 Measurement of Lipid Peroxides

Thiobarbituric acid-reacting substances (TBARS) measure primarily malondialdehyde derived from lipid peroxidation, as well as other breakdown products from oxidatively modified proteins, carbohydrates, and nucleic acids [25]. Various commercially available kits can be used. In this assay, formation of malondialdehyde is estimated by the thiobarbituric acid method. About 1 mL of each sample is mixed with 2 mL of trichloroacetic acid (15 %), thiobarbituric acid (0.375 %) and hydrochloric acid (0.25 N) and then heated in a boiling water bath for 15 min. After cooling, the precipitate is centrifuged at 1,000 g for 10 min. The absorbance of the supernatant is measured at a 535 nm wavelength by using a spectrophotometer. The concentration of thiobarbituric acid-reactive substances is determined by considering the coefficient of molar absorptivity of the product. A standard curve is constructed by using a stock solution of 10 mM MDA prepared from tetramethoxypropane (Sigma Chemical Co., St. Louis, MO). The assay is performed in duplicate, and the results are reported as nmol MDA [26]. Sometimes a lipid peroxidation promoter such as ferrous sulfate (2.5 mM) and sodium ascorbate (12.5 mM) is also used [27, 28].

2.10.5 Measurement of Protein Oxidation

The most commonly used marker of protein oxidation is protein carbonyl content [29]. Redox cycling cations such as Fe^{2+} or Cu^{2+} can bind to cation binding locations on proteins and with the aid of further attack by H_2O_2 or O_2 can transform side-chain

amine groups on several amino acids (i.e., lysine, arginine, proline, or histidine) into carbonyls. The most convenient procedure is the reaction between 2, 4-dinitrophenylhydrazine (DNPH) and protein carbonyls. DNPH reacts with protein carbonyls, forming a Schiff base to produce the corresponding hydrazone, which can be analyzed spectrophotometrically at 360–385 nm [30]. This assay is available as a kit (Catalog No. 10005020, Cayman Chemical, Ann Arbor, MI). Protein carbonyl content can be measured both in fluids and tissue and is expressed as protein carbonyl content (nmol/ml) or as protein carbonyl content (ng/mg of protein).

2.10.6 Total Plasma Lipid Hydroperoxides

Total plasma lipid hydroperoxides (LHP) are determined using the ferrous oxidation in Xylenol Orange (FOX) assay [31]. The method is based on the principle of the rapid peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} under acidic conditions. The latter, in the presence of xylene orange, forms a Fe^{3+} -xylenol orange complex that can be measured spectrophotometrically at 560 nm.

2.10.7 Measurement of Total (Free and Esterified) 8-F2-Isoprostane

8-F2-isoprostane measures stable end product of oxidized lipids derived from arachidonic acid [32–34]. Total (free and esterified) 8-F2-isoprostane can be measured using a commercial kit (cat. No. 516351; Cayman Chemical, Ann Arbor, MI). Samples (250 μL) are treated with potassium hydroxide and extracted with ethanol and purified through an affinity column (Catalog No. 416358, Cayman Chemical, Ann Arbor, MI) eluted and analyzed by enzyme immunoassay technology and concentration is expressed in pg/mL.

2.10.8 Measurement of Fat-Soluble Antioxidants

Fat-soluble antioxidants (vitamin A, vitamin E, beta-carotene, and lycopene) confer micronutrient antioxidant protection. These can be measured by HPLC. Samples (300 μL) are mixed with equal volumes of ethyl alcohol containing an internal standard (a-tocopherol acetate) and extracted twice with 2 mL of hexane. The upper organic phase is removed, evaporated to dryness under nitrogen, reconstituted in 300 μL of mobile phase containing 60 % acetonitrile–25 % methanol and 15 % ethylene chloride, and sonicated. Sixty μL is next injected onto a Supelco C18 column with Supelco guard precolumn and the vitamins separated [35]. Absorbance data are obtained from a photodiode array spectrometer set to simultaneously record

at 292 nm for α -tocopherol, 326 nm for retinol and 452 nm for carotenoids and then quantified and concentration is expressed in mg/mL.

2.10.9 Measurement of Oxidative Stress by Metabolomics

The study of metabolomics involves quantifying the composition of small molecules within a tissue or biofluid sample to identify the metabolites present within a cellular system and a metabolite profile is generated [36, 37]. Multiple methods of metabolically analyzing fluids have been developed. Morphological indicators alone are not efficient in determining the viability of an embryo. Determining a metabolic profile of embryonic culture media for IVF embryos can be used to assess the metabolic differences between those that implant and those that do not implant [36–39]. Metabolomic analysis can accurately determine if an embryo is likely to be viable. The presence of these metabolites can be indicative of not only the viability of embryos, as is the case when testing embryonic culture media, but also can be used as a diagnostic tool when testing other fluids within the female reproductive system.

Depending on the type of fluid being analyzed, some methods are preferred over others. Some of the platforms for metabolic analysis are as follows: nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography–mass spectrometry (LC–MS) capillary electrophoresis–mass spectrometry (CE–MS), and gas chromatography–mass spectrometry (GC–MS) [36]. NMR technique is useful when analyzing tissue extracts or fluids and requires no fluid separation, but it has a low sensitivity. LC–MS is useful when analyzing thermolabile and polar compounds while CE–MS is useful when analyzing volatile and nonvolatile compounds [36]. CE–MS is most useful when the concentration or the volume of the fluid/tissue is very small.

2.11 Near-Infrared Spectroscopy

Biological fluids can also be subjected to near infrared (IR) spectroscopy. This procedure analyzes the sample with a reflective spectrograph that has a large dynamic range photodiode detector. Results are generally in the range of 700–1,050 nm [37]. The sample is subjected to IR signaling and the machine records the sensitivity of each molecule. The results of IR spectroscopy can show the presence of—thiol or sulfhydryl groups, (–SH), hydroxyl groups (–OH), carbogens (–CH), and amines (–NH) groups based on their corresponding vibrational sensitivities that are indicative of OS [38]. Using the NMR method, Seli et al. [38] observed that cultures with higher concentrations of glutamate and lower levels of pyruvate had embryos that were significantly more likely to result in pregnancy. Glutamate is important in protecting the embryo from damage associated with exposure to concentrations of ammonium [39].

2.12 Raman Analysis

Raman analysis allows to accurately ‘fingerprint’ biological species within a fluid based on the vibration and molecular motion of biological/chemical species when subjected to spectroscopy [40]. The composition of the material can be determined using Raman analysis. This analysis records the cells sensitivity to the exposure of certain wavelengths and records the results from 50 to $3,450\text{ cm}^{-1}$ [38]. Although this method conducts signals at lower intensities compared to infra red spectroscopy. Raman analysis is useful to detect the presence of fatty acids, lactate, and glucose in addition to the presence of sulfhydryls [38].

Utilizing Raman and near-IR spectroscopy method, an increase in $-\text{SH}$ and a simultaneous decrease in $-\text{CH}$, and $-\text{NH}$ was seen. All these groups are associated with OS [38].

2.13 Proton Nuclear Magnetic Resonance

The broad application of Proton NMR in gynecology and obstetrics allows the in vivo detection of ROS in the tissue [39, 41]. This method requires a tissue or fluid sample to be analyzed with a spectrometer in which the reabsorbance of the magnetic energy is used to determine the molecules present. Typically, the values are in a range of 0–10 ppm.

2.14 Oxidative Stress in Reproductive Diseases

OS either due to the production of excessive ROS or due to the limited ability of antioxidants to scavenge these ROS results in oxidative stress. OS affects a variety of body fluids, tissues, organs; as well, a host of female infertility diseases are attributed to the production of pathological ROS levels. Here we briefly describe the more important effects of ROS and/or its end products on various body fluids, tissues, and certain diseases attributed to OS [7, 8, 11, 42].

2.15 Ovaries

2.15.1 Oocytes and Follicles

OS is involved in the physiological aspects of ovarian function [43, 44]. Various studies have confirmed the role of ROS in follicular maturation, folliculogenesis, function of the corpus luteum, as well as ovulation. Parenchymal steroidogenic

cells, endothelial cells, and phagocytic macrophages are among the producers of ROS within the ovaries [7, 8, 45]. In addition, normal ovaries often express many of the common biomarkers of OS [46]. Markers of OS such as Cu-SOD, Zn-SOD, and Mn-SOD, GPx γ glutamyl synthetase, and lipid peroxides have been measured by immunohistochemical staining, mRNA expression, and thiobarbituric acid methods [44, 47]. A decrease was reported in the antioxidant levels, specifically GPx, and these had a negative impact on the fertilization rates of gametes [48]. Another study reported that GPx and Mn-SOD can actually be used as markers to detect oocyte maturation, thereby implying exposure to OS [49]. NO also contributes to ovarian function. High levels of NO have adverse effect on the rate of cleavage, overall embryo quality, and implantation rates. Physiological levels of NO in follicular fluid were reported to be beneficial and these correlated with follicles containing mature oocytes that eventually fertilized [50]. A study by Bedaiwy et al. [51] found that patients presenting with peritoneal factor infertility also had elevated levels of serum NO.

2.15.2 Follicular Fluid and ART Outcomes

The environment of the follicular fluid is thought to play a critical role in oocyte maturation and the eventual development of an embryo [6, 52–54]. The follicular fluid is known to be metabolically active and contains steroid hormones, growth factors, cytokines, granulosa cells, and leukocytes [6, 28, 55]. Additionally, there are many antioxidants found in follicular fluid, including vitamin E, carotene, ascorbate, cysteamine, taurine, hypotaurine, transferrin, thioredoxin, and dithiothreitol, which promote healthy oocyte maturation, and oocyte viability, however, the results are conflicting [48, 54–57]. Increased ROS levels have been associated with poor oocyte quality, low fertilization rate, and impaired embryo development [6, 58, 59]. ROS levels of the follicular fluid in women who had undergone a successful IVF treatment were significantly higher than the ROS levels of the fluid in women who did not have success in their IVF treatment [59–61]. This indicates that while an imbalance of prooxidants and antioxidants can cause a disturbance in natural female reproductive tendencies, ROS within the follicular fluid is essential for different phases of oocyte development and maturation, although the exact function remains unknown [28, 53, 54, 62, 63].

Current knowledge of the origin of ROS in follicular fluid remains unclear. It is speculated that ROS may be generated from the metabolic environment surrounding the embryo and that oxidative phosphorylation, NADPH oxidase and xanthine oxidase are sources of ROS from within the oocyte [28]. Steroid hormones, growth factors, cytokines granulosa cells, and leukocytes are present components of this environment that are known to increase ROS production [6, 60]. It is speculated that the intra-follicular microenvironment and the condition therein have an important role in the development of the oocyte. Reports suggest that a decrease in ROS within the follicular fluid and an increase in total antioxidant capacity—will increase the viability of the embryo prior to

implantation [53, 54, 58, 61]. Furthermore, detecting ROS within the follicular fluid could help predict the chances of pregnancy or explain some of the underlying causes of some female reproductive diseases.

2.16 Amniotic/Placental Fluid and ART Outcomes

During pregnancy, the mother and fetus can be exposed to high levels of OS [64]. It is important to analyze the composition of the amniotic fluid to determine the effect of ROS in fetal development. Amniotic fluid reaches its highest volume in the second and third trimester of gestation. It is composed mostly of fetal urine but also of fetal lung secretions [65]. While the exact source of OS during pregnancy is not well understood, it is possible that the increasing volume of amniotic fluid as the gestation period progresses plays a role in its production. However, additional sources are speculated to contribute to the increased total antioxidant capacity (TAC) noticed in amniotic fluid. These sources are the intramembranous exchange of nutrients between the chorionic plate and amniotic-chorionic membranes, as well as the transmembranous exchange of nutrients between the amniotic-chorionic membranes and the uterine wall [65]. Measuring ROS in amniotic fluid may help study the common conditions of OS such as fetal growth restriction and preeclampsia that result in increased levels of ROS possibly caused by the increased volume of amniotic fluid.

2.17 ROS and IVF Culture Media

Generally, when a couple undergoes IVF, the embryos chosen for implanting are selected by morphological criteria. The current morphological assessment examines the rate of cleavage, fragmentation, inclusion bodies, and the allocation of inner cell mass [36]. It is important to test the culture media because eight of ten transferred embryos will not result in a successful implantation and two of three ART cycles will not result in pregnancy suggesting that morphological criteria alone do not provide enough information when determining the viability of an embryo [38].

Metabolomic analysis provides a method to assess and search for products within the culture media, including metabolites resulting from OS, which may help signal the fertilization potential of the embryos examined [38]. The in vitro culture environment contains a higher concentration of oxygen as opposed to the conditions of in vivo embryos [53]. Assessing the concentration of pyruvate within the culture media can be a helpful in determining viability and the presence of ROS. Pyruvate is thought to be important because of its metabolic significance and implications during the Krebs's cycle in addition to balancing the oxidation and reduction interactions [66].

2.18 Fallopian Tubes

2.18.1 Tubal Factors

The fallopian tubes contain an internal NO system. NO is a vasodilator and acts to improve tubal contractions and motility. A decrease in the production of NO can hamper tubal motility and subsequently cause sperm transport delay, ovum retention, as well as general infertility problems [4, 8]. A significant increase in the levels of NO within the fallopian tubes can be toxic to any invading microbes, including human spermatozoa [8, 67]. The presence of NO synthase has been reported in human tubal cells.

2.18.2 Hydrosalpinx

Hydrosalpinx is defined as a blocked, dilated, and fluid filled fallopian tube that has usually also been affected by a previous tubal infection. This disease is commonly associated with female factor infertility as it is correlated with lower pregnancy and implantation rates as well as an increased rate of miscarriages. OS has often been associated as an underlying factor causing characteristic embryotoxicity in hydrosalpingeal fluid.

Low levels of ROS are beneficial for blastocyst development and may denote the physiological amounts that are present in a normal endosalpinx, high levels are pathological, and result in deleterious effects on the embryo [6, 67, 68]. However, a complete lack of detection of ROS in hydrosalpingeal fluid has a higher correlation with endosalpinges that have undergone a greater amount of damage and deterioration. Embryotoxicity normally associated with the presence of hydrosalpingeal fluid is not necessarily caused by an excess of ROS. ROS levels detected in hydrosalpingeal fluid may originate as byproducts from other natural body processes such as cellular respiration. Higher levels of ROS may also be generated by the inflammatory response resulting from chronic salpingitis [69].

Few studies have been conducted on human subjects with regard to hydrosalpingeal fluid and the ART outcomes. In a study using mouse as a model, Bedaiwy et al. [68] reported that exposure to hydrosalpingeal fluid containing higher concentrations of ROS resulted blastocyst with an increased rate of development. This correlation suggests that in healthier subjects, the granulosa cells will have an increased metabolic activity, which will produce more ROS. However, these authors also noted that the level of ROS detected was most likely not high enough to be considered detrimental to the developing embryo [68].

The removal of the damaged tube via salpingectomy was also shown to improve the implantation rates of the embryo [70]. This suggests that a healthy salpingeal tubal environment correlates with healthy implantation rates. There have been few human studies, which have analyzed the affect of ROS on ART outcomes. The adverse effects of hydrosalpinges have been shown to be reversible by salpingectomy prior to IVF [68].

2.18.3 Amniotic Sac/Placenta

The placenta is an organ that connects the developing fetus to the uterine wall of its mother in order to facilitate nutrient uptake, excretion, and gas exchange. Sufficient uteroplacental circulation is necessary in order to ensure a healthy pregnancy. Early in the pregnancy, the ends of the spiral arteries are blocked by endovascular trophoblast cells, however between weeks 10 and 12, presence of trophoblasts opens up the arteries allowing circulation thereby significantly increasing the oxygen tension. At this point, there is surge in placental OS. However, when the trophoblasts cells are prematurely dislodged from the arteries due to thinning, fragmentation, or reduced endovascular invasion, it results in OS that has been linked to spontaneous abortions and/or recurrent pregnancy loss [71]. The biomarkers of OS in the placenta include: LHP, intracellular ROS, TAC, and DNA adducts-8-hydroxy 2-deoxyguanosine [72].

2.18.4 Reactive Oxygen Species and Endometrium

ROS appear to play a regulatory role in the endometrial cycle [9]. Various studies have suggested a link among OS and the propagation of the proliferative, secretory, and menstrual phases of the monthly cycle in the endometrium. NO regulates endometrial microvasculature and significantly increased concentrations of NO have been linked with implantation failure and subsequent decrease in pregnancy rates [73]. NO synthase is secreted by the endothelial cells on the surface of the endometrium and it helps to prepare for implantation by an embryo. Inducible NOS and endothelial NOS expression have been demonstrated in human endometrium and endometrial vessels [5, 74]. An increase in endothelial NO synthase production and ROS end products has also been reported, specifically in lipid peroxidation concentrations towards the end of the secretory cycle, just prior to the onset of menstruation. Also a decrease in SOD levels was reported during the same stage of this cycle suggesting that OS contributes to the breakdown and subsequent shedding of the endometrial lining. Reduced expression of SOD leads to failed pregnancy [47, 75]. Studies have also suggested that ROS can activate nuclear factor kappa B that promotes the production of prostaglandin F₂ alpha and COX-2 mRNA, which further promotes the shedding of the endometrium [71, 76–78].

2.18.5 Reactive Oxygen Species and Peritoneal Fluid

Peritoneal fluid is located in the peritoneal cavity and lubricates the pelvic cavity, uterus, ovaries and fallopian tubes, and contains hormones secreted from the ovarian follicles and corpus luteum [6, 79]. Leukocytes (macrophages) within the

peritoneal tissue secrete cytokines, such as interleukins (IL) and tumor necrosis factor (TNF)- α , in a healthy peritoneal environment [80]. However, the macrophages are activated in response to inflammatory diseases, such as endometriosis, and secrete increased amounts of the cytokines [81]. Elevated levels of macrophages induce OS within the peritoneal cavity, thus altering the delicate environment of the peritoneal fluid and promoting infertility [32]. While the exact mechanism of the idiopathic infertility remains unclear, it is thought that there is a correlation between elevated numbers of macrophages present in the peritoneal fluid and infertility [82].

OS and antioxidant biomarkers are present in both serum and peritoneal fluid [83]. In peritoneal fluid, OS is initiated in inflammatory cells and the cellular debris serves as a substrate. In the serum/plasma, the oxidized metabolites such as ox-LDL are incorporated into carriers and they modify lipids, proteins, and carbohydrates in the peripheral circulation.

2.18.6 Reactive Oxygen Species and Endometriosis

Endometriosis is associated with chronic inflammation, and ROS are proinflammatory mediators that modulate cell proliferation [84, 85]. Development of OS in the local peritoneal environment may be one of the links in the chain of events leading to endometriosis-associated infertility [86]. Elevated ROS are produced from erythrocytes and apoptotic endometrioma cells, as well as the activated polymorphonuclear leukocytes and macrophages that are recruited to phagocytize the apoptotic cells [2, 87].

Markers of OS have been found to be elevated both in serum and peritoneal fluid of patients with endometriosis, [88–90].

2.18.7 Levels of Antioxidants

Peritoneal fluid is more susceptible to OS than serum. Markers in peritoneal fluid provide a more localized measure of OS related to endometriosis. Significantly, lower levels of vitamin E were reported in the peritoneal fluid than in plasma. Murphy et al. [83], suggesting that the peritoneal cavity has less antioxidant protection than serum. High amounts of enzymatic antioxidants such as GPx, SOD, Cu, Zn, and Mn-SOD and xanthine oxidase have been reported in women with endometriosis, suggesting ROS generation, and OS activity. [32, 74, 90].

2.18.8 Lipid Peroxides

Women with endometriosis had significantly higher levels of lipid peroxides such as malondialdehyde-modified low-density lipoprotein, and oxidized low-density lipoprotein, 8-F2-isoprostane, paraoxonase activity, 8-hydroxy 1-deoxyguanosine, 8-hydroxy-2-nonenal, and 4-hydroxy-2-nonenal than women without endometriosis [32, 89, 91].

2.18.9 Nitric Oxides

Increased levels of NO and NOS have been reported in the endometrium and in the peritoneal fluid of patients with endometriosis [22, 23, 92].

2.18.10 ROS and Sperm in Peritoneal Fluid

A link has been suggested between elevated ROS levels and the toxicity of the peritoneal fluid to the sperm, although evidence suggests that this is an unlikely cause for women with mild endometriosis [93]. Another hypothesis as to why elevated ROS within the peritoneal fluid impacts fertility is that women with endometriosis are more likely to have elevated levels of proinflammatory cytokines (macrophages) within their peritoneal fluid which could be a contributing factor to their infertility, however, the pathway remains unclear [93]. It is hypothesized that peritoneal fluid diffuses into the fallopian tubes where it may cause damage to sperm [91, 94, 95]. Higher concentrations of ROS were reported in the peritoneal fluid of women with idiopathic infertility compared with fertile controls but were not different from women with endometriosis [81]. However, the sample size was small in this study. Although there have been many reports in support of the hypothesis that elevated ROS levels contribute to idiopathic infertility and infertility related to endometriosis, there have also been some studies suggest that there is no correlation between the two pointing out the continuing controversy in the medical literature [52, 96, 97].

2.18.11 ROS and Immune System

An increase in the production of ROS in endometriosis is attributed to the activation of the immune system [98, 99]. ROS may play a role in the regulation of the expression of genes encoding some immunoregulators, cytokines, and cell adhesion molecules which are involved in the pathogenesis of endometriosis [100, 101]. Women with endometriosis exhibit an increased titer of autoantibodies

related to OS that result in an increase in serum autoantibody titers to oxidatively modified low-density lipoproteins [83, 89].

It is not clear whether endometriosis associated OS is due to a lack in antioxidants or an increase in the production of ROS. However, there appears to be an increased amount of enzymatic antioxidant expression by the endometrial cells of women developing endometriosis. Even so, the amount of ROS present in these patients seems to outnumber the defense mechanisms available to prevent them from causing damage [101].

2.18.12 Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) affects 5–10 % of the reproductive aged female population [11]. Women with PCOS may also suffer from metabolic syndrome. Hyperinsulinemia is a characteristic of this disorder and increased presence of insulin circulating in the blood stream as well as TNF- α induced OS on theca cell proliferation [102]. While the exact role of ROS in folliculogenesis remains unclear, it is speculated that the origin of ROS is from the mononuclear cells of hyperglycemic patients [103, 104]. Folliculogenesis is a complex process in which ovarian follicles and the oocyte within develop to maturity. In normal women, the process of folliculogenesis involves many complex endocrine and intra ovarian paracrine reactions, which sustain a suitable intra follicular environment for the developing oocyte [105]. On a molecular level, the women experience androgen and LH hypersecretion, frequent insulin resistance because of hyperinsulinemia, and polycystic ovaries. OS may cause further proliferation of ovarian mesenchymal cells in patients with polycystic ovarian syndrome [105]. Because ROS are known to damage the lipids within cell membranes by lipid peroxidation, they can permanently damage the follicle and oocyte. For this reason, ROS are considered an integral factor in the etiology of PCOS [12]. Higher DNA damage (strand breaks) is seen in PCOS subjects compared to controls [106]. The susceptibility of DNA to OS in these patients may help explain the link between PCOS and ovarian cancer. Higher levels of protein carbonyl (a biomarker of protein oxidation) and C- reactive protein (biomarker of inflammation) as well as increase in levels of malondialdehyde have been reported in PCOS women [72].

2.18.13 Pre-Eclampsia

Pre-eclampsia is usually identified by presence of endothelial cell dysfunction and large amounts of lipid peroxidation activity [107]. Although the etiologic origin of pre-eclampsia is unknown, it continues to one of the leading causes of maternal and neonatal mortality [108, 109].

Women who are diagnosed with pre-eclampsia during pregnancy often have abnormally large amounts of ROS production with respect to NO and superoxide specifically as well as increased levels of antioxidants and high placental lipid peroxidation [107, 110]. An increase in the amount of activated neutrophils in women with pre-eclampsia contributes to the production of ROS. This also contributes to the typical vascular endothelial damage usually associated with the disease [111]. A study by Lee et al. [24] found that neutrophils isolated from pregnant women in their third trimester who were diagnosed with pre-eclampsia produced significantly more ROS than those isolated from healthy mothers. Neutrophil activation in pre-eclampsia may also lead to a greater amount of lipid peroxidation from the excessive production of reactive oxygen species. ROS have also been implicated in the pathogenesis of pre-eclampsia as seen by increased presence of endothelium derived NO levels [112, 113]. High levels of peroxynitrite formation have been associated with reports of increased endothelial NO synthase and decreased SOD, an enzymatic antioxidant, in pre-eclamptic patients [114, 115]. Molecules known as advanced glycation end products (AGE) are generators of ROS and can simultaneously cause vascular dysfunction through an association with cell surface receptors. Elevated levels of these molecules are present in women with pre-eclampsia [114].

A possible explanation for the etiologic origin of pre-eclampsia could involve the perfusion of the placenta in expectant mothers. Poorly perfused placental tissue may trigger free radical process and initiate lipid peroxidation, which is one of the main identifiable characteristics of pre-eclampsia [116]. O_2^- has been reported to initiate lipid peroxidation [117]. This combined with an oxidative imbalance in the blood are mechanisms leading to endothelial cell injury. An increase in plasma thiobarbituric acid reactive substances and decrease in the activities of both enzymatic and nonenzymatic antioxidants is seen in subjects during pregnancy and pre-eclampsia [118]. OS has been suggested as a link between the two-stage model of the pre-eclampsia syndrome; reduction of placental perfusion caused by maternal factors (stage 1) and activation of the maternal endothelium with multi-system disorders (stage 2).

2.18.14 Maternal Diabetes

Pregnant mothers who are diabetic face a higher risk of embryopathies, spontaneous abortions, and perinatal mortality that is associated with excessive OS [119]. Diabetic mothers give birth to a higher number of offspring with congenital malformations compared to healthy mothers. Increased rates of lipid peroxidation and protein carbonylation were reported in experimentally induced diabetic pregnancy [120, 121]. An excess of ROS has been observed when diabetes-induced embryopathy was blocked by antioxidants in vivo and in vitro [2, 8, 42] indicating that addition of antioxidants reduced the potential amount of damage caused by ROS. Siman et al. [122] showed the involvement of ROS in diabetes-induced malformations. In this study, antioxidant treatment with butylated hydroxytoluene, vitamin E or C resulted in a reduction of the appearance of congenital malformations from

approximately 25 % to less than 8 %. Diabetes itself is classified as a state of OS and low-density lipoproteins from pregnant diabetic women are highly susceptible to oxidation. In a study by Trocino et al. [123] embryos that have been cultured under hyperglycemic conditions showed increased production levels of ROS and significant reduction in glutathione synthesis and as well as in currently existing levels of the antioxidants. The presence of ROS is increased in embryos that have been exposed to high levels of glucose, possibly due to an increase in O_2^- generation or oxidative metabolism. The prevalence of congenital malformations preceded by embryonic dysmorphogenesis in vitro can be minimized by reducing the exposure of oxidative substrates to embryonic mitochondria or by improving ROS scavenging ability [124].

2.18.15 Recurrent Pregnancy Loss

Recurrent pregnancy loss (RPL) affects 0.5–3 % of reproductive age women and is defined as three or more consecutive spontaneous abortions occurring before 5 months of gestation [125]. Although the etiology of RPL can be attributed to many factors including gynecologic disease, anatomic abnormalities of the uterus, genetic anomalies in the fetus, and sperm DNA damage, almost 50–60 % of cases involving RPL have been observed to implicate the presence of OS [67, 70, 72, 126]. There is an increase in OS during the earlier parts of pregnancy from the trophoblast inside the placenta, as measured by various biomarkers such as TAC and ROS [72]. If there is an excess of OS and a lack of adequate antioxidant levels to protect the surrounding lipids, DNA, and proteins from damage, significant harm could be caused to the developing embryo [127, 128]. Women who are suffering from RPL have been found to possess weakened antioxidant defense systems as well as increased levels of OS biomarkers; therefore, it seems logical to assume that an excess in the presence of ROS may contribute to the pathological basis of the disease [129, 130]. Male partners with large amounts of leukocytes in their semen, morphologically abnormal sperm, or sperm with significant amounts of DNA damage can also contribute to ROS production levels and subsequent spontaneous abortions [131–134]. It is important to be able to detect the various biomarkers of OS in pregnant women in order to effectively predetermine chances for developing RPL and to combat the disease using appropriate treatment methods.

2.19 Conclusions

The detection of ROS in the female reproductive system is important for a variety of reasons. We can analyze the different fluids and determine that lower levels of ROS can be indicative of healthy women, such as in hydrosalpingeal fluid, or that the elevated levels indicate that something is wrong such as in amniotic, peritoneal, and

follicular fluid. Additionally, the ROS levels can be used to determine if there is a link between gynecological diseases and OS as well as how likely an oocyte will get fertilized or if an embryo will implant. In short, it is important to detect OS for the continuing understanding of the female reproductive cycle and health.

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