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Influence of ROS on Ovarian Functions

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Abstract

High level of ROS (Reactive Oxygen Species), due to an increased production of oxidant species and/or a decreased efficacy of antioxidant system, can lead to oxidative stress (OS) an emerging health risk factor involved in the aging and in many diseases, either in humans or in animals. ROS are a double-edged sword – they serve as key signal molecules in physiological processes, but also have a role in pathological processes involving the female reproductive tract.

ROS affect multiple physiological processes in reproduction and fertility, from oocyte maturation to fertilization, embryo development and pregnancy. Several studies indicate that follicular atresia in mammalian species due to the accumulation of toxic metabolites often results from oxidative stress. It has been suggested that ROS under moderate concentrations play a role in signal transduction processes involved in growth and protection from apoptosis. Conversely, increase of ROS levels is primarily responsible for the alteration of macromolecules, such as lipids, proteins and nucleic acids, that lead to significant damage of cell structures and thereby cause oxidative stress. To prevent damage due to ROS, cells possess a number of non-enzymatic and enzymatic antioxidants. Non-enzymatic antioxidants include vitamin C, glutathione and vitamin E. Enzymatic antioxidants consist of superoxide dismutases (MnSOD and Cu/ZnSOD) that convert superoxide into hydrogen peroxide; glutathione peroxidase (GPX) and catalase (CAT) which neutralize hydrogen peroxide. Intracellular homeostasis is ensured by the complex interaction between pro-oxidants and antioxidants.

This chapter describes gathering evidence that oxidative stress is involved in ovarian physio-pathology caused by diverse stimuli. There is strong evidence that ROS are involved in initiation of apoptosis in antral follicles caused by several chemical and

physical agents, in the fluid follicular environment, influencing the folliculogenesis and the steroidogenesis. Although less attention has been focused on the roles of ROS in primordial and primary follicle death, several studies have shown protective effects of antioxidants and/or evidence of oxidative damage, suggesting that ROS may play a role in these smaller follicles as well. Oxidative damage to lipids in the oocyte has been implicated as a cause of persistently poor oocyte quality. Developing germ cells in the fetal ovary have also been shown to be sensitive to toxicants and ionizing radiation, which induce oxidative stress. Recent studies have begun to elucidate the mechanisms by which ROS mediate ovarian toxicity. It has been investigated the role of antioxidant enzymes, such as catalase, glutathione peroxidase and the SOD isoforms in maintaining low levels of oxidative stress.

The literature provides some evidence of oxidative stress influencing the entire reproductive cycle. OS plays a role in multiple physiological processes from oocyte maturation to fertilization and embryo development. An increasing number of published studies have pointed towards increased importance of the role of OS in female reproduction. Of course, there is much to learn about this topic, whereby it cannot be underestimated.

Keywords: Assisted reproductive technologies (ART), reactive oxygen species, ovary functions

1. Introduction

High level of ROS (Reactive Oxygen Species), due to an increased production of oxidant species and/or a decreased efficacy of antioxidant system, can lead to oxidative stress (OS) an emerging health risk factor involved in the aging and in many diseases, either in humans or in animals. ROS are a double-edged sword – they serve as key signal molecules in physiological processes, but also have a role in pathological processes involving the female reproductive tract.

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2. Follicular development and ovary functions

The study of folliculogenesis and factors involved in its function is important in order to develop techniques able to increase the effectiveness of therapies or biotechniques included in assisted reproductive technologies (ART).

The follicle and oocyte development in mammals starts in fetal life. Briefly the primordial germinal cells undergo to mitosis until the ovogonias formed become primary oocytes. The meiotic development starts and at the birth the progression stops to the diplotene phase of the first meiotic division [1]. It will continue at the puberty. During the period of meiosis interruption the chromosomes become relaxed and nuclear structure so formed is named germinal vesicle (GV). At the puberty the GV disappears, the chromatin is recondensed, the pairs of homologous chromosomes are separated and half of them are expelled forming the first polar body. At this point the meiosis is interrupted again (metaphase II - MII). In this moment the oocyte is mature and fertile [2-4]. Luteinizing hormone (LH) is responsible of resumption of meiosis [5, 6]. The oocytes included in primordial follicles form a finite stock which leave this stage just when they are stimulated [7]. However, it was found that young adult rats have mitotic activity in germinative cells in order to maintain the follicular pool. The mechanisms involved in growing are not yet known [8].

During folliculogenesis the ovarian steroids, estradiol (E2) and progesterone (P), and the peptide hormone, inhibin, are synthesized in the granulosa cells and theca cells. These hormones feed back to regulate the synthesis and secretion of GnRH, LH, and FSH. The

majority of ovarian follicles do not ovulate, but undergo an apoptotic process of degeneration called atresia at the small antral follicle stage [9].

Growth of the antral follicles, in most cases, can be divided into two phases. In the first phase, characterized by slow growth stage, early growth of follicles can be attributed to an increase in the number of granulosa cells and therefore an increase in the surface of the granulosa layer [10]; this stage is critical for the development of oocyte capacity, in which it reaches the final size and competence [11, 12]. In the second phase, characterized by fast growth, in follicles larger than 2-5 mm, follicular growth appears to result from antrum development rather than an increase of the number of granulosa cells. This exponential increase in the antrum surface extends up to a possible ovulation of this follicle [13]. Modest are the information about the endocrine dependence or influence on the growth of small antral follicles. Several were the experiments performed to determine which hormone(s) is involved in this process. In cows, the immunization of GnRH, hence inactivation of the hormone, demonstrated that the first stage of the antral follicular growth can occur in an environment characterized by basal levels of follicle stimulation hormone (FSH) and without luteinizing hormone (LH) pulses [14-16]. It has not been demonstrated how the growth of small antral follicles is possible under basal levels of FSH. In mice the follicular wall is not responsive to FSH up to follicles develop from pre-antral stage to small antral follicles [17]. In any case, the second phase is absolutely under FSH control and adequate pulse of LH [18]. Stimulation of preovulatory follicle development in rodents via injection of equine chorionic gonadotropin (eCG, also called pregnant mare's serum gonadotropin), which has FSH and LH receptor-binding activity, followed 46-48 h later by an ovulatory dose of human chorionic gonadotropin (hCG), which has only LH receptor-binding activity, is commonly used in experiments assessing the effects of gonadotropin hormones on ovarian gene expression and other endpoints and for generating preovulatory follicles or ovulated oocytes for other studies [19].

In mammalian species, the main function of the corpus luteum (CL) is the synthesis of progesterone which is required for the establishment of a uterine environment suitable for the development of peri-implantation conceptus (embryo and associated extra-embryonic membranes) and the successful progression and maintenance of pregnancy [20]. Progesterone acts on the endometrium to regulate the synthesis of growth factors, cytokines, transport and adhesion proteins, protease inhibitors, hormones and enzymes which are primary regulators of conceptus implantation, survival and development [21]. Thus, compromised CL progesterone production Although the mechanisms of CL rescue from cell death and maintenance of progesterone production are very complex and vary among mammalian species [22], there is substantial evidence that reactive oxygen species (ROS) are key factors in determining the CL lifespan [23] and that antioxidants play significant roles in CL physiology during the oestrous/menstrual cycle [24-27]. Luteal ROS production and propagation depends upon several regulating factors, including luteal antioxidants, steroid hormones and cytokines, and their crosstalk. However, it is unknown which of these factors have the greatest contribution to CL function. In addition, the sequence of events leading to the functional and structural luteal regression at the end of the oestrous/menstrual cycle is still not clear. The scarce in-vivo reports studying the CL of rats [29], women [28] and sheep [28, 29] have shown the importance

of antioxidant enzymes in the control of CL function during the peri-implantation period. As a luteal phase defect can impact fertility by preventing implantation and early conceptus development in livestock and humans, this review attempts to address the importance of ROS-scavenging antioxidant enzymes in the control of mammalian CL function and integrity [30].

3. Reactive Oxygen Species (ROS): Chemical and Oxidative Stress (OS)

Free radicals are believed to play an important role in regulating the metabolic activity and functioning of some organs. There is a complex interaction of the pro-oxidants (free radicals) and antioxidants, resulting in the maintenance of the intracellular homeostasis. Whenever there is an imbalance between the pro-oxidants and antioxidants, favorable to free radicals, a state of oxidative stress (OS) is initiated. It is an emerging health risk factor involved in the aging and in many diseases, either in humans or in animals. Under normal conditions, paired electrons create stable bonds in biomolecules. A free radical is defined as any species capable of independent existence that contains one or more unpaired electrons in the outer orbit, independently upon the expressed electric charge. Depending on the distribution of the charge (electron cloud) and/or of its redox potential, free radicals have a more or less marked reactivity, linked to the spontaneous tendency to exist as entities having all the electrons arranged in pairs. This state corresponds to the chemical stability. The radicals are not equally reactive, in general the increase of charge and volume ratio of free radicals is directly proportional to their reactivity, therefore, they will tend to reach their own stability stripping electrons to any chemical species with which they are in contact and oxidize them [31].

Free radicals are classified on the basis the nature of the atom to which it belongs the orbital with the unpaired electron. There are, therefore, free radicals centered on oxygen, carbon, nitrogen, or chlorine, and so on. The present chapter, however, will reference mainly to free radicals centered on the oxygen, known more simply as oxygen free radicals. The latter, in fact, besides being one of quantitatively the most important elements of living matter, as well as the primary source of life itself, through a variety of mechanisms – not last the same cellular respiration – induces continuously the formation of chemical species with reactivity characteristics.

The oxygen free radicals are included into more large family of reactive oxygen species (ROS). This term indicates a class of reactive chemical species derived from oxygen, not necessarily radical, all united by more or less marked tendency to oxidize various organic substrates (carbohydrates, lipids, amino acids, proteins, nucleotides, etc.). Classic examples of radical origin of ROS are singlet oxygen and hydroxyl radical. The ozone and hydrogen peroxide, however, are not radical reactive oxygen species.

In living organisms, ROS are generated during normal cellular metabolic activity; some exogenous agents, however, can increase production, even with direct mechanism. It is possible to identify at least five sources of primary metabolic free radicals, in relation to the cellular site mainly interested in the production of ROS: the plasma membrane, mitochondria, peroxisomes, the smooth endoplasmic reticulum (microsomes) and the cytosol. In each of these

locations ROS are produced either spontaneously or as a result of reactions catalyzed by enzymes or by transition metals (eg. iron or copper) [31].

The free radicals can be generated by different mechanisms and, once formed, generally give rise to a series of chain reactions, in the course of which the radicalic site can be transferred or inactivated [31, 32].

Free radicals are mainly generated by homolytic cleavage or interaction with the transition metals. The term homolytic cleavage refers to the division of the covalent bond of a molecule as effect of the administration energy (thermal or radiant), with generation of two new chemical species, each one with an unpaired electron, distinctive element of free radicals. A classic example of homolytic cleavage is the radiolysis or photolysis of water that generates an atom of hydrogen and a hydroxyl radical. This chemical reaction is different from the ionization observed, for example, after dissolved in water molecules having at least one covalent bond polarized (eg. HCl). In this case, the water molecules, because of their polarity and without any administration of energy, are able to crack one of the polarized covalent bonds of the molecule solute generating two chemical species loaded of opposite sign, a cation and an anion (H^+ and Cl^- , respectively, in the example considered). The ionization, unlike the homolytic cleavage, the doublet electronic binding of the original molecule is not separated but remains in one of the new ionic species (anion) [33].

In the interaction with the transition metals, the electron generated by oxidation of a metal transition in ionic form (eg. from Fe^{2+} to Fe^{3+} or Cu^+ to Cu^{2+}) breaks a covalent bond to a target molecule generating a radical free and an anion. Alternatively, the electron required for reducing a transition metal in ionic form (eg. from Fe^{3+} to Fe^{2+} or Cu^{2+} to Cu^+) is extracted from the covalent binding of a target molecule, which is decomposed into a free radical and a cation. Through this mechanism, for example, iron (Fe^{2+}/Fe^{3+}) or copper (Cu^+/Cu^{2+}) act as catalysts in a sequence of redox reactions generating alkoxy radicals (RO^*) and peroxy ($R-O-O^*$) from peroxides ($R-O-O-R$). In the simplest case - described for the first time by Fenton - one ferrous ion (Fe^{2+}), oxidizes to ferric ion (Fe^{3+}), transfers its electron to a molecule of hydrogen peroxide (H_2O_2) and it breaks one of covalent bonds, generating a free radical (the hydroxyl radical, HO^*) and an anion (hydroxyl ion). In turn, the ferric ion (Fe^{3+}) is reduced - regenerating as any catalyst - to ferrous ion (Fe^{2+}), ripping an electron from a second molecule of hydrogen peroxide, which is split into a free radical (radical perhydroxyl (HOO^*)), and a cation (a hydrogen ion, H^+). Similarly, the hydroperoxides are split, for catalytic action of the iron, in the radical alkoxy (RO^*) and peroxy (ROO^*). In the absence of catalysts, the split of peroxides - which gives rise to a single species radical, the alkoxy - can take place only with energy consumption. A method of great biological relevance that gives rise to the formation of free radicals, includes the decomposition of nitrocompounds. In fact, alkyl radicals originate following the removal of molecular nitrogen (N_2) [31].

Once a radical reaction is triggered, it tends to propagate chain. There are four basic mechanisms of propagation of radical reactions: transfer, addition, fragmentation and rearrangement. The most common among these is the transfer. In this mode, the free radical - generated by one of previous reactions - attacks a molecule subtracting to it one of its atoms (generally a hydrogen atom). The result is the formation of a new reactive species and, in practice, radical

site has been transferred. With this mechanism, for example, the hydroxyl radical (HO^*), attacking an organic molecule (R-H), rips to this one atom of hydrogen and generates, with a molecule of water (H_2O), an alkyl radical (R^*). With this mechanism, the radical site is transferred from the hydroxyl radical to the alkyl one.

Finally, a radical reaction chain may stop (term) by two mechanisms: combination or disproportion. In particular, in the combination, which is the homolytic cleavage of the reverse reaction, two radicals react with each other giving rise to a molecule not more reactive. The first radical acts as the oxidant, while the second acts as a generic antioxidant. This mechanism is exploited to block a radical reaction, and in general, any radical process chain can be interrupted by the intervention of agents called, generically, antioxidants.

In living organisms ROS are generated during normal cellular metabolic activity; some exogenous agents, however, may increase production, even with direct mechanism (figure 1).

As mentioned above, it is possible to identify at least 5 of primary metabolic free radical sources, in relation to cellular site: the plasma membrane, the mitochondria, peroxisomes, smooth endoplasmic reticulum (microsomes) and the cytosol (figure 2).

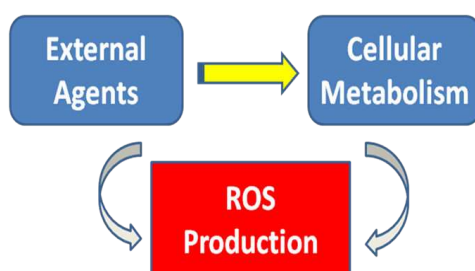


Figure 1. General mechanism of ROS production.

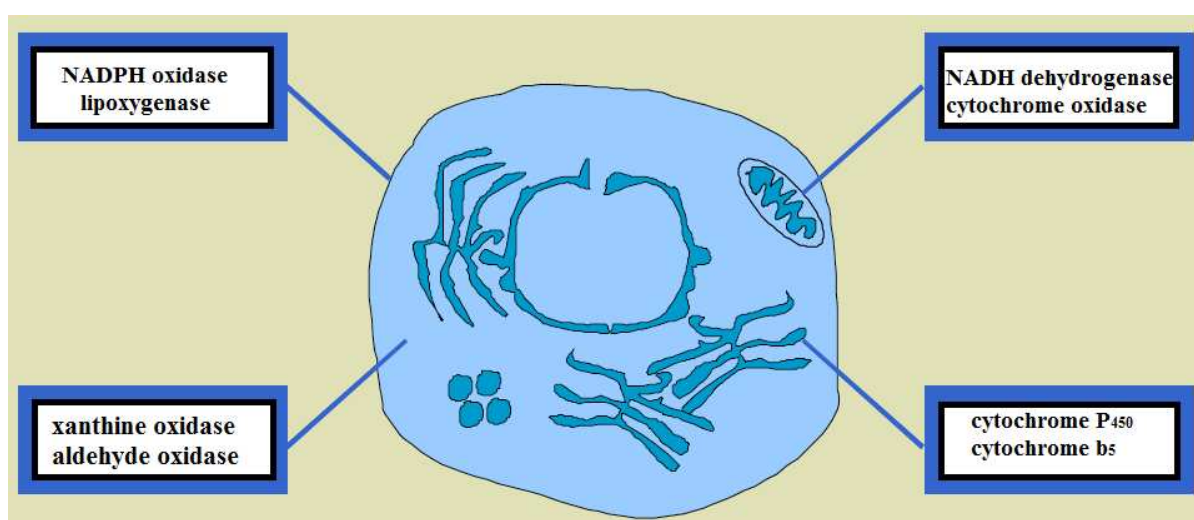


Figure 2. Primary source of ROS cell production

The plasma membrane is one of the most important sources of ROS, particularly (but not exclusively) in polymorphonuclear leukocytes (PMNs). In fact, in the plasma membrane of PMNs are located several enzymes, such as the NADPH oxidase and lipoxygenase, whose activation is accompanied by the production, respectively, of superoxide anion and metabolic intermediates with chemical characteristics of peroxides. The NADPH oxidase is an enzyme that catalyzes the formation of superoxide anion by NADPH (H^+) and molecular oxygen, after specific stimulation of PMNs, due, for example, to endotoxins, bacteria, or antibodies).

The reaction is made possible by the increased availability of NADPH (H^+), for the increased oxidation of glucose through the shunt of hexoses, and of molecular oxygen, under the so-called "respiratory burst". The system of lipoxygenase, localized also at the level of the plasma membrane, includes three enzymes, the 5-, 12-, and 15- lipoxygenase, which catalyze the formation, from arachidonic acid, of 5-, 12-, and 15-HPETE (hydroperoxyeicosatetraenoic acid), respectively. These substances are chemically hydroperoxides acids, they belong to a group of ROS named ROM (reactive oxygen metabolites, ie metabolites or derived reactive oxygen). The production of ROS at the level of PMNs plasma membrane for activation NADPH oxidase and/or lipoxygenase, takes place, typically, in the course of reactive processes (eg. infections, immunoreactions pathogenic, inflammation) [31].

The mitochondria are the primary metabolic source of ROS because the enzyme complexes of respiratory chain are localized on their crests and are involved in oxidative phosphorylation. Ideally, the transfer of electrons from reduced NAD to cytochrome C and from the latter to oxygen should end with the production of H_2O , once synthesized ATP, (reduction tetravalent of molecular oxygen). However, already in normal conditions, this process is not perfect so, for not easily controllable reasons, a certain amount of electrons (1-2%) escapes the system transport of various coenzymes (eg. ubiquinone, flavoproteins, cytochromes, etc.) and reacts directly with molecular oxygen, generating, thus, superoxide anion and/or hydrogen peroxide (reduction uni- and bivalent molecular oxygen). In fact, this process, during a intense exercise in skeletal muscle, this electronic shunt can reach 15% of the oxygen used by mitochondria due to the intense stimulation of cellular metabolism. The phenomenon of the reduction in one or bivalent molecular oxygen takes place, in the mitochondria, without the intervention of enzymes, as opposed to what is observed in other cell locations. In other words, from a purely chemical point of view, the production of free radicals during oxidative phosphorylation is not just a mode of enzymatic production of reactive species. In fact, as it has just been mentioned, the generation of free radicals in living organisms is closely related to vital phenomena and, therefore, constitutes a "physiological" phenomenon that takes place continuously in the course of redox reactions through both enzymatic and non-enzymatic mechanisms. It should be stressed that, in addition to mitochondria, there are other sources of non-enzymatic free radicals in cells. For example, peroxynitrite spontaneously generates hydroxyl and nitroxide radicals. However, the most important non-enzymatic reactions from a biological standpoint for the production of free radicals are those catalyzed by transition metals. In these reactions, which generally require iron or copper in the reduced state (respectively Fe^{2+} and Cu^+), hydrogen peroxide is split into hydroxyl radical and hydroxyl ion for incorporation of the electron ripped to transition metal, which is released in the oxidized form (Fe^{3+} and Cu^{2+} ,

respectively), according to the mechanism discussed above of the interaction with transition metals. Hydroperoxides undergo a similar reaction, which generate the alkoxy radical. The enzymes that regenerate the transition metals in the reduced state constitute a complex indicated with MCO (metal-catalyzed oxidation systems). They include xanthine oxidase, NADPH and NADH oxidase, nicotinic acid hydroxylase, the cytochrome P450 system, the NADH reductase (with coenzyme quinone), the succinic-reductase (with coenzyme quinone) and an amount of iron-sulfur proteins non-heme. The quinones and reduced flavin prosthetic groups generated by these enzymes in their turn reduce the transition metals, resulting in the direct reduction of molecular oxygen to hydroxyl radical and/or peroxide hydrogen (through the mediation or not of superoxide anion).

In addition to the plasma membrane and mitochondria, peroxisomes also represent an important source of ROS. In these cell organelles, in fact, a particular process of fatty acid oxidation takes place, which is different from the conventional way (beta-oxidation). In the first stage of this sequence of reactions, a flavoprotein extracts a pair of hydrogen atoms from one molecule of activated fatty acid (acyl-CoA) by transferring it directly to molecular oxygen, with the formation of hydrogen peroxide (subsequently inactivated by catalase).

In the endoplasmic reticulum (microsomes) production of reactive species passes through the cytochrome P450. The latter plays a major role in detoxification processes. The cytochrome P450 acts as immediate donor of electrons in many reactions of hydroxylation, particularly those that take place within the hepatocytes and that are aimed to inactivation of hormones (eg. steroid) and not physiological compounds (xenobiotics, such as toxic and hydrophobic drugs which are thereby made more soluble and less toxic). The P450 is a heme iron protein localized not only in the endoplasmic reticulum of the liver but also in the mitochondria of the adrenal cortex that, in a process very complex and not yet fully clarified, acts as connection between NADPH (H^+) (electron donor) and the substrate that should be hydroxylated. In this complex reaction, a substrate able to be hydroxylated (SH) reacts with NADPH (H^+) and molecular oxygen (O_2) to form the corresponding hydroxylated derivative (S-OH), plus NADP⁺ and water. A production of free radicals in the cell also occurs in the course of many other biochemical reactions, such as during oxidation of hypoxanthine to xanthine and xanthine to uric acid, which mark the final phase of the catabolism of purine nucleotides. Both of these reactions are catalyzed by xanthine dehydrogenase, a molybdenum enzyme. Under special conditions, such as during the so-called ischemia-reperfusion, xanthine dehydrogenase is converted to xanthine oxidase (probably for proteolytic cleavage calcium-dependent). The latter, using as a final electron acceptor the oxygen, generates hydrogen peroxide and superoxide anion, starting, respectively, from hypoxanthine and xanthine.

Other reactions that generate free radicals are described in the synthesis of catecholamines.

From the above, it is clear that ROS represent intermediate obligated cellular metabolism. And since their production is closely linked to the vital phenomena, they have been called "irreplaceable companions" of our existence.

It appears evident that in each cell site, the production of reactive species has its own specific function. In fact, it has been recognized that ROS play an important role "in the service of life"

because they are not only involved in cell metabolism but also in the "reactive processes" such as infection and inflammation. Actually, the superoxide anion and other ROS are generated on the outer surface of the plasma membrane of activated leukocytes. These reactive species attack extraneous components such as bacteria, weakening the wall and making them more readily accessible to phagocytosis and, ultimately, to their destruction. These "immunological" activities are expressed not only in respect of extraneous components but also against "self" components, such as tissues or transplanted organs (rejection reaction). This strategy is also used in the course of healing of organs or tissues subject to trauma. In fact, the leukocytes migrate to the injured, are activated and begin bombing damaged cells with free radicals, that accelerate their destruction, remove lysis products, and promote the recovery (regeneration). The production of free radicals by the cells may sometimes undergo a considerable increase depending on external stimuli. In fact, physical, chemical and biological agents, alone or in combination, may also induce the generation of ROS or increase the "physiological" production through a specific metabolic stimulation. Ionizing and UV radiation are reported to be physical agents. Both these sources of energy can induce the phenomenon of homolytic cleavage of water, also called radiolysis or photolysis, depending on the type of radiation involved.

In this reaction, the water molecule absorbs energy and uses it to break one of its two covalent bonds with the hydrogen: the products will be two free radicals, the hydroxyl radical and the hydrogen atom. Considering that a living organism is made up primarily of water and he spends most of his life under the influence of radiation (UV or ionizing they are) it is clear how this phenomenon affects substantially the production of free radicals.

As chemical agent, capable of stimulating the production of free radicals, ozone (ROS) is to be quoted. It directly generates peroxy radicals by interaction with phenolic compounds. The two cases considered so far (radiation and ozone) are examples of direct production of reactive species. Other chemical agents, however, such as polycyclic aromatic hydrocarbons, or certain drugs, induce increased production of free radicals through an indirect mechanism, activating the cytochrome P450 microsomal level. Biological agents that typically lead to increased production of ROS for metabolic activation are bacteria, as part of the physiological process of defense against infection, and certain antibodies, as part of some reactions immune-pathogen. In these cases, as mentioned with regard to the plasma membrane, the PMNs are directly implicate. They, in fact, possess NADPH oxidase and a series of enzymes directly involved in the production and, in part, inactivation of reactive chemical species, such as superoxide dismutase (SOD), myeloperoxidase (MPx), catalase (CAT) and glutathione peroxidase (GPx).

SOD catalyzes the conversion of superoxide anion into hydrogen peroxide which, in turn, can be inactivated to water by CAT or GPx. However, the availability of chlorides - even at physiological concentrations - makes the hydrogen peroxide a substrate for MPx. The end result is the production of a highly oxidising agent, the hypochlorous acid (HClO). The HClO can attack numerous organic substrates and, in particular, amino acids and proteins, to produce chloramines, a potential source of alkoxyl and peroxy radicals. Finally, an increase in free radical production may be observed in "physiological " situations, such as after an intense muscular effort or in the course of many diseases. In the latter case, often, it is not clear how far the ROS are the cause or the effect of a certain pathology [31].

4. The antioxidant defense system

ROS are chemical species potentially detrimental. For this reason, living organisms have developed over millennia of evolution a complex antioxidant defense system, consisting of a set of enzymes, vitamins, trace elements and other vitamin-like substances. These antioxidants may be classified according to different criteria: on the basis of the origin, in endogenous and exogenous, on the basis of the chemical nature, in the enzymatic and non-enzymatic, and on the basis of the solubility in fat-soluble and water-soluble. On the basis, however, of the mechanism of action prevalent, physiological antioxidants can be easily assembled into four main groups: preventive antioxidants, scavenger, shelter agents and adaptation agents [34].

Preventive antioxidants are agents that, through various mechanisms, such as the chelation of transition metals, prevent the formation of reactive species.

The scavengers act through different mechanisms. They may be of hydrophilic nature (albumin, urate, ascorbate, urate) or lipophilic (carotenoids, vitamin E, ubiquinol). According to some researchers, the scavenger should be distinguished from antioxidants proper. In fact, while the scavenger (eg. A-tocopherol) are agents that reduce the concentration of free radicals removing them from the medium in which they are located, antioxidants (eg. Diphenylamine) are agents that inhibit the auto-oxidation process, e.g. the fat rancidity. This phenomenon, well known in food science, is called auto-oxidation since it occurs through a sequence of autocatalytic radical reactions in the presence of oxygen. Alternatively, you can use the term peroxidation, as the same process generates intermediates with characteristics of peroxides (R-O-OR).

Through this process some dietary fat rancid and cellular membranes of living organisms are oxidized.

Shelter agents include only enzymes involved after the damage from reactive species has been established. Their action - often sequential - provides first the identification of the molecular segment oxidized, then the separation of the fragment unusable and, finally, the synthesis and the insertion of a new segment in substitution of the damaged one. The category of shelter antioxidants includes hydrolases (glycosidases, lipases, proteases), and the transferase and polymerases, all essential for the repair of free radical damage of important molecules or cellular structures (eg. DNA, membranes, etc.).

Finally, the agents of adaptation include all substances or techniques or procedures through which it is possible to strengthen the physiological antioxidant system of an organism. For example, a proper physical exercise or the adoption of a proper and balanced diet are measures by itself able to check the oxidative metabolism by reducing the production of reactive species, and induction of enzymes with antioxidant activity.

The antioxidant defense system is regularly distributed in the body, both at the extracellular and intracellular levels.

In plasma, the set of substances potentially able to give equivalent reducing (hydrogen atoms or single electrons) so as to meet "the greed of electrons" that makes free radical constitutes

unstable is the so-called barrier antioxidant. In the plasma, all protein and, in particular, albumin, bilirubin, uric acid, cholesterol, and various exogenous antioxidants introduced with food or in the form of dietary supplements (ascorbate, tocopherol, polyphenols etc.) are part of it. The thiol groups (-SH), commonly found in the cysteine side chain, play a role of particular importance in the context of this barrier. In addition, thiol groups, are the most chemically reactive sites on proteins, such as albumin, and have strong reducing properties [35, 36].

Inside the cells, the antioxidant system of cell defense has its precise compartmentalization (figure 3). The antioxidant system includes some enzymes (glutathione, superoxide dismutase, catalase) and a series of substances taken from outside (vitamins and substances similar to antioxidant activity, such as polyphenols, trace elements etc.). Some of these agents are fat-soluble (eg. tocopherols) and, entering the team of biomembranes, constitute the first line of defense against the attack of free radicals. Others, however, are water soluble (eg. ascorbate) and intervene especially in the context of soluble matrix of the cytoplasm and cellular organelles.

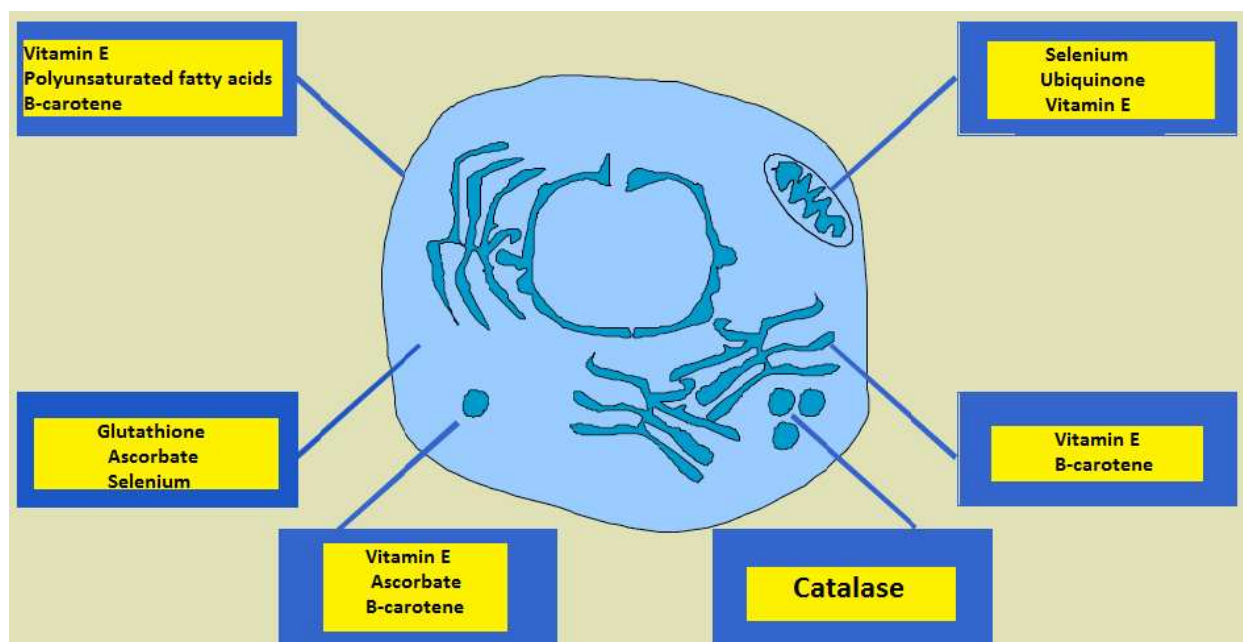


Figure 3. Compartmentalization of antioxidant system

Glutathione (GSH) is a tripeptide (L-g-glutamyl-L-cysteinyl-glycine, with multiple biological functions and that has been found in all mammalian cells [37-39]. Its biological activity is primarily related to the active thiol group of the cysteine residue [40]. The reduced and oxidized forms of glutathione (GSH and GSSG) act in concert with other redox-active compounds (e.g., NAD(P)H) to regulate and maintain cellular redox status. It is an abundant low-molecular-mass thiol antioxidant, which either interacts directly with reactive oxygen and nitrogen species (ROS and RNS, respectively) or serves as a cofactor for many antioxidant and associated enzymes such as peroxidases and transferases [41]. The chemical structure of GSH determines its potential functions and its broad distribution among all living organisms reflects

its important biological role. Probably most importantly, GSH is responsible for protection against ROS and RNS, and detoxification of endogenous and exogenous toxins of an electrophilic nature. Depletion of GSH results in DNA damage and increased H_2O_2 concentrations; as such, GSH is an essential antioxidant. During the reduction of H_2O_2 to H_2O and O_2 , GSH is oxidized to GSSG by glutathione peroxidase (GPx). Glutathione reductase participates in the reverse reaction, and utilizes the transfer of a donor proton from NADPH to GSSG, thus, recycling GSH [42]. Vitamin E (α -tocopherol) protects GPx4-deficient cells from cell death. In addition, glutathione is (1) a storage form of cysteine in the cells and for interorgan transfer; (2) a storage form and transporter of nitric oxide (as GSNO); (3) involved in the metabolism of estrogens, leukotrienes, and prostaglandins, reduction of ribonucleotides to deoxyribonucleotides, and maturation of iron-sulfur clusters of proteins; (4) involved in the regulation of certain transcription factors from the environment to cellular transcription machinery; (5) involved in the detoxification of many endogenous compounds and xenobiotics (the mercapturate pathway); and (6) copper and iron transfer. Glutathione also can be used even for the detoxification of ions of transition metals such as chromium [43, 44].

Five isoforms of glutathione peroxidase exist in the body: GPx1, GPx2, GPx3, GPx4, and GPx5. GPx1 is the cytosolic isoform that is widely distributed in tissues, while GPx2 encodes a gastrointestinal form with no specific function; GPx3 is present in plasma and epididymal fluid. GPx 4 specifically detoxifies phospholipid hydroperoxide within biological membranes. Free glutathione exists in vivo mostly as two forms, reduced (GSH) and oxidized (glutathione disulfide; GSSG). GPx5 is found in the epididymis [39].

Superoxide dismutase (SOD): Other enzymes directly detoxify ROS. SOD reacts with superoxide anion radicals to form oxygen and H_2O_2 . The enzyme SOD exists as three isoenzymes: SOD 1, SOD 2, and SOD 3. SOD 1 contains Cu and zinc (Zn) (Cu, Zn-SOD) as metal co-factors and is located in the cytosol. SOD 2 (Mn-SOD) is a mitochondrial isoform containing manganese (Mn), and SOD 3 encodes the extracellular form (ECSOD). SOD 3 is structurally similar to Cu, Zn-SOD, as it contains Cu and Zn as cofactors [45, 46].

Catalase (CAT) is a heme-containing homotetrameric protein. CAT can decompose hydrogen peroxide (H_2O_2) in reactions catalyzed by two different modes of enzymatic activity: the catalatic mode of activity ($2\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O}$) and the peroxidatic mode of activity ($\text{H}_2\text{O}_2 + \text{AH}_2 \rightarrow \text{A} + 2\text{H}_2\text{O}$). Although several substrates such as methanol and ethanol can be oxidized by the peroxidation reaction, the physiological significance of this catalase function is not understood. Decomposition of H_2O_2 by the catalatic activity of catalase follows the fashion of a first-order reaction, and its rate is dependent on the concentration of H_2O_2 . In fact, catalase belongs to the group of enzymes that catalyze reactions at a rate near kinetic perfection; the reaction rate is only limited by the rate at which the enzyme collides with the substrate. Catalase is ubiquitously present in all prokaryotes and eukaryotes. With the exception of erythrocytes, it is predominantly located in peroxisomes of all types of mammalian cells where H_2O_2 is generated by various oxidases. However, a certain amount of catalase has also been found in mitochondria of rat heart. Since H_2O_2 serves as a substrate for Fenton reaction to generate the highly reactive hydroxyl radical, catalase is believed to play a role in cellular antioxidant defense mechanisms by limiting the accumulation of H_2O_2 [47-49].

The non-enzymatic antioxidants consist of dietary supplements and synthetic antioxidants such as vitamin C, GSH, taurine, hypotaurine, vitamin E, Zn, selenium (Se), betacarotene, and carotene [41]. Vitamin C (ascorbic acid) is a known redox catalyst that can reduce and neutralize ROS. Its reduced form is maintained through reactions with GSH and can be catalyzed by protein disulfide isomerase and glutaredoxins. Glutathione is a peptide found in most forms of aerobic life as it is made in the cytosol from cysteine, glutamate, and glycine [42]; it is also the major nonenzymatic antioxidant found in oocytes and embryos. Its antioxidant properties stem from the thiol group of its cysteine component, which is a reducing agent that allows it to be reversibly oxidized and reduced to its stable form [42]. Levels of GSH are regulated by its formation *de-novo*, which is catalyzed by the enzymes gamma-GCS and glutathione synthetase [4, 11]. Glutathione participates in reactions, including the formation of glutathione disulfide, which is transformed back to GSH by glutathione reductase at the expense of NADPH [17].

Cysteine and cysteamine (CSH) increase the GSH content of the oocyte. Cysteamine also acts as a scavenger and is an antioxidant essential for the maintenance of high GSH levels. Furthermore, CSH can be converted to another antioxidant, hypotaurine [43, 44].

The concentrations of many amino acids, including taurine, fluctuate considerably during folliculogenesis. Taurine and hypotaurine are scavengers that help maintain redox homeostasis in gametes. Both neutralize lipid peroxidation products, and hypotaurine further neutralizes hydroxyl radicals [44].

Like GSH, the Thioredoxin (Trx) system regulates gene functions and coordinates various enzyme activities. It detoxifies H_2O_2 and converts it to its reduced state via Trx reductase [45]. Normally, Trx is bound to apoptosis-regulating signal kinase (ASK) 1, rendering it inactive. However, when the thiol group of Trx is oxidized by the SO anion, ASK1 detaches from Trx and becomes active leading to enhanced apoptosis. ASK1 can also be activated by exposure to H_2O_2 or hypoxia-reoxygenation, and inhibited by vitamins C and E. The Trx system also plays a role in female reproduction and fetal development by being involved in cell growth, differentiation, and death. Incorrect protein folding and formation of disulfide bonds can occur through H^+ ion release from the thiol group of cysteine, leading to disordered protein function, aggregation, and apoptosis [2].

Vitamin E (α -tocopherol) is a lipid soluble vitamin with antioxidant activity. It consists of eight tocopherols and tocotrienols. It plays a major role in antioxidant activities because it reacts with lipid radicals produced during lipid peroxidation [42]. This reaction produces oxidized α -tocopheroxyl radicals that can be transformed back to the active reduced form by reacting with other antioxidants like ascorbate, retinol, or ubiquinol.

The hormone melatonin is an antioxidant that, unlike vitamins C and E and GSH, is produced by the human body. In contrast to other antioxidants, however, melatonin cannot undergo redox cycling; once it is oxidized, melatonin is unable to return to its reduced state because it forms stable end-products after the reaction occurs (see below for functions).

5. Commonly used markers of ROS-induced modification of cellular components

It seems that despite their high chemical reactivity most generated ROS do not lead to serious negative physiological consequences for organisms. That is mainly due to the action of highly efficient systems of ROS neutralization operating in concert with reparation and elimination of ROS-modified molecules always exists, that may be called the basal steady-state (stationary) level [37, 50]. Reactive oxygen species can modify most types of biomolecules including proteins, lipids, carbohydrates, nucleic acids, metabolic intermediates, etc. It is widely accepted that the use of only one type of modification to assess oxidative damage during oxidative stress is not sufficient. That is due to the different sensitivity, dynamics, and nature of ROS-promoted modifications. Instead, in order to evaluate the intensity of ROS-involving processes, several approaches for the evaluation of particular oxidatively modified molecules have been selected. They reflect the level of products of interaction between ROS and cellular components of different natures. "Classically", several essential markers are used. They are: (i) for lipids – the formation of malonic dialdehyde (MDA), isopsoralens, and lipid peroxides; (ii) for proteins – protein carbonyl groups; and (iii) for DNA – 8-oxoguanine. Malonic dialdehyde is commonly measured via its reaction with thiobarbituric acid (TBA). However, this reaction is not specific and many other compounds react with TBA under the assay conditions. The array of products formed is collectively called thiobarbituric acid reactive substances (TBARS) to reflect this low specificity. Certain amino acids, carbohydrates, aldehydes and other compounds interfere with the reaction measurement and, therefore, this method should be used with precaution and discussed taking into account the highlighted issues [50]. In the last decade, an HPLC technique was applied to evaluate MDA levels and this method, along with immunochemical identification [51] can now be recommended as more reliable than the TBARS assay. There are also many other approaches to evaluate the intensity of ROS induced lipid peroxidation and the measurement of lipid peroxides [51], 4-hydroxynonenal [52] are just some of them. Selection of methods depends on many things, particularly tools available [33]. Probably the most popular method for detection of ROS-modified proteins is the one based on the formation of additional carbonyl groups with their visualization due to their interaction with 2, 4-dinitrophenylhydrazine [53]. The hydrazones formed are measured spectrophotometrically. Specific antibodies that interact with carbonyl groups on proteins [54] have also been developed. In some cases, there is also the possibility to evaluate the amount of dityrosines and other products of free radical induced oxidation of proteins. Oxidation of nucleic acids also forms an array of products, but in this case there are some favorites that are relatively easy to quantify. These are mainly oxidatively modified guanine derivatives, of which 8-hydroxyguanine (8-OHG) is the most commonly used, but 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-oxo-7, 8-dihydroguanine (8-oxoGua) can also be measured. Certainly, there are many more different markers of ROS-induced modification of cellular constituents, but those listed here are the most widely used and applied approaches.

6. Influence of ROS on reproductive functions

ROS affect multiple physiological processes in reproduction and fertility, from oocyte maturation to fertilization, embryo development and pregnancy. Several studies indicate that follicular atresia in mammalian species due to the accumulation of toxic metabolites often results from oxidative stress. It has been suggested that ROS under moderate concentrations play a role in signal transduction processes involved in growth and protection from apoptosis. Conversely, increase of ROS levels is primarily responsible for the alteration of macromolecules, such as lipids, proteins and nucleic acids, that lead to significant damage of cell structures and thereby cause oxidative stress. To prevent damage due to ROS, cells possess a number of nonenzymatic and enzymatic antioxidants. Nonenzymatic antioxidant include Vitamin C, glutathione, cysteamine, vitamin E. Enzymatic antioxidants consist of superoxide dismutases (MnSOD and Cu/ZnSOD, which are in the mitochondria and cytosol, respectively), that convert superoxide into hydrogen peroxide; glutathione peroxidase (GPX) and catalase (CAT) which neutralize hydrogen peroxide. Intracellular homeostasis is ensured by the complex interactions between pro-oxidants and antioxidants.

This chapter describes gathering evidence that oxidative stress is involved in ovarian pathology caused by diverse stimuli. There is strong evidence that ROS are involved in initiation of apoptosis in antral follicles caused by several chemical and physical agents, in the fluid follicular environment, influencing the folliculogenesis and the steroidogenesis. Although less attention has been focused on the roles of ROS in primordial and primary follicle death, several studies have shown protective effects of antioxidants and/or evidence of oxidative damage, suggesting that ROS may play a role in these smaller follicles as well. Oxidative damage to lipids in the oocyte has been implicated as a cause of persistently poor oocyte quality. Developing germ cells in the fetal ovary have also been shown to be sensitive to toxicants and ionizing radiation, which induce oxidative stress. Recent studies have begun to elucidate the mechanisms by which ROS mediate ovarian toxicity. It has been investigated the role of antioxidant enzymes, such as catalase, glutathione peroxidase and the SOD isoforms in maintaining low levels of oxidative stress.

The literature provides some evidence of oxidative stress influencing the entire reproductive cycle. OS plays a role in multiple physiological processes from oocyte maturation to fertilization and embryo development. An increasing number of published studies have pointed towards increased importance of the role of OS in female reproduction. Of course, there is much to learn about this topic, whereby it cannot be underestimated.

7. Role of ROS in folliculogenesis, ovulation, and corpus luteum function

The ROS should not always be coupled with negative effects [56]. Accumulating data have recently shown that reactive oxygen species can regulate cell function by controlling production or the activation of substances that have biological activities.

Numerous genes related to inflammation are induced in preovulatory follicles by the LH surge. The analogy of ovulation with an acute inflammation may suggest a role for ROS along this process. Because ROS are massively generated during the inflammatory process hypothesized that ROS could be involved in the signaling cascade leading to ovulation. The findings were that H₂O₂ mimicked the effect of LH, bringing about an extensive mucification/expansion of the follicle-enclosed cumulus-oocyte complexes; impaired progesterone production was observed in isolated follicles incubated with LH in the presence of antioxidant agents; furthermore, LH-stimulated up-regulation of genes, the expression of which is crucial for ovulation, was substantially attenuated upon ROS ablation. Together, these results provide evidence that ovarian production of ROS is an essential for preovulatory signaling events, most probably transiently triggered by LH [56].

The increase in steroid production in the growing follicle causes an increase in P450, resulting in ROS formation. Reactive oxygen species produced by the pre-ovulatory follicle are considered important inducers for ovulation. Oxygen deprivation stimulates follicular angiogenesis, which is important for adequate growth and development of the ovarian follicle. Follicular ROS promotes apoptosis, whereas GSH and follicular stimulating hormone (FSH) counterbalance this action in the growing follicle. Estrogen increases in response to FSH, triggering the generation of catalase in the dominant follicle, and thus avoiding apoptosis [26].

In ovaries, the corpus luteum is formed after ovulation and produces progesterone, which is necessary for the establishment and maintenance of pregnancy. When pregnancy occurs, the rescue of the corpus luteum and subsequent progesterone production are important for the maintenance of pregnancy. In contrast, when pregnancy does not occur after ovulation, the decline of progesterone production is important for the follicle development of the next reproductive cycle. The chance of conception occurring as soon as possible and as often as possible depends on how rapidly progesterone production declines. Therefore, the strategy for reproduction in the ovary is the rapid rescue of the corpus luteum when pregnancy occurs, and the rapid termination of the corpus luteum function when pregnancy does not occur after ovulation. Corpus luteum regression is defined as that the corpus luteum declines in function, decreases in volume, and thereafter disappears from the ovary. Corpus luteum regression consists of two stages of regression, functional luteolysis and structural luteolysis. Structural luteolysis is defined as structural involution of the corpus luteum, and is clearly distinguished from functional luteolysis which is characterized by depletion of progesterone production without structural changes such as loss of luteal cells and blood vessels. Rapid decline in progesterone production is important for follicle growth in the next reproductive cycle. It is therefore of interest to study the mechanism of functional luteolysis. ROS and SOD are involved in functional luteolysis. ROS are produced in the corpus luteum [26]. There are several potential sources of ROS in the corpus luteum. Macrophages and neutrophils, that are clear sources of reactive oxygen species, are well documented as residing in the corpus luteum [57-61]. The increase in ROS in the corpus luteum is involved in functional luteolysis. The decrease in Cu, Zn-SOD expression could be one of the causes for the increase in reactive oxygen species in the regressing corpus luteum. It seems there is another possible mechanism

able to increase ROS. PGF2 α has been well recognized as a luteolysin since it increases in the corpus luteum during the regression phase [62] and inhibits the production of progesterone by luteal cells. A number of reports have shown so far that the inhibitory effect of PGF2 α on progesterone production by the corpus luteum is, in part, mediated through the increase of ROS [63, 64]. ROS can activate phospholipase A2 activity and cyclooxygenase-2 expression in the corpus luteum which are key enzymes for PGF2 α synthesis. Thus, there seems to be a close interrelation between PGF2 α and ROS [65, 66].

Steroidogenic cells are also potential sources of reactive oxygen species because reactive oxygen species are generated as byproducts of normal metabolism. Intracellular sources of ROS include mitochondrial electron transport, endoplasmic reticulum, nuclear membrane electron transport systems and plasma membranes [67]. There is a significant co-relationship between Cu, Zn-SOD activities and serum progesterone concentrations. In contrast, lipid peroxide levels increase in the corpus luteum during the regression phase in the both rat models and show an opposite change from serum progesterone concentrations [68, 69]. Reactive oxygen species generated normally during steroidogenesis restrict the capacity of the corpus luteum to produce progesterone [70]. In pregnancy, the decrease in Cu, Zn-SOD expression causes the inhibition of progesterone production via the increase in ROS. Therefore, the increase in ability to scavenge ROS may be associated with the maintenance of luteal cell integrity and prolonged life span of the corpus luteum [71]. In other animals, such bovines, SOD and CAT have been reported to be correlated with progesterone production by the corpus luteum [72]. It is plausible that the luteotropic substances, usually synthesized by placenta during pregnancy, stimulate the expression of molecules that protect luteal cells from ROS. Finally, the increase in Cu, Zn-SOD by placental luteotropins is an important mechanism to rescue the corpus luteum and maintain progesterone production [73].

Aerobic metabolism utilizing oxygen is essential for energy requirements of the gametes, and the free radicals play a significant role in physiological processes within the ovary. Many studies have demonstrated involvement of ROS in the follicular-fluid environment, folliculogenesis, and steroidogenesis [74]. The immunohistochemical distribution of the copper-zinc superoxide dismutase (Cu, Zn-SOD) in the human ovary was given by [74]. They found, for the first time, that the gestational corpus luteum, theca and granulosa lutein cells showed intensive and moderate staining activity, respectively, to Cu, Zn-SOD. Furthermore, they suggested that, as SOD catalyses the dismutation reaction of superoxide anion radicals, the theca interna cells play an important role in the protection of the developing oocyte from oxygen radicals by acting as a blood-follicular barrier during follicle maturation, [76] underlined the presence of manganese superoxide dismutase (Mn-SOD) and Cu, Zn-SOD in human ovaries and fallopian tubes, with different localizations and actions. The superoxide radical-SOD system might play an important role in ovulation and in the luteal function of the human ovary in the human ovary and fallopian tube, and to examine the role of superoxide radicals and SODs in the human ovulatory process. These enzymes can be considered as markers of cytoplasmic maturation [77].

Culture of small and large (preovulatory) antral rat follicles without gonadotropin support leads to apoptotic death within 24 h, while FSH suppresses apoptosis [78]. To investigate if oxidative stress plays a role in granulosa cell apoptosis during follicular atresia in the immature rat ovary, healthy antral follicles obtained from rats were in the absence or presence of FSH, SOD, ascorbic acid (a free radical scavenger), N-acetyl-L-cysteine (a free radical scavenger and stimulator of endogenous glutathione peroxidase activity), or CAT. The results showed that each antioxidant was able to protect against apoptosis in rat large antral follicles cultured without gonadotropin support [79].

Markers of peroxidation were measured in follicular fluids and sera of women attending an in vitro fertilization (IVF), to assess the pro or anti oxidative status and the effects of the administration of antioxidants. The substances in follicular fluid were all significantly lower than those in serum, both in the presence or absence of antioxidants. In conclusion, the intensity of peroxidation in the Graafian follicle is much lower than that in serum. This gradient is the result of the lower rate of initiation of peroxidation in the follicular fluid due to, probably, the presence of efficient antioxidant defense systems in the direct milieu of the oocyte before ovulation [80].

The role of ROS and antioxidant enzymes was provided using immunohistochemical localization, mRNA expression, and thiobarbituric acid methods that suggested a complex role in ovulation and luteal function in the human ovary [80]. Oxidative stress has been shown to affect the midluteal corpus luteum and steroidogenic capacity both in vitro and in vivo. In a very interesting study, using corpora lutea collected from pregnant and nonpregnant patients, it was observed that during normal situations, Zn-SOD expression parallels the levels of progesterone, with a rise from early luteal to midluteal phase and decrease during regression of the corpus luteum. The mRNA expression, however, of Cu, Zn-SOD in the corpus luteum during pregnancy was much higher than those of midcycle corpora lutea. This factor enhanced SOD expression during pregnancy, possibly caused by increased human chorionic gonadotropin (HCG) levels, and may be the cause of apoptosis of the corpora lutea. Similarly, the antioxidant enzymes glutathione peroxidase and MnSOD are considered the markers for cytoplasmic maturation, as these are expressed only in metaphase II oocytes [6]. Decreased developmental potential of oocytes from poorly vascularized follicles has also been attributed to low intrafollicular oxygenation [8]. Studies demonstrate intensified lipid peroxidation in the preovulatory Graafian follicle and that glutathione peroxidase may help in maintaining low levels of hydroperoxides inside follicle, suggesting an important role of oxidative stress in ovarian function. Oxidative stress and inflammatory process have roles in the pathophysiology of polycystic ovarian disease and drugs such as Rosiglitazone maybe effective by decreasing the levels of oxidative stress [81].

Two groups have developed Cu, Zn-SOD null mice, and both groups reported that the female mice were subfertile; however, the mechanistic basis for the reduced fertility of female Cu, Zn-SOD null mice remains unclear. [82] reported that ovaries of adult female Cu, Zn-SOD null mice had reduced numbers of preovulatory follicles and corpora lutea. They concluded that these mice were subfertile because of a defect in late follicular development or ovulation. In contrast, [83] reported that Mn-ZnSOD null female mice had normal ovarian histology and

ovulated similar numbers of ova during a natural estrous cycle but displayed increased postimplantation embryonic lethality. Perhaps the different genetic backgrounds of these two Cu, Zn-SOD knockout models accounts for these different findings. A study by [84] on copper chaperone for superoxide dismutase null mice, which have decreased ability to incorporate copper into Mn-ZnSOD, found a similar phenotype as [85], with abnormal development of antral follicles and no corpora lutea. Taken together, the evidence seems to support a role for Cu-ZnSOD in antral follicle development. Cu, Zn-SOD knockout is lethal prior to puberty. However, transplantation of ovaries from Mn-SOD knockout juvenile mice to the ovarian bursa of wild-type mice, in which the ipsilateral ovaries had been removed and the contralateral oviducts had been cut, resulted in all stages of follicular development, ovulation, and fertility, suggesting that this enzyme is not critical for ovarian function.

Superoxide, hydrogen peroxide and lipid peroxides are generated in luteal tissue during natural and prostaglandin-induced regression in the rat, and this response is associated with reversible depletion of ascorbic acid. ROS immediately uncouple the luteinizing hormone receptor from adenylate cyclase and inhibit steroidogenesis by interrupting mitochondrial cholesterol transport. The cellular origin of oxygen radicals in regressing corpora lutea is predominantly from resident and infiltrated leukocytes, especially neutrophils. ROS are also produced within the follicle at ovulation and, as the corpus luteum, leukocytes are the major source of these products. Antioxidants block the resumption of meiosis, whereas the generation of reactive oxygen induces oocyte maturation in the follicle. Although oxygen radicals may serve important physiologic roles within the ovary, the cyclic production of these damaging agents over years may lead to an increased cumulative risk of ovarian pathology that would probably be exacerbated under conditions of reduced antioxidant status [87].

Melatonin appears to have some kinds of functions at different stages of follicle development, oocyte maturation, and luteal stage. Melatonin concentration in the growing follicle may be an important factor in avoiding atresia, because melatonin in the follicular fluid reduces apoptosis of critical cells. Melatonin also has protective actions during oocyte maturation reducing intrafollicular oxidative damage. An association between melatonin concentrations in follicular fluid and oocyte quality has been reported. In the ovarian follicle, melatonin impacts the function of numerous cells, especially granulosa cells and the ovum (oocyte). The actions of melatonin in these cells are mediated via membrane receptors and also possibly via binding sites in the nucleus and in the cytosol. In addition to its receptor-mediated actions, melatonin also functions as a direct free radical scavenger to reduce oxidative stress at the level of the ovary; this beneficial action is carried out without an interaction with a receptor. Additional antioxidant functions of melatonin are achieved when the indole stimulates enzymes which metabolize free radicals to less toxic products. The antioxidative enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in thecal cells, granulosa cells and in the follicular fluid. Via these actions, melatonin reduces free radical damage, which would be especially bad for the ovum, and maintains these elements in an optimally functional state. The origin of melatonin in the follicular fluid is the blood and from its local synthesis in granulosa cells [87-89].

8. Assisted Reproductive Techniques (ART) and ROS

Assisted reproductive techniques (ART) are advanced technological procedures, which are the treatments of choice in many cases of female and male infertility or assisted fertilization, included the use of medical techniques, such as drug therapy, artificial insemination, or in vitro fertilization, to enhance fertility. Expanded ART include any directed action taken by humans to enhance reproduction in animals, both through 1) Assisted reproduction with a technical component (mostly mammals), 2) assisted reproduction using various forms of population management. The two are not mutually exclusive.

ART include:

1. Artificial Insemination
2. Embryo transfer
3. In vitro fertilization
4. Semen/embryo sexing
5. Intra cytoplasm sperm injection (ICSI)
6. Gamete/embryo micromanipulation
7. Somatic cell nuclear transfer (SCNT)
8. Genome resource banking.

They function, in humans, as an alternative to overcome causative factors of infertility, such as endometriosis, tubal factor infertility, male factor infertility. They can be used in the veterinary field also [90]. ART, in fact, were recently accepted into the programs for the safeguard of endangered species from extinction [90-93]. In a feasible program it is necessary proceed in the following five steps: 1) Technique development in a domestic animal counterpart, if available; 2) characterization of species-specific reproductive biology in a targeted non-domestic animal; 3) assessment of technique feasibility for producing offspring; 4) demonstration of adequate efficiency for applied usage; 5) application of new tool for population management [90] Figs 4, 5, and 6 show cumulus oocyte complexes (COCs) from mare explanted ovaries: these tools are employed in ART to have genetic improvement in horses,

Oxidative stress is involved in ovarian physio-pathology caused by diverse stimuli caused by several chemical and physical agents: ROS are involved in initiation of apoptosis in antral follicles in the fluid follicular environment, influencing the folliculogenesis and the steroidogenesis. ROS may play a role in these smaller follicles as well. Oxidative damage to lipids in the oocyte has been implicated as a cause of persistently poor oocyte quality. Developing germ cells in the fetal ovary have also been shown to be sensitive to toxicants and ionizing radiation, which induce oxidative stress. Recent studies have begun to elucidate the mechanisms by which ROS mediate ovarian toxicity. It has been investigated the role of antioxidant enzymes, such as catalase, glutathione peroxidase and the SOD isoforms in maintaining low levels of oxidative stress [46]. It was demonstrated for the first time by [94] that high oxygen concen-

tration compromises nuclear maturation rates and worsens the oxidative stress during in vitro maturation (IVM) of canine oocytes.

Incubated oocytes showed severely high quantities of superoxide dismutase (SOD), glutathione reductase (GSR), glutathione peroxidase (GPX1) and catalase (CAT) mRNA and this effect results in a protective mechanism against oxidative stress [95].

[45] studied the effect of ovary transport media supplementation with SOD on ovarian cell viability and apoptosis and in vitro embryo production (IVEP). They proposed, as mechanism of action, the intervention of SOD in inactivating the atmospheric O₂, potential deleterious precursor of free radicals.

With IVF, sperm-oocyte interaction occurs in culture media, leading to fertilization [32]. Reactive oxygen species may develop as a consequence of increased oocyte number per dish, spermatozoa, and cumulus cell mass. Cumulus cells demonstrate higher antioxidant activity at the beginning of culture than denuded oocytes do [96]. In ICSI, a single sperm is injected into an oocyte's cytoplasm [142]. It bypasses natural selection, thus allowing for the injection of damaged spermatozoon into the oocyte. Alternatively, the IVF process prevents fertilization by DNA-damaged spermatozoa [97].

Recently, OS has been identified as an important factor in ART success. Oocyte metabolism and a lack of antioxidants combined with the follicular and oviductal fluid of the embryo causes an increase in ROS levels [384]. Follicular fluid is the net result of both the transfer of plasma constituents to follicles and the secretory activity of granulosa and theca cells [385]. The oocyte develops within the FF environment and this intimately affects the quality of oocytes and their interaction with sperm, thus affecting implantation and embryonic development [98]. Oxidative stress contributes to oocyte quality, and its degree can be assessed by biomarkers of lipid peroxidation [99]. The effects of OS may be further altered by environmental factors. A hyperoxic environment augments SO radical levels by promoting enzyme activity. Particularly in IVF, increased incubation time heightens exposure to O₂ concentration [100]. As in biological systems, metallic cations act as exogenous sources of OS by stimulating ROS formation in ART culture media, and metal chelators such as EDTA and transferrin can ameliorate the production of ROS [43]. Furthermore, visible light can cause ROS formation, thereby damaging DNA [101]. Fertilization success in ART is determined by the quality of spermatozoa involved [32]. Although ROS contribute to normal sperm functions such as oocyte fusion, capacitation, and acrosome reaction, OS produced by spermatozoa may provoke oxidative damage to the oocyte, decreasing the likelihood for fertilization [81].

The in vitro environment exposes gametes and embryos to an excess of ROS with the absence of enzymatic antioxidant protection normally present during in vivo fertilization and pregnancy. Free radicals are thought to act as determinants in reproductive outcomes

due to their effects on oocytes, sperm, and embryos [95]. Oxidative stress disturbs human oocyte intracellular Ca²⁺ homeostasis as well as oocyte maturation and fertilization. During ovulation, ROS are produced within the follicles, however, the excessive production of ROS may increase the risk for poor oocyte quality since oxidative stimulation promotes oocyte maturation and wall rupture within the follicle [390]. A physiologic amount of ROS in follicular

fluid is indicative of a healthy developing oocyte [102]. In vitro fertilization can disturb the oxidant-antioxidant balance, rendering the culture media less protected against oxidation. The adverse effects of sustained OS and resulting loss of oocyte antioxidant content were shown to be improved by adding lipophilic and hydrosoluble antioxidants to the culture media to lessen OS [103]. Oral vitamin and mineral supplementation have been shown to increase serum concentrations of GSH and vitamins C and E; these antioxidants have been suggested to play a significant role in IVF outcomes [104].

Much research on IVEP has focused on the damaging effects of an oxidative environment and the inherent creation of reactive oxygen species that may impair embryo development. There are evidences that endoplasmic reticulum (RE) is significantly less reducing, consequently, excessive supplementation of reducing agents in media to offset oxidative damage has resulted in controversial outcomes as slight redox imbalances are detrimental for embryo development [73]. Conversely, an excess of ROS produced without sufficient antioxidant protection may lead to disequilibrium of the redox balance versus oxidative stress characterized by damaging DNA, RNA, protein and lipids [74]. Studies have been performed under high and low oxygen tension conditions and have resulted in controversial findings. Studies using antioxidants on swine model, indicated that the effect of the combination of GSH, β -ME and cysteine on embryo development. Treatment groups had a greater number of developing embryos than the control and the favorable result depended on the high O₂ culture conditions were used [105].

In contrast, guaiazulene (a component of various chamomile species with antioxidant properties) had no positive effect on embryo development under low oxygen tension (5 % O₂) [106]. Furthermore, [94] found that low oxygen gas composition improves nuclear maturation rates and alleviates the oxidative stress for canine oocytes during in vitro maturation.

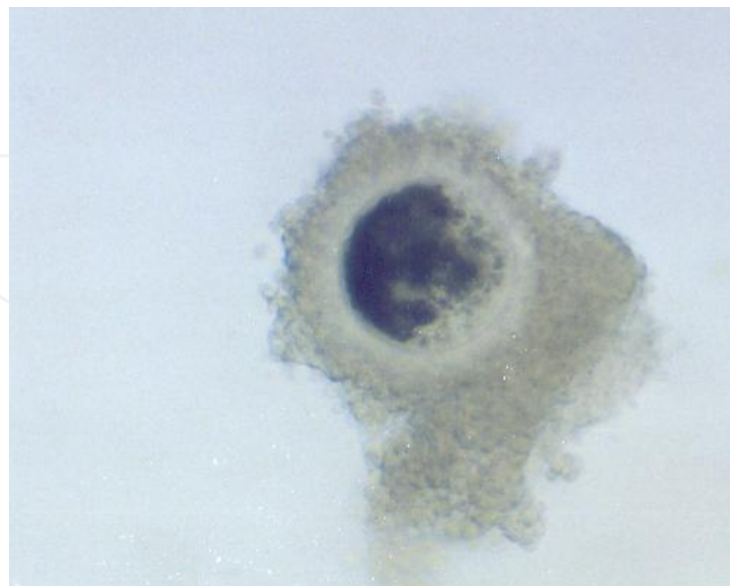


Figure 4. Cumulus Oocyte Complexes (COCs) of Pre Antral Follicle from explanted mare ovaries. Ooplasm bipolarisation with a dark and a clear portion (ptical microscope, 100x)

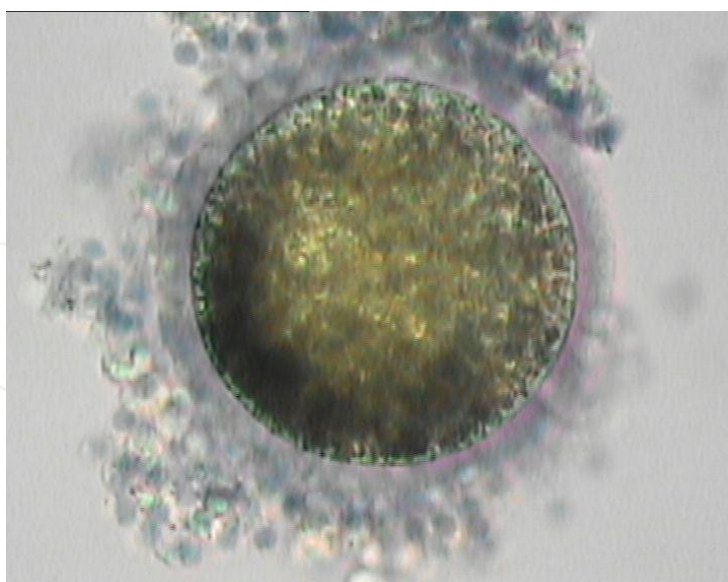


Figure 5. Cumulus Oocyte Complexes (COCs) of Pre Antral Follicle from explanted mare ovaries. COC stained with 5-carboxyfluorescein diacetate (cFDA) and trypan blue (with unviable cumulus cells and viable oocyte) (optical microscope 200x).

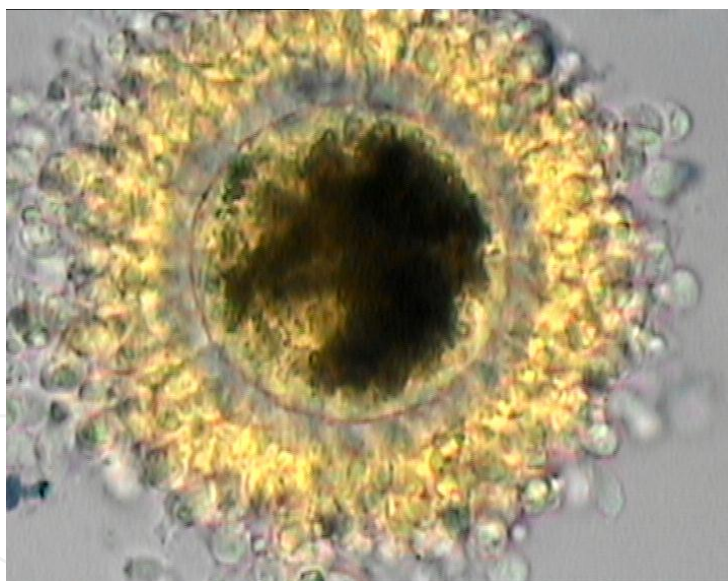


Figure 6. Cumulus Oocyte Complexes (COCs) of Pre Antral Follicle from explanted mare ovaries. Viable COC stained with 5-carboxyfluorescein diacetate (cFDA) and trypan blue (optical microscope 200x)

9. Conclusions

Oxidative stress has been extensively studied for about four decades. Substantial progress has been achieved to date – from descriptive characterization of this process to delineation of

molecular mechanisms underlining adaptive responses and targeted manipulations of expected responses. In recent years, the importance of ROS synthesis in ovarian functions has been established also. Several data have recently shown that reactive oxygen species can regulate cell function by controlling production or the activation of substances that have biological activities. It has been suggested that ROS under moderate concentrations play a role in signal transduction processes involved in growth and protection from apoptosis. Conversely, increase of ROS levels is primarily responsible for the alteration of macromolecules, such as lipids, proteins and nucleic acids, that lead to significant damage of cell structures and thereby cause oxidative stress. Oxidative damage to lipids in the oocyte has been implicated as a cause of persistently poor oocyte quality after early life exposure to several toxicants. Developing germ cells in the fetal ovary have also been shown to be sensitive to toxicants and ionizing radiation, which induce oxidative stress. Recent studies have begun to elucidate the mechanisms by which ROS mediate ovarian toxicity. To prevent damage due to ROS, cells possess a number of nonenzymatic and enzymatic antioxidants. that include Vitamin C, glutathione, cysteamine, vitamin E, superoxide dismutases (SOD1, SOD2, and SOD3), glutathione peroxidase, and catalase. Intracellular homeostasis is ensured by the complex interactions between pro-oxidants and antioxidants. The bulk of evidence in support of therapeutic effects of antioxidants to date, has been observed through experimental studies on animals and humans ART, whose aim is depth knowledge of human reproductive functions, conservation of species in danger of extinction, and acceleration of life cycles using reproduction for purposes of genetic and productive.

In the future, the hope is to clarify the efficacy of antioxidants as potential therapies for infertility and in ART the use of specific antioxidants to improve multiple physiological processes from oocyte maturation to fertilization, embryo development and pregnancy.

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