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The Impact Of Myeloperoxidase And Its Related Oxidants On Metaphase Ii Mouse Oocyte Quality

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THE IMPACT OF MYELOPEROXIDASE AND ITS RELATED OXIDANTS ON METAPHASE II MOUSE OOCYTE QUALITY

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DEDICATION

I devote my thesis to my precious parents, Nuri and Sabah, my amazing husband, Osama, and my two adorable sons, Tamim and Jehad, for their unremitting support and genuine love.

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LIST OF ABBREVIATIONS

MPO	myeloperoxidase
ROS	reactive oxygen species
OS	oxidative stress
MLT	melatonin
H_2O_2	hydrogen peroxide
$^{\bullet}OH$	hydroxyl radical
$O_2^{\bullet -}$	superoxide
HOCl	hypochlorous acid
Cl^-	chloride
Fe(II)	ammonium ferrous sulfate
MLT	melatonin
AFMK	N^1-acetyl-N^2-formyl-5-methoxykynuramine
COC	cumulus oocyte complex
MII	metaphase II mouse oocyte stage
MT	microtubules morphology
CH	chromosomal alignment
CCs	cumulus cells
Cx	connexin
HPLC	High performance liquid chromatography analysis

CHAPTER 1

INTRODUCTION

Poor oocyte quality may be the root cause of infertility or poor reproductive outcomes [1-4]. Oocyte deterioration results from oxidative stress mediated by enhancement of macrophage activity in areas with inflammation, which is known to be involved in the pathogenesis of different fertility disorders [2, 5-13]. The deleterious actions of activated macrophages, the major source for reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and hypochlorous acid (HOCl), as well as the pro-inflammatory enzyme myeloperoxidase (MPO), are secondary to their ability to migrate to any site in the female genital tract such as fallopian tubes, uterine cavity, and peritoneal cavity as well as their cellular effects at the level of the oocyte [8, 14-28]. Indeed, macrophages have been found in the cumulus mass surrounding oocytes retrieved from normal healthy woman [23, 26]. Several studies have demonstrated that non-activated macrophages excrete growth factors which benefit the embryo growth [29]. However, once activated, macrophages have detrimental effects on embryo development as well as embryonic pre-implantation, either through enhancement of inflammatory pathways or through ROS overproduction [2, 30-33]. Higher levels of MPO and ROS, as well as the deficiency of antioxidants such as glutathione and ascorbate, have been found in the peritoneal fluid and follicular fluid of women suffering from chronic inflammatory diseases such as endometriosis, polycystic ovary syndrome (PCOS), chronic pelvic infection and unexplained infertility,all of which are associated with poor reproductive outcomes [31, 34-43].

One of the main factors contributing to poor reproductive outcomes is decreased oocyte quality [4, 5, 44-46]. Metaphase II (MII) oocytes are known to be exposed to some ROS during ovulation [47-50], however increased levels may impair their proper fertilization, development, maturation and compromise the genetic material [1, 2, 7]. The major functional parameters used

to assess oocyte quality are spindle microtubule morphology (MT), chromosomal alignment (CH) [5, 8, 11, 51] and organization of the cumulus oocyte complex (COC) [5, 44, 52] as it has been established that they can be affected by changes in the oocyte microenvironment such as increased ROS [5, 52]. In addition to impairment of oocyte quality, overproduction of ROS can cause damage to the cellular proteins, lipids, and DNA [5, 53, 54]. The toxicity of ROS depends in part on their bioavailability, rate of production, stability, and the bioavailability of antioxidants [5, 8, 55-58].

The generation of reactive oxygen species

Once macrophages are triggered by pro-inflammatory signals, the oxidase activity of NADPH oxidase, a plasma membrane enzyme from triggered macrophage cells, begins to generate $O_2^{\bullet-}$ [5, 8, 19, 59-61]. Superoxide generates H_2O_2 as an end product non-enzymatically, or through superoxide dismutase-catalyzed reaction [19, 59, 62]. The physiological intracellular lifetime of H_2O_2 is moderately long and it is the precursor of the more toxic $HO^{\bullet}$ through a well-known Fenton reaction ($Fe^{2}+ + H_2O_2 + H^{+} \rightarrow Fe^{3+} + HO^{\bullet} + H_2O$) [5, 8, 14, 15, 63, 64]. Hydrogen peroxide is freely diffusible through biological membranes [55, 65], and its overproduction is extremely destructive to cells and tissue [5, 64, 66-68]. Hydrogen peroxide reacts with MPO, which is abundantly expressed in macrophages, in the presence of chloride (Cl^{-}) to generate the toxic oxidant HOCl [19-21, 56, 69]. Hypochlorous acid can, in turn, destroy the MPO heme moiety causing free iron release, which propagates more oxidant formation such as $HO^{\bullet}$ [16, 70].

Effect of reactive oxygen species on oocyte-cumulus cells complex

Translational research has shown that patients with inflammatory conditions have higher rates of adverse reproductive outcomes through poorer oocyte quality compared to those without, suggesting that ROS enhancement may be central to the decrease in fertility potential [2, 4, 5, 71,

72]. Indeed, ROS have been shown to decrease the density of the cumulus cells surrounding the oocyte, as well as induce cumulus cells apoptosis, which disturbs the defensive antioxidant machinery of the cumulus oocyte complex [3, 52, 73]. A variety of antioxidant treatments have been studied to mitigate the negative effects of the toxic oxidants [51, 74-77]. Further research has suggested that melatonin (MLT), a potent MPO inhibitor and HOCl scavenger, may function to protect oocyte quality against oxidative stress (OS) [51]. Since the underlying factors in the balance between damaging oxidants and innate and exogenous protective mechanisms in the oocyte remain poorly understood, the hypothesis of this dissertation is that ROS and specifically MPO related oxidants negatively affect oocyte quality, and that MLT may protect against these effects.

Myeloperoxidase (MPO) and its related oxidants

Myeloperoxidase, a heme-containing enzyme, is one of the major enzymes secreted upon activation of neutrophils and macrophages [19, 20, 69]. Its molecular mass is ~146 KDa [78]. It is composed of two identical subunits connected together by a single disulphide bridge, operating the structural design of the heme pocket (containing the heme prosthetic group), which is the catalytic site of the dimeric form of the enzyme [70, 78, 79]. Myeloperoxidase is considered as one of the major alternative pathways to generate ROS [8, 70]. The catalytic cycle of MPO is depicted in Figure 1. In this cycle, a ferryl π cation radical, Compound I (MPO–Fe (IV) = $O^{\bullet+\pi}$) is considered to be the first intermediate of the reaction of the MPO ferric form, MPO–Fe(III), with H_2O_2 in the presence of chloride (Cl^-). Compound I is converted back to MPO–Fe (III) with the concomitant

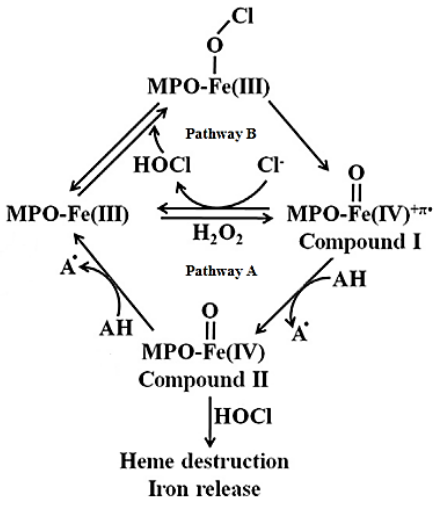

Figure 1: A working kinetic model for the competition between H_2O_2 and HOCl at the catalytic site of MPO during steady MPO state catalysis [70].

two-electron oxidation of Cl^- to HOCl [19, 21, 56, 80]. Alternatively, in the absence of Cl^-, Compound I is converted back to MPO–Fe(III) through a two-step one-electron (1e−) oxidation pathway involving organic or inorganic one-electron substrates such as MLT [18, 81, 82]. Compound II is the catalytically inactive form of the enzyme and the long-lived intermediate in the cycle [70]. It can execute only 1e− oxidation reactions. Thus, formation of HOCl is not possible with this intermediate [70]. During inflammation, the amount of HOCl produced from activated neutrophils is around 150−425 μM HOCl/hr, whereas at sites of inflammation, the HOCl level is estimated to be as high as 5 mM [83]. Under these circumstances, HOCl can damage the host tissue by the same mechanism used to destroy invading pathogens [19, 20, 83].

Myeloperoxidase, a source of free iron

Recent studies by Maitra *et. al.* have demonstrated that MPO may be regulated by feedback inhibition via HOCl [70]. The accumulation of the released HOCl in the solution mixture permits the competition with H_2O_2 on the catalytic site of MPO, which in this case is the heme prosthetic group resulting in heme destruction and free iron release [70]. It has been established that higher MPO activity and HOCl levels, combined with the higher free iron that exists in peritoneal fluid of chronic inflammatory diseases such as in advanced stages of endometriosis can set the stage for generation of a more toxic molecule, •OH through a known Fenton reaction mechanism [8, 39, 84]. The toxicity of HO• and HOCl are mainly due to their capacity to participate in serious pathological consequences through cellular mitochondria poisoning, lipid peroxidation, and uncoupling of oxidative phosphorylation [14, 85-87]. The wide range of MPO properties strongly suggests that MPO is one of the main factors that contribute to deteriorate oocyte quality under oxidative stress environment. The above information draws our attention to use MLT as a potent MPO inhibitor and antioxidant scavenger to overcome the detrimental effect of oxidative stress on oocyte quality.

Melatonin

Melatonin, a known pineal hormone to regulate circadian rhythms, is secreted in most mammalian tissues including the female reproductive tissues such as ovary and follicular fluid [76, 77, 88-94]. It has been shown to have multiple properties specifically in the antioxidants field compared to other antioxidants [76, 91, 95]. Melatonin has not only a unique ability to significantly increase the enzymatic antioxidant gene expression and levels of Cu, Zn-superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT) [76, 77, 90, 95, 96], but also scavenges and /or prevents the formation of a variety of ROS such as HOCl and •OH, and inhibits pre inflammatory enzymes such as MPO [16, 81] and thus preventing the oxidation of lipids and proteins as well as DNA damage [16, 81, 91]. Not only is MLT characterized by the broad spectrum ability to scavenge ROS but its metabolites such as cyclic 3-hydroxmelatonin, N(1)-acetyl-N(2)-formyl-5-methoxykynuramine (AFMK) and N(1)-acetyl-5-methoxykynuramine (AMK) also exhibit multi-antioxidant properties and have no biologically harmful consequence similar to MLT [76, 96-103]. Melatonin scavenges HOCl like methionine and taurine but in a fundamentally different way [104]. Taurine binds with HOCl to form toxic taurine chloramines [105]. Taurine chloramine is a less active oxidant that oxidizes thiols and heme proteins, which extend the reactivity of HOCl [105-107]. Furthermore, MLT, unlike other HOCl scavengers, displays a high affinity towards transition metal binding (e.g. iron (III), copper and zinc), and subsequently reduces their cytoplasmic availability, thus preventing more ROS generation mediated by Fenton reaction [108-110]. The capacity of MLT to scavenge •OH is greater than other antioxidants such as vitamin E and mannitol [76, 96, 103, 111].

Along with melatonin's antioxidants properties, melatonin has shown distinguished anti-inflammatory effects when it is administered either exogenously in-vivo or when added to cultured cells via regulation of cellular pathways [51, 91, 94, 112-117]. Melatonin has the ability

through its action on 2 G-protein coupled receptors, MT1 and MT2 to suppress many different inflammatory cell types including macrophage cells [114, 115, 117]. Thus, different doses of synthetic MLT have been used in medical scenarios in which inflammation plays a role such as a weak immune system due to stress, oxidative hemolysis, and even cancer progression [116, 117].

Melatonin a potent inhibitor of myeloproxidase

Melatonin has the ability to inhibit MPO chlorinating activity at two different points [16, 81]. First, it binds to MPO to form the inactive melatonin-MPO-Cl complex [16]. Under these circumstances the MPO catalytic activity is limited by the dissociation of melatonin from the complex [16, 81]. Second, it competes with Cl^- by serving as a 1e- substrate for MPO Compounds I and II [16, 81]. Under these conditions, MPO maintains its ability to consume H_2O_2 without the generation of HOCl [16, 56, 81]. Thus, understanding this unique function of melatonin on MPO activity may represent a new therapeutic a treatment support to control the inflammatory pathways where MPO is elevated. In addition, melatonin prevents HOCl-mediated heme destruction and subsequent free iron release for most hemoprotein compounds including MPO [16].

Melatonin's role in female reproduction

Melatonin has been found in the follicular environment surrounding the oocyte [76, 77, 88-94], which could be the result of the pumping activity of channels located in the follicular wall; therefore, its antioxidant properties could benefit the oocyte. Clinical prospective trials have found an increase in the pregnancy rate for a group of women suffering from PCOS when their treatment regimen was supplemented with MLT compared to a group who did not receive melatonin [77, 118, 119]. Clear direct evidence linking MLT supplementation with oocyte quality improvement have recently been provided through the demonstration that a significant decrease in the ROS level improved oocyte maturation in oocyte media supplemented with MLT

compared to those without MLT [91, 120-122]. Furthermore, MLT (1mM) supplemented media of murine oocytes collected 8 hours after ovulation showed a significant increase in the percentage of fertilization [123]. Recently, it has been shown that MLT has the ability to protect the spindle's morphology when exposed to higher concentrations of HOCl [51].

Microtubule and chromosomal alignment marker for oocyte quality

Several markers for oocyte quality have been used to evaluate deterioration in the postovulatory MII oocyte quality using in-vitro studies such as zona pellucid a dissociation time (ZPDT), oocyte microtubular dynamics (OMD), and cortical granule (CG) degradation, as oocyte aging markers [4, 5, 44]. Oocytes at MII phase, the arrested stage of ovulated oocyte before fertilization, demonstrated a dense array of filaments with bundles forming the meiotic spindle [124-126]. Moreover, this is the stage where the meiotic spindles are attached with the chromosomes at the equator of the spindles [124-127].

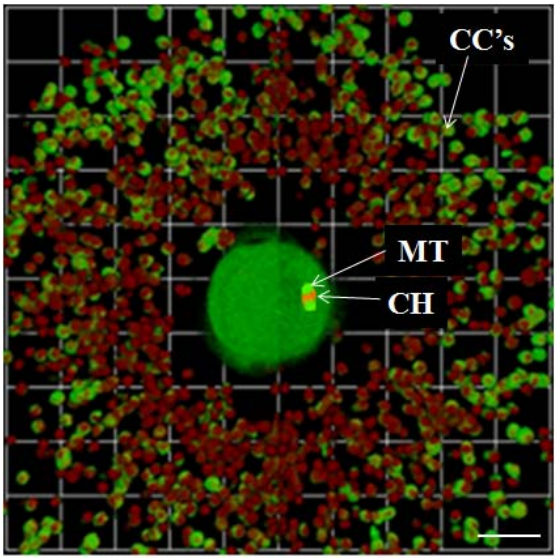

Figure 2: Image of cumulus oocyte complex (COC) obtained using Confocal Zeiss LSM 510 META NLO microscope. Control COC with normal microtubule (MT) and chromosomal (CH) alignment with a good organization of cumulus cells (CC's) surrounding the oocyte. Scale bars: 1 pixel, 3 mm. Results depict observations made after three experiments.

In mammals, the oocyte is typically surrounded by numerous layers of cumulus cells (CC's), derived from the granulosa cells (GCs), forming the COC (cumulus oocyte complex) [23, 73, 128] (Figure 2). Their presence in sufficient numbers and organization may serve as a predictive marker for oocyte quality [23, 73, 128]. The cumulus cells provide several important functions to the oocyte such as protection, and nutrient delivery through their gap junction proteins channels, a network of intercellular membrane channels mediating

communication within the COC [23, 73, 129-131]. The oocyte, under pathological conditions, protects itself against the toxicity of ROS through enzymatic (e.g., catalase and glutathione peroxidase) and nonenzymatic antioxidants (e.g., ascorbic acid and reduced glutathione) provided by the surrounding CC's [132, 133]. Increases in the rate of fertilization and early embryo development have been found when the human oocyte or the embryo had been cultured in the presence of cumulus cells [134]. Therefore, derangements in COC organization, cumulus cell number, and cumulus-cumulus cell interaction through staining of the gap junction protein, connexin 43, were the parameters used to determine the mechanism of oocyte quality deterioration secondary to direct ROS insult.

Despite the structural simplicity of COC, preserving the intact communication between the oocyte and its surrounding CCs is important to the oocyte to withstand the unstable environment, namely OS, which may negatively impact oocyte quality [52, 135].

In this study we focus on the use of meiotic spindle structure, MT and CH, as an important oocyte quality marker because they are essential for proper oocyte division and subsequent embryo formation [11, 136, 137]. Moreover, spindle disruption will lead to improper CH segregation leading to an imbalance in the genetic material of the daughter nucleus with detrimental effect on fertility as well as in assisted reproduction outcomes [137]. We and others have developed a 1-4 scoring system depending on the oxidative damage effect on the meiotic spindle structure, MT and CH, as an important oocyte quality marker (Figure 3) [8, 11, 51, 52]. Briefly, a good spindle configuration was coded for (score 1, 2) where

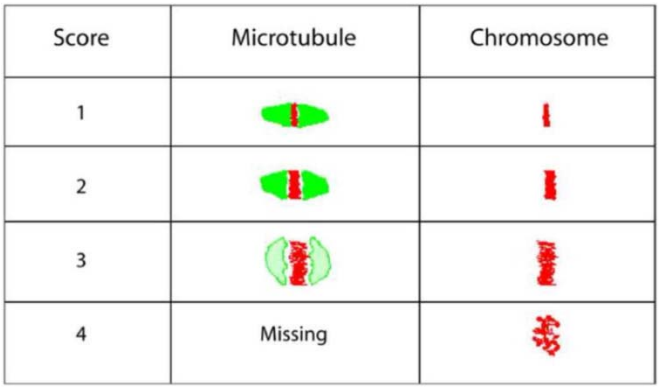

Figure 3: Schematic diagram demonstrating scoring system of MT and CH alteration based on previous study by Choi *et al* and Banerjee *et al* [8, 11, 51]

microtubules were organized in a barrel-shape, whereas, abnormal or poor (score 3, 4) were for spindle length reduction, disorganization and complete absence of spindle. Chromosomal configuration was considered as good (score 1, 2) when chromosomes are normally arranged at the equator of the spindle, while poor (score 3, 4) when the chromosomes are dispersed or show aberrant or less condensed appearance [8, 11, 51, 52]. Studying oxidative stress-mediated spindle structural damage could serve as a feasible approach for further assessments to search for the therapeutic options to preserve the good quality for the oocyte under inflammatory states.

Aims of dissertation:

1) Determine the direct effect of ROS on metaphase II mouse oocyte quality
2) Determine the effect of MPO (purified and naturally secreted from activated macrophages) related oxidants on metaphase II mouse oocyte quality and whether melatonin can protect against this effect.
3) Define the mechanism of melatonin protection.

CHAPTER 2

THE DEFENSIVE ROLE OF CUMULUS CELLS AGAINST REACTIVE OXYGEN SPECIES INSULT IN METAPHASE II MOUSE OOCYTES

(This chapter contains previously published material. See Appendix A)

Abstract

We investigated the ability of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and hypochlorous acid (HOCl) to overcome the defensive capacity of cumulus cells, and elucidate the mechanism through which ROS differentially deteriorate oocyte quality. Metaphase II mouse oocytes with (n=1634) and without cumulus cells (n=1633) were treated with increasing concentration of ROS and the deterioration in oocyte quality was assessed by the changes in the microtubule morphology (MT) and chromosomal alignment (CH). Oocyte and cumulus cell viability and cumulus cell number were assessed by indirect immunofluorescence, staining of gap junction protein, and trypan blue staining. The treated oocytes showed decreased quality as a function of increasing concentrations of ROS as compared to controls. Cumulus cells show protection against H_2O_2 and $^{\bullet}OH$ insult at lower concentrations, but this protection was lost at higher concentrations ($\geq$50 μM). At higher H_2O_2 concentrations treatment dramatically influenced the cumulus cell number and viability with resulting reduction of the antioxidant capacity making the oocyte more susceptible to oxidative damage. However, cumulus cells offered no significant protection against HOCl at any concentration used. In all circumstances in which cumulus cells did not offer protection to the oocyte, both cumulus cell number and viability were decreased. Therefore, the deterioration in oocyte quality may be caused by one or more of the following: a decrease in the antioxidant machinery by the loss of cumulus cells, the lack of scavengers for specific ROS, and/or the ability of the ROS to overcome these defenses.

Introduction

Oocytes are surrounded by tightly packed, highly organized layers of cumulus cells (CCs) that exist in spatial and temporal heterogeneity, forming the cumulus-oocyte complex (COC) [23, 73, 128]. In addition to their role in providing nutrition to the oocyte, the CCs provide a communication network between the oocyte and its extracellular microenvironment [23, 73, 129, 130]. The manner through which CC's communicate within the COC are specialized connections called gap junctions, which are aggregates of protein-based intercellular channels that directly connect adjacent cells, allowing the bi-directional movement of molecules [131]. These channels known as connexins (Cx) have been described in many tissues; however Cx 37 is the only one identified on the oocyte, and forms connection with CC's, while Cx 43 is the main gap junction protein found on CC's. Loss of Cx proteins has been attributed to disrupted folliculogenesis, CC dysfunction, and altered cell and tissue viability [131, 138-140]. Therefore, Cx's are critical as they function as primary means of disseminating information to and from the oocyte, and also serve the important function of anchoring the CC's within the COC to protect the oocyte. It is known that oocytes, under pathological conditions, protect themselves against the toxicity of reactive oxygen species (ROS) through a scavenging enzymatic (e.g., catalase and glutathione peroxidase) and nonenzymatic antioxidant (e.g., ascorbic acid and reduced glutathione) network provided by the surrounding CC's [132, 133]. Translational research has shown that patients with these conditions have higher rates of adverse reproductive outcomes and poor oocyte quality compared to those without such disorders suggesting that inflammation may be central to the decrease in fertility potential [2, 4, 5, 71, 72].

Reactive oxygen species such as superoxide ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) are highly disruptive to cellular function [5, 8, 51]. The major intracellular sources of H_2O_2 are spontaneous production, superoxide dismutase-

catalyzed reaction of $O_2^{\bullet-}$ [62, 141], generation through the mitochondrial electron transport chain, and the NADPH oxidase system in the cellular plasma membrane [5, 66, 142]. Hydrogen peroxide is found in physiologic concentrations ranging from 10-20 μM and up to 100 μM in pathologic circumstances [143]. Exposure of mouse oocytes to higher H_2O_2 concentrations (200 μM) completely inhibited cleavage, and caused arrest of the zygote at the 1-cell stage [144]. A link between the concentration of endogenous H_2O_2 and the occurrence of apoptosis in human embryos has been suggested [144-147]. There are other ways that H_2O_2 can indirectly affect oocyte quality, for example, we have recently shown that $^{\bullet}OH$ generated by the H_2O_2-induced Fenton reaction caused instantaneous oocyte damage, which has been estimated indirectly in plasma at levels of 250-500 μM/L [8, 148]. More recently we have demonstrated that the diffused intraoocyte H_2O_2 in the presence of chloride, which are normally found in the oocyte and oocyte microenvironment respectively, can trigger the catalytic activity of MPO generating the toxic oxidant, HOCl [58, 141], a substance known to deteriorate oocyte quality [51]. Activated neutrophils, the major cellular releaser of MPO, generate around 150−425 μm HOCl/hr, whereas at sites of inflammation, the HOCl level is estimated to reach as high as 5 mM [83]. Previously, it has been shown that higher levels of MPO exist in the peritoneal and follicular fluid of women with inflammatory conditions such as endometriosis [39, 43]. Macrophages, neutrophils and monocytes are the major cellular sources of MPO, which function to generate HOCl and other ROS [20]. The current study investigates the ability of the antioxidant system of the CC's to deflect the H_2O_2-mediated oxidative damage from mouse oocytes, and highlights the mechanism through which H_2O_2 deteriorates oocyte quality. Our results provide a previously undescribed mechanistic link between excess H_2O_2 accumulation and poor oocyte quality, namely, through the disassembly and decreased viability of the protective CC cloud, which allows the dismantling of the spindle and chromosomal alignment.

These effects may cause poor oocyte quality and thus poor reproductive outcomes, which are associated with various inflammatory conditions.

Materials and Methods

Materials

All the materials used were of the highest grade of purity and without further purification. Hydrogen peroxide, sodium hypochlorite (NaOCl), ammonium ferrous sulfate (Fe(II)), Human tubular fluid (HTF) media, anti-α tubulin antibody, FITC conjugate anti-goat antibody, propidium iodide, 1% BSA (Bovine Serum Albumin), 0.1% M Glycine, and 0.1% Triton X- 100 were obtained from Sigma–Aldrich (St. Louis, MO, USA). Normal Goat Serum (2%) was from Invitrogen (Grand Island, NY) and 0.2% Powder Milk from grocery. Metaphase II oocytes (with and without cumulus cells) from a B6C3F1 mouse crossed with a B6D2F1 mouse were obtained commercially (Embryotech Inc.) in cryopreserved straws using ethylene glycol-based slow freeze cryopreservation protocol. We used mature MII oocytes for the purpose of understanding the defense rendered by the cumulus cells at the mature cell stage level. Though it is understood that there may be some loss of antioxidant defense during the expansion as a physiologic mechanism for preparation for ovulation, we believe that pre-existence of intact gap junctions for delivery of antioxidant defense is essential for prevention of spindle damage during maturation process in inflammatory states. It is known that the oocyte spindle repolymerizes to normal structure when incubated in media for 60 -120 min at 37°C and 5% CO_2 [149, 150] prior to induction of oxidative stress. This mechanism actually has helped support utilization of frozen oocytes for studying spindle damage. The use of frozen-thawed oocytes is well accepted as many studies have been published in the past utilizing frozen thawed oocytes and effects of oxidative stress on spindle morphology [151, 152].

Methods

Metaphase II mouse oocytes with and without cumulus cells, in triplicate for each ROS experiment, were transferred from straws to phosphate buffer saline (Dulbeco's PBS) and washed to remove excess cryoprotectant for 3 minutes. Oocytes were then transferred to HTF media and incubated at 37º C and 5% CO_2 for 60 minutes to allow spindle repolymerization and attainment normal oocyte architecture [153, 154]. The oocytes were then screened for the presence of the polar body confirming their Metaphase II stage. Ten – twenty oocytes from each group were discarded as they were found to be immature or displayed disrupted zona pellucidas. In each ROS experiment metaphase II oocytes with and without cumulus cells were divided into three groups: H_2O_2, $^{\bullet}OH$, and HOCl treatments.

For H_2O_2 treatment (experiments performed in triplicate), oocytes were divided into four different groups; (group 1, n=620) oocytes without cumulus cells incubated with increasing concentrations of H_2O_2 (10, 17, 25, 50 and 100 µM); (group 2, n=611) Oocytes with cumulus cells incubated with increasing concentrations of H_2O_2 (10, 17, 25, 50 and 100 µM); (group 3, n=62) untreated oocytes with and (group 4, n=62) without cumulus cells.

For •OH treatment (experiments performed in triplicate), oocytes were divided equally into eight groups: oocytes with (group 1, n=171) and without (group 2, n=171) cumulus cells treated with increasing concentrations of H_2O_2 (5, 10, 20 µM); oocytes with (group 3, n=60) and without (group 4, n= 61) cumulus cells treated with a fixed concentration of Fe(II) (100 µM); oocytes with (group 5, n= 173) and without (group 6, n= 191) cumulus cells pre-incubated with a fixed concentration of Fe (II) (100 µM) and treated with increasing concentrations of H_2O_2 (5, 10, 20 µM) under these circumstances all H_2O_2 was converted to $^{\bullet}OH$; and untreated oocytes with (group 7, n= 60) and without (group 8, n= 73) cumulus cells. Due to the instability of $^{\bullet}OH$ and its instant effect on oocyte quality, all sets were exposed for less than 10 minutes. This short

incubation time also eliminates the effect of H_2O_2 and Fe(II) alone as previously described [8]. The concentration of Fe (II) (100 μM) used in the current study to establish the •OH generating system has been widely used in previous studies [155].

For HOCl treatment (experiments performed in triplicate), oocytes were divided into four different groups: (group 1, n=324) oocytes with cumulus cells and (group 2, n=391) oocytes without cumulus cells treated with increasing concentrations of HOCl (10, 25, 50 and 100 μM); and (group 3, n=73) untreated oocytes with cumulus cells and (group 4, n=64) untreated oocytes without cumulus cells. The treated and untreated oocytes were incubated with HOCl for 15 min to ensure maximum effect.

Immunofluorescence staining and fluorescence microscopy

All treated and untreated oocytes were fixed in a solution prepared from 2% formaldehyde and 0.2 % Triton X-100 for 30 minutes at 25° C [3]. The fixed oocytes were treated with blocking solution (PBS, 0.2% Powdered Milk, 2% Normal Goat Serum, 1% Bovine Serum Albumin (BSA), 0.1 M Glycine and 0.1% Triton X-100) for 30 minutes then washed with PBS for 3 minutes [3, 8]. Subsequently, the oocytes were subjected to indirect immunofluorescence staining by incubating in mouse primary anti-α tubulin antibody against the MT for 60 minutes and secondary fluorescein isothiocyanate (FITC) conjugated anti-goat antibody for 30 minutes [3]. The chromosomes were stained using propidium iodide (PI) and incubated for 15 minutes [3]. Stained oocytes were loaded into an anti-fade agent on slides with two etched rings and cover slips were affixed using nail varnish. The alterations in the MT and CH were compared with controls and scored by three blinded observers based on a previously published scoring system (Figure 2) [11, 126]. Scores of 1-4 were assigned for both MT and CH alterations, with scores 1 and 2 combined for good outcomes meaning microtubules were organized in a barrel-shaped with slightly pointed poles formed by organized microtubules

crosswise from pole to pole, and chromosomes were normally arranged in a compact metaphase plate at the equator of the spindle [11, 126]. Scores of 3 and 4 signified poor outcomes and consisted of spindle length reduction, disorganization and/or complete spindle absence, and chromosome dispersion or aberrant condensation appearance [11, 126]. Images were obtained utilizing both immunofluorescence and confocal microscopy.

Confocal microscopy, assessment of microtubules and chromosomal alignment

Slides were examined with the Axiovert 25 inverted microscope (Zeiss, Thornwood, NY) using Texas Red (red) and FITC (green) fluorescent filters with excitation and emission wavelengths of 470 and 525 nm, and 596 and 613 nm, respectively. Confocal images were obtained utilizing a Zeiss LSM 510 META NLO (Zeiss, Germany) microscope. Oocytes were localized using a 10 x magnification lens and spindle alterations assessed using 40 x oil immersion lens. The MT was stained fluorescent green, which was distinct from the fluorescent red staining of the chromosomes. Following completion of the experiments each oocyte was closely examined for spindle status by three independent observers blinded to the assigned treatment groups. Observers used comprehensive evaluation of the individual optical sections and the 3-D reconstructed images.

Viability assay (Measurement of COC viability):

We used oocytes with cumulus cells (n=100) exposed to 10 and 25 μM of each ROS before fixing followed by addition of 10 μL of trypan blue dye (Sigma) into the HTF media for 4 minutes. The untreated oocytes were also subjected to trypan blue dye to determine the number of viable cells (the dye exclusion test) in the media. This test is based on the fact that living cells possess intact cell membranes that will keep out certain dyes (trypan blue and propidium), whereas dead cells will not. Both control and exposed groups were examined under the Axiovert 25 light microscope and cumulus cells were counted for staining with images obtained.

Connexin 43 immunostaining and fluorescence confocal microscopy:

In this experiment, we grouped the metaphase-II mouse cumulus oocytes (n=30) in two sets in the HTF culture media, A) control cumulus oocytes, B) cumulus oocytes incubated with 100 μM H_2O_2 oocytes for 45 minutes. Then as mentioned before, oocytes were fixed in a solution prepared from 2% formaldehyde and 0.2 % Triton X-100 for 30 minutes. The fixed oocytes were treated with blocking solution as previously described for 1 hour, and then washed with PBS for 3-5 minutes. Subsequently, the oocytes were subjected to indirect immunostaining for connexin 43 (Cx 43) by incubating them in Monoclonal Anti-Connexin-43 antibody produced in mouse (1:100) ((C8093) from Sigma-Aldrich) over night at 4ºC follow by secondary FITC conjugated antigoat antibody (1:50) for 30 minutes (green color). The chromosomes were stained using propidium iodide (1:50) by incubating for 15 minutes to count the granulosa cells before and after H_2O_2 treatment. Stained oocytes were loaded into an antifade agent on slides with two etched rings and cover slips were placed using nail varnish.

Solutions preparation

The H_2O_2 solution was prepared fresh in phosphate buffer (PH 7.4), while the concentrations of the working solutions were determined spectrophotometrically (extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm) [156, 157].

The HOCl was prepared as previously described with some modifications [158]. Briefly, a stock solution of HOCl was prepared by adding 1 ml of NaOCl solution to 40 ml of 154 mM NaCl and the pH was adjusted to around 3 by adding HCl. The concentration of active total chlorine species in solution, expressed as $[HOCl]^T$ (where $[HOCl]^T = [HOCl]+[Cl_2]+[Cl_3^-]+[OCl^-]$) in 154 mM NaCl, was determined by converting all the active chlorine species to OCl^- by adding a bolus of 40 μl of 5 M NaOH and measuring the concentration of OCl^-. The concentration of OCl^- was determined spectrophotometrically at 292 nm (ε = 362

M^{-1} cm^{-1}). As HOCl is unstable, the stock solution was freshly prepared on a daily basis, stored on ice, and used within 1 hr of preparation. For further experimentation, dilutions were made from the stock solution using 200 mM phosphate buffer, pH 7.0, to give working solutions of lower HOCl concentrations [70]. During and after the preparation process, all solutions were kept on ice to minimize decomposition.

Statistical analysis:

Statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA procedures were performed to compare the percentage of oocytes with poor outcomes (scores 3 and 4) for MT and CH between controls and oocytes treated with various oxidants concentrations. No transformation was used because a good portion of the data ranged between 0.2 and 0.8. The Tukey's post-hoc procedure was used for pairwise comparisons among treatment groups. Statistical significance was indicated by $P< 0.05$. Independent *t*-tests were conducted to compare the cumulus and non-cumulus oocytes for each oxidant concentration.

Results

Effect of ROS on cumulus enclosed and denuded oocytes

The majority of both cumulus-enclosed and denuded control oocytes had good-scores (98% and 90% respectively) (Figure 4).

Exposure to H_2O_2, $^{\bullet}OH$, and HOCl resulted in various detrimental effects on oocyte quality, as assessed by the poor scoring of MT and CH. These detrimental effects depended on the presence or absence of cumulus cells, relative strength of the oxidizing agent, and its concentration.

As shown in Figure 4 A and Figure 5 (upper panel), in the absence of cumulus cells, increasing H_2O_2 concentrations (10, 17, 25, 50 and 100 µM) were associated with significant

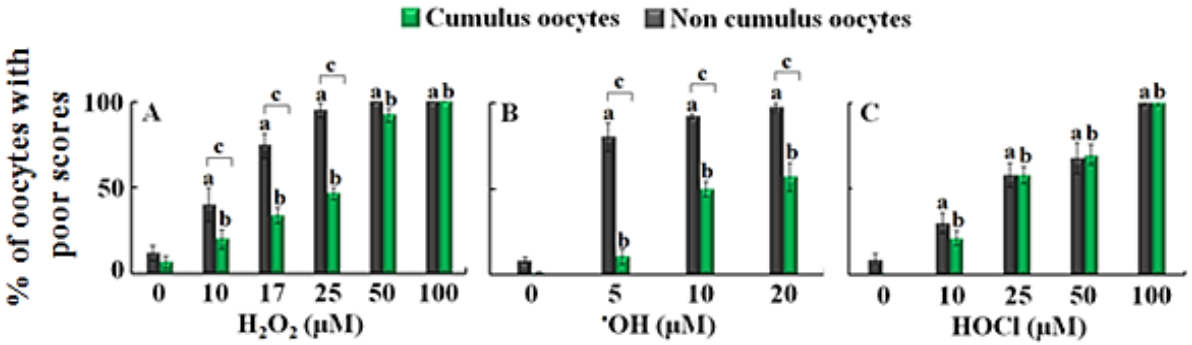

Figure 4: The effect of increasing concentration of H_2O_2, •OH, and HOCl on MT of metaphase-II mouseoocytes in the absence (gray bars) and the presence (green bars) of cumulus cells. (A) The percentage of oocytes with poor scores in MT treated with 0, 10, 17, 25, 50 and 100 µM H_2O_2. (B) The percentage of oocytes with poor scores in MT treated with 0, 5, 10, and 20 µM •OH. (C) The percentage of oocytes with poor scores in MT treated with 0, 10, 25, 50 and 100 µM HOCl. One-way ANOVA and independent t-test employing SPSS 21.0 were used for statistical analysis. $^{a}p< 0.05$ non-cumulus oocytes as compared to control, $^{b}p< 0.05$ cumulus oocytes as compared to control, and $^{c}p< 0.05$ cumulus compared to non-cumulus oocytes at each concentration. The experiments were conducted with three replications; the error bars represent the standard error of the mean.

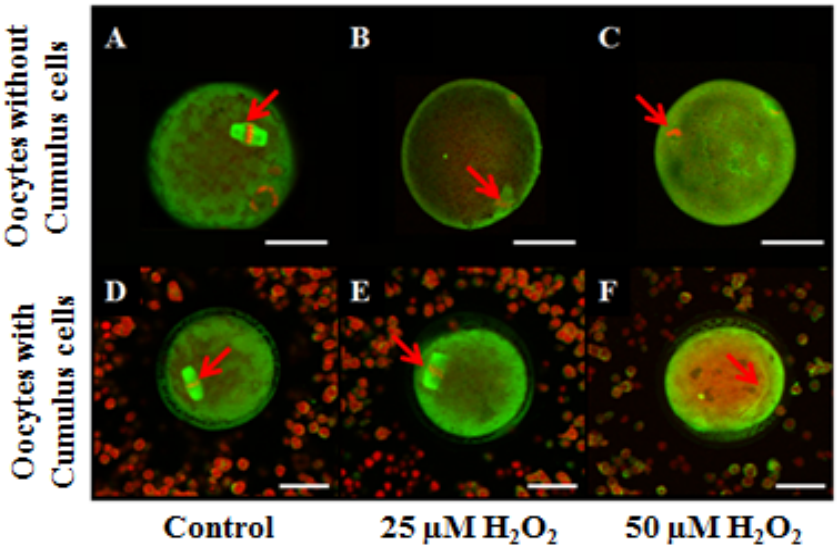

Figure 5: Images showing the effect of different H_2O_2 concentrations on oocyte quality obtained using Confocal Zeiss LSM 510 META NLO microscope. (A) Control oocyte without cumulus cells with normal MT and CH. (B) Oocyte without cumulus cells with altered MT and CH exposed to 25 µM H_2O_2. (C) Oocyte without cumulus cells treated with 50 µM H_2O_2. (D) Control oocyte with cumulus cells with normal MT and CH. (E) Oocyte with cumulus cells with normal MT and CH exposed to 25 µM H_2O_2. (F) Oocyte with cumulus cells treated with 50 µM H_2O_2 with altered MT and CH. Scale bars: 1 pixel, 5 mm for images A–C and 1 pixel, 3 mm for images D–F. Red arrows show the MT and the CH alterations. The experiments were conducted with tree replications.

increases in poor scores of MT (45%, 74.4%, 93.5%, 100% and 100% respectively) ($p< 0.001$). In contrast as shown in Figure 5 lower panel, in the presence of cumulus cells, the frequency of poor MT scores also increased when subjected to similar concentrations of H_2O_2, but to a lesser extent: 19.8% ($p>0.05$), 33.9% ($p<0.05$), 46.7% ($p<0.001$), 96.7% ($p<0.001$) and 100%

($p<0.001$), respectively (Figure 4A and Figure 5). Similar results were observed for CH. A similar trend was observed for oocytes treated with increasing concentration of H_2O_2 in the presence of fixed amounts of Fe(II) (100 μM). Under these circumstances, H_2O_2 was immediately converted to $^{\bullet}OH$ in a 1:1 ratio [8]. Poor scores of MT were higher in the non-cumulus groups at 5, 10, and 20 μM (80.3%, 92.2% and 96.6% respectively ($p<0.001$)) compared to oocytes with cumulus cells (10.5% ($p>0.05$), 49.8% ($p<0.001$) and 56.6% ($p<0.001$) respectively) suggesting cumulus cell protection ($p<0.001$) (Figure 4B). In contrast to H_2O_2 and $^{\bullet}OH$, non-significant independent *t*-tests revealed that cumulus cells do not offer protection against HOCl at concentrations of 10-100 μM (Figure 4C). Poor scores were noted following increasing HOCl concentrations (10, 25, 50, 100 μM) for both cumulus (20.7%, 57.9%, 66.1% and 100% respectively) and non-cumulus oocytes (30%, 58.3%, 64.6% and 100%, respectively) compared to controls ($p<0.001$). Thus, antioxidant machinery provided by cumulus cells may have selective protection against ROS.

Effect of ROS on cumulus cell number, dispersion, and connexin 43

To determine the mechanism by which different concentrations of ROS overwhelm the protective antioxidant machinery provided by cumulus cells, we next investigated the effect of increasing concentrations of H_2O_2, $^{\bullet}OH$, and HOCl on COC organization, cumulus cell number, and cumulus-cumulus cell interaction through staining of gap junction protein, connexin 43, utilizing confocal imaging as well as cumulus oocytes viability using trypan blue dye exclusion method.

As shown in Figure 6A, untreated oocytes were surrounded by tightly packed highly organized layers of cumulus cells. After treatment with lower concentrations of H_2O_2 (<25 μM) the COC remained intact and organized with similar number of cumulus cells observed as compared to controls, reflecting the preservation of oocyte quality (Figure 5B and C). Treatment

with high H_2O_2 concentrations changed the organized compact cumulus cell mass into a dispersed structure of cells (Figure 6D). In some cases, these clouds of cumulus cells are small, scattered, and remain loosely linked to the oocyte (Figure 6). Collectively, these alterations in the oocyte microenvironments upon exposure of the oocytes with cumulus cells to higher ROS concentrations could explain the damaging effect of H_2O_2 and $^{\bullet}OH$ to oocyte quality.

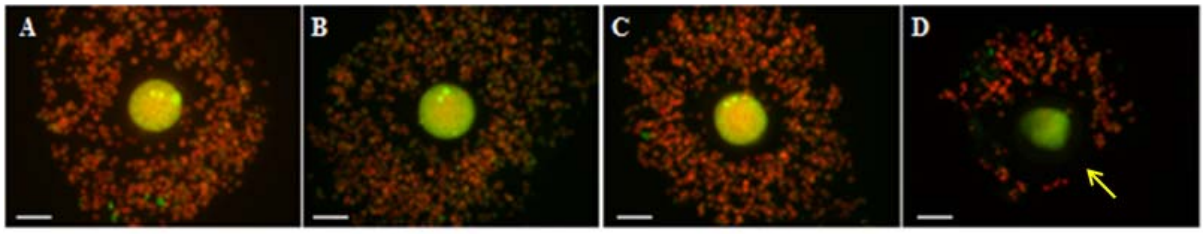

Figure 6: Images of cumulus-oocyte complexes as a function of increasing concentrations of H_2O_2 obtained using amicroscope-mounted Axiocam camera with Axiovision software (Zeiss). Panel A is a control of COC with normal MT, CH, and good organized cluster of cumulus cells surrounding the oocyte. Panels B-D oocytes treated with 10, 25, and 50 µM H_2O_2, respectively. Scale bar: 1 pixel, 2 mm. The experiments were conducted with tree replications.

As shown in Figure 7A, the control COC, stained for Cx 43 (green) and CH (red), showed organized clustering of cumulus cells surrounding the oocyte. Oocyte with cumulus cells exposed to a higher H_2O_2 concentration (e.g. 100 µM) showed significant decrease in the cumulus cells number or in some cases complete removal of the cumulus cells compared with untreated COCs (data not shown). However, the intensity of Cx 43 staining appears similar to control.

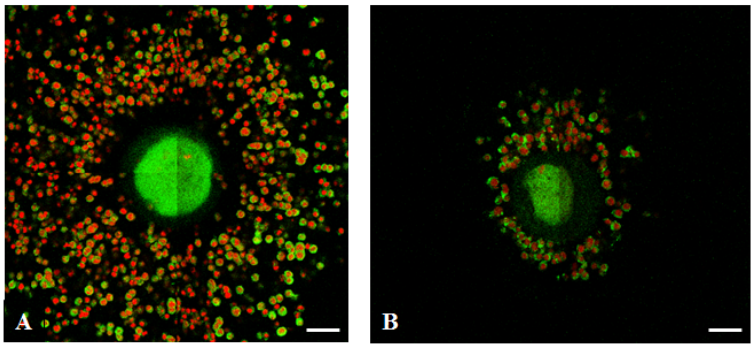

Figure 7: Metaphase-II mouse cumulus oocyte complexes (COCs) images obtained with Confocal Zeiss LSM 510 META NLO microscope showed the effect of H_2O_2 on Connexin (Cx) 43 density (n=20). A) Control COC stained for Cx 43 (green) with CH (red) with organized clustering of cumulus cells surrounding the oocyte. (B) COC exposed to 100 µM of H_2O_2 with significant decrease in the cumulus cells number with decrease in Cx 43 staining compared with untreated COC. Results depict observations from three experiments. Scale bar: 1 pixel, 2 mm.

Finally, the trypan blue dye exclusion method was used to determine the viability of the cells after exposure to HOCl, and to explain the failure of cumulus cells to protect oocyte against HOCl. Oocytes without cumulus cells exposed to higher HOCl concentrations (>25 µM) had a higher intensity of staining compared with untreated controls and oocytes exposed to lower concentrations of HOCl (Figure 8, upper panels).

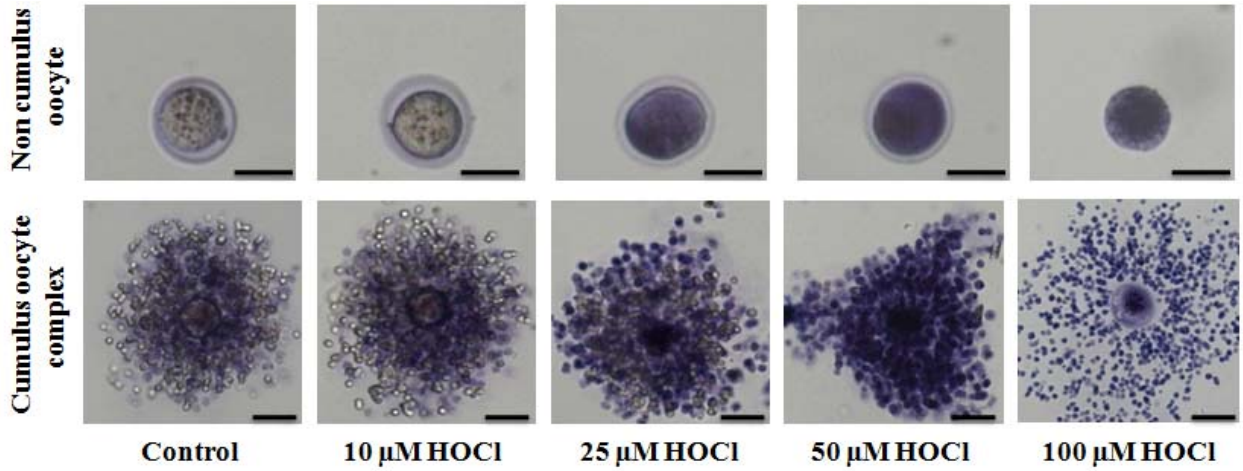

Figure 8: Images of oocytes without (upper panel) and with (lower panel) cumulus cells as a function of increasing concentrations of HOCl obtained using a microscope- mounted Axiocam camera with Axiovision software (Zeiss). Trypan blue viability staining with increased stain uptake in oocytes exposed to 0, 10, 25, 50, and 100 µM HOCl). Scale bars: 1 pixel, 4 mm for images A–C and 1 pixel, 2 mm for images D–F. The experiments were conducted with tree replications.

Cumulus oocytes showed greater intensity of staining and significant decrease in cumulus cell number at higher concentrations of HOCl, specifically at 100 µM (Figure 8 lower panels). This signifies that exposure to HOCl rendered the cumulus cells nonviable, which could explain the failure of the cumulus cells to provide antioxidant defense as trypan blue staining was similar between cumulus and noncumulus oocytes (Figure 8). Collectively, the mechanism through which cumulus cells lose their ability to defend against the effects of ROS is largely through the partial or complete loss of cumulus cells, which can be explained by the loss of cumulus cell viability.

Discussion

In this work, we investigated the ability of cumulus cells to protect the oocyte against

ROS and elucidate the mechanism and details of this action. Our results showed that oocytes, with and without the surrounding cumulus cells, treated with increasing concentrations of various ROS exhibited deterioration in oocyte quality as a function of concentration, when compared to untreated controls. Cumulus cells demonstrated protection against H_2O_2 and $^{\bullet}OH$ insult at low concentrations, but this protection was lost at higher concentrations. Cumulus cells offered no statistically significant protection against HOCl at any concentration. In all circumstances in which cumulus cells did not offer protection to the oocyte, both cumulus cell number and viability were decreased as judged by confocal immunofluorescence and viability staining. Collectively, the deterioration in oocyte quality may be caused by a decrease in the antioxidant machinery of the COC by loss of cumulus cells or the lack of scavengers for specific ROS, and/or the ability of the ROS to overwhelm these defenses.

Hydrogen peroxide is known to generate cellular toxicity both independently and through its involvement in the production of other ROS [63, 85, 141]. Hydroxyl radical, produced by the H_2O_2-driven Fenton reaction, is known to be associated with disorders of iron overload, such as endometriosis and hemochromatosis, in which reproductive failure linked to oxidative stress is common [8, 63, 159-161]. Our current investigation supports the notion that CC's, the major components of the cellular layers directly surrounding the oocyte, provide protection against ROS only to a certain extent. Indeed, oocyte exposure to low concentrations of H_2O_2 in the absence or in the presence of Fe(II), where H_2O_2 is instantly converted to $^{\bullet}OH$, induced little or no effect on the organized arrangement of surrounding cumulus cells. Under these circumstances, the oocyte's protection against $H_2O_2/^{\bullet}OH$ insult is due to the antioxidant machinery provided by the cumulus cells rather than that provided locally by the oocyte. This conclusion is based on a significant decrease in the percentage of oocytes with poor scores in the presence versus absence of cumulus cells as a function of increasing concentration of $H_2O_2/^{\bullet}OH$

(Figure 2 A & B). In addition to a number of non-enzymatic small molecule antioxidants that are known to be present in the intact COC, there are a number of enzymes responsible for H_2O_2 detoxification including catalase, glutathione peroxidase and peroxiredoxin [162-166]. These enzymes display the capacity to scavenge lower concentrations of both H_2O_2/$^{\bullet}OH$ and protect the oocytes from their damaging effects [162, 163, 165].

We have also shown that treatment with high H_2O_2 and $^{\bullet}OH$ concentrations changed the organized compact cumulus cell mass into a dispersed structure of cells. With high but physiological concentrations of these oxidants the compact clouds of cumulus cells are scattered and remain loosely linked to the oocyte. At pathological H_2O_2 and $^{\bullet}OH$ concentrations, the cumulus cells are stretched further and finally disconnected from the oocyte. Once the cumulus cells are dispersed or disconnected from the oocyte, the oocyte has lost its protection and therefore becomes as susceptible to ROS insult as non- cumulus oocytes [73, 167]. The loss of CC protection is due to the partial or complete loss of cumulus cell number, which could be caused by either the loss of cumulus cell viability or by the disruption of cumulus cell-cell interactions (Cx 43). Our results show the major factor is the loss of cumulus cell viability, which may explain the mechanism for oxidative stress associated reproductive failure.

Hydrogen peroxide is also known to be involved in the production of HOCl mediated by MPO [58, 70, 141]. At all concentrations tested, HOCl treatment was harmful and directly affected the viability and the number of cumulus cells through its ability to react with a range of biological molecules, particularly those with thiol, thiolether, heme proteins, and amino groups leading to tissue injury [168, 169]. Recently we showed that HOCl, in a feedback mechanism, degraded the heme-ring in MPO which released free iron [70] and led to $^{\bullet}OH$ generation [8, 63]. In contrast to treatment with H_2O_2 and $^{\bullet}OH$, CC's showed no significant sign of protection against HOCl at any concentration. We have recently shown that $ONOO^-$, like HOCl, mediates

damage to MT and CH alignment [3]. After exposure to both reagents, cumulus cells were stripped from the oocyte and oocyte viability was significantly compromised. Such compromise could occur either due to the deficiency of specific enzymatic and non-enzymatic antioxidants that help to scavenge ROS throughout the female reproductive tract [74, 170].

The CC's undergo cyto-differentiation, proliferation, and expansion, and are important during early oocyte growth and development, maturation, ovulation, and fertilization [73, 171-173]. Most infertility disorders are associated with decline in cumulus cell number, spindle abnormalities, and an altered cumulus oocyte association, leading to poor oocyte quality, as well as, poor reproductive outcomes [66, 129, 134, 145, 146, 174, 175]. Therefore, the presence of cumulus cells maintained in correct organization relative to the oocyte appears necessary for the protection and function of the oocyte. Fatehi *et al*, have demonstrated that intact cumulus cells during *in vitro* fertilization protected bovine oocytes against oxidative stress and improved first cleavage [134]. In addition, incomplete denudation of oocytes prior to ICSI enhances embryo quality and blastocyst development [134]. Furthermore, the removal of cumulus cells before complete oocyte maturation showed a premature migration with partial exocytosis of cortical granules [176] as well as adversely affects early embryonic development [177, 178].

It has also been shown that apoptosis rates of human CC from morphologically abnormal oocytes were significantly higher than morphologically normal oocytes examined under transmission electron microscopy [73]. An increase in CC apoptosis has also been associated with immaturity of human oocytes, impaired fertilization, and suboptimal embryo development [73]. The mutual dependency of the oocyte and the CCs involves a complex and varied set of interactions, and the functionality of COC depends on the individual competence and cooperation of both the CC's and the oocyte [73].

In conclusion, the intact arrangement of viable, functional cumulus cells around the

oocyte is paramount to the quality and reproductive capacity of the oocyte. Enhancement in the production or defective elimination of ROS and subsequent oxidative stress may be associated with infertility through a mechanism that involves the COC dysfunction and deterioration in oocyte quality. The mechanisms through which the different ROS affect oocyte quality are through cumulus cell apoptosis, causing decreased number of cumulus cells, or decrease in cumulus cell viability. H_2O_2 like •OH, decreased the viability of cumulus cells; however at high concentrations decreased the number as well. HOCl, like $ONOO^-$, stripped the cumulus cells from the oocyte as well as dissolved the zona pellucida. The severity of the insult of ROS on cumulus-cumulus and cumulus-oocyte interaction depends mainly on the bioavailability of the antioxidant machinery provided by cumulus cells, and the scavenging ability of these antioxidants. When increasing ROS concentration overwhelm the antioxidant machinery provided by the oocyte and /or cumulus cells, the mechanism of damage is most likely to be similar in both cumulus cells and oocyte.

CHAPTER 3

THE IMPACT OF MYELOPEROXIDASE AND ACTIVATED MACROPHAGES ON METAPHASE II MOUSE OOCYTE QUALITY

Abstract

Myeloperoxidase (MPO), an abundant heme-containing enzyme present in neutrophils, monocytes, and macrophages, is produced in high levels during inflammation, and associated with poor reproductive outcomes. MPO is known to generate hypochlorous acid (HOCl), a damaging reactive oxygen species (ROS) utilizing the hydrogen peroxide (H_2O_2) and chloride (Cl^-). Here we investigate the effect of the MPO system and activated macrophages on oocyte quality. Mouse metaphase II oocytes with and without cumulus cells were divided into the following groups: 1) Incubation with a catalytic amount of MPO (40 nM) for different incubation periods in the presence of 100 mM Cl^- with and without H_2O_2 and in the absence and presence of melatonin (100 μM), at 37°C (n=648/648); 2). Co-cultured with activated mouse peritoneal macrophage cells (1.0 x 10^6 cells/ml) in the absence and presence of melatonin (200 μM), an MPO inhibitor and ROS scavenger, for different incubation periods in HTF media, at 37°C (n=200/200); 3). Oocytes receiving no treatment and incubated for 4 hrs as controls (n=73/64). After treatment, oocytes were fixed, stained and scored based on the microtubule morphology (MT) and chromosomal alignment (CH). Treatment with MPO and activated macrophages were all found to negatively affect oocyte quality in a time dependent fashion as compared to controls. In all cases the presence of cumulus cells offered no protection; however significant protection was offered by melatonin. This work provides a direct link between MPO and decreased oocyte quality leading to poor reproductive outcomes. Therefore, strategies to decrease the inflammation caused by MPO or its mediators may influence clinical outcomes in infertility patients.

Introduction

There are many challenging questions and issues surrounding poor reproductive outcomes. Many of these problems have come to the forefront of the medical field with greater expectations from medical science. A substantial body of literature has proposed a link between oxidative stress and poor reproductive outcomes [2, 66, 120]. Oxidative stress, generated by reactive oxygen species (ROS) overproduction [120, 179] or myeloperoxidase (MPO) activity [56, 180], plays a central role in inflammation that causes these conditions [13, 181]. The deleterious actions of activated macrophages, the major source for ROS and MPO, are secondary to their ability to migrate to any site in the female genital tract and their cellular effects at the level of the oocyte [24-27, 182]. Under inflammatory conditions, activated macrophages are found in the cumulus cell mass within the cumulus oocyte complex (COC) [26, 182]. At sites of inflammation, the amount of MPO generated has been reported to reach a concentration of 1–2 mM [28]. High levels of MPO have been found in the collected peritoneal fluid samples of patients with chronic genital diseases [35, 36], polycystic ovarian syndrome (PCOS) [37, 38], advanced stages of endometriosis [35, 39, 40], and pelvic inflammatory disease [35, 41, 42]. Moreover, elevated MPO levels have also been found in the follicular fluid of women with chronic anovulation [43], which correlated to a decline in their fertility.

Myeloperoxidase generates hypochlorous acid (HOCl) through MPO activity in the presence of chloride (Cl^-) and hydrogen peroxide (H_2O_2) [19, 21]. Activated neutrophils generate around 150−425 μM HOCl/hr, while at areas of inflammation, the HOCl level can be reach as high as 5 mM [83, 183]. Under these conditions, HOCl not only destroys invading pathogens but can also cause damage through its capacity to react with other biomolecules, including aromatic chlorination, aldehyde generation, chloramine formation, and oxidation of thiols [179, 184]. Accumulation of HOCl can also mediate hemoproteins heme destruction and

subsequent free iron release and protein aggregation through a feedback mechanism involving MPO deterioration [56]. Both, HOCl and increased iron levels have been involved in several inflammatory conditions such as endometriosis [39, 84]. HOCl is a much more powerful oxidant in accelerating oocyte aging than other ROS through its ability to deteriorate the oocyte microtubule morphology (MT) and chromosomal alignment (CH), which are markers of oocyte quality [179]. Although MPO and HOCl are found in large amounts during inflammation contributing to poor reproductive outcomes, little is known about the exact mechanisms through which MPO affects oocyte quality.

Recently, utilizing HPLC and amperometric integrated H_2O_2-selective electrode, our group demonstrated real time in vivo measurements of intracellular H_2O_2 and its ability to diffuse outside the oocyte to activate extracellular MPO generating HOCl [55]. The ability of this investigation to provide a precise measurement of *in situ* H_2O_2 was secondary to limiting reactivity with nearby biological processes and minimizing loss caused by diffusion, and demonstrated through the use of catalase that the measurements were that of H_2O_2 and not an unknown substance in our system [55]. Our results showed that the diffused H_2O_2 triggered MPO chlorinating activity, which in turn facilitated oocyte quality deterioration, which was shown to be preventable if oocytes were pre-treated with melatonin. Melatonin, a known pineal hormone involved in the regulation of circadian rhythms [88, 89] has identified as a potent inhibitor of MPO chlorination activity and a potent scavenger of its final product, HOCl [16, 81, 185]. The beneficial effect of melatonin on oocyte quality and fertilization has been previously described [76, 77, 185, 186].

The current study demonstrates that MPO has a detrimental effect on oocyte quality through its chlorination activation, and defines the link between MPO (purified and naturally secreted from macrophages) and oocyte quality (MT and CH) deterioration; a mechanism that

can be prevented by using melatonin. These results may help in designing treatment plans for assisted reproductive technologies for patients with inflammatory conditions.

Materials and Methods

Materials

Hydrogen peroxide, melatonin, Human tubular fluid (HTF) media were purchased life technology, anti-α tubulin antibody, and Alexa Fluor® 488-AffiniPure Goat Anti-Mouse IgG (H+L) were purchased Jackson ImmunoResearch. Propidium iodide (PI), 1% bovine serum albumin (BSA), 0.1% M glycine, 0.1% Triton X-100, sodium nitrite, and trypan blue, lipopolysaccharide (LPS) were purchased from Sigma Aldrich (St. Louis, MO, USA). Normal goat serum 2% was purchased from Invitrogen and powdered milk, 0.2%, was obtained from grocery. Peritoneal macrophage cells (non-stimulated, adherent, and non-dividing) derived from female C57BL/6 mice were obtained from Astarte Biologics, LLC (Bothell, WA) (1 x 10^6 /1ml), macrophage cell media and other supplements were also obtained from ScienCell Research Laboratories, Inc. (Carlsbad, CA). The macrophage media (DMEM) with its supplemented materials and 10% Fetal Bovine Serum (FBS) were obtained from Science Cell Research Laboratories (Carlsbad, CA). Other chemicals and reagents were of the highest purity grades available and obtained from Sigma Aldrich.

Methods

Myeloperoxidase Purification:

Myeloperoxidase was purified initially from detergent extracts of human leukocytes by sequential lectin affinity and gel-filtration chromatography [187-189]. Trace levels of eosinophil peroxidase that may be contaminating were then removed by passage over a sulfopropyl Sephadex column [188]. Purity of isolated MPO was established by demonstrating a Reinheitzal (RZ) value of 0.85 (A430/A280), SDS–PAGE analysis with Coomassie blue staining, and gel

tetra- methylbenzidineperoxidase staining to the absence of eosinophil peroxidase activity. Enzyme concentration was determined spectrophotometrically utilizing extinction coefficients of 89,000 M^{-1} cm^{-1}/heme of MPO [190].

Hydrogen peroxide solution:

The H_2O_2 solutions were prepared fresh in phosphate buffer (PH 7.4), after which the concentration of the working solutions was determined spectrophotometrically (extinction coefficient of 43.6M ˉ1 cm ˉ1 at 240 nm) [191, 192]. During the preparation process, all the solutions were kept on ice to minimize decomposition.

Melatonin solution:

A stock solution of melatonin was dissolved in dimethylformamide (DMF) and diluted to the required concentration with phosphate buffer (pH = 7.4). The final concentration of DMF in all melatonin solutions was less than 1% and did not interfere with MPO activity or have any effect on oocyte quality [2].

Oocyte preparation:

Metaphase II mouse oocytes (with and without cumulus cells) were obtained from a B6C3F1 mouse crossed with a B6D2F1 mouse in cryopreserved straws using ethylene glycol-based slow freeze cryopreservation protocol (Embryotech Lab). We chose to use frozen-thawed oocytes instead of fresh as both our group and others have performed many experiments on both and found that treatment of fresh and frozen oocytes with reactive oxygen species had yielded similar and reproducible results [3, 7, 44, 151, 152, 179]. Institutional Review Board approval was not required, as the oocytes were obtained from Embryotech. Oocytes were transferred from straws to phosphate-buffered saline (Dulbeco's PBS) and washed to remove excess cryoprotectant for 5 minutes. Oocytes were then transferred to HTF media and incubated at 37ºC and 5% CO_2 for 60 minutes to allow repolymerization of spindles. The oocytes were then

screened for the presence of polar bodies confirming their metaphase II stage [56]. Ten to twenty oocytes from each group were discarded as they were found to be immature or displayed disrupted zona pellucida (ZP).

Purified myeloperoxidase treatment on oocytes (with and without cumulus cells)

Using the same processes for oocytes handling as mentioned in the previous section, metaphase II mouse oocytes with (n = 648) and without cumulus cells (n = 648) were divided into the following groups, which were performed in triplicate.

Group 1: oocyte incubated with fixed concentration of MPO (40 nM) at different incubation period (3, 6, 12, and 24 hrs); group 2: oocytes incubated with fixed concentration of MPO (40 nM) + 20 μM H_2O_2 at different incubation period (3, 6, 12, and 24 hrs); group 3: oocytes incubated with fixed concentration of MPO (40 nM) at different incubation period (3, 6, 12, and 24 hrs) preincubated with melatonin (100 μM); group 4: oocytes incubated with fixed concentration of MPO (40 nM) + H_2O_2 (20 μM) at different incubation period (3, 6, 12, and 24 hrs) on oocytes preincubated with melatonin (100 μM); group 5: Untreated oocytes were used as a control; and group 6: oocytes with melatonin (100 μM) alone for 24 hr incubation period. All oocytes were fixed at the time points (3, 6, 12, and 24 hrs) and evaluated for alteration of the following: MT structure and CH alignment. All experiments were carried out in HTF media containing 100 mM Cl^- which is similar to the physiological oviduct Cl^- concentration [193]. All cell transfers were performed by using 200-mm micropipette tips (ORIGIO, Cooper Surgical).

Macrophage cells co-cultured with oocytes (with and without cumulus cells)

We followed the protocol of macrophage cell co-culture as described by Honda et al (1994) [29] with some modifications. The 1 ml vials containing macrophage cells (1.0 x 10^6 cells/vial) were thawed at 37ºC then centrifuged at 1800 rpm at 4ºC for 5 min then the cryopreservative solution was removed and replaced with the macrophage media, mixed, and 1

µl of the media containing macrophage cells was used to test the cell viability using Trypan blue dye exclusion assay. Cells were recounted before utilization and were placed into 30 mm dishes (Falcon). The macrophage concentration per dish was chosen secondary to previous data which showed a significant reduction in fertilization rate in co-culture group as compared to control [29]. Macrophage cells were stimulated with lipopolysaccharide (LPS) (10 ng/ml) for maximal MPO secretion [194, 195]. Cells were allowed to rest for 16 hr at 5% CO_2, 37ºC to allow the cells to adhere to the base of culture dishes. The following day, the macrophage media was removed, washed with PBS twice, and then with HTF twice to remove the unadherent cells and replaced with HTF media. Cells were then reincubated at 5% CO_2, 37ºC to be ready for co-cultured with the oocytes.

In the triplicate experiment, the oocytes (with n = 200 and without cumulus cells n = 200, total) were divided into the following groups: group 1: oocytes with and without cumulus cells incubated with stimulated macrophage cells for 1, 2, 3, and 4 hrs; group 2: oocytes with and without cumulus cells incubated with stimulated macrophage cells preincubated with melatonin (200 µM) for 1, 2, 3, and 4 hrs; group 3: oocytes with and without cumulus cells incubated with 100 µM melatonin alone for 4 hrs; group 4: control oocytes receiving no treatment and incubated for 4 hrs. All groups were incubated in HTF for 4 hrs, 37ºC, 5% CO_2. We chose 4 hrs of incubation as previous studies stated that 4-6 hrs is the time for optimal fertilization [196, 197]. The doses of melatonin, and HOCl were selected on the basis of our preliminary results and our previous studies [16, 185]. All oocytes were fixed at the time points (1, 2, 3, and 4 hrs) and evaluated for alteration of the following: MT structure and CH alignment.

Immunofluorescence staining and fluorescence microscopy

Oocytes were fixed in a solution prepared from 2% formaldehyde and 0.2 % Triton X-100 for 30 minutes and then treated with blocking solution (PBS, 0.2% Powdered Milk, 2%

Normal Goat Serum, 1% BSA, 0.1 M Glycine and 0.1% Triton X-100) for 1 hr followed by PBS washing for 3 minutes. The oocytes were then subjected to indirect immunostaining using mouse primary anti-α tubulin antibody against the MT (1:100, overnight) and secondary Alexa Fluor® 488-AffiniPure Goat Anti-Mouse IgG (H+L) (1:50, 1 h). The chromosomes were stained using PI for 10 min. Stained oocytes were loaded into anti-fade agent on slides with two etched rings. Images were obtained utilizing both immunofluorescence and confocal microscopy.

Confocal microscopy, assessment of microtubules and chromosomal alignment

Confocal microscopy, assessment of microtubules morphology and chromosomal alignment slides were examined with the Zeiss LSM 510 META NLO (Zeiss LSM 510 META) microscope using PI (red) and Alexa Fluor® 488 (green) fluorescent filters with excitation and emission wavelengths of 470 and 525 nm, 496 and 519 nm, respectively. Oocytes were localized using a 10x magnification lens and spindle alterations assessed using 100 x oil immersion lens. The MT was stained fluorescent green, which was distinct from the fluorescent red staining of chromosomes. The alterations in the MT and CH were compared with controls and scored by three different observers blinded to treatment groups based on a previously published scoring system using comprehensive evaluation of the individual optical sections and the 3-D reconstructed images (Figure 2) [185, 198]. Scores of 1-4 were assigned for both MT and CH alterations, with scores 1 and 2 combined for good outcomes meaning microtubules were organized in a barrel-shaped with slightly pointed poles formed by organized microtubules crosswise from one pole to and chromosomes were normally arranged in a compact metaphase plate at the equator of the spindle. Scores of 3 and 4 signified poor outcomes and consisted of spindle length reduction, disorganization and/or complete spindle absence, and chromosome dispersion or aberrant condensation appearance.

Statistical analysis:

Statistical analyses were performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA analyses were performed to compare the percentage of oocytes with poor outcomes (scores 3 and 4) for MT and CH between controls with various time intervals and each treatment group applied to cumulus and non-cumulus oocytes. The same ANOVA procedures were also performed to investigate the effects of different treatments on cumulus and non-cumulus oocytes at different time intervals. Pair wise comparisons made using Tukey's post hoc test following significant ANOVA tests, which defined as $P<0.05$. Independent t tests were conducted to compare the cumulus and non-cumulus oocytes for each treatment and time interval combination.

Results

Effect of purified MPO/melatonin on MT and CH of metaphase II oocytes without and with cumulus cells

To test whether MPO activation through the extra-oocyte diffusion of H_2O_2 could deteriorate the mouse metaphase II oocyte quality, we investigated the time dependent effect of MPO, in the absence and presence of melatonin, a potent MPO inhibitor, on oocyte MT and CH in the absence and presence of cumulus cells. Incubation of the oocytes with MPO alone (40 nM) showed significant deterioration in oocytes quality in a time depended manner as judged by alterations in the MT and CH in the treatment groups and cumulus cells show no protection (Figure 9A & B-upper Panels).

Whereas the presence of 100 µM melatonin showed significant protective effect on MT and CH when incubated with MPO for shorter time (3 hrs) and this protection was lost by longer time of incubation (Figure 9A & B-lower Panels). Pre-supplementing the oocyte medium with more melatonin concentrations (400 µM), showed more protection in the oocyte quality up to 6

hrs of incubation with MPO (data not shown). These results concluded that preservation of normal MT and CH under MPO activity can be achieved by using the antioxidant and MPO inhibitor, melatonin.

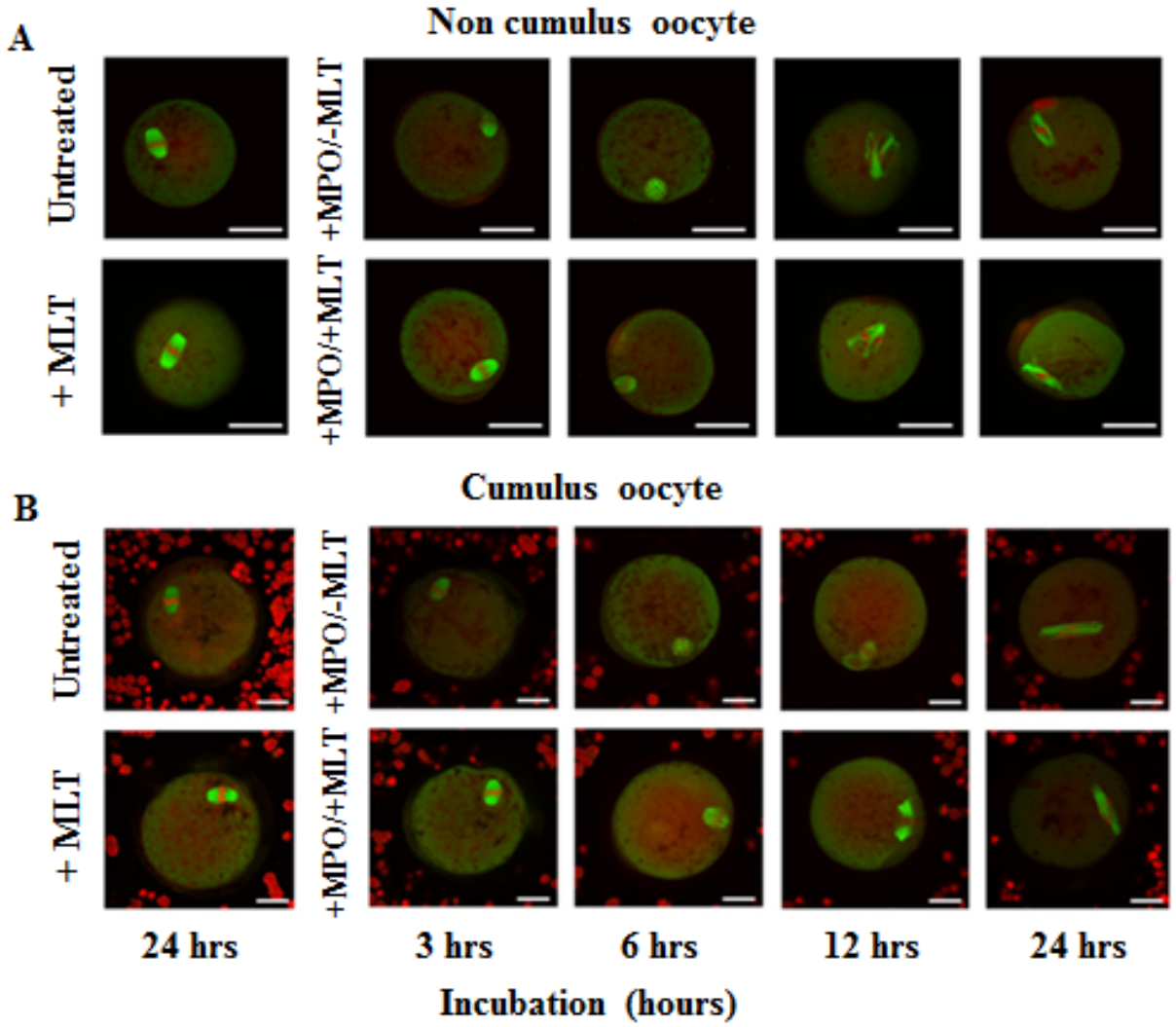

Figure 9: Images of oocytes microtubule morphology (MT) and chromosomal alignment (CH) obtained using Confocal Zeiss LSM 510 META NLO microscope. A) Oocytes without cumulus cells showed: -Upper Panel: Detrimental time dependent effect of myeloperoxidase (MPO) (40 nM) on MT and CH. -Lower Panel: Melatonin (MLT) supplementation (100 µM) showed normal MT and CH in the presence MPO at 3 hrs of incubation similar to controls and alterations in MT and CH by increasing the incubation time. B) Oocytes with cumulus cells showed similar observations that have been seen in A for MPO effect in the absence and presence of MLT. Collectively, cumulus cells failed to offer significant protection against MPO catalytic activity. Scale bars: 1 pixel, 5 mm for images A and 1 pixel, 3 mm for images B. Results depict observations made after three experiments.

For comparison, the effect of MPO, in the absence and presence of melatonin, on MT and CH were quantitated based on our well established 1-4 scoring system (see method section for

more details) and the percentages of poor scores were blotted as a function of time (Figures 10 and 11).

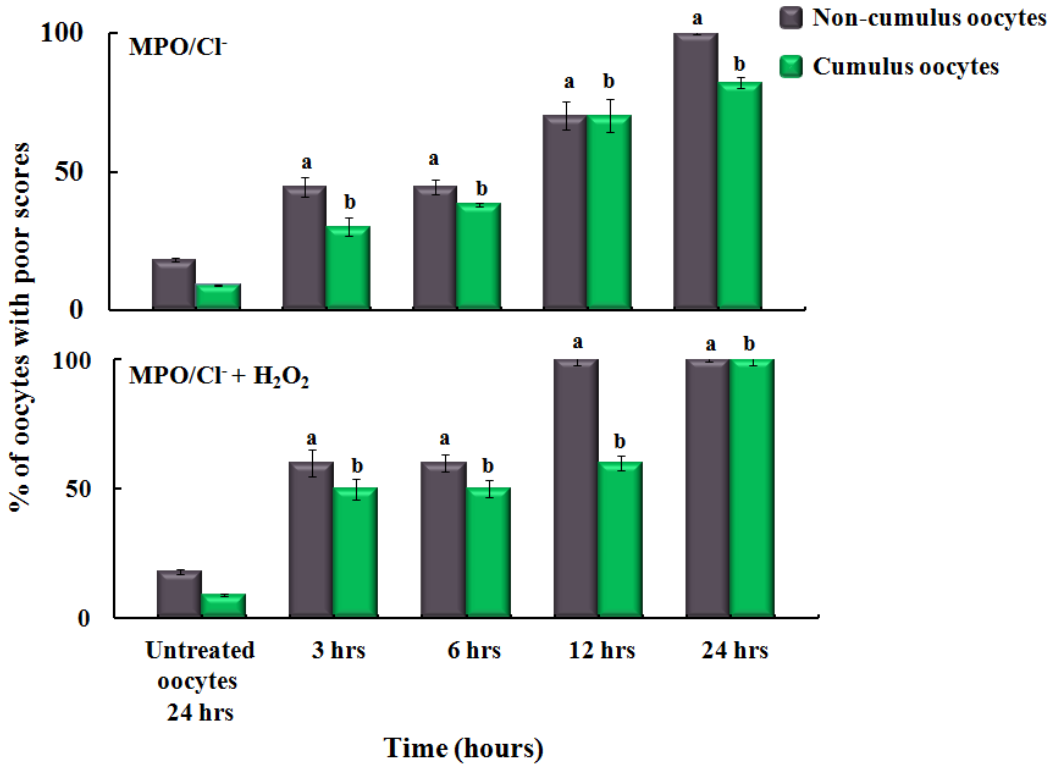

Figure 10: The effect of MPO/HOCl system on oocytes quality in the presence and absence of cumulus cells. Both panels show triplicate experiments of the percentage of oocytes with cumulus (n=648) (green bars) versus those without (n=648) (gray bars) with poor microtubule morphology (MT) scores observed in the untreated oocytes compared to oocytes treated with a fixed catalytic MPO concentration (40 nM) without addition of H_2O_2 (20 µM) (upper panel) and after addition of H_2O_2 (20 µM) (lower panel). All oocytes were incubated to different times of incubation (3, 6, 12 and 24 hrs) followed by indirect immunofluorescence staining method to observe MT and CH. Human tubulin fluid (HTF) media contains similar chloride (Cl^-) concentration (~100 mM) to the oviduct fluid. There is a time dependent effect of MPO activity on oocytes quality in the presence and absence of cumulus cells ($p < 0.05$). Cumulus cells fail to protect MT against damage from MPO activity ($p > 0.05$). Similar results were observed for the chromosomes alignment (CH). One-way ANOVA and independent t-test using SPSS 22.0 used to analyzed the results as following: (a) $P < 0.05$ non-cumulus oocytes as compared to control. (b) $P < 0.05$ cumulus oocytes as compared to control. (c) $P < 0.05$ between cumulus and non-cumulus oocytes. H_2O_2 addition on the oocytes in the lower panel showed no significant difference compared to oocytes in the upper panel. The standard error for each point was estimated to be less than 10%.

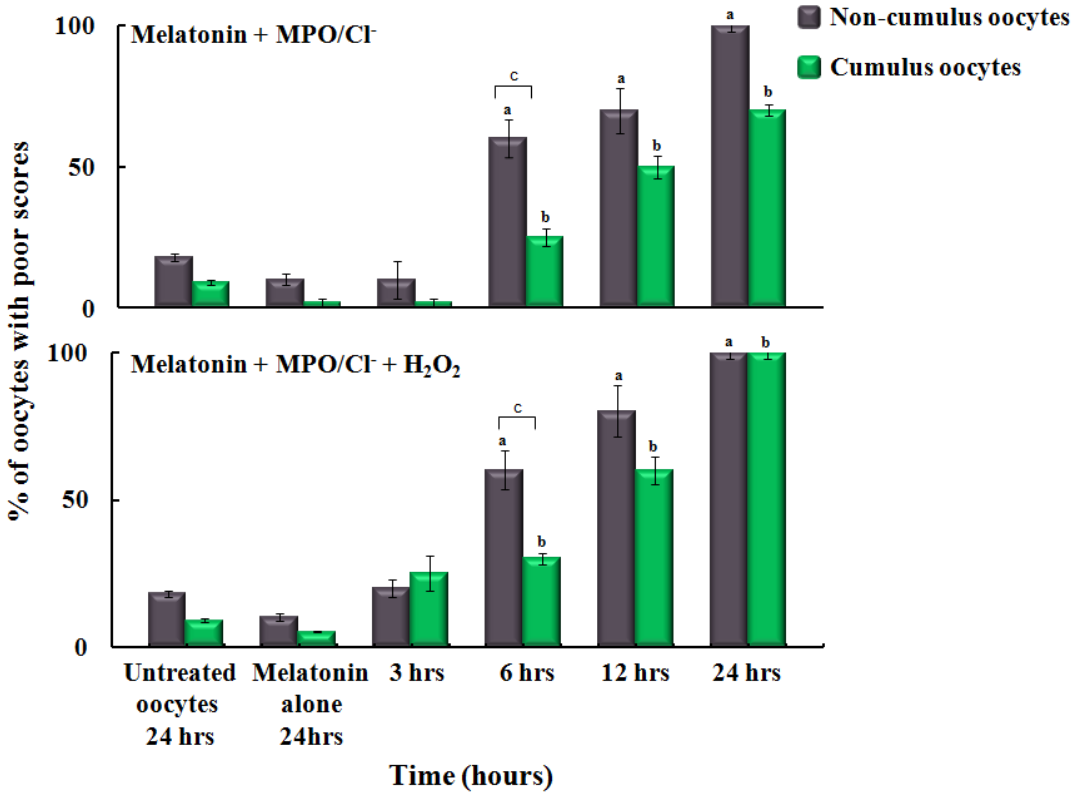

Figure 11: The protective effect of melatonin against MPO/HOCl system on oocytes quality in the presence and absence of cumulus cells. Both panels show triplicate experiments of the percentage of oocytes with cumulus (n=648) (green bars) versus those without (n=648) (gray bars) with poor microtubule morphology (MT) scores observed in the untreated oocytes compared to oocytes treated with a fixed catalytic MPO concentration (40 nM) without addition of H_2O_2 (20 µM) (upper panel) and after addition of H_2O_2 (20 µM) (lower panel) after pre-supplement the oocytes media with melatonin (100 µM). All oocytes were incubated to different times of incubation (3, 6, 12 and 24 hrs) followed by indirect immunofluorescence staining method to observe MT and CH. Human tubulin fluid (HTF) media contains similar chloride (Cl^-) concentration (~100 mM) to the oviduct fluid. Melatonin showed a significant protection against MPO activity at 3 hrs incubation ($p < 0.05$) as it works as direct HOCl scavenger and MPO inhibitor. The poor scores in MT showed by increasing the time of incubation caused as melatonin have been consumed. Cumulus cells fail to protect MT against damage from MPO activity ($p > 0.05$). H_2O_2 addition on the oocytes in the lower panel showed no significant difference compared to oocytes in the upper panel. Similar results were observed for the chromosomes alignment (CH). One-way ANOVA and independent t-test using SPSS 22.0 used to analyzed the results as following: (a) $P < 0.05$ non-cumulus oocytes as compared to control. (b) $P < 0.05$ cumulus oocytes as compared to control. (c) $P < 0.05$ between cumulus and non-cumulus oocytes. The standard error for each point was estimated to be less than 10%.

Figure 10 upper Panel showed the time dependent increase in the percentage of poor scores for MT and CH for oocyte with and without cumulus cells incubated with MPO. In the absence of cumulus cells, 3 hrs incubation with MPO showed 48% poor scoring and stayed almost the same at 6 hrs incubation and increased to 70%, and 100% at 12 and 24 hrs respectively, compared to control group 18.6%. Almost similar results were observed for oocytes MT and CH with cumulus cells incubated with the same amount of MPO for the same

incubation periods (36.6%, 38.7%, 70%, and 82% poor scores, respectively, compared to control groups 9.0%).

Figure 11 showed the protective effect of melatonin on oocyte quality against MPO treatment. Oocytes without cumulus cells incubated with MPO/melatonin showed a significant decrease in the percentages of poor scores MT and CH at 3 hr (7%) of incubation compared to longer incubation periods 6, 12 and 24 hrs (57%, 70%, and 100%, respectively) ($p<0.001$) (Figure 11 upper panel/gray bars). Whereas, the poor scores for cumulus oocytes incubated for the 3, 6, 12, and 24 hrs with melatonin/MPO were approximately 1.3%, 23.3%, 50%, and 70%, respectively as compared with the control group score average of 1.0% (Figure 11 upper Panel/green bars). In control experiments, oocytes incubated with melatonin alone for 24 hr showed poor scores of ~10.0% similar to untreated oocytes. Cumulus cells showed a sign of protection against MPO in the presence of melatonin at 6 hrs of incubation ($p<0.05$).

To determine whether MPO activation is the major cause for oocyte quality deterioration, we repeated the same experiments in the presence of exogenously added H_2O_2 (20 μM) to the oocytes media immediately after MPO addition and incubated the oocytes for the same incubation times (3, 6, 12 and 24 hrs) in the absence and presence of 100 μM melatonin (Figures 10 and 11-lower Panels). Our results showed that in the absence of cumulus cells, the poor scores for MT and CH for 3, 6, 12 and 24 hrs of incubation periods with MPO/H_2O_2 alone were 60%, 63.3%, 100% and 100% respectively (Figure 10-lower Panel/gray bars). In the presence of cumulus cells, the percentage of poor scores for 3, 6, 12, and 24 hrs of incubation periods with MPO/H_2O_2 alone were 50%, 53%, 60%, and 100% respectively (Figure 10-lower Panel/green bars). Results in this section mirrored those of the above experiment (Figure 10, upper Panel) in which H_2O_2 supplementation had no additional effect on oocyte quality ($p>0.05$), and again cumulus cells did not appear to provide protection to the oocyte ($p>0.05$). The poor scores of

non-cumulus oocytes (for MT and CH) incubated with melatonin/MPO/H_2O_2 for 3, 6, 12, and 24 h incubation were 20%, 63.3%, 73.3%, and 100% respectively (Figure 11 lower Panel/gray bars). Under these circumstances, melatonin also showed a protection at 3 hrs of incubation; but not in the other incubation periods. In the presence of cumulus cells, the poor scores for oocytes MT and CH incubated with the same treatments for the same incubation periods were 25%, 60%, 60%, and 100% respectively (Figure 11 lower Panel/green bars). Results in this section mirrored results without addition of H_2O_2, in that the presence of melatonin protected against MPO with H_2O_2 in the 3 hrs group ($p<0.001$); but not in the 6, 12, or 24 hrs groups ($p>0.05$). Cumulus cells did not showed a protective effect after 6 hrs of incubation in the presence of melatonin and MPO with H_2O_2 ($p>0.05$). Collectively, inhibiting MPO activity by using melatonin preserve the quality of the oocytes, thus MPO activity is the major cause of poor oocyte quality.

Effect of stimulated macrophages on MT and CH of metaphase II oocytes without and with cumulus cells

Since purified MPO activity was responsible for oocyte quality deterioration, then we test whether the exposure to stimulated macrophages could mediate deterioration of the oocytes quality through a mechanism that involves the MPO catalysis. To test this hypothesis, we co-cultured the oocytes with stimulated macrophages as function of time, in the presence and absence of melatonin (200 μM).

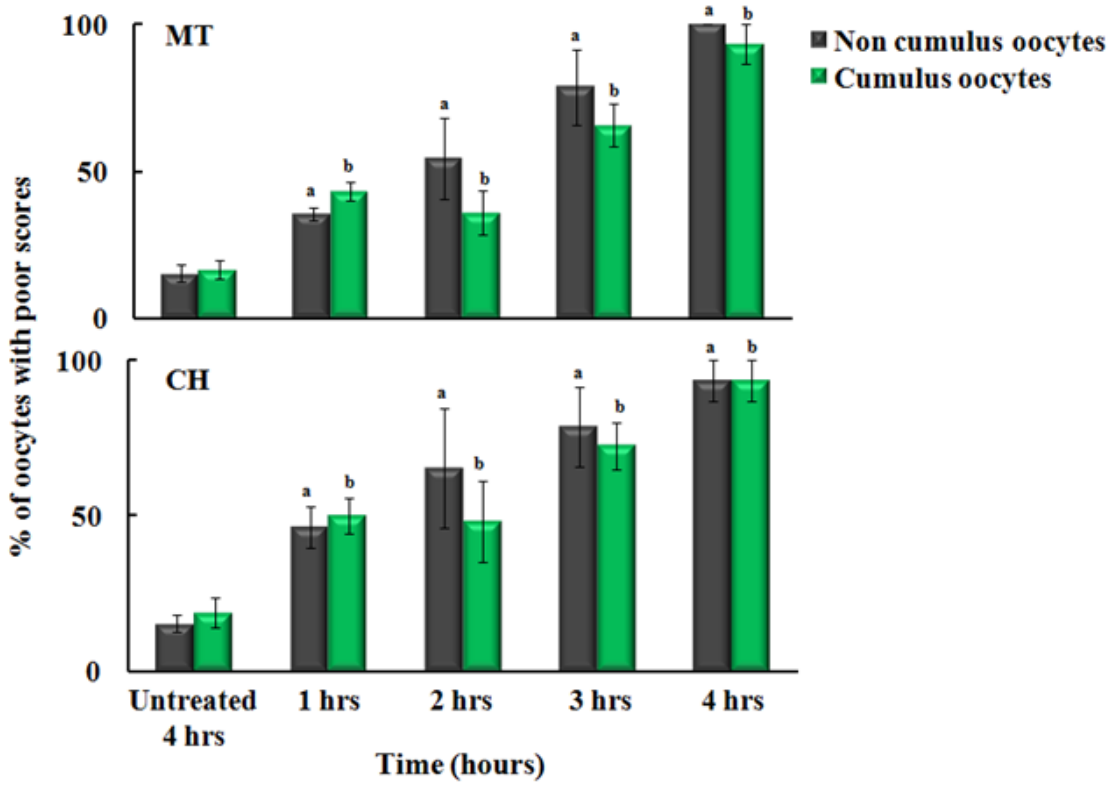

Figure 12: The direct effect of stimulated macrophages on oocyte quality, microtubule morphology (MT) and chromosomal alignment (CH), in the absence (gray bars) and presence (green bars) of cumulus cells. There was a significant time dependent effect of stimulated macrophages on MT and CH ($p<0.05$). Cumulus cells did not offer significant protection against macrophages activity ($p>0.05$). The experiments were conducted with three replications and the error bars represent the standard error of the mean.

As showed in Figure. 12, in the absence of cumulus cells, the poor scores for the oocytes incubated with stimulated macrophage for 1, 2, 3 and 4 hrs were approximately 35.5%, 54.4%, 78.5% and 100% for MT and CH (Figure 12). In the presence of cumulus cells, the average poor scores for the oocytes incubated with stimulated macrophage cells for 1, 2, 3 and 4 hrs was approximately 43.3%, 36.1%, 65.7% and 93.3% for MT and CH (Figure 12). Overall, as showed in Figure. 10, increasing the incubation time increased significantly the poor scores for MT and CH ($p<0.05$).

Figure 13 demonstrated the power of melatonin to inhibit the activity of naturally MPO secreted form stimulated macrophages. The poor scores for noncumulus oocytes MT and CH incubated with melatonin/stimulated macrophage cells for 1, 2, 3 and 4 hrs were approximately 20.5%, 63.8%, 93.3% and 100% (Figure 13). While, in the presence of cumulus cells, the poor

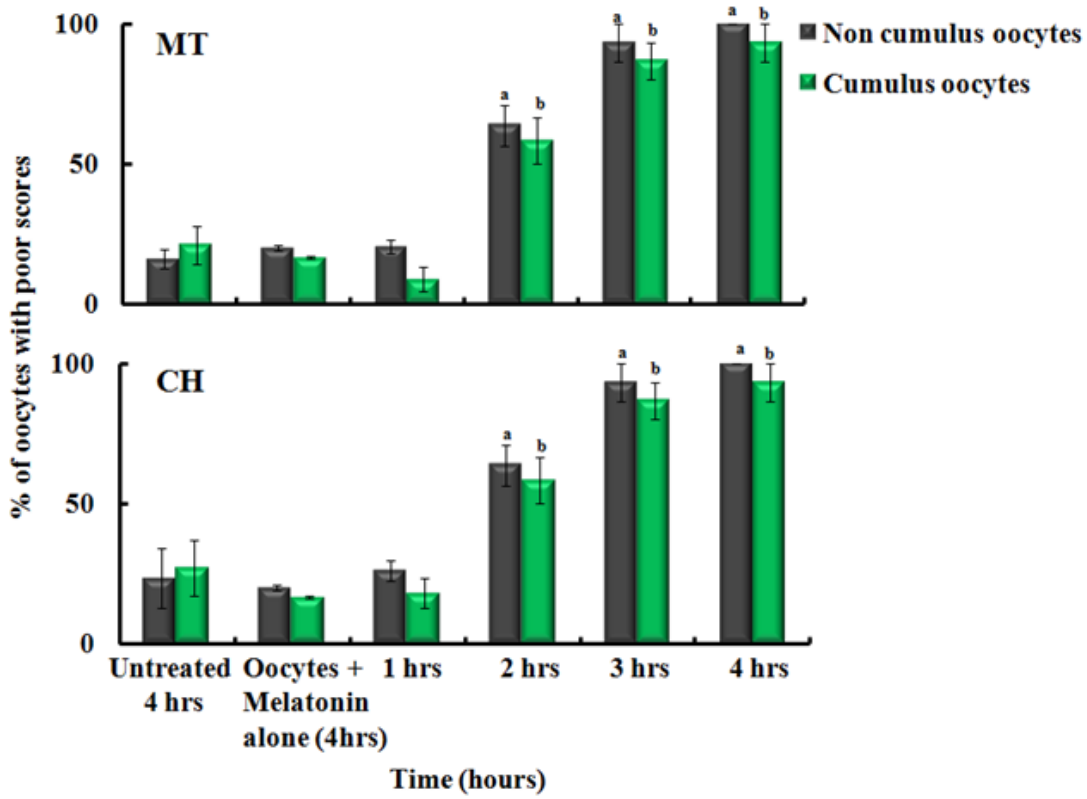

Figure 13: The protective effect of melatonin (MLT) against stimulated macrophages activity on oocytes quality (MT and CH) in the absence (gray bars) and presence (green bars) of cumulus cells. The presence of melatonin (200 μM) showed significant protection for MT and CH at 1 hr incubation ($p<0.05$). In general, cumulus cells did not offer significant protection against macrophages activity ($p>0.05$). The experiments were conducted with three replications and the error bars represent the standard error of the mean.

scores for MT and CH for 1, 2, 3 and 4 hrs were approximately 8.9%, 58.3%, 86.6% and 93% (Figure 13). Overall, melatonin showed a significant protection of MT and CH quality at 1 hr of incubation with macrophage cells ($p<0.05$) compared to longer time periods. The control group had poor scores for approximately 20% of noncumulus and 16.6% for cumulus oocytes ($p>0.05$). Cumulus cells showed a non-significant protective effect against MPO secreted from stimulated macrophages in the presence and absence of melatonin at 2, 3, and 4 hrs of incubation ($p>0.05$). Collectively, the major cause for oocytes quality deterioration is the activation of macrophages as well as MPO that can be successfully inhibited by using melatonin.

Discussion

Recent studies have shown that intra-oocyte H_2O_2 concentration is relatively high and

diffuses to the extracellular environment of the oocyte [55, 199]. Our current study confirms and extends these results and indicates that the diffused H_2O_2 deteriorates oocyte quality through MPO activation independent of cumulus cells and exogenously added H_2O_2, and could be prevented by treatment with melatonin, a potent inhibitor of MPO chlorinating activity [16, 81, 185]. Similarly, stimulated macrophages were also found to deteriorate oocyte quality independent of cumulus cells presence in a time dependent fashion, and could be prevented by melatonin. Macrophages are one of the principal defense mechanisms of innate immunity [200, 201] as a source of MPO and other toxic molecules used in controlled environments to degrade invading pathogens [19, 20]. Although, the association between macrophages and infertility has been repeatedly reported [6, 12, 202], the current work is the first to mechanistically link the MPO activity with the deterioration in the oocyte quality which adversely influences infertility.

All the indications point to diffused intra-oocyte H_2O_2 being sufficient to trigger the MPO chlorinating activity (generation of HOCl), which was responsible for the loss of oocyte quality. Hydrogen peroxide is a naturally occurring molecule within the oocyte and high a portion appears to diffuse outside the oocyte [55]. Hydrogen peroxide is an uncharged stable molecule, and permits through biological membranes in a fashion similar to water [203, 204] via limited diffusion and transport through specialized proteins known as aquaporins [205]. MPO activated through intraoocyte diffused H_2O_2, was found to negatively affect oocyte quality in time dependent manner in a similar trend to that recently observed when oocytes treated with increasing concentration of exogenous HOCl (Figure 2C) (Shaeib et al., in press. 2015). Treatment with HOCl disturbs the antioxidant capacity of cumulus cells by decreasing the number and/or viability of these protective cells (Figure 2C) (Shaeib et al., in press. 2015). Indeed, MPO treatment was found to mediate cumulus oocyte damage to almost the same extent as that in the absence of cumulus cells. HOCl may mediate oxidative damage and/or oocyte

fragmentation through its ability to undergo numerous reactions with biomolecules, including aromatic chlorination, chloramine formation, aldehyde generation, and oxidation of thiols [179, 184]. Preservation of oocyte quality by melatonin provides further evidence for the involvement of MPO activation in causing oocyte quality deterioration [16, 185]. The ability of MPO to utilize melatonin as a one electron substrate to produce less antioxidant substances, such as N1-acetyl-N2-formyl-5-methoxynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), limits the duration of the oocyte protection by the amount of the melatonin provided [97, 98]. Previously, we have shown that pre-incubation of oocytes with increasing concentrations of melatonin prior to HOCl treatment significantly prevented HOCl-mediated deterioration of oocyte quality [185]. It has been further demonstrated that, under specific condition, melatonin treatment could significantly improve fertilization and pregnancy rates [90, 123]. This work provides a direct link between MPO and deterioration of oocyte quality leading to poor reproductive outcomes.

Several inflammatory diseases such as endometriosis, polycystic ovarian syndrome, diabetes, are not only associated with increased ROS production but also increased MPO levels [35, 37-40, 206, 207]. In these disorders, elevated MPO levels have also been linked directly or indirectly with decline in fertility [2, 208]. We have previously showed that oocytes obtained from women with endometriosis display granulosa cells apoptosis, positive for nitrotyrosine, premature cortical granule exocytosis in oocytes, disrupted microtubular morphology, and disrupted chromosomal alignment [209]. The deterioration of these oocyte quality parameters is not only caused by ROS but may also occur through MPO catalysis consistent with our current results [55, 56, 185]. Elevated MPO activity shifts the environment from one of host defense to one capable of host damage directly through the generation of ROS or indirectly through hemoprotein heme destruction and subsequent free iron release [56]. Free

iron (most commonly Fe^{2+}) through H_2O_2 driven Fenton reaction yields •OH, which propagates deterioration in oocyte quality contributing to the development of infertility [14, 15, 210]. MPO can also consume NO as a physiological one electron ($1e^-$) substrate [211]. Direct quantitative NO measurements utilizing NO-selective electrodes revealed that there is a significant amount of NO inside the oocyte [212]. NO deficiency has been shown to deteriorate oocyte quality and accelerate oocyte aging [7, 44]. Thus, MPO may damage the oocytes through multiple pathways: generation of oxidants such as HOCl and •OH, serving as a source of free iron, and depleting NO.

MPO is produced in high levels during inflammation throughout of the female reproductive tract from stimulated inflammatory cells such as neutrophils, monocytes, and macrophages [40, 120, 213, 214]. The distribution of macrophages in the ovary during different stages of oocyte development, as well as their presence in peri-ovulatory human follicular fluid, suggest that macrophages play important roles in folliculogenesis and tissue restructuring at ovulation [25, 26]. Indeed, during oocyte development in mice, rats, and humans, macrophages are recruited into the cellular layers of the follicle causing their numbers to be greatest just prior to ovulation [25, 182]. Activation of these macrophages or recruitment of other immune cells in the presence of inflammation for any reason can therefore contribute to deterioration in oocyte quality. Activated macrophages may mediate oocyte quality damage not only through triggering of MPO chlorinating activity, but also through reactive oxygen species and cytokine cascades. Previously, we have shown that ROS such as $O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$, and $ONOO^-$, as well as, IL-6, generated in the process of oxidative stress, not only regulates the inflammatory setting and contributions in keeping of chronic inflammatory state but also directly or indirectly affects the metaphase-II oocyte spindle and significantly contributes to infertility [2, 3, 56, 179]. Similarly, treatment with melatonin (MPO inhibitor and ROS scavenger) highlights the culpability of

macrophages in affecting oocyte quality.

Parallel to increased expression of MPO in inflammation, research has demonstrated elevations in macrophage concentration and activity in conditions such as polycystic ovarian syndrome and endometriosis related infertility [26, 215-218]. Non-activated peritoneal macrophages when co-cultured with the oocyte post fertilization leads to higher rates of development in in-vitro embryos when compared to the control group [29]. In support of the variable role of macrophages based on activation status, an association has been demonstrated between endometriosis and increased numbers of macrophages; however concluded based on colorimetric assay of MPO activity that impaired function or abnormal activation, and not macrophages population size is important for endometriotic tissue proliferation [208]. Similarly, high concentrations of inflammatory cytokines (TNF and IL-1) secreted from activated macrophages have been shown to cause deleterious effects on pre-implantation embryos [219]. Therefore, increased macrophage activation in the follicular fluid may cause a disruption in folliculogenesis and the deterioration in oocyte quality observed in pathologic conditions causing infertility [6, 24, 220, 221]. As shown in *in vitro* studies, oxidative states generated upon activating macrophages may also compromise oocyte quality by affecting the meiotic spindle [2, 3, 55, 56, 179, 185, 198]. Thus, irrespective of whether purified MPO or activated macrophages are utilized, this works provides an initial mechanistic link between MPO activity and deterioration in oocyte quality.

In conclusion, our current work showed for the first time the link between stimulated-macrophages, a major source of MPO, and oocyte quality deterioration, highlighting the implications of these cells in infertility caused by inflammatory conditions. Melatonin has potential therapeutic effects in preserving oocyte quality, thus improving reproductive outcomes in patients with chronic inflammation.

CHAPTER 5

MELATONIN PREVENTS MYELOPEROXIDASE HEME DESTRUCTION AND THE GENERATION OF FREE IRON MEDIATED BY SELF-GENERATED HYPOCHLOROUS ACID

(This chapter contains previously published material. See Appendix B).

Abstract

Myeloperoxidase (MPO) generated hypochlorous acid (HOCl) formed during catalysis is able to destroy the MPO heme moiety through a feedback mechanism, resulting in accumulation of free iron. Here we show that the presence of melatonin (MLT) can prevent HOCl-mediated MPO heme destruction using a combination of UV-visible photometry, hydrogen peroxide (H_2O_2)-specific electrode, and ferrozine assay techniques. High performance liquid chromatography (HPLC) analysis showed that MPO heme protection was at the expense of MLT oxidation. The full protection of the MPO heme requires the presence of a 1:2 MLT to H_2O_2 ratio. Melatonin prevents HOCl–mediated MPO heme destruction through multiple pathways. These include by competing with chloride, the natural co-substrate; switching the MPO activity from a two electron oxidation to a one electron pathway causing the buildup of the inactive Compound II, and its subsequent decay to MPO-Fe(III) instead of generating HOCl; binding to MPO above the heme iron, thereby preventing the access of H_2O_2 to the catalytic site of the enzyme; and direct scavenging of HOCl. Collectively, in addition to acting as an antioxidant and MPO inhibitor, MLT can exert its protective effect by preventing the release of free iron mediated by self-generated HOCl. Our work may establish a direct mechanistic link by which MLT exerts its antioxidant protective effect in chronic inflammatory diseases with MPO elevation.

Introduction

Melatonin (MLT) is naturally synthesized from the amino acid tryptophan in the pineal

gland, but also by other non-endocrine organs (e.g., cerebellum, cerebral cortex, retina, skin, ovary, liver, pancreas, kidneys, and immune competent cells), and acts through 2 G-protein coupled receptors, MT_1 and MT_2 [91, 222-224]. In humans, like most vertebrates, MLT operates as a modulator of circadian rhythms, and displays an oscillatory pattern through its unique ability to function as a signal, which organisms use to synchronize their circadian system [91, 225]. Through its ability to scavenge a wide range of reactive oxygen species (ROS), multiple studies have shown distinct antioxidant and anti-inflammatory effects when MLT is administered either exogenously *in vivo* or when added to cultured cells via regulation of cellular pathways [51, 91, 112-114, 226]. The effects and action mechanisms of MLT belong to or take part in many different cell types including inflammatory cells such as monocytes–macrophages, neutrophils, eosinophils, basophils, mast cells, and natural killer cells [114, 115]. Therefore, various doses of synthetic MLT supplements have been used to treat a variety of medical scenarios such in which inflammation plays a role such as weakened immune system due to stress, oxidative hemolysis of red blood cells, and cancer progression [116, 117]. Recently, we have shown that MLT is a potent inhibitor of the inflammatory enzymes myeloperoxidase (MPO) and other related peroxidases (e.g. eosinophil peroxidase) [81, 104, 227].

Myeloperoxidase is a heme protein, present in the neutrophils, which utilizes chloride (Cl^-) in the presence of H_2O_2 to generate HOCl [141, 228]. This process occurs through H_2O_2 reduction that leads to the formation of MPO Compound I (ferryl porphyrin π cation radical, Fe(IV)=O(+π•)), which oxidizes Cl^- to HOCl [229]. Myeloperoxidase compound I is also capable of oxidizing various organic and inorganic substrates by two successive 1e− transfers generating compound II (MPO-Fe(IV)=O) and MPO-Fe(III), respectively. The rate limiting step in a typical peroxidase cycle is the reduction of compound II to MPO-Fe(III).

Furthermore, physiological reductants such as superoxide, nitric oxide, MLT, and ascorbic acid are known to accelerate this process [81, 230-234]. Hypochlorous acid is a potent oxidant that is capable, under normal circumstances, of functioning as a powerful antimicrobial agent [141, 228]. However, under a number of pathological conditions such as inflammatory diseases, in which ROS production can become excessive, HOCl is capable of mediating tissue damage [141, 235]. Interestingly, many inflammatory disorders such as ovarian cancer and atherosclerosis, in which MPO/HOCl has been known to be elevated, are also associated with significant free iron accumulation [15, 84, 236, 237]. Recently, we have highlighted the potential link between elevated HOCl and hemoprotein heme destruction, and subsequent generation of free iron [57, 229, 238]. Detailed mechanistic insight into how exogenously added or self-generated HOCl mediates the MPO heme moiety has recently been elucidated [57, 70]. Therefore, factors that influence rates of HOCl removal are of growing interest [156, 185, 228, 239-242]. Here, we examine the ability of MLT to prevent HOCl-mediated heme destruction and subsequent iron release. These findings may have therapeutic repercussions as they elucidate the mechanism behind the rationale for addition studies on MLT supplementation for patients with chronic inflammatory conditions in which MPO is elevated. Additionally, this work may open the door for the development of other treatment interventions in this patient population.

Materials and Methods

Materials

All the materials used were of highest-grade purity and used without further purification. Sodium hypochlorite (NaOCl), H_2O_2, ammonium acetate (CH_3COONH_3), ferrozine, MLT, ascorbic acid, and dimethylformamide, were obtained from Sigma Aldrich (St. Louis, MO, USA).

General Procedures

MPO purification

MPO was initially purified from detergent extracts of human leukocytes by sequential lectin affinity and gel filtration chromatography [58, 187, 188]. Trace levels of contaminating eosinophil peroxidase (EPO) were then removed by passage over a sulfopropyl Sephadex column [188]. Purity of isolated MPO was established by demonstrating a Reinheitszahl (RZ) value of >0.85 (A_{430}/A_{280}), SDS−PAGE analysis with Coomassie Blue staining, and gel tetramethylbenzidine peroxidase staining to confirm no contaminating EPO activity. Enzyme concentration was determined spectrophotometrically utilizing extinction coefficients of 89,000 M^{-1} cm^{-1}/heme of MPO [190].

H_2O_2-selective electrode measurements

Hydrogen peroxide measurements were carried out using an H_2O_2-selective electrode (Apollo 4000 free radical analyzer; World Precision Instruments, Sarasota, FL, USA). Experiments were performed at 25°C by immersing the electrode in 3 ml of 0.2 M sodium phosphate buffer, pH 7.0. Experiments were carried out under two different conditions: sequential additions of 10 μM H_2O_2 to a continuously stirred buffer solution supplemented with 40 nM MPO and 100 mM Cl^- in the absence and presence of 200 μM MLT during which the change of H_2O_2 concentration was continuously monitored.

Absorbance measurements

The absorbance spectra were recorded using a Cary 100 Bio UV–visible photometer, at 25 C, pH 7.0. Experiments were performed in 1 ml phosphate buffer solution supplemented with MPO (1.0-1.5 μM), 100 mM Cl^-, and incremental addition of 180 μM of H_2O_2 (20 μM; 2 μl) in the absence and presence of increasing MLT concentrations (0-200 μM). After each H_2O_2 addition, the reaction mixture was left 10 min for reaction completion and absorbance spectra

were then recorded from 300 to 700 nm.

Free iron analysis

Free iron release was measured colorimetrically using ferrozine, following a slight modification of a published method [243]. To 100 µl of the sample (MPO–HOCl reaction mixture) 100 µl of ascorbic acid (100 mM) was added. After 5 min of incubation at room temperature, 50 µl of ammonium acetate (16%) and the same volume of ferrozine (16 mM) were added to the mixture and mixed well. Subsequently, the reaction mixture was incubated for 5 min at room temperature and the absorbance was measured at 562 nm. A standard curve prepared using ammonium Fe(III) sulfate was used for the calculation of free iron concentration. Final concentrations of the additives were as follows: ascorbic acid, 33.33 µM; ammonium acetate, 5.3%; and ferrozine, 5.3 µM.

High Performance Liquid Chromatography (HPLC)

HPLC analyses were performed using a Shimadzu HPLC system equipped with an SCL-10A controller, LC-10 AD binary solvent delivery pumps, SIL-10 AD autosampler, SPD-M10 A diode array detector, and an RF-10 A XL fluorescence detector. An Alltech 5 um particle size column was used with a 4.6 x 150 mm reverse phase octadecylsilica (C18). To monitor the chromatogram, the RF fluorescence detector was set at 321 nm for excitation and 465 nm for emission and the SPD diode array detector was set at 400 nm. HPLC grade solvents were prepared as follows: solvent A, 0.1% TFA in water and solvent B, 0.1% TFA in 80% acetonitrile. Solvent gradients were set as follows: 0-10 min 55-65% B, 10-14 min 65-90% B, followed by reducing solvent B composition to 55% within 14-24 min. The column elution was carried out at flow rate of 0.8 ml/min with a linear gradient of solvents. After treatment of MLT with MPO in presence of H_2O_2 for 24 hr, the reaction mixture was filtered through an Amicon Ultra-15 centrifugal filter unit with Ultracel-10 membrane (from Millipore) 3-kDa cut-off by

centrifuging at 14,000 rcf rate for 30 min at 4°C; then 50 µl of filtered sample was injected for analysis. At the end of the run the system was equilibrated with 45% solvent A; each sample was analyzed in triplicate.

N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) synthesis

The AFMK was synthesized following the method of Tan *et al.* [244] with slight modification. Briefly, 5 mg MLT were dissolved in 100 µL methanol and the reaction mixture was mixed with 500 µL H_2O_2 (30%). The formation of AFMK was followed by the increase in absorbance at 340 nm. AFMK was isolated and confirmed by HPLC analyses.

Solution preparation

HOCl preparation

HOCl was prepared as previously described with some modifications [158]. Briefly, a stock solution of HOCl was prepared by adding 1 ml of NaOCl solution to 40 ml of 154 mM NaCl and the pH was adjusted to around 3 by adding HCl. The concentration of active total chlorine species in solution, expressed as $[HOCl]_T$ (where $[HOCl]_T = [HOCl] + [Cl_2] + [Cl_3^-] + [OCl^-]$) in 154 mM NaCl, was determined by converting all the active chlorine species to OCl^- by adding a bolus of 40 µl of 5 M NaOH and measuring the concentration of OCl^-. The concentration of OCl^- was determined spectrophotometrically at 292 nm ($\varepsilon = 362\ M^{-1}\ cm^{1}$). As HOCl is unstable, the stock solution was prepared on a daily basis, stored on ice, and used within 1 hr of preparation. For further experimentation, dilutions were made from the stock solution using 200 mM phosphate buffer, pH 7, to give working solutions of lower HOCl concentrations.

Melatonin solution

A stock solution of MLT was dissolved in dimethylformamide (DMF) and then diluted to the required concentrations with phosphate buffer (pH = 7.00). The concentration of DMF in all MLT solutions was less than 1% and did not interfere with MPO activity.

Results

Melatonin prevents MPO inactivation by HOCl generated during MPO steady-state catalysis

The ability of MLT to prevent HOCl damage to MPO catalytic activity was determined by two methods. The first involved the use of an H_2O_2-selective electrode, which measured the first step in the MPO catalytic cycle in which H_2O_2 is consumed by MPO. The second method measured HOCl –mediated MPO heme destruction utilizing UV-Visible and free iron release using ferrozine assay.

The H_2O_2-selective electrode measurements revealed that addition of an aliquot of H_2O_2 (10 μM; 3.5 μL) to the continuously stirred reaction solution supplemented with 40 nM MPO and 100 mM Cl^- demonstrated an instant consumption of H_2O_2, as previously reported [156, 234, 239]. Subsequent multiple additions of the same amount of H_2O_2 to the MPO/Cl^- solution mixture caused MPO inhibition, as judged by the accumulation of H_2O_2 (amplitude of H_2O_2 signal) and a slower rate of its consumption (longer duration) (Figure 14A).

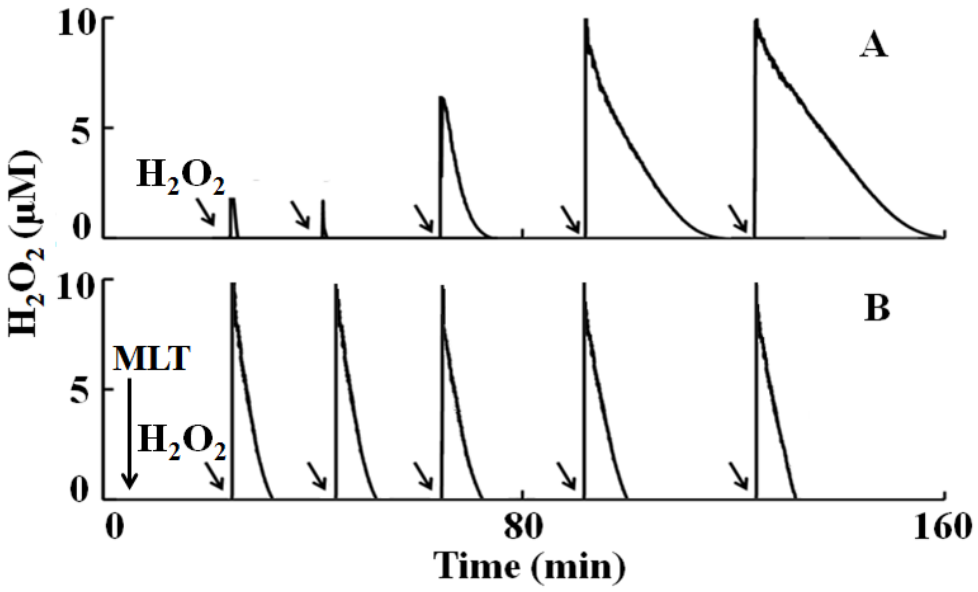

Figure 14. Melatonin inhibits MPO chlorination activity and prevents MPO heme destruction and iron release mediated by MPO self-generated HOCl. (A) A typical recording by an H_2O_2-selective electrode demonstrating the dramatic MPO feedback inhibition mediated by self-generated HOCl after addition of equal amounts of H_2O_2 (10 μM, 1–2 μl in 3 ml reaction mixture) five consecutive times (denoted by the arrows) to a continuously stirred phosphate buffer (200 mM, pH 7.4) containing 40 nM MPO and 100 mM Cl^-, at 25°C. (B) Similar experiment was repeated in the presence of MLT (100 μM), showing a significant protection of peroxidation activity of MPO. Under these circumstances, MLT inhibits the chlorinating activity of MPO and no heme destructions have been observed [81]. The data shown are representative of three independent experiments.

Under these circumstances, self-generated HOCl inhibited MPO through a mechanism that involves heme destruction, precluding the enzyme from functioning at maximum activity (80-90% inhibition) [70] (Figure 14A). We used 40 nM MPO as the catalytic concentration. The pathophysiological effect of MPO was shown at concentrations of 5 nM [245]. To examine whether MLT could prevent the feedback heme destruction mediated by HOCl, an identical experiment was repeated in the presence of a saturating amount of MLT. Addition of H_2O_2 (10 μM) to a continuously stirred buffer solution supplemented with 40 nM MPO, 100 mM Cl^-, and 200 μM MLT caused a much slower rate of H_2O_2 consumption compared to control (Figure 13B), which indicated that MLT inhibited the MPO catalytic activity [246]. The repeated addition of the same amounts of H_2O_2 to the MPO/Cl^-/MLT reaction mixture showed that the degree of MPO inhibition remained the same for all of the five trials. Thus under these conditions, MLT protected the peroxidation activity of MPO, but inhibited the chlorinating activity of the enzyme by serving as 1 e^- substrate for both MPO compounds I and II [81].

We next performed UV-visible photometry to correlate the degree of catalytic inhibition with HOCl-mediated heme destruction.

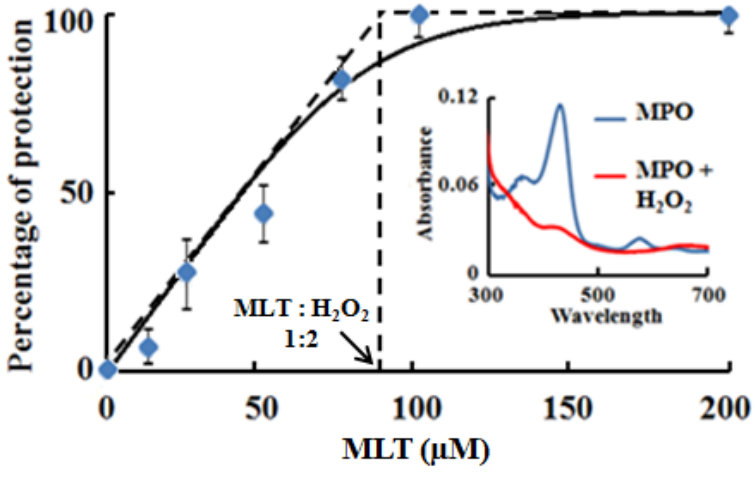

Figure 15. Melatonin prevents MPO heme destruction mediated by self-generated HOCl during steady state catalysis. Fixed amount of MPO (1 μM) was incubated with fixed amount of Cl^- (100 mM) and increasing concentration of MLT (12 -200 μM), and the reaction mixtures were incrementally received fixed amount of H_2O_2 (20 μM, total concentration of 180 μM). After reaction completion, the spectra of the reaction mixtures were scanned from 300 - 700 nm. The percent recovery in MPO Soret peak (430 nm) plotted as a function of melatonin concentration. The full protection of the MPO heme contents required the presence of a ratio 1: 2 MLT: H_2O_2. The inset shows the absorbance spectra of MPO ferric form before (blue trace) and after the last incremental addition of H_2O_2 (red trace). The flattening in the MPO spectrum indicates MPO heme destruction. The data points are the average of three independent experiments.

As shown in the Figure. 15 inset; blue trace, MPO-Fe(III) as isolated displays a Soret absorbance peak centered at 430 nm, with three additional beaks at 573, 630, and 694 nm. Since the addition of a high molar ratio of H_2O_2 to MPO causes the conversion of MPO to Compound (III) (MPO-FeII-O_2 complex) [247], the oxidation of the MPO heme moiety mediated by self-generated HOCl was monitored by sequential addition of H_2O_2 (20 μM; 3 μl) (180 μM H_2O_2 total) to the MPO-Fe(III)/Cl^- mixture. With each incremental addition of H_2O_2, there was a proportional decrease in the MPO Soret peak, indicating that HOCl-mediated MPO feedback inhibition is associated with MPO heme destruction. After the last addition of H_2O_2 (180 μM total) solution to enzyme mixture, the spectrum recording showed a flattening in the Soret peak at 430 nm indicating MPO heme destruction (Figure 15 inset; red trace). This flattening in the Soret peak region occurred solely in the presence of Cl^-, signifying HOCl to be the major cause of MPO heme destruction. To confirm that MLT prevents HOCl-mediated MPO heme destruction, a fixed amount of MPO/Cl^- mixture was preincubated with increasing concentrations of MLT prior to incremental additions of H_2O_2 to the reaction mixture. Figure 15 shows the percentage recovery of MPO heme content, measured at 430 nm after the last addition of the incremental H_2O_2 to the enzyme solution, as a function of MLT concentration. In the presence of a saturating amount of MLT (>100 μM), spectral analysis indicated no losses in the heme content. Under these conditions, the MPO- H_2O_2 system utilized MLT as a $1e^-$ substrate for the formation and subsequent decay of Compound II. The accumulation and stability of MPO Compound II (characterized by a Soret absorbance peak at 450 nm) during catalysis depended on the MLT concentration. In the presence of lower MLT levels, addition of limiting amounts of H_2O_2 (10 μM) to the solution mixture caused immediate appearance of MPO Compound II, which then decayed to the ferric form in the next few seconds. In the presence of higher MLT concentrations (e.g. 100-400 μM), no significant change in absorbance was

observed upon the addition of an H_2O_2 solution to the MPO mixture, indicating that the rate of MPO compound II decay exceeded the rate of formation, which was consistent with previous results [248]. In the presence of 50 µM MLT, only 50% recovery was noted in the MPO Soret absorbance peak of the total enzyme. As shown in Figure. 15, the full protection of the MPO heme contents required the presence of a ratio 1:2 MLT: H_2O_2 ratio. Collectively, our results showed that heme destruction did not occur in the presence of MLT, where MPO began reducing H_2O_2 without generating HOCl, indicating that self-generated HOCl is the major cause of MPO inactivation.

To investigate how the flattening in the Soret absorbance peak at 430 nm in H_2O_2-treated samples, is linked to MPO heme depletion, and if MLT can prevent this finding, we studied the free iron release after H_2O_2 treatment in the absence and presence of saturating amounts of MLT. By comparing to the free iron content of the untreated control, treatment with H_2O_2 led to a significant increase in free iron content as compared to control (Figure 16).

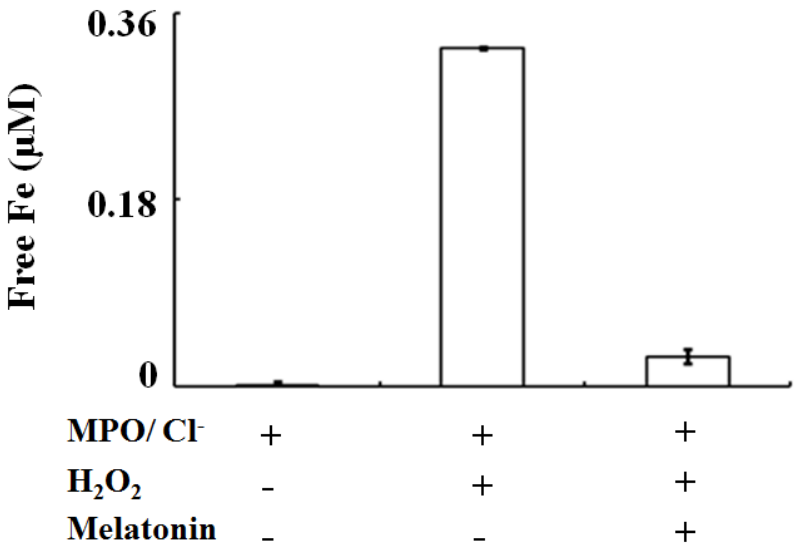

Figure 16. Melatonin prevents HOCl mediated MPO heme destruction and subsequent free iron release during MPO catalysis. MPO (1.2 µM) was incubated with 100 mM Cl^- in the absence and presence of 400 µM MLT followed by the addition of aliquots of H_2O_2 (in increments of 20 µM) to the reaction mixture. The free iron released was measured using ferrozine assay as detailed under Materials and methods. No free iron was detected before the addition of H_2O_2. The data are the averages of three independent experiments with the error bars representing the standard error of measurement.

Additionally, in the same figure, we noted around 25% free iron detection. This finding is likely secondary to the fact that not all iron was detached from the heme fragments, and

therefore not able to be detected by the assay. The accumulation of free iron significantly decreased in the presence of saturating amounts of MLT, confirming the above spectrophotometric studies. Thus, MLT not only inhibits MPO catalytic activity, but also prevents heme destruction and subsequent free iron release mediated by self-generated HOCl.

The protection of MPO heme destruction mediated by self-generated HOCl occurred at the expense of melatonin oxidation

Finally, HPLC analysis (anion exchange) was utilized to investigate in depth the mechanism by which MLT presence prevents MPO heme destruction mediated by self-generated HOCl. Using this method, we observe an accumulation of two major MLT metabolites when concentrations of MLT used were sufficient to produce dramatic effects on the rates of Compound II formation, duration, and decay. The population of these metabolites is varied and depending on the H_2O_2 concentration used (Figure 17).

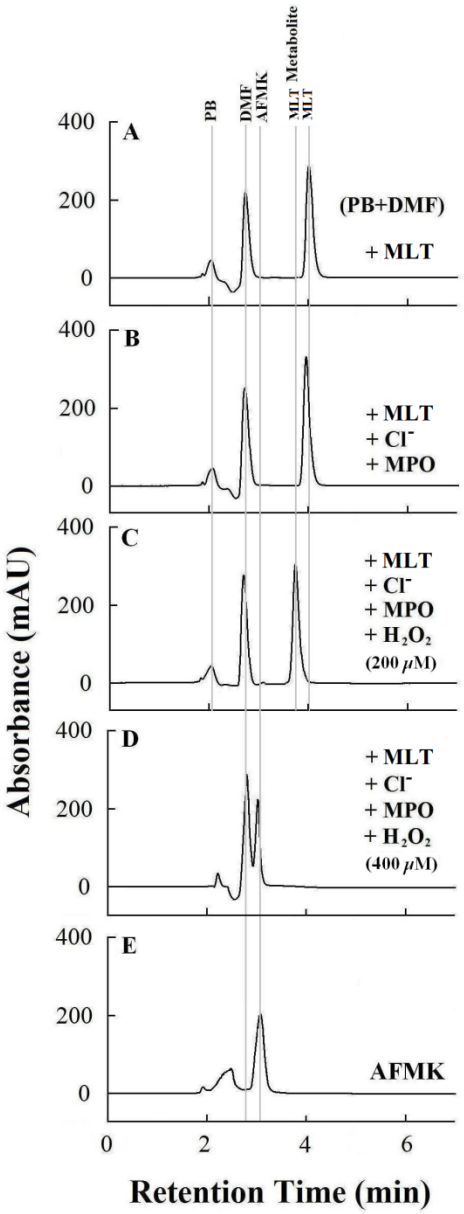

Figure 17: HPLC analysis shows MLT oxidation thereby preventing MPO heme destruction and generation of free iron. A) HPLC trace for MLT (elution time 3.98 min) dissolved in DMF (elution time 3.31 min) and phosphate buffer (elution time 2.48 min). B) Addition of MPO and Cl^- causes no significant change in MLT peak intensity and/or retention time. C) Addition of H_2O_2 (sequential addition of 20 μM, total 200 μM) results in a significant shift in MLT retention time elution time (3.71 min) as well as the appearance of a small peak around 3.57 min. D) Increasing levels of H_2O_2 (400 μM) resulted in the domination of the MLT metabolite eluted at 3.57 min showing the retention and absorbance properties of AFMK (elution time 3.57 min) as shown in panel (E).

HPLC analyses were conducted under five different conditions: MLT alone; MPO (40 nM) pre-incubated with MLT (100 μM) alone; the solution mixture of MPO pre-incubated with MLT (100 μM) which received sequential additions of 20 μM H_2O_2 (to total either 200 or 400 μM H_2O_2); and finally AFMK alone. After reaction completion, the reaction mixtures were filtered to eliminate MPO and the supernatants were then injected into the HPLC system. Under our experimental conditions phosphate buffer and DMF were eluted at 2.48 and 3.31 min, respectively (Figure 17). MLT alone was eluted at 3.95 min (Figure 17A) while AFMK alone was eluted at 3.57 min (Figure 17E), and both were identified by their characteristic spectra observed from the photodiode array detector at 222 and 236 nm, respectively. Pre-incubation of MLT (100 μM) with a catalytic amount of MPO (40 nM) and Cl^- (100 mM), in the absence of H_2O_2, fails to generate any detectable MLT metabolite (Figure 17B). HPLC analysis also indicated that relatively short time incubation of H_2O_2 at the different concentrations employed (200-400 μM) in the experiment for 2h has no effect on the MLT (100 μM) moiety (data not shown). However, long incubation of high concentration of H_2O_2 (30%) with MLT in the presence of methanol generates AFMK (Figure 17E) [244].

However, incremental addition of H_2O_2 to the enzyme- MLT mixture resulted in MLT oxidation to a lower elution time indicating formation of MLT metabolites with lower hydrophobicity. The enzyme sample that was pre-incubated with 100 μM MLT and treated with 200 μM H_2O_2 (total) led to the production of two main MLT metabolites with elution times of 3.71 and 3.57 min with the first being the most abundant and attributed to the formation of hydroxyl melatonin metabolite (Figure 17C). Although we provide no direct evidence for the hydroxyl melatonin formation, we do note corresponding $1e^-$ heme reduction steps for MPO Compounds I and II in the presence of MLT. The MPO sample that was incubated with 100 μM MLT and treated with 400 μM H_2O_2 (total) led to the production of a MLT metabolite with

elution time of 3.57 min, which was similar to elution time of AFMK alone (Figure. 17D). Consistent with these studies, Ximenes *et al.* similarly have observed two MLT metabolites (hydroxyl melatonin and AFMK) when MLT was incubated with MPO-H_2O_2 system or with stimulated neutrophils [249]. Thus, MLT prevents MPO heme destruction either by directly scavenging HOCl and/or inhibiting MPO chlorinating activity.

Discussion

In this work, we show that MLT largely prevents MPO catalytic inhibition, attributed to MPO heme destruction and the generation of free iron associated with HOCl synthesis, through its function as a potent MPO inhibitor and/or a HOCl scavenger. Thus, MLT may contribute to the reduction of the inflammatory process not only by inhibiting MPO and consuming HOCl, but also by diminishing the release and accumulation of free iron.

Recently, we have characterized an irreversible inhibition that is related to MPO heme destruction and the generation of free iron, when appropriate concentrations of self-generated HOCl are reached in the enzyme milieu [70]. These findings were recently confirmed by Paumann-Page et *al* [250]. The accumulation of the released HOCl in the solution mixture permits the competition with H_2O_2 on the catalytic site of MPO, which is in this case is the heme prosthetic group [70]. Hypochlorous acid interacts with both MPO-Fe(III) and Compound I and accelerates their conversion to Compound II [70, 251], or forms a relatively stable MPO-Fe(III)-OCl complex, which also converts to Compound II prior to heme destruction [70]. Compound II is a long-lived intermediate so would be predominantly susceptible to HOCl assault leading to heme destruction [57]. In the absence of an MPO inhibitor, HOCl scavenger, or both, the degree of MPO heme destruction is significantly high in that only a small portion of the total enzyme (5-10%) is estimated to remain active after multiple cycles of HOCl synthesis [229]. Our current results demonstrate that the degree of MPO heme pocket alterations (e.g., by changes in the

hydrogen bonds) mediated by MLT is not only sufficient to affect the interaction of Compound I with Cl^- preventing the generation of HOCl, but also prevents HOCl access to the heme moiety; thereby avoiding HOCl-dependent heme destruction. These notions are consistent with our previous detailed kinetic studies, which showed the ability of MLT to inhibit MPO chlorinating activity despite the presence of high concentrations of Cl^- [81]. Melatonin competes with Cl^- and switches the MPO catalytic activity from a $2e^-$ oxidation to a $1e^-$ oxidation pathway. Under these conditions, MPO did not generate HOCl but still consumed H_2O_2 at slower rates. H_2O_2-selective measurements showed that MLT presence inhibits MPO peroxidase activity. This observation appears relevant even in the presence of alternative substrates because peroxidases like MPO are not saturated under physiological conditions [252]. These notions are also supported by theoretical modeling, which showed that indole compounds could be accommodated in the narrow regions of the active site pockets of MPO when the indole ring is situated parallel to the heme plane and close enough to the D pyrrole ring. Under the circumstances the side chain of the indole compound is directed toward the outside of the distal cavity [253].

It is clear from the MLT presence that the MPO chlorinating activity, but not H_2O_2, is implicated in MPO heme destruction and free iron release. This conclusion is consistent with previous studies by Paumann-Page et *al.* who showed that the MPO inactivation mediated by H_2O_2 is unlikely to take place in the presence of reducing substrates (100 mM Cl^-), and where the concentration of H_2O_2 does not accumulate [254]. The amount of MLT used (100 μM) in the current work is sufficient to inhibit MPO. Studies on the effect of MLT on HOCl production by neutrophils and purified MPO have showed that the concentration of MLT that inhibited HOCl production by 50% (IC_{50}) was estimated to be 18 μM and reduced to 4 μM when superoxide was removed by addition of superoxide dismutase [249]. In contrast, the IC_{50} value, calculated from the initial rate of H_2O_2 consumption, as a function of the MLT concentration was 3 μM [81]. Our

HPLC analysis showed that the protection of MPO heme destruction mediated by self-generated HOCl occurred at the expense of MLT oxidation, which depends on the concentration of H_2O_2 used. Ximenes *et al.* showed the elution of two MLT metabolites when MLT was exposed to neutrophils [249]. In their system, the major and minor peaks were AFMK and a hydroxlyated melatonin metabolite, respectively. We similarly observed 2 peaks in our system; however, we believe the major peak was the hydroxylated intermediate when a lower concentration of H_2O_2 (1:2, MLT: H_2O_2) was used. In contrast, AFMK predominated when MPO was exposed to higher concentrations of H_2O_2 (1:4, MLT: H_2O_2). This alteration in the peroxidation turnover resulted in the reversal of the populations of the two MLT metabolites. Thus, prevention of MPO heme destruction depends on multiple factors including the bioavailability of HOCl, the presence of a capable $1e^-$ substrate that can compete with Cl^- switching the reaction from a $2e^-$ to a $1e^-$ oxidation pathway (e.g. ascorbic acid, superoxide, and nitric oxide), and the presence of HOCl scavengers.

Melatonin prevention of HOCl-mediated heme destruction is not limited to MPO, but also applies to other hemoprotein model compounds, such as hemoglobin, lactoperoxidase, catalase, as well as isolated human red blood cells [57, 70, 238, 249, 255, 256]. Earlier kinetic measurements have indicated that HOCl initially mediates the sequential formation of ferryl peroxidase-like intermediates, compounds I and II, followed by heme degradation [70, 238, 251, 257]. Hypochlorous acid can also mediate tetrapyrrole ring destruction independent of the iron molecule that resides in the porphyrin center [57]. A general chemical mechanism that describes the tetrapyrrole ring destruction resulting from the direct attack of HOCl and generation of multiple heme degradation products is well documented [57, 185, 228, 229, 238]. Because of MLT's ability to inhibit MPO, destabilize the Compound II intermediate and/or directly scavenge HOCl, MLT could be considered an ideal component for prevention against HOCl mediated

oxidative damage.

Although experiments that utilized methionine or taurine as scavenger of HOCl showed that they could prevent HOCl-mediated MPO heme destruction [104] similar to MLT, there are important differences in the fundamental aspects. Melatonin and its precursors, unlike other HOCl scavengers, display a high affinity towards transition metal (e.g. iron (III), copper and zinc) binding, and subsequently reduce their cytoplasmic availability [108-110]. In addition, several *in vivo* studies have shown that administration of MLT directly or indirectly neutralizes a variety of ROS, resulting in the reduction of lipid peroxidation, protein oxidation, and DNA damage, thus helping the immune system [51, 108, 109, 258, 259]. One other factor that distinguishes MLT from other HOCl scavengers (e.g. taurine, cystine, cysteine and uric acid) is that its oxidation products have no biologically harmful sequelae [104, 105]. Melatonin reacts with HOCl to produce 2-hydroxymelatonin [47] at a rate sufficient to protect catalase against inactivation by this molecule [260]. Melatonin's presence during MPO catalysis is associated with a significant diminution of free iron release, decrease in the intensity of the fluorescent heme degradation products, and reduction in different profiles of protein aggregation [47]. In contrast, taurine reacts with HOCl to form a less active oxidant taurine chloramine. It is important, however, to note that while chloramines are less reactive than HOCl, they can still oxidize thiols, thioethers and heme proteins, and thus extend the reactivity of HOCl [105-107].

The association between enhanced MPO expression and increased levels of free iron is characteristic of many inflammatory disorders including cardiovascular diseases such atherosclerosis, pulmonary diseases such as cystic fibrosis, neurodegenerative diseases such as Alzheimer's disease as well as arthritis, diabetes, and has been found to be risk factor for various cancers [15, 21, 236, 237, 261-265]. As free iron accumulates, it disturbs body processes by replacing certain vital minerals such as zinc, copper, and manganese in many enzymes, depleting

vitamins such as vitamin E and D, and may lead to chronic infection, and inflammation [266]. Due to its properties as an excellent oxygen transporter, iron tends to stimulate the growth of tumor cells and bacteria [267, 268]. Therefore, blocking the MPO chlorination machinery (MLT, tryptophan, and tryptophan analogs) [81, 156, 239] or scavenging HOCl (MLT, methionine, lycopine, taurine, and glutathione) might be a useful therapeutic approach in reducing free iron release in a wide variety of inflammatory conditions.

CHAPTER 5

CONCLUSIONS

This work is the first to link the effect of activated macrophages, simulating inflammation, through a mechanism involving MPO catalytic activity and ROS generation with the deterioration in the oocyte quality, which may contribute to poor reproductive outcome.

Our results from the Chapter 2 definitively confirm the deleterious effect of increasing ROS concentration on oocyte quality. Importantly, the supportive surrounding cumulus cells armed with antioxidant machinery showed variable ability to protect oocyte quality against ROS assault. Cumulus cells showed limited protection against H_2O_2 and $^{\bullet}OH$ insult at low concentrations, however they lost their ability to protect at higher concentrations. Moreover, cumulus cells offered no significant protection to the oocyte against HOCl at any concentration. Further investigation of these findings indicate that ROS compromise cumulus cell function by decreasing both cell number and viability, which is ultimately associated with the loss of oocyte viability.

Reactive oxygen species are known to be alternatively generated through the catalytic activity of MPO, which is synthesized and secreted from activated macrophages. In Chapter 3 we successfully showed the impact of activated macrophages on oocyte quality, which mirrored and then exceeded the impact of purified MPO, in a time dependent fashion. Similarly, HOCl, the MPO related oxidant also deteriorated oocyte quality irrespective of cumulus cells presence (please see Chapter 2 results). Melatonin showed a powerful protective ability against MPO, either purified or naturally secreted from activated macrophages, and against ROS by functioning as a potent inhibitor of MPO chlorinating activity and ROS scavenger.

Recent studies demonstrated that MPO is the major source of HOCl as well as free iron release under a HOCl feedback mechanism, which degraded the heme-ring in MPO. Under these

conditions, free iron reacts with H_2O_2, through a known Fenton reaction, generates $^{\bullet}OH$, thus worsening the oocyte microenvironment. Further research has found that the intra-oocyte H_2O_2 diffuses to the extracellular environment of the oocyte activating MPO and deteriorating oocyte quality. It is highly likely that HOCl is generated in the oocyte–macrophages culture media (see Chapter 3) and mediates oocyte quality deterioration.

Chapter 4 elucidated the mechanism of HOCl generation through the MPO catalytic system and confirmed that generated HOCl has the ability to oxidize and thus consume MLT, as judged by the appearance of the melatonin intermediates utilizing HPLC analysis. Briefly, we have described, in the absence of any inhibitor or scavenger, that sufficient amounts of self-generated HOCl have the ability to irreversibly inhibit MPO activity by destroying the heme moiety of the MPO molecule and subsequently generate free iron. While repeating the experiment in the presence of MLT, we found that melatonin can with Cl^- and interact with Complex I to obstruct MPO chlorinating ability and prevent HOCl generation. Melatonin also acts as a direct HOCl scavenger and therefore prevents HOCl accumulation thereby avoiding HOCl-mediated MPO heme destruction. Furthermore, using ferrozine assays we revealed that the accumulation of free iron significantly decreased in the presence of MLT (see Chapter 3). Therefore, MLT preserves MPO peroxidation activity (by consuming H_2O_2 at slower rates) and prevents further oxidant generation and free iron release. This study is important because we are the first to link activated macrophages, a major source of MPO, with oocyte quality deterioration, highlighting the effects of activated macrophages in infertility caused by inflammation. As MLT can disable and scavenge MPO, and displays beneficial therapeutic effects in preserving oocyte quality, it may improve reproductive outcomes in patients with chronic inflammation.

APPENDIX A

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(Chapter 1)

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Author: Faten Shaeib, Sana N. Khan, Iyad Ali, Mili Thakur, Mohammed G. Saed, Jing Dai, Awoniyi O. Awonuga, Jashoman Banerjee, Husam M. Abu-Soud

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(Chapter 3)

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ABSTRACT

THE IMPACT OF MYELOPEROXIDASE AND ITS RELATED OXIDANTS ON METAPHASE II MOUSE OOCYTE QUALITY

by

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Inflammatory reactions mediated by oxidative stress (OS) have been implicated in the deterioration of oocyte quality, which may lead to subfertility. Oxidative stress generated from enhancement of activated macrophages secondary to an inflammatory response are the major source of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and hypochlorous acid (HOCl), as well as, the pro-inflammatory enzyme myeloperoxidase (MPO). Previously, it has been shown that these ROS have deleterious effect on oocytes; however the link between inflammation through macrophage activity and oocyte quality remains unclear. In this work, we investigated: 1) the mechanism through which direct exposure of ROS and MPO, or through their generation by activated macrophages, deteriorate oocyte quality and whether melatonin (MLT), a potent MPO inhibitor and ROS scavenger, can protect oocyte quality; and 2) the mechanism through which MLT inhibits MPO catalytic activity.

Our results indicated that ROS differentially deteriorate oocyte quality in a dose dependent manner possibly secondary to the overwhelming of the defense antioxidant capacity of the cumulus oocyte complex (COC). Cumulus cells demonstrated protection against H_2O_2 and $^{\bullet}OH$ insult at low concentrations, but this protection was lost at higher concentrations and all

concentrations of HOCl as judged by changes in the organized compact cumulus cell mass into a dispersed mass of cells with decreased cumulus cell number and viability. Therefore, increasing ROS concentration overpowered the antioxidant machinery provided by the oocyte and /or cumulus cells, through loss of cumulus cells, or the lack of scavengers for specific ROS. This mechanism of damage may be associated with infertility related to COC dysfunction and thus deterioration in oocyte quality.

Myeloperoxidase as well as activated macrophages negatively affected oocyte quality in a time dependent fashion. In all circumstances cumulus cells did not offer protection to the oocyte; however significant protection was offered by MLT. Kinetic studies have shown that MLT inhibits the MPO chlorinating (generation of HOCl) activity through its ability to compete with Cl^-, the natural substrate of MPO, and serve as a one electron substrate of MPO Compounds I and II. Thus, MLT preserves the MPO peroxidation activity (by consuming H_2O_2 at slower rates) without the generation of HOCl through a two-step one-electron ($1e^-$) oxidation pathway.

This study is the first to link activated macrophages, a major source of MPO and ROS, and oocyte quality deterioration, highlighting the effects of activated macrophages in infertility caused by inflammation. MLT has beneficial therapeutic effects in preserving oocyte quality, thus improving reproductive outcomes in patients with chronic inflammation.

AUTOBIOGRAPHICAL STATEMENT

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Education

M.B.Ch.B.: University of Zawia Medical School, Zawia, Libya (06/2005)
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Peer Reviewed Publications

1- **Shaeib F**, Khan SN, Ali I, Najafi T, Maitra D, Abdulhamid I, Saed GM, Pennathur S, Abu-Soud HM. Melatonin prevents myeloperoxidase heme destruction and the generation of free iron mediated by self-generated hypochlorous acid. PLoS One. 2015, PMID: 25835505.
2- Khan SN, **Shaeib F**, Najafi T, Kavdia M, Gonik B, Saed GM, Goud PT, Abu-Soud HM. Diffused Intra-Oocyte Hydrogen Peroxide Activates Myeloperoxidase and Deteriorates Oocyte Quality. PLoS One. PLoS One. 2015, PMID: 26197395.
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6- Banerjee J, **Shaeib F**, Maitra D, Saed GM, Dai J, Diamond MP, Abu-Soud HM.. Peroxynitrite affects the cumulus cell defense of metaphase II mouse oocytes leading to disruption of the spindle structure in vitro. Fertil Steril. 2013, PMID: 23721714.
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8- Maitra D, Banerjee J, **Shaeib F**, Souza CE, Abu-Soud HM. Melatonin can mediate its vascular protective effect by modulating free iron level by inhibiting hypochlorous acid-mediated hemoprotein heme destruction. Hypertension. 2011, PMID: 21464394.

Poster Presentation (selected)

1- **Shaeib F**, Banerjee J, Thakur M, Diamond MP, Abu-Soud HM. (2013). *Melatonin prevents myeloperoxidase heme destruction and the generation of free iron mediated by self-generated hypochlorous acid.* Poster presented at: SFRBM's 20th Annual Meeting; San Antonio, TX.
2- **Shaeib F**, Banerjee J, Thakur M, Diamond MP, Abu-Soud HM. (2013). *Oxidative stress induces metaphase-II mouse oocyte spindle damage.* Poster at: SFRBM's 20th Annual Mtg; San Antonio, TX.
3- **Shaeib F**, Banerjee J, Thakur M, Diamond MP, Abu-Soud HM. (2013). *Confocal 3-dimensional reconstruction can serve as a useful tool to quantify oxidative stress induced oocyte spindle damage.* Poster presented at: SFRBM's 20th Annual Meeting; San Antonio, TX.
4- **Shaeib F**, Jashoman B, Maitra D, Diamond M, Abu-Soud HM. (2012). *Hydroxyl radical and mouse oocyte quality* Poster: 4th Ann. Mich. Alliance for Reprod. Tech. & Sci. (MARTS); Ann Arbor, MI.
5- **Shaeib F**, Khan SN, Abu-Soud HM. (2015). *The impact of activated macrophages on metaphase II mouse oocyte quality* Poster presented at: SFRBM's 21st annual meeting; seattle, WA.
6- **Shaeib F**, Khan SN, Abu-Soud HM. (2015). *The impact of activated macrophages on metaphase II mouse oocyte quality* Poster presented at: ASRM; Baltimore, MD.
7- **Shaeib F**, Khan SN, Ali I, Dai J, Drewlo S, Saed GM, Abu-Soud HM. (2014). *The Impact of Myeloperoxidase on Metaphase II Mouse Oocyte Quality* Poster presented at: ASRM; Honolulu, HI.
8- **Shaeib F**, Khan SN, Banerjee J, Thakur M, Dai J, Awonuga AO, Saed GM, Abu-Soud HM. (2014). *Role of Cumulus Cells in Defense Against Reactive Oxygen Species Insult in Metaphase II Mouse Oocytes* Poster presented at: ASRM; Honolulu, HI.