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Impact of hydrogen peroxide driven Fenton reaction on mouse oocyte quality

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Abstract

Here we show that hydroxyl radical ($\bullet\text{OH}$) generated through the Fenton reaction alters metaphase-II mouse oocyte microtubules (MT) and chromosomal alignment (CH). Metaphase-II mouse oocytes, obtained commercially, were grouped as follows: control, hydrogen peroxide (H_2O_2), Fe (II), and combined (Fe (II) + H_2O_2) treatments. After 7–10 minutes of incubation, at 37 °C, MT and CH were evaluated on fixed and stained oocytes and scored by two blinded observers. Pearson Chi-square test, Fisher's Exact test were used to compare outcomes between controls and treated groups, and also amongst each group. Our results showed that poor scores for MT and CH increased significantly in oocytes treated with combination of H_2O_2 and Fe(II) ($p < 0.001$); oocytes treated with H_2O_2 alone or Fe(II) alone showed no or little changes compared to control. Comparison of oocyte groups that received increasing concentrations of H_2O_2 and fixed amount of Fe (II) showed that 70 – 80 % demonstrated poor scores both in MT and CH when pretreated with 5 μM H_2O_2 , and increased up to 90–100% when treated with 10–20 μM of H_2O_2 . Hydroxyl radical generated by H_2O_2 driven Fenton reaction deteriorates the metaphase-II mouse oocyte spindle and CH alignment, which is thought to be a potential cause of poor oocyte quality. Thus, free iron and/or ROS scavengers could attenuate the $\bullet\text{OH}$ -mediated spindle and chromosomal damage, thereby serving as a possible approach for further examination as a therapeutic option in inflammatory states.

Keywords

hydroxyl radical; hydrogen peroxide; free iron; Fenton reaction; reactive oxygen species; pelvic inflammation; endometriosis; oocyte aging; oxidative stress; myeloperoxidase; heme protein

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INTRODUCTION

In peritoneal fluid, reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) are elevated in response to ongoing acute or chronic inflammatory states such as, endometriosis, the effect of certain medications, radiation, and/or pollutants causing tissue injury thereby possibly contributing to impaired fertility [1, 2]. These molecules, unlike nitric oxide, significantly deteriorate the postovulatory metaphase-II oocyte quality and integrity, and accelerate oocyte aging, as judged by markers such as alteration in ooplasmic microtubule dynamics, premature release of cortical granules, and an increase in zona pellucida dissolution time [3–6]. These findings not only explain the “age-enhancing” effects of ROS on the oocytes, but also support the theory of ROS in physiological regulation of the oocyte temporal window for optimal fertilization [3–6]. Reactive oxygen species may, therefore, be considered as mediators of the adverse influence exerted by disorders that affect reproduction.

Hydroxyl radical ($\bullet OH$) is a highly reactive molecule that can cause severe damage to the host cell by oxidatively modifying amino acids, purine and pyrimidine bases of DNA, and lipids [7]. It has been established that higher reactive oxygen species (ROS), myeloperoxidase (MPO) activity, combined with higher free iron exists in peritoneal fluid in advanced stages of endometriosis can set the stage for the generation of $\bullet OH$ [8–10]. The major pathways that lead to the generation of $\bullet OH$ depends mainly on the existence of the three components as shown in Figure 1. Mitochondria are the major intracellular sites of generation of $O_2^{\bullet-}$, which is sequentially reduced to H_2O_2 and $\bullet OH$ [11]. Superoxide could be generated enzymatically by NADPH oxidase, cytochrome P450-dependent oxygenases, and xanthine oxidase which are found in neutrophils, eosinophils, monocytes, and macrophages or non-enzymatically, when a single electron is directly transferred to O_2 [11–14]. Alternatively, xanthine oxidoreductase, a major source of ROS, converts hypoxanthine and xanthine to uric acid with simultaneous production of $O_2^{\bullet-}$ [15]. Most of generated $O_2^{\bullet-}$ undergoes a spontaneously [16] or superoxide dismutase (SOD)-catalyzed reaction [13] generating H_2O_2 as an end product. Hydroxyl radical can be generated by the reaction of H_2O_2 with reduced iron, which is the well known as a Fenton reaction [10, 17, 18].

Previously, we have demonstrated that MPO can serve as a source of free iron under conditions where both peroxynitrite and H_2O_2 are elevated [19]. Recently, we have shown that HOCl, the final product of MPO, could mediate-hemoprotein heme destruction and release free iron [20–22]. Alternatively, MPO and other related enzymes (e.g. lactoperoxidase and eosinophil peroxidase) could generate $\bullet OH$ through their reaction with H_2O_2 or HOCl, that lead to subsequent generation of the corresponding ferryl complexes (Compound I; $Fe(IV)=O^{\pi+}$ and Compound II; $Fe(IV)=O$) [23], which in turn reacts with HC-R molecules (e.g. xenobiotics substrates for cytochrome P450) to generate $\bullet OH$ [10, 24]. Alternatively, MPO Compound I reacts with chloride to generate HOCl which directly reacts with Fe (II) to generate $\bullet OH$ [10, 25–27].

Within ovarian follicles, enzymatic and non-enzymatic antioxidants protect oocytes from oxidative stress that are generated during ovulation [1, 2]. As a consequence, attempts have been made to prevent deterioration in oocyte quality by supplementing the culture media

with antioxidants, such as caffeine, vitamin C and reduced glutathione (GSH) [28–30]. Recently, we have shown that melatonin pre-treatment prevents HOCl-mediated damage to the MT and CH alignment of metaphase-II mouse oocytes [31].

Although, •OH has been implicated to play a role in a number of pathological conditions such as inflammatory diseases, endometriosis, atherosclerosis, respiratory distress, acute vasculitis, rheumatoid arthritis, glomerulonephritis, and cancer (see ref. [10] for detailed review), it is a less studied molecule on oocyte equality and infertility. Here we show that •OH mediates damage to the MT and CH alignment of the metaphase-II mouse oocytes.

MATERIALS AND METHODS

Materials

Metaphase-II mouse oocytes were obtained commercially (Embryotech Inc.) in cryopreserved straws. Hydrogen peroxide (H_2O_2), ammonium ferrous sulfate (Fe (II)), and 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 purchased from Sigma Aldrich (St. Louis, MO). 2% Normal Goat Serum from Invitrogen (Grand Island, NY) and 0.2% Powder Milk from grocery. Other chemicals and reagents were of the highest purity grades available and obtained from Sigma Aldrich (St. Louis, MO).

Methods

Metaphase-II mouse oocytes ($n = 200$) were obtained commercially and were transferred in phosphate buffer saline (Dulbecco's PBS) and washed to remove excess cryoprotectant for 3 minutes. This was followed by transferring the oocytes in HTF media and incubated at 37°C and 5% CO_2 for 60 minutes. The oocytes were then screened for presence of polar body confirming their Metaphase-II stage. The oocytes were divided equally into four experimental sets: *Set 1*. Study of the effect of increasing concentration of H_2O_2 (5, 10, 20 μM) on oocytes; *Set 2*. Study of the effect of fixed concentration of Fe(II) (100 μM) on oocytes; *Set 3*. Study the effect of Fe (II) (100 μM) on oocytes pre-incubated with increasing concentration of H_2O_2 (5, 10, 20 μM); *Set 4*. Untreated oocytes were used as a control. The end points of the experiments involved the morphological assessment of MI and CH alignment. Results obtained were compared in each experimental set between different groups using appropriate statistical tests. Concentration of Fe (II) (100 μM) used in the current study to establish the •OH generating system, have been widely used in previous studies [32]. The effect of varying incubation time on oocyte exposed to fixed concentration of H_2O_2 (25 μM). Metaphase-II oocytes were exposed to 25 μM of H_2O_2 for different time periods (15, 30, 45 and 60 min) followed by indirect immunostaining to determine changes in the spindle and chromosomal alignment after fixing the cells as described in previous work by Choi et al [28].

Immunostaining and fluorescence microscopy

Oocytes were fixed in a solution prepared from 2% formaldehyde, 0.2 % Triton X-100 for 30 minutes. The fixed oocytes were treated in blocking solution (PBS, 0.2% Powder Milk,

2% Normal Goat Serum, 1% BSA, 0.1% M Glycine and 0.1% Triton X-100) for 30 minutes then washed with PBS for 3 minutes. Subsequently the oocytes were subjected to indirect immunostaining using mouse anti- α tubulin antibody against the MT as the primary and FITC conjugate anti goat antibody as the secondary antibody. The chromosomes were stained using propidium iodide. Stained oocytes were loaded into anti fade agent on slides with two etched rings. The alterations in the MTs and chromosomes were compared with controls and scored by two different observers based on the scoring system published in previous studies (Figure 2) [28, 31, 33]. Scores of 1–4 were assigned for both MT and CH alterations, with scores 1 and 2 combined for good outcomes, and 3 and 4 for poor outcomes. Images were obtained both utilizing immunofluorescent 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 ; 596 and 613 nm respectively. Confocal images were obtained utilizing a Zeiss LSM 510 META NLO (Zeiss LSM 510 META) microscope. 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. Individual treated and control oocytes in each experiment set were closely examined for spindle status. The categorization of oocytes based on MT and CH status was performed by an independent observer blinded to treatment group assignment, who used comprehensive evaluation of the individual optical sections and the 3-D reconstructed images.

H₂O₂-selective electrode measurements

Hydrogen peroxide consumption and the generation of $\bullet$ OH measurements were carried out using an H₂O₂-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 HTF media, pH 7.4. H₂O₂ (10 μ M) was added to a continuously stirred media during which the rise and fall of H₂O₂ concentration was continuously monitored. Where indicated, Fe (II) (100 μ M, final) was added to the reaction mixture.

Solution preparation

The H₂O₂ solutions were prepared fresh in phosphate buffer (pH = 7.0) and the concentration of the working solutions was determined spectrophotometrically (extinction coefficient of 43.6 M⁻¹cm⁻¹ at 240 nm) [34, 35]. During preparation, all the solutions were kept on ice to minimize any decomposition.

Statistical Tests

Statistical analysis was performed using SPSS® version 19.0 (SPSS Inc., Chicago, IL). The frequency data in each test and control subgroup were analyzed using the Chi Square test. Frequencies of MT dynamics, CH structure, as well as spindles in individual subgroups with

various exposures within groups were compared to their respective control oocyte subgroups using the Fisher's exact test.

RESULTS

Our initial experiment was designed to study the effect of $\bullet\text{OH}$ on oocyte spindle structure and CH alignment in mouse metaphase-II oocytes utilizing H_2O_2 – driven Fenton reaction. To provide additional evidence that the observed alteration in oocytes quality was due to $\bullet\text{OH}$ generation, and not to H_2O_2 alone or Fe(II) alone, we first studied the effect of H_2O_2 as a function of time on mouse metaphase-II oocytes. As shown in Figure 3 left panel, increasing incubation time above 15 minutes significantly increased poor scores in both MT and CH confirming previous report [28, 31, 33]. Although approximately in 20% of the control groups, oocytes had poor outcomes, exposure of the oocytes for < 10 minutes to H_2O_2 did not significantly increase the odds of a poor outcome (Figure 3 left Panel). But increasing the exposure time appeared to significantly alter the spindle structure as evident by changes in MT morphology and CH alignment in the MII mouse oocytes. Prior to studying the effects of $\bullet\text{OH}$ on metaphase-II oocyte spindle and CH alignment, we thought to measure the rate of H_2O_2 consumption driven by Fenton reaction utilizing H_2O_2 -selective electrodes. As shown in Figure 3 right panel, following addition of an aliquot of 10 μM H_2O_2 to a continuously stirred buffer solution, the H_2O_2 signal rose rapidly, achieved a maximum after ~30 s, and fell gradually as H_2O_2 was depleted by auto-reduction ($2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$). Subsequent addition of 100 μM Fe(III) to the reaction mixture caused a rapid decay in the H_2O_2 signal (Figure 3, right Panel), indicating that $\bullet\text{OH}$ is released within the next 3 minutes.

Based on our finding from the above experiment, < 10 min (Figure 3 left panel highlighted zone) of incubation, where H_2O_2 has minimal effect on oocyte quality, would be ideal to distinguish the effects of $\bullet\text{OH}$ from H_2O_2 on the oocyte spindle and CH alignment. Collectively, exposure of oocytes to Fe(II) 100 μM or increasing concentration of H_2O_2 from 5–20 μM alone showed minimal effect on both spindle structure and CH alignment compared to control, consistent with previously reported studies [28] (Figures 4 and 5, Table 1). The treatment combination caused almost three folds as many cells with poor scores as H_2O_2 -5 μM alone (chi-square 5.06 with Fisher's exact $p=0.038$). The treatment combination also caused over 80% more poor scores in cells than Fe alone (chi-square=11.556 and Fisher's exact $p=0.002$). Around 70–80% of poor scores for the spindle and chromosome were observed for the oocytes treated with 100 μM Fe (II) combined with 5 μM H_2O_2 which increased from 90–100% upon increasing concentration of H_2O_2 to 10–20 μM respectively. Pearson Chi-square value of 20 with $p=0.000$ for MT and Chi-square of 20 with $p=0.000$ for CH was observed when 100 μM Fe (II) was combined with 10 μM of H_2O_2 . Pearson Chi-square value of 18 with $p=0.000$ for MT and Chi-square value of 14.4 with $p=0.000$ was observed for CH when oocytes were treated with 20 μM of H_2O_2 compared to controls (Table 1). Oocytes treated with H_2O_2 at 5, 10 and 20 μM alone had no significant differences in poor scores for MT and CH when compared to each other. Oocytes treated with 100 μM Fe (II) had no increased poor outcomes compared to controls. (Figure 5, Table 1).

DISCUSSION

Our results showed that the time dependent H₂O₂ mediated damage to MT and CH alignment of oocyte had a prolonged lag phase before increasing significantly (Figure 3). This lag phase can be extended to longer time by decreasing H₂O₂ concentration. Within the same lag phase time frame (0–15 min), poor scores in the MT and CH increased significantly when oocytes were exposed to H₂O₂ and Fe (II) in combination. Thus, •OH mediated damage to the MT and CH alignment of the metaphase-II mouse oocytes. The poor scores in MT and CH (10–20%) that was occasionally observed in the control group could be attributed to the remnant effect of cryopreservation even after repolymerization of the spindle by incubating for an hour with 5% CO₂ at 37°C which is similar to previously conducted studies [28].

A growing body of evidence implicates ROS in the pathogenesis of various diseases that are associated with infertility in women [1, 2]. Recently, we have shown that ROS such as O₂•⁻, HOCl, and H₂O₂ display the potential capacity to disrupt the spindle structure, zona pellucida dissolution time, ooplasm MT dynamics, and cortical granule status of the oocyte, which are essential for the proper oocyte quality, cell division and subsequent embryo formation [3, 4, 31, 33]. Disturbance of one or more of these elements may lead to improper chromosomal segregation affecting the ploidy of the daughter nucleus with detrimental effect on the fertility. Under normal physiological conditions, enzymatic and non-enzymatic antioxidant minimizes ROS-mediated cellular oxidations [1, 2]. Under a given pathological condition the extent of ROS-mediated oocyte damage depend mainly on its diffusion rate to reach its target and the bioavailability of ROS scavengers which limit their ability to exert biological effects. Though •OH have been implicated as an important player in ROS induced disease states, our current data provides direct evidence for the first time that •OH driven by H₂O₂ induced Fenton reaction caused instant oocyte damage. Hydroxyl radical, like other ROS, contributes to oxidative modification/fragmentation of oocytes through its ability to undergo numerous reactions and target small organic biomolecules, proteins, nucleic acids, and unsaturated fatty acids. During the persistent oxidative stress that occurs under pathological conditions when endogenous antioxidant defense systems may not provide sufficient protection, •OH and other ROS may be considered as major contributors in the pathogenesis of infertility [1, 2, 4, 17, 26].

In biological system, the source of free iron that is capable of catalyzing free radical reactions is still not known, but it thought to be generated from iron storage proteins, mitochondria and heme proteins such as MPO, hemoglobin and myoglobin [17–19, 21, 22, 36–38]. Recent studies have shown that MPO-HOCl system not only play an important role in generating •OH [10] through the formation of ferryl ion intermediate, but it also serves as a source of free iron through its ability to mediate hemoprotein heme destruction [20–22]. As reviewed in ref. [8], high neutrophil activity with expression of MPO, the source of HOCl during inflammation, is higher in disorders such as advanced endometriosis and ovarian cancer, and diseases associated with higher levels of free iron. Studies by Yamaguchi *et. al.* [39] and Iizuka *et. al.* [40] have reported that endometriotic cyst iron content ranges from 100–1.7 mM. Patients with endometriosis who develop endometriomas may have heavy or prolonged bleeding into these cysts and peritoneal implants thus

exposing more heme proteins to HOCl generated locally by MPO leading to the generation of free iron mediated by HOCl-hemeprotein heme destruction [8, 31, 33, 41]. Circumstances of increased ROS generation and elevated iron set the stage for •OH production via the Fenton reaction.

The sources of •OH generation in different disease setting are of great interest, especially in relation to intensifying the oxidative damage. An important source of •OH is from the reaction of mammalian peroxidases ferryl complex (Fe(IV)=O, Compound II) with HC-R reagents [10, 24]. Hydroxyl radical is extremely labile (an estimated half-life of 10^{-9} s) and it quickly and indiscriminately reacts with mitochondrial DNA, synthesis and accumulation of oxidized proteins, oxidative stress through hemoproteins heme destruction, protein aggregation, lipid peroxidation, change the membrane lipid composition and cause lysis of the cell membrane and death of the oocyte [10, 42]. It also compromises antioxidant machinery e.g., decreased GSH/GSSG ratio, which could affect optimal chromatin decondensation at fertilization and consequently altered gene expression [43, 44]. This could also explain the acceleration of oocyte aging and deterioration of the oocyte quality known to occur with various disorders that contribute significantly to reproductive failure, infertility associated with endometriosis, advancing age, diabetes mellitus, and a myriad of other clinical conditions [5, 8]. It has been shown that iron chelator (e.g. desferoxamine) and/or ROS scavenger (GSH, ascorbate, melatonin, and lycopene) can significantly benefit women in their advanced reproductive ages with poor oocyte quality and low ovarian reserve to increase their fertility outcomes [31, 45–50].

Previous studies from our laboratory have shown that ROS such as $O_2^{\bullet-}$, HOCl, and H_2O_2 can alter the oocyte quality manifested by hypergranulated cytoplasm, absence of perivitelline space, abnormal spindle dynamics [4]. We have also shown that melatonin, a potent scavenger of HOCl, can prevent HOCl-induced alterations in MT and CH structure of metaphase-II mouse oocytes as heme degradation and free iron release from hemoglobin [31, 51, 52]. Choi et al. demonstrated that H_2O_2 initiated the deterioration in the MT and CH structure in dose and time dependent manners [28]. More recently, we have demonstrated similar changes assessed by the same scoring technique for the alterations of the spindle and chromosomal structure by IL-6 [33]. Based on these studies and our current results, we can conclude that uncontrolled action of •OH could be considered as the most damaging and effective reactive oxygen species by far, which can have devastating effects within the oocyte. These results underline the importance of developing methods targeting the overproduction of •OH and prevention and treatment of diseases associated with oxidative stress and elevated iron. In particular, therapeutic strategies that focus on modulating Fenton reaction conditions by removing catalytic iron, superoxide $O_2^{\bullet-}$, or H_2O_2 could provide beneficial effects by diminishing the formation of •OH.

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Figure 1.

Major routes of generation of $•OH$. Abbreviations; NOX - NADPH Oxidase, O_2 - oxygen, $O_2^{\bullet-}$ - superoxide, SOD – superoxide dismutase, MPO – myeloperoxidase, H_2O_2 – hydrogen peroxide, HOCl – hypochlorous acid, CH-R – xenobiotics substrates for cytochrome P450 .

Score	Microtubule	Chromosome
1
2
3
4	Missing

Figure 2.
Schematic diagram demonstrating scoring system of MT and CH alteration based on previous study by Choi et al and Banerjee et al [22, 25, 26].

Figure 3.

Optimizing H_2O_2 driven Fenton reaction conditions. Percentage of oocytes with poor outcomes in MT (score 3, 4) at different incubation time point when oocytes were treated with $25 \mu\text{M}$ H_2O_2 . The highlighted time frame is the time of incubation selected to minimize the impact of H_2O_2 on oocyte quality. The values shown are the average of duplicate measurements taken from five separate experiments. The left panel dashed line, a typical recording by a H_2O_2 -selective electrode demonstrating the auto-reduction of H_2O_2 $10 \mu\text{M}$ following addition to a stirred media solution (pH 7.0) at 25°C , *solid line*, addition of H_2O_2 followed by $100 \mu\text{M}$ Fe(II) resulted in a dramatic acceleration in the rate of H_2O_2 consumption and the generation of $\bullet\text{OH}$.

Figure 4.

Images of oocytes obtained using microscope mounted 'Axiocam' camera with Axiovision software (Zeiss): (A) control oocyte with normal spindle and chromosomal (CH) alignment, (B) oocyte treated with 100 μM of Fe (II) only (C) altered spindle and CH alignment with exposure to 10 μM of hydrogen peroxide (H_2O_2), (D) oocyte exposed to 100 μM Fe (II) pretreated with 10 μM of H_2O_2 . Scale – 1 pixel, 5 mm. Results depict observations made after five experiments.

Figure 5.

The percentage of oocytes with poor scores in MT and CH (n = 200) observed in the untreated oocytes, oocytes treated with 10 μ M H₂O₂, oocyte treated with 100 μ M Fe(II) only, and oocytes treated with a combination of 10 μ M H₂O₂ and 100 μ M Fe(II). The experiment was conducted with three replications and the error bars represent the standard error of the mean.

Table 1

Percentages of poor scores in MT and CH when exposed to H₂O₂ (5, 10 and 20 µM), Fe (II) (100 µM) and combinations of H₂O₂ and Fe (II) compared to unexposed controls.

Treatment	Oocyte component	Score 3 and 4 (Poor)	Pearson Chi-Square P- Value
Control	MT	24 %	NS*
	CH	20%	
Fe (II) (100 µM)	MT	16 %	NS*
	CH	24 %	
H ₂ O ₂ (5 µM)	MT	20%	NS*
	CH	12%	
H ₂ O ₂ (10 µM)	MT	34%	NS*
	CH	19%	
H ₂ O ₂ (20 µM)	MT	60%	NS*
	CH	60%	
H ₂ O ₂ (5 µM) + Fe (II) (100 µM)	MT	80%	0.001
	CH	80%	0.001
H ₂ O ₂ (10 µM) + Fe (II) (100 µM)	MT	100%	0.000
	CH	100%	0.000
H ₂ O ₂ (20 µM) + Fe (II) (100 µM)	MT	100%	0.000
	CH	100%	0.000

NS*- not significant