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Original Contribution

Impact of hydrogen peroxide-driven Fenton reaction on mouse oocyte quality

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ABSTRACT

Here we show that hydroxyl radical ($^{\bullet}OH$) 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 min of incubation at 37 °C, MT and CH were evaluated on fixed and stained oocytes and scored by two blinded observers. Pearson χ^2 test and Fisher exact test were used to compare outcomes between controls and treated groups and also among the treated groups. Our results showed that poor scores for MT and CH increased significantly in oocytes treated with a 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 few changes compared to control. Comparison of oocyte groups that received increasing concentrations of H_2O_2 and a fixed amount of Fe(II) showed that 70–80% demonstrated poor scores in both MT and CH when pretreated with 5 μM H_2O_2, and this increased up to 90–100% when treated with 10–20 μM 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 $^{\bullet}OH$-mediated spindle and chromosomal damage, thereby serving as a possible approach for further examination as a therapeutic option in inflammatory states.

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 effects 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 post-ovulatory metaphase-II oocyte quality and integrity and accelerate oocyte aging, as judged by markers such as alterations in ooplasmic microtubule dynamics, premature release of cortical granules, and increase in zona pellucida dissolution time [3–6]. These findings not only explain the 'aging' effects of ROS on the oocytes, but also support the theory of ROS in the 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.

The 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 ROS and myeloperoxidase (MPO) activity, combined with the higher free iron that 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 $^{\bullet}OH$ depend mainly on the existence of the three components as shown in Fig. 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, 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 the generated $O_2^{\bullet-}$ undergoes either a spontaneous [16] or a superoxide dismutase-catalyzed

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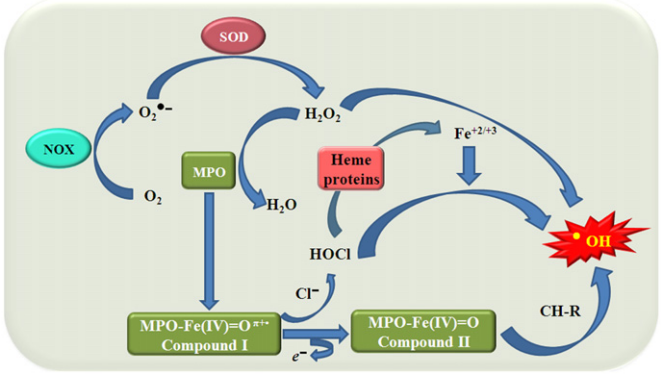

Fig. 1. Major routes of generation of •OH. Abbreviations: NOX, NADPH oxidase; O_2, oxygen; $O_2^{\bullet-}$, superoxide; SOD, superoxide dismutase; MPO, myeloperoxidase; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; CH-R, xenobiotic substrates for cytochrome P450.

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 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 •OH through their reaction with H_2O_2 or HOCl, which leads to subsequent generation of the corresponding ferryl complexes (Compound I, Fe(IV)=$O^{\pi+\bullet}$, and Compound II, Fe(IV)=O) [23], which in turn react with HC–R molecules (e.g., xenobiotic substrates for cytochrome P450) to generate •OH [10,24]. Alternatively, MPO Compound I reacts with chloride to generate HOCl, which directly reacts with Fe(II) to generate •OH [10,25–27].

Within ovarian follicles, enzymatic and nonenzymatic antioxidants protect oocytes from oxidative stress that is generated during ovulation [1,2]. As a consequence, attempts have been made to prevent deterioration in oocyte quality by supplementing the culture medium with antioxidants, such as caffeine, vitamin C, and reduced glutathione (GSH) [28–30]. Recently, we have shown that melatonin pretreatment prevents HOCl-mediated damage to the microtubule (MT) and chromosomal (CH) alignment of metaphase-II mouse oocytes [31].

Although •OH has been implicated as playing 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 in oocyte quality and infertility. Here we show that •OH mediates damage to the MT and CH alignment of metaphaseII mouse oocytes.

Materials and methods

Materials

Metaphase-II mouse oocytes were obtained commercially (Embryotech, Inc.) in cryopreserved straws. H_2O_2, ammonium ferrous sulfate (Fe(II)), human tubular fluid (HTF) medium, anti-α-tubulin antibody, FITC-conjugated anti-goat antibody, propidium iodide, BSA (bovine serum albumin), glycine, and Triton X-100 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Normal goat serum was purchased from Invitrogen (Grand Island, NY, USA) and powdered milk from a grocery store. Other chemicals and reagents were of the highest purity grade available and obtained from Sigma–Aldrich.

Methods

MetaphaseII mouse oocytes (n =200) were obtained commercially and were transferred in Dulbecco's phosphate-buffered saline (PBS) and washed to remove excess cryoprotectant for 3 min. This was followed by transferring the oocytes to HTF medium and incubating at 37 °C and 5% CO_2 for 60 min. The oocytes were then screened for the presence of polar bodies confirming their metaphase II stage. The oocytes were divided equally into four experimental sets: Set 1, study of the effect of increasing concentrations 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 preincubated with increasing concentration of H_2O_2 (5, 10, 20 μM); Set 4, untreated oocytes used as a control. The end points of the experiments involved the morphological assessment of MT and CH alignment. Results obtained were compared in each experimental set between groups using appropriate statistical tests. The concentration of Fe(II) (100 μM) used in this study to establish the •OH-generating system has been widely used in previous studies [32].

To study the effect of varying incubation time on oocytes exposed to a fixed concentration of H_2O_2 (25 μM), metaphase II oocytes were exposed to 25 μM H_2O_2 for various time periods (15, 30, 45, and 60 min), followed by indirect immunostaining to determine changes in the spindle and chromosomal alignment after the cells were fixed as described in a 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 min. The fixed oocytes were treated in blocking solution (PBS, 0.2% powdered milk, 2% normal goat serum, 1% BSA, 0.1 M glycine, and 0.1% Triton X-100) for 30 min and then washed with PBS for 3 min. Subsequently the oocytes were subjected to indirect immunostaining using mouse anti-α-tubulin antibody against the MT as the primary and FITC-conjugated 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 observers based on the scoring system published in previous studies (Fig. 2) [28,31,33]. Scores of 1–4 were assigned for both MT and CH alterations, with

Score	Microtubule	Chromosome
1		
2		
3		
4	Missing	

Fig. 2. Schematic diagram demonstrating the scoring system for MT and CH alterations based on previous studies by Choi et al. and Banerjee et al. [28,31,33].