

RESEARCH ARTICLE

Diffused Intra-Oocyte Hydrogen Peroxide Activates Myeloperoxidase and Deteriorates Oocyte Quality

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Abstract

Hydrogen peroxide (H₂O₂) is a relatively long-lived signaling molecule that plays an essential role in oocyte maturation, implantation, as well as early embryonic development. Exposure to relatively high levels of H₂O₂ functions efficiently to accelerate oocyte aging and deteriorate oocyte quality. However, little precise information exists regarding intra-oocyte H₂O₂ concentrations, and its diffusion to the oocyte milieu. In this work, we utilized an L-shaped amperometric integrated H₂O₂-selective probe to directly and quantitatively measure the real-time intra-oocyte H₂O₂ concentration. This investigation provides an exact measurement of H₂O₂ in situ by reducing the possible loss of H₂O₂ caused by diffusion or reactivity with other biological systems. This experiment suggests that the intra-oocyte H₂O₂ levels of oocytes obtained from young animals are reasonably high and remained constant during the procedure measurements. However, the intra-oocyte H₂O₂ concentration dropped significantly (40-50% reduction) in response to catalase pre-incubation, suggesting that the measurements are truly H₂O₂ based. To further confirm the extracellular diffusion of H₂O₂, oocytes were incubated with myeloperoxidase (MPO), and the diffused H₂O₂ triggered MPO chlorinating activity. Our results show that the generated hypochlorous acid (HOCl) facilitated the deterioration in oocyte quality, a process that could be prevented by pre-incubating the oocytes with melatonin, which was experimentally proven to be oxidized utilizing HPLC methods. This study is the first to demonstrate direct quantitative measurement of intracellular H₂O₂, and its extracellular diffusion and activation of MPO as well as its impact on oocyte quality. These results may help in designing more accurate treatment plans in assisted reproduction under inflammatory conditions.

Introduction

Hydrogen peroxide (H_2O_2) is a signaling molecule that comprises an important component of the oocyte microenvironment, and its overproduction mediates oocyte deterioration and dysfunction [1, 2]. Hydrogen peroxide is typically generated from a nonenzymatic substrate decay or superoxide dismutase-catalyzed reaction of superoxide ($\text{O}_2^{\bullet-}$) [3]. The biological effects of H_2O_2 are governed in part, by its intrinsic stability in the intracellular space, reactivity, oxidizing and reducing properties, permeability through cell membrane, function as a precursor of the more toxic hydroxyl radical, and a substrate for multiple enzymes [3, 4]. We and others have previously shown the critical role of this molecule in the female reproductive system, in that exogenously added H_2O_2 accelerates the aging process and deteriorates oocyte quality in concentration and time dependent manners [1, 5, 6]. These findings suggest that direct and precise intracellular measurements of H_2O_2 and its diffusion through the oocyte membrane are critically important, specifically under certain inflammatory conditions.

Given the importance of physiologic concentrations of H_2O_2 in signaling and defense as well as the potential for damage at elevated concentrations, the balance of the production and removal, either by scavenging or transportation out of the cell is paramount to the maintenance of the overall biological redox status [7]. Previously it was widely believed that H_2O_2 was able to freely cross biological membranes, this was likely to explain observed phenomenon as opposed to experimental data [7]. Recently; however, newer data suggests that membranes have limited permeability to H_2O_2 , which suggests that the molecular transportation must occur via means other than unhindered diffusion [8, 9]. The permeability of a membrane to H_2O_2 could be altered by changes in membrane composition, stage of cell cycle or by mechanical forces exerted on biological membranes [7]. Although numerous studies have focused on the exogenous addition of H_2O_2 in the deterioration of oocyte quality [1, 5, 6] information focused on its intracellular production or its transportation in the oocyte microenvironment and how these factors affect oocyte quality are lacking.

Since myeloperoxidase (MPO), an inflammatory extracellular enzyme, utilizes H_2O_2 as a substrate, the ability of H_2O_2 to diffuse to the extracellular environment is of critical importance when inflammatory changes disrupt the normal milieu [10–12]. Myeloperoxidase is a homodimeric heme-containing enzyme found in azurophilic granules of neutrophils [13, 14]. In inflammatory conditions, MPO is released from the neutrophils in the extracellular environment where, in the presence of H_2O_2 , it generates the toxic oxidant, hypochlorous acid (HOCl) through a catalytic cycle common to the mammalian peroxidase family. Derangements in MPO have also been implicated in many inflammatory conditions such as diabetes and cardiovascular disease as well as reproductive disorders such as polycystic ovary syndrome, endometriosis, and ovarian cancer [10, 11, 15–19], although an understanding of the mechanism of these damaging effects is unclear.

Melatonin is known to play an important role in the homeostasis of various neuroendocrine systems including circadian sleep rhythms, blood pressure, immunity, and reproduction [20, 21]. Importantly, melatonin also has antioxidant functions as it is known to prevent lipid peroxidation and lipoprotein modification [21]. As a protective antioxidant, melatonin can prevent HOCl insult by directly scavenging HOCl or by modulating the catalytic activity of MPO by the catalytic oxidation of melatonin [10, 22, 23]. The capacity of melatonin to compete with Cl^- leads to the inhibition of the MPO chlorinating activity while maintaining the peroxidation activity of MPO [22–25]. We have recently shown that HOCl is able to accelerate oocyte aging and deteriorate oocyte quality to a higher degree than other oxidants such as $\text{O}_2^{\bullet-}$ or H_2O_2 [1, 26].

The present studies evaluate whether intracellular H_2O_2 can serve as an extracellular substrate for MPO to produce HOCl thus deteriorating oocyte quality, and whether melatonin can

protect against this damage. To accomplish these goals, an L-shaped amperometrically integrated H_2O_2 -selective electrode has been modified to directly measure the H_2O_2 concentration continuously within non-untreated oocytes or oocytes treated with catalase. High performance liquid chromatography (HPLC) was used to understand the diffusion of H_2O_2 , and its reaction with MPO to generate HOCl as well as the oxidation of melatonin. These findings have important implications for our understanding of the pathogenesis of inflammatory diseases and for the development of novel therapeutic strategies to combat inflammatory damage in the conditions with poor reproductive outcomes.

Materials and Methods

Materials

All the materials used were of the highest grade of purity and without further purification. Hydrogen peroxide, Human tubular fluid (HTF) media, melatonin (MEL), dimethylformamide (DMF), 3,3',5,5'-tetramethylbenzidine (TMB), all the solvents used in HPLC experiment, 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. The study involved the use of oocytes obtained from super-ovulated 8–14 week-old mice B6D2F1 ($n = 20$), which was approved by Wayne State University's Animal Investigation Committee.

Intra-oocyte H_2O_2 measurement

Cumulus oocytes retrieved from the oviductal ampullae were treated with 0.1% hyaluronidase (w/v) in Human tubular fluid (HTF) media (Sigma-Aldrich (St. Louis, MO, USA)) for 2–4 minutes at 37°C. Oocytes were subsequently denuded to remove all cumulus cells with a narrow bore pulled glass Pasteur pipette, thoroughly rinsed in M2 media (Sigma-Aldrich), then the oocytes screened for the presence of the polar body confirming their Metaphase II stage. Oocytes then kept in HTF medium (Sigma-Aldrich) pre-equilibrated with 5% CO_2 in air at 37°C in a common pool before randomly transferred into test and control groups. Twenty non cumulus oocytes were used for H_2O_2 electrode experiment.

Non cumulus oocytes were pre-incubated with 100 μM melatonin in HTF media and treated with 40 nM MPO for 24 h. Of note, HTF media contains Cl^- levels akin to the oviductal fluid (~100mM). Treated oocytes were then subjected to indirect fluorescence immunocytochemistry to assess the alterations in metaphase-II mouse oocyte microtubules morphology (MT) and chromosomal alignment (CH) (markers of oocytes quality), and compared to untreated oocytes and oocytes incubated with melatonin (100 μM) alone for 24 h. In the same experiment, all the HTF media from the all treated and untreated groups was filtered and investigated using HPLC.

Immunofluorescence staining and fluorescence microscopy

Immunofluorescence staining and fluorescence microscopy were performed as previously described [5, 27]. Images were obtained utilizing both immunofluorescence and confocal microscopy.

Confocal microscopy, assessment of oocyte quality

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 as previously described [27]. Three independent observers blinded to the assigned treatment groups performed the categorization of oocytes based on MT and CH status. Observers used comprehensive evaluation of the individual optical sections and the 3-D reconstructed images.

Myeloperoxidase Purification

Myeloperoxidase (MPO) was initially purified from detergent extracts of human leukocytes by sequential lectin affinity and gel-filtration chromatography [28–30]. Trace levels of contaminating eosinophil peroxidase were then removed by passage over a sulfopropyl Sephadex column [29]. Purity of isolated MPO was established by demonstrating a Reinheitszahl value of 0.85 (A430/A280), SDS-PAGE analysis with Coomassie blue staining, and gel tetra- methylbenzidineperoxidase staining to the absence of contaminating eosinophil peroxidase activity. Enzyme concentration was determined spectrophotometrically utilizing extinction coefficients of $89,000 \text{ M}^{-1} \text{ cm}^{-1}$ /heme of MPO [31].

Melatonin solution

A stock solution of melatonin was dissolved in dimethylformamide (DMF) and then diluted to the required concentration with phosphate buffer (pH = 7.4). The final concentration of DMF in all melatonin solutions was fixed (less than 1%) and did not interfere with MPO activity nor did it have any effect on oocyte quality [26].

Electrode Description

H₂O₂ measurements were performed utilizing an Apollo-4000 H₂O₂ meter (World Precision Instruments, Sarasota, FL) equipped with an L-shaped H₂O₂ electrode (ISO-HPO-100). The features of this electrode have been previously described [32]. The absolute H₂O₂-reactive part of the needle electrode compromises its proximal 5–15 μm with a diameter of 0.8–5 μm , which is insulated by glass and has 0.5 μM limit of detection of H₂O₂. The H₂O₂ sensor uses an Ag/AgCl reference electrode and the H₂O₂-selective membrane is a WPI (Worcester Polytechnic Institute—Worcester, MA USA) membrane and was not disclosed.

Instrument Design

The electrode used in this study had a 10 μm H₂O₂ reactive part and a 2 μm diameter. The instrument calibration was performed at 37°C. The electrode was equilibrated and polarized along the recommendations of the manufacturing company (WPI). Special microtools were utilized for this procedure; these pipettes were made to meet the requirements of the measurements. For calibration, known amounts of H₂O₂ (0–2 μM) were used. The H₂O₂ (Sigma–Aldrich) solutions were prepared fresh in phosphate buffer (pH 7.4), then the concentration of the working solutions were determined spectrophotometrically (extinction coefficient of $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ at 240 nm [5, 33, 34]). During the preparation process, all solutions were kept on ice to minimize decomposition. The calibration curve was constructed by plotting the signal output (pA) versus the concentration of the H₂O₂ (μM) added at that time ($R^2 = 0.997$). The slope was then determined and entered into the Apollo 4000 software program to observe data in μM concentration mode.

A manipulated form of the aforementioned electrode was designed with a 45° angle curvature in the insulated part of electrode before insertion to increase solidification of the electrodes to prevent breakage of extremely fragile electrodes. By this method the response of the electrode is relatively fast due to its close proximity to the source of H₂O₂.

The H₂O₂-electrode was immersed into the phosphate buffered saline (PBS) buffer solution, the vial was placed over a plate stirrer, and the electrode allowed to stabilize for 3–5 min. Aliquots of different concentrations of H₂O₂ were added to the PBS buffer. The current (pA) output from the H₂O₂ electrode was increased rapidly. Within a few seconds the response reached a plateau and the second aliquot of H₂O₂ was then added. Successive additions of the remaining aliquots of H₂O₂ were made in a similar way.

Procedure for in situ measurement

The entire procedure from oocyte preparation to H₂O₂ electrode insertion has been previously reported with the use of the oocyte media (human tubular fluid (HTF) media) or PBS buffer surface as the "zero point" [32]. The zona pellucida (ZP) was slit open using a partial zona dissection (PZD) micropipette, and the probe was inserted through the ZP opening deep into the ooplasm. The oolemma was broken after deep invigilation using a technique similar to Intra-cytoplasmic sperm injection (ICSI). The picoampere difference for the H₂O₂ signal and the corresponding micromolar difference in concentration were read off the mean of the H₂O₂ calibration curves. In addition to the control group of oocytes, an additional group (n = 20) was incubated with 100 nM catalase for 30–40 min during intraoocyte H₂O₂ measurement to ensure that the measurements were truly H₂O₂ based. To minimize errors, three factors were taken in consideration for catalase treatment: time of incubation, catalase concentration, and possible contamination with catalase. Reducing the catalase concentration to half with the same incubation time had little effect on the intra-oocyte H₂O₂ concentration. Therefore, the catalase concentration and incubation time were selected to assure maximum effect on the intra-oocyte H₂O₂ concentration. Additionally, dilution of the catalase solution to a lower concentration (< 5 nM) by adding fresh media prior to electrode insertion had no effect on the results indicating no contamination upon electrode insertion. Furthermore, in our experiments, no leaks occurred, and had clear membrane damage occurred, the oocyte would be excluded from the study.

High-performance liquid chromatography analysis

HPLC analyses were performed using a Shimadzu HPLC system equipped with a 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. The column used was an Alltech 5 μm particle size, 4.6 x 150 mm reverse phase octadecylsilica (C18). To monitor the chromatogram, the 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 (Trifluoroacetic acid) in 80% acetonitrile. Moreover solvent gradient was 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 a flow rate of 0.8 ml/min with the linear gradient of solvents. After incubation of melatonin (100 μM) with MPO (40 nM) in the medium containing mouse oocytes (n = 41) for 24 h, the reaction mixture was filtered through an Amicon Ultra-15 centrifugal filter unit with Ultracel-10 membrane (from Millipore) with a 3-kDa cut-off by centrifuging at 14,000 relative centrifugal force (rcf) for 30 min at 4°C [35]; then 50 μl of the filtered sample was injected. At the end of the run the system was equilibrated with 45% solvent A; each sample was analyzed in triplicate.

Taurine Chloramine Assay

Eighty noncumulus oocytes were preincubated at 37°C in a buffer (containing 10 mM phosphate buffer, 10 mM potassium chloride and 140 mM sodium chloride, 1 mM calcium chloride, 0.5 mM magnesium chloride and 1 mg/ml glucose) with 5mM taurine [23] and 40 nM

MPO. The control solution contained the same materials in the absence of oocytes. After 30 minutes, the solutions were centrifuged and the supernatants were put on ice. Formation of HOCl indicated MPO activity, and was assessed by a change in absorbance measurements using the taurine chloramine assay. Taurine Chloramine was assayed by adding 200 μl of the oocyte supernatant to 50 μl of a reagent solution containing 10 mM TMB and 100 μM sodium iodide in 50% DMF and 400 mM acetic acid [23]. Under these conditions taurine chloramine oxidizes TMB to a blue product with an absorbance maximum at 655 nm. A standard curve was performed by adding 50 μl of reagent to 200 μl of 10 mM phosphate buffer solution pH 7.4 containing taurine and different HOCl concentrations (0–50 μM). The absorbance measurements were detected in Spectra Max 190 plate reader (Molecular Devices).

Statistical Analysis

Independent t-tests were performed using SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA) to compare groups of oocytes treated with versus without catalase, statistical significance was indicated by $P < 0.05$.

Results

An H_2O_2 -sensitive electrode tip was inserted directly into untreated oocytes and oocytes treated with catalase, and the real-time profiling of intra-oocyte H_2O_2 *in vivo* were recorded. The arrows in Fig 1 show the time of insertion and withdrawal from two individual oocytes; incubated with 100 nM catalase for 30–40 min (Fig 1; right trace) versus untreated oocyte (left trace). As shown in Fig 2, in oocytes incubated with catalase ($n = 20$), the H_2O_2 fell significantly by ~50% compared with untreated oocytes ($n = 20$). These results confirm measurement of H_2O_2 . The mentioned comparison was made using independent t-test for equality of means and Levene's test for equality of variances. The mean intracellular H_2O_2 levels for control and catalase groups showed significant difference ($P < 0.001$) (Fig 2).

The viability of oocytes was judged visually under 600 \times magnification using Nomarsky contrast during the measurement process. The parameters of oocyte quality [36–38] evaluated included: lack of intactness of their shape, darkness in cytoplasm membrane for oocytes

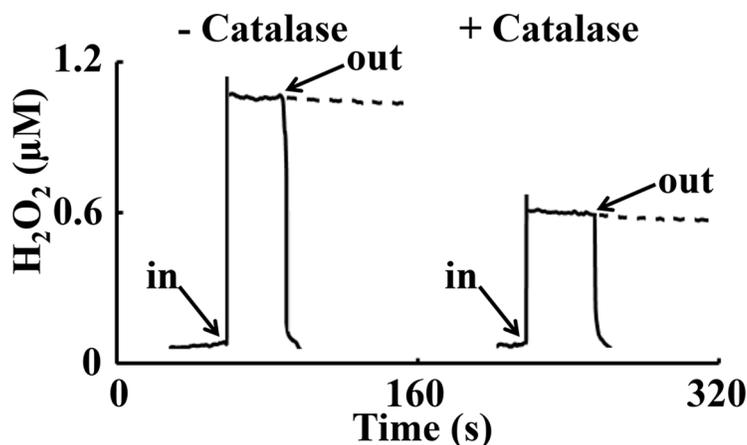


Fig 1. Real-time measurements of intra-oocyte H_2O_2 concentration utilizing H_2O_2 -selective electrode. With the use of the oocyte media or PBS buffer surface as the “zero point”, the H_2O_2 electrode tip was inserted directly into the ooplasm. The pic o ampere differences were recorded and the stable intra-oocyte reading was taken as the H_2O_2 signal. The arrows show the time of insertion and withdrawal from the oocyte. The inset shows the insertion process.

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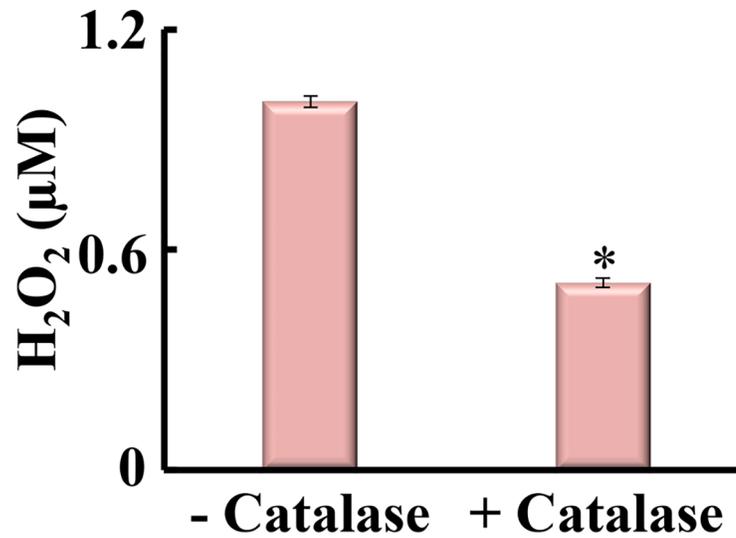


Fig 2. Effect of catalase, an H₂O₂ scavenger, on intra-oocyte H₂O₂ concentration (n = 20). The error bars represent the standard errors of mean.

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undergoing lysis, and turgidity immediately after the electrode insertion after culture at 37°C under 5% CO₂ in air for 1 hour. None of these signs were observed among the oocytes.

Hypochlorous acid, the final product of MPO-Cl⁻-H₂O₂ system, is thought to be damaging to the oocyte, thus we thought to exploit the diffusion capacity of H₂O₂ and its ability to activate MPO that subsequently mediates deterioration of oocyte quality. The oocyte quality and viability was assessed visually before and after MPO/melatonin treatments. Our results showed poor outcomes for MT and CH in oocytes treated with MPO (40 nM) as compared to controls independent of presence of cumulus cells (Fig 3). No changes as compared with controls were visualized among the oocytes treated with a combination of MPO and melatonin for 3 hours (data not shown). Thus, a sufficient amount of H₂O₂ is diffused from the oocyte that is capable of activating MPO. Melatonin, a potent MPO inhibitor and HOCl scavenger, could attenuate these damages.

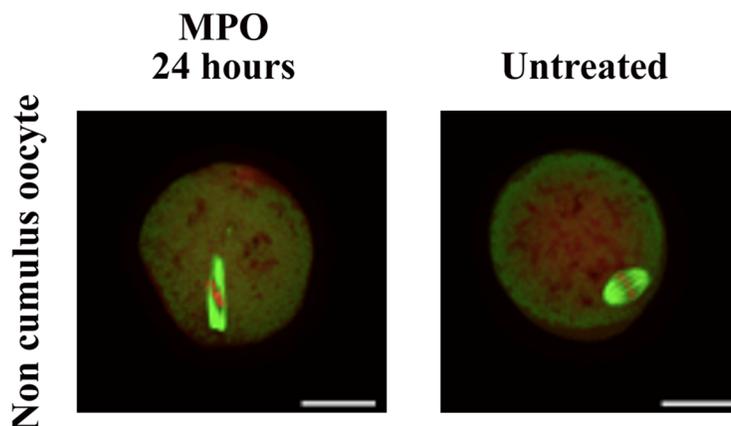


Fig 3. Effect of MPO on oocyte quality: The upper panel represents a noncumulus control oocyte with good quality scoring. The lower panel shows a noncumulus oocyte after incubation with MPO that received poor scoring.

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To investigate the mechanism of melatonin protection, HPLC analysis was performed under different experimental conditions; (1) melatonin alone (100 μM), (2) melatonin (100 μM) with MPO (40 nM), (3) melatonin (100 μM) with MPO (40 nM) treated with 100 μM H_2O_2 , and (4) MPO (40 nM) with oocytes with melatonin (100 μM). Under our experimental conditions, melatonin (222 nm) eluted at 3.98 min and was identified by its characteristic spectra observed from the photodiode array detector (Fig 4A). MPO alone had no effect on melatonin elution time (Fig 4B). MPO incubated with melatonin in the presence of oocytes there was a progressive reduction in the melatonin signal along with the formation of new peak eluting at an earlier time (Fig 4C). Similar elution times were observed when melatonin was treated with MPO-Cl⁻- H_2O_2 system. Thus, our results suggest that, in the presence of oocytes, oxidation of melatonin is the result of the activation of the MPO system caused by the diffused H_2O_2 . The appearance of new and earlier eluting peaks (3.47 min) in the chromatograms could be due to the formation of melatonin metabolite products with more hydrophobicity and lower polarity than N(1)-acetyl-N(2)-formyl-5-methoxykynuramine (AFMK) (3.57 min) that could be achieved by treating MPO/melatonin with higher concentrations of H_2O_2 . A hydroxylated intermediate in addition to AFMK was reported when melatonin was treated with H_2O_2 or neutrophils [23]. Additionally, activation of MPO by the diffused intra-oocyte H_2O_2 was confirmed utilizing the taurine chloramine assay. Indeed, incubation of 80 oocytes for 30 minutes with MPO in the presence of taurine caused the formation of a deeper blue color compared to control.

Discussion

Here, we utilized a custom made H_2O_2 -selective probe (L-shaped 5–15 μm tip) to precisely measure and quantitate the intra-oocyte cellular levels of H_2O_2 , a signaling molecule that affects biological and physiological function of the oocyte. Our findings suggest that the H_2O_2 concentration of oocytes obtained from young animals (super-ovulated 8–14 week-old mice) is relatively high (1.0 ± 0.07 μM), and that a significant portion appears to diffuse outside the oocyte. While the H_2O_2 levels remain reasonably constant during measurements, the intra-oocyte H_2O_2 concentration was reduced significantly (40–50%) when oocytes were pre-incubated with catalytic amounts of catalase, suggesting that these measurements were truly H_2O_2 centered rather than caused by an unknown interfering substance in our system. H_2O_2 diffusion out of the oocytes is also demonstrated through its ability to trigger the chlorinating activity of MPO in which HOCl alters metaphase-II mouse oocyte quality parameters of MT and CH. These alterations can be prevented by pre-incubation of oocytes with melatonin, a potent MPO inhibitor and HOCl scavenger [22]. These findings, in part, contribute to our establishment of the hypothesis that elevated levels of reactive oxygen species (ROS) such as H_2O_2 and HOCl as well as the MPO system directly or indirectly play a significant role in deteriorating oocyte quality.

In general, H_2O_2 is known to play a crucial role in both signaling and cellular regulation, and can be produced in all cellular compartments (mitochondria, cytosol, and peroxisome) from the enzymatic dismutation of $\text{O}_2^{\bullet -}$ [39]. After reversible diffusion between the cells compartments, a substantial portion of the intra-cellular H_2O_2 (60–70%) is either converted enzymatically through glutathione peroxidase and/or catalase to water, consumed by non-enzymatic low molecular weight antioxidants such as reduced glutathione, or converted to other ROS in the presence of electron donors [8, 40]. Based on the model of the semipermeable membrane, which is applied to biological systems, molecular transport through membranes depends on the size, charge of molecules to be transported and membrane composition [41]. Hydrogen peroxide, unlike $\text{O}_2^{\bullet -}$, is an uncharged and stable molecule, and therefore displays permeability in biological membranes. Hydrogen peroxide is thought to pass through

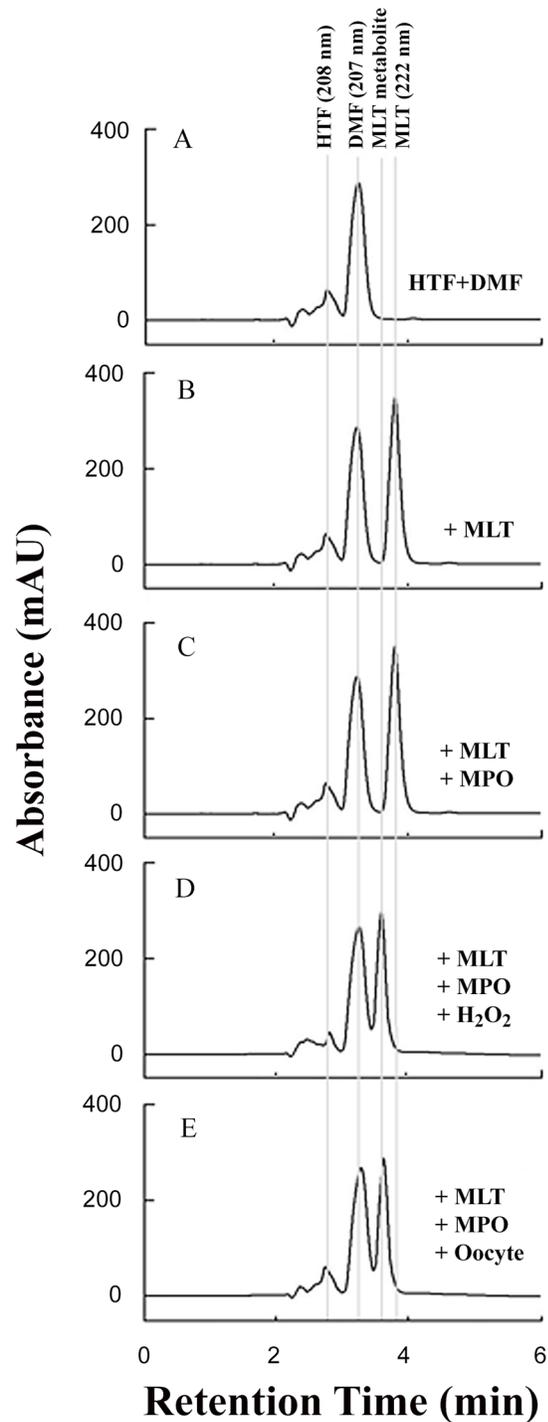


Fig 4. HPLC analysis shows evidence of the release of H_2O_2 , which activates the catalytic function or MPO causing melatonin oxidation. A) HPLC trace DMF (elution time 3.31 min) and phosphate buffer (elution time 2.48 min). B) Trace for Melatonin (3.98 min) dissolved in DMF. C) Addition of MPO causes no significant change in melatonin peak intensity and/or retention time. D) Addition of exogenous H_2O_2 (sequential addition of 20 μM , total 200 μM) results in a significant shift in melatonin retention time elution time to a hydroxylated intermediate (3.71 min). E) Incubation of MPO and melatonin in the presence of oocytes produces similar peaks and retention time to exogenously added H_2O_2 , signifying that H_2O_2 released from the oocytes reacts with MPO causing melatonin oxidation.

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biological membranes in a fashion similar to water, via limited diffusion as well as through specialized transport proteins called aquaporins [42]. Aquaporins similarly aid in the transportation of numerous other small uncharged and partially charged molecules including glycerol, urea, CO₂, polyols, purines, pyrimidines, and nitrate [43, 44]. The rate of movement through these proteins is regulated and variable depending on a variety of factors including gradients [45]. Newer evidence also suggests certain aquaporins may be specific to certain molecules; H₂O₂-specific aquaporins have been described in yeast cells [46]. Based on theoretical modeling work it appears that under physiologic conditions, approximately 30–40% of intracellular H₂O₂ diffuses out of the cell [9, 46].

Quantitative measurement of H₂O₂ in rat liver cells was calculated using catalase levels to range from ~0.001 to ~0.1 μM in periods of low and high H₂O₂ generation respectively [47, 48]. In mitochondrial cells, mathematical modeling gave an estimate of 0.04 μM [49]. Similarly, steady-state levels of H₂O₂ measured from the extracellular environment of mammalian cell suspensions have ranged from 0.02 to 2 μM. Calculations based on the permeability and gradient of H₂O₂ estimated that intracellular concentrations would be approximately 0.002–0.2 μM [50, 51]. The use of an amperometric microsensor has also been described in the measurement of H₂O₂ levels in rat brain tissue, estimating the extra and intracellular concentration of H₂O₂ to be 2.0–4.0 μM and 0.2–0.4 μM respectively [52]. In summation, these results suggest an intracellular mammalian physiologic range of H₂O₂ to be 0.001–0.7 μM [53]. These levels have also been studied in their relationship to adverse cellular effects. Hydrogen peroxide levels above the 0.5–0.7 μM range were found to be associated with apoptosis in Jurkat T cells [54]. Earlier studies by different groups utilizing different methods have shown that the intracellular H₂O₂ concentration ranging from ~0.001 μM to a higher of ~0.1 μM during peak H₂O₂ generation [47, 48]. Our measured intra-oocyte H₂O₂ concentration was estimated to be ~1.0 μM and reduced to the half when the oocyte was pre-incubated with catalase. Therefore, our findings are consistent with results obtained from other cell types. However, these results are in contrast to findings by Tripathi et al. in which the mouse intra-oocyte levels of H₂O₂ were estimated to be approximately 80 ng/oocyte. Based on an oocyte volume of 249 pL [55], this H₂O₂ concentration at 9.45 M is an improbably high value and is not consistent with life [56]. Low concentration of exogenously added H₂O₂ exposed to the oocyte for short duration has no or little effect on oocyte quality whereas higher concentration and longer exposure to H₂O₂ deteriorates oocyte quality [1, 5]. Cumulus cells show some protection against lower H₂O₂ concentration, but this protection is lost at higher concentrations (Shaeib *et al.* unpublished results). As oocytes have the additional protective antioxidant machinery of the cumulus cell, a feature, which is not seen in other cell types, the oocyte may be able to accommodate or withstand higher concentrations of H₂O₂ than other cell types.

We believe that sufficient amounts of H₂O₂ are released from the oocyte to the extracellular milieu and triggered the chlorinating activity of MPO. The amount of HOCl generated from MPO under these conditions is known to be cytotoxic as indicated by its capability to deteriorate oocyte quality [26]. Melatonin has been shown to significantly protect oocyte quality against HOCl assault either through the inhibition of MPO or through the direct scavenging of HOCl [26]. Previously, we have shown the melatonin can inhibit the catalytic activity of MPO through its ability to compete with Cl⁻ and switch the MPO catalytic activity from a 2e⁻ oxidation of Cl⁻ to a 1e⁻ oxidation pathway [22]. In these conditions the enzyme maintains its peroxidase activity but it loses its chlorinating activity and the net result is melatonin oxidation [22]. Thus, the protection is limited by melatonin concentration and the rate of its consumption by MPO. Consistent with these findings, we recently have shown that exogenously added HOCl deteriorates oocyte quality in a manner comparable to our current observed results independent of the presence of cumulus cells [26]. Elevated levels of HOCl have many damaging effects

including, but not limited to, the loss of mitochondrial DNA, the loss of the functional electron transport chains, as well as protein oxidation, and cause oxidative stress via hemoproteins heme destruction, protein aggregation, lipid peroxidation, change the membrane lipid composition, and lysis of the cell membrane leading to oocyte death [57]. Hypochlorous acid could also mediate the dysregulation of the overall antioxidant defense machinery, which could impact optimal chromatin decondensation at fertilization and, therefore, vary gene expression [23, 58–60]. Importantly, we have found that addition of melatonin protects against exogenous HOCl mediated damage [10].

Our evidence suggests that the deterioration of oocyte quality is mediated by MPO chlorinating activity rather than direct H₂O₂ insult. Hydrogen peroxide is a less powerful oxidant compared to HOCl. In physiologic circumstances, the exact nature of the relationship between H₂O₂ released from the oocyte and its interaction with cumulus cells is still under investigation. However, recently, we have shown that in the absence of cumulus cells, H₂O₂, through a mechanism that involves alteration in the MT and CH, deteriorates oocyte quality in a concentration dependent manner [5]. Cumulus cells provide protection against H₂O₂ insult at lower concentrations (>50 μM), but fail to protect the oocyte against higher concentrations (Shaeib *et al.* unpublished results). Therefore, we can speculate that disturbance in antioxidant machinery either by decreasing antioxidant enzymatic activity or exhaustion of small antioxidant molecules increases intra-oocyte H₂O₂ concentration and its diffusion to oocytes' surrounding. These phenomena may explain failures in the fertilization process in some oocyte during intracytoplasmic sperm injection (ICSI), which requires in part good oocyte quality. Uncontrolled generation of intracellular H₂O₂ may lead to atresia, poorly formed zona pellucida, or abnormal eggs that have limited or no potential of further development [5, 61]. Our findings also link inflammatory conditions related to poor reproductive outcomes such as diabetes, endometriosis and others with oocyte aging or deterioration of oocyte quality from elevated MPO levels [62, 63]. For example, it has been shown that in advanced stage endometriosis compared with early stage, neutrophil activity with expression of MPO, and thus HOCl are higher secondary to either suppression of phagocytic activity or establishment of neovascularization [64]. Efforts have therefore been made to prevent deterioration in oocyte quality by supplementing the culture media with antioxidants, such as melatonin, caffeine, vitamin C and reduced glutathione (GSH) [65].

In conclusion, this investigation presents an oocyte-exclusive method for quantitation of H₂O₂ which is aimed at reducing interfering effects and providing the highest sensitivity and precision in H₂O₂ detection in a single oocyte. Finally, we link MPO with poor oocyte quality and poor reproductive outcomes in the setting of inflammatory conditions. Additionally, with growing evidence that melatonin can protect oocytes against deterioration; melatonin may be a potential target for therapeutic intervention.

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Author Contributions

Conceived and designed the experiments: SNK GMS PTG HMAS. Performed the experiments: SNK FS TN. Analyzed the data: SNK FS HMAS. Contributed reagents/materials/analysis tools: BG. Wrote the paper: SNK FS MK HMAS.

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