Free Radical Biology and Medicine 91 (2016) 275–280

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Peroxynitrite deteriorates oocyte quality through disassembly of microtubule organizing centers

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ARTICLE INFO

Article history:
Received 16 September 2015
Received in revised form
23 December 2015
Accepted 29 December 2015
Available online 31 December 2015

Keywords:
Pericentrin
Microtubule organizing center
Peroxynitrite
Oocyte quality
Spindle morphology

ABSTRACT

Previous theoretical studies have suggested that utilization of 3-D imaging to acquire morphologic parameters of meiotic spindles may be useful in infertility related procedures as an assessment of oocyte quality. However, our results show that treatment of oocytes with increasing concentrations of peroxynitrite ($ONOO^-$) caused a dramatic alteration in spindle shape in which morphologic parameters are not measurable or are uninformative in terms of oocyte quality. Metaphase II mouse oocytes ($n=520$) were treated with increasing concentrations of $ONOO^-$, after which all oocytes were fixed and subjected to indirect immunofluorescence. Oocyte quality was assessed by alterations in the microtubule-organizing center (MTOC), pericentrin location, microtubule morphology, and chromosomal alignment. In untreated oocytes, pericentrin is primarily assembled utilizing the acentrosomal MTOC, which appears as a condensation at both spindle poles. The spindle has a symmetrical pointed barrel shape, assembled around the chromosomal plate at the spindle equator. Oocytes treated with low concentrations of $ONOO^-$ (<2.5 μM) showed shortening of the spindle apparatus, while pericentrin scatters from a tight condensation to a dispersed cluster around each spindle pole. At higher $ONOO^-$ concentrations (>2.5 μM) the central attachments between microtubules are strained and bend or unevenly break, and the MTOC proteins are further dispersed or undetectable. Peroxynitrite mediated MTOC damage, which deranges the chromosomal scaffold at the time of assembly and separation, caused the deterioration in oocyte quality. These results provide a link between reactive oxygen species and poor reproductive outcomes and elucidate the underlying etiology, which could be used as a superior biomarker for oocyte quality compared to existing assessment tools.

1. Introduction

Infertility is a growing problem worldwide. Causes of poor reproductive outcomes include broad inflammatory conditions resulting in the inability of an oocyte to be properly fertilized or the loss of an early embryo secondary to an abnormal genetic makeup [1–3]. Thus, poor oocyte quality may be the root cause of infertility or poor reproductive outcomes [2–6]. A major factor associated with oocyte deterioration is oxidative stress mediated by enhancement of reactive oxygen species (ROS) such as superoxide ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), hydroxyl radical, hypochlorous acid, and peroxynitrite ($ONOO^-$) as well as the deficiency of antioxidants such as glutathione and nitric oxide (NO) [1,5,7,8]. Peroxynitrite, a short-lived molecule, is a powerful oxidant that is generated from the near diffusion-controlled reaction of NO with $O_2\bullet^-$ [9–11]. Because of its multiple pathways of production and reaction, $ONOO^-$ concentrations are difficult to calculate in vivo, however, the rate of $ONOO^-$ production in specific compartments has been estimated to be as high as 50–100 μM/min [12]. The toxicity of $ONOO^-$ comes from the generation of free radicals (carbonate radical/nitrogen dioxide radical) when it reacts with carbon dioxide and hydronium ion [11,13]. Peroxynitrite is known to damage many cellular components, including direct deterioration of oocyte quality, in addition to, reacting with thiols and iron–sulfur centers, and initiating lipid peroxidation [5,9,12]. Peroxynitrite is a major source of nitrite (NO_2^-) and nitrate (NO_3^-) production. Indeed, follicular fluid NO_2^-/NO_3^- levels have been

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http://dx.doi.org/10.1016/j.freeradbiomed.2015.12.033
0891-5849/

linked with poor oocyte quality and poor reproduction outcome in women with endometriosis [6].

Spindle sensitivity to alterations in the oocyte microenvironment makes it an ideal marker of oocyte quality. Previously, our group and others have shown that exposure to ROS causes deterioration of oocyte quality markers microtubule morphology (MT) and chromosomal alignment (CH) [3,5,8]. Exposure of the metaphase II oocyte spindle to toxic environmental components not only disturbed the symmetrical barrel-shaped structure formed from individual microtubule spindle fibers, but also the chromosomes equatorially aligned in preparation for chromatid disjunction and segregation. The paired pericentrin proteins, which are components of the microtubule organizing center (MTOC) scaffolding, are attached to the distal ends of the microtubule spindles, which form the structural frame around the chromosomal plate [14]. The proximal ends of microtubule spindles are attached to the kinetochore proteins, which are embedded within either side of the chromosomal plate. Given the peripheral location of the MTOC at the spindle poles, these proteins are more susceptible to insult or injury than proteins embedded within the spindle.

This susceptibility in spindle structure is not limited to animal models, as it also occurs in human cells. Previous studies have found that while the spindle structure does maintain a standard bipolar organization, it does manifest a variety of morphologic variations of uncertain functional significance [15]. Due to the viscoelastic nature of the spindle, some perturbations causing minor changes to the length and width may be reversible; however, when the spindle force balance is deregulated, for example, by ROS insult, these abnormal spindle morphologies may become permanent [16]. Therefore, the spindle movement is a dynamic process in which spindle geometry could be used as a marker, as opposed to volume, which potentially remain constant or could be nonmeasurable or misleading.

We hypothesize the spindle alterations are mediated by either disruption of the spindle force balance or pericentrin derangement caused by the $ONOO^-$, and that characterizing the pattern of pericentrin movements in response to different exposures provides more information about the quality and performance of the spindle and genetic material contained within than a simple assessment of spindle dimensions. These associations carry great significance for the understanding of oocyte structure and function as they relate clinically to human fertility treatments and demonstrate our lack of understanding regarding the relationship between spindle geometry and the functional conservation of the chromosome segregation process.

2. Materials and methods

2.1. Materials

All the materials were of the highest purity grade and were used without further purification. Peroxynitrite was obtained from Cayman Chemical. Sodium nitrite, Human tubular fluid (HTF) media, anti-α tubulin antibody, DAPI, 1% Bovine Serum Albumin (BSA), 0.1% M Glycine, 0.1% Triton X-100 and propidium iodide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Purified Mouse anti-mouse pericentrin was purchased from BD Biosciences, Alexa 633 Fluor goat anti-mouse IgG were purchased from Life Technology. Fab Fragment Affinity Purified Antibodies for Blocking and Alexa Fluor® 488-AffiniPure Goat Anti-Mouse IgG (H+L) were purchased from Jackson ImmunoResearch. Normal Goat Serum (2%) was from Invitrogen (Grand Island, NY) and 0.2% Powdered Milk was obtained from a local grocer.

2.2. Solution preparation

The peroxynitrite solutions were prepared fresh daily by diluting needed volume in 0.002 M NaOH solution. The concentration of the working solutions was determined spectrophotometrically (extinction coefficient of $1705\ M^{-1}\ cm^{-1}$ at 302 nm) [17]. No changes in the pH of the media were detected after the addition of basic $ONOO^-$. During preparation, all the solutions were kept on ice to minimize any decomposition.

2.3. Methods

Metaphase II mouse oocytes without cumulus cells ($n=320$) were divided into the following groups. Group 1: oocytes incubated with increasing concentrations of $ONOO^-$ (2.5, 5, 10, 25, and 50 μM). Group 2: oocytes incubated with NO_2^-:NO_3^- (50/50 μM), the stable final products of $ONOO^-$, and decomposed $ONOO^-$ (2.5, 5, 10, 25, and 50 μM) in which $ONOO^-$ was initially added to the oocyte media (HTF) and after 4–5 min oocytes were transferred to the same media for the incubation period (as positive controls); Group 3: untreated oocytes were used as a control. All oocytes were fixed at 15 min of incubation and evaluated for alteration of MT structure and CH alignment [4,5,8,18]. To investigate the effect of $ONOO^-$ on pericentrin location and organization at the spindle poles, metaphase II mouse oocytes with ($n=100$) and without cumulus cells ($n=100$) were treated with increasing concentrations of $ONOO^-$ (0, 2.5, 5, 10, 25, and 50 μM). All oocytes were fixed at 15 min of incubation and evaluated for alteration of the MT structure and CH alignment with further evaluation for pericentrin. Results obtained were compared in each experimental set between different groups. All cell transfers were performed using 200-μM micropipette tips (ORIGIO, Cooper Surgical). Numerous experiments have shown that ROS treatment of fresh and frozen oocytes yielded similar and reproducible results [1,7,19] both in terms of spindle dynamics and cumulus cell viability and DNA integrity [20,21]. Institutional Review Board approval was not required, as the oocytes were obtained from Embryotech.

2.4. Immunofluorescence staining and fluorescence microscopy

Oocytes were fixed in a solution prepared from 2% formaldehyde and 0.2% Triton X-100 for 30 min. The fixed oocytes were 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 h then washed with PBS for 2–3 min. Subsequently, the oocytes were subjected to indirect immunostaining using Purified Mouse Anti-Mouse Pericentrin (1:100, overnight at 4 °C) as a primary antibody against pericentrin and Alexa 633 Fluor goat anti-mouse IgG (1:200, 2 h at 25 °C) as secondary antibody. Oocytes were then washed three times followed by blocking with Fab Fragment Affinity Purified Antibody (20 μg/ml) for 30 min at 25 °C. Following the last blocking, oocytes were rewashed for 2–3 min followed by mouse primary anti-α tubulin antibody against the MT (1:300, overnight at 4 °C) and secondary Alexa Fluor® 488-AffiniPure Goat Anti-Mouse IgG (H+L) (1:50, 1 h at 25 °C). The chromosomes were stained using propidium iodide (red color) for 10–15 min or DAPI (blue color) for 1–2 min. Stained oocytes were loaded into anti-fade agent on slides with two etched rings. Images were obtained utilizing both immunofluorescence and confocal microscopy.

2.5. Confocal microscopy, assessment of microtubules and chromosomal alignment

Confocal microscopy, assessment of microtubule morphology, chromosomal alignment and pericentrin slides were examined