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Detection of DNA Double-Stranded Breaks in Mouse Oocytes

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Abstract

Oocytes are amongst the biggest and most long-lived cells in the female body. They are formed in the ovaries during embryonic development and remain arrested at the prophase of meiosis I. The quiescent state may last for years until the oocytes receive a stimulus to grow and obtain the competency to resume meiosis. This protracted state of arrest makes them extremely susceptible to accumulating DNA-damaging insults, which affect the genetic integrity of the female gametes and, therefore, the genetic integrity of the future embryo.

Consequently, the development of an accurate method to detect DNA damage, which is the first step for the establishment of DNA damage response mechanisms, is of vital importance. This paper describes a common protocol to test the presence and progress of DNA damage in prophase-arrested oocytes during a period of 20 h. Specifically, we dissect mouse ovaries, retrieve the cumulus-oocyte complexes (COCs), remove the cumulus cells from the COCs, and culture the oocytes in M2 medium containing 3-isobutyl-1-methylxanthine to maintain the state of arrest. Thereafter, the oocytes are treated with the cytotoxic, antineoplasmic drug, etoposide, to engender double-strand breaks (DSBs).

By using immunofluorescence and confocal microscopy, we detect and quantify the levels of the core protein γH2AX, which is the phosphorylated form of the histone H2AX. H2AX becomes phosphorylated at the sites of DSBs after DNA damage. The inability to restore DNA integrity following DNA damage in oocytes can lead to infertility, birth defects, and increased rates of spontaneous abortions. Therefore, the understanding of DNA damage response mechanisms and, at the same time, the establishment of an intact method for studying these mechanisms are essential for reproductive biology research.

Introduction

The process of meiosis in mammalian female germ cells is initiated in the ovaries before birth. The total number of oocytes is established in the ovaries primarily during embryogenesis. Oocytes enter meiosis and remain arrested at prophase I[1]. After the onset of puberty and the production and endocrine action of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), oocytes may reinitiate and complete meiosis[2]. In humans, prophase arrest can last for up to 50 years[3]. The cell divisions following the entry into meiosis I are asymmetric, resulting in the production of a small polar body and an oocyte that retains its size. Thus, most cytoplasmic components are stored in the ooplasm during early embryogenesis[4]. Then, the oocytes enter meiosis II, without reforming their nucleus or decondensing their chromosomes, and remain arrested at metaphase II until fertilisation[5].

A unique characteristic that distinguishes oocytes from somatic cells is the state of arrest in prophase I, when the oocyte possesses an intact nucleus (germinal vesicle [GV] arrest), referred to as the GV stage[6]. Based on the chromatin organization, GV-stage oocytes are classified into two categories: non-surrounded nucleolus (NSN) and surrounded nucleolus (SN)[7,8]. In NSN GV-stage oocytes, the chromatin spreads throughout the whole nuclear region, and transcription is active, while in SN oocytes, the chromatin forms a compact ring that surrounds the nucleolus, and transcription is silent[9]. Both types of GV-stage oocytes show meiotic competence; they enter meiosis at the same rate, but the NSN oocytes present low developmental capacity and cannot develop beyond the two-cell stage embryo[10].

The protracted state of prophase I arrest increases the incidence of DNA damage accumulation[11]. Therefore, DNA damage response mechanisms in oocytes are essential for allowing the production of gametes with genetic integrity and for ensuring that the resulting embryo has a physiological chromosomal content.

A central aspect of the DNA damage response is DNA repair. The main pathways for DSB repair in eukaryotic cells include non-homologous end joining (NHEJ), homologous recombination (HR), and alternative NHEJ[12,13,14,15]. NHEJ is a faster but more error-prone mechanism, while HR requires more time to be completed but has high fidelity[16].

There is not enough knowledge about the mechanisms that oocytes use for DNA damage repair. Studies have shown that DNA damage induced in fully grown mammalian oocytes by the use of genotoxic agents, such as etoposide, doxorubicin, or UVB or ionizing radiation, does not affect the timing and rates of exit from prophase I arrest[17]. Oocytes can undergo GV breakdown (GVBD) even in the presence of elevated levels of damage. This damage can be determined by the observation of γH2AX. This phosphorylated form of H2AX (γH2AX) is a DSB marker, which is located at the site of breaks and functions as a scaffold to help repair factors and proteins to accumulate at broken ends[18].

The absence of cell cycle arrest following DNA damage is due to an insufficient DNA damage checkpoint that allows oocytes with unrepaired DNA to re-enter meiosis. Following high levels of DNA damage, a checkpoint can maintain prophase arrest through the activation of an ATM/ Chk1-dependent pathway. The limited checkpoint response to DSBs is due to the limited activation of ATM[17,19]. In the M-

phase of meiosis I, research has shown that DNA damage may activate a spindle assembly checkpoint (SAC)-induced meiosis I checkpoint, which prevents the activation of the E3 ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C) and, therefore, M-phase exit. Moreover, the ablation of SAC proteins overcomes the state of M-phase arrest, thus underlining the importance of the SAC in the establishment of the meiosis I chekpoint[20].

As previous research clearly shows, DSBs cannot induce a robust prophase checkpoint in mouse oocytes. If such damage is left unrepaired, it could lead to embryos carrying chromosomal abnormalities. Therefore, it is important to study the DNA damage response at different stages of female gametogenesis to better understand the unique pathways that oocytes use to cope with potential genetic insults.

Protocol

All mice experiments were approved by the local authorities (Region of Ioannina, Greece) and conducted in accordance with the European Communities Council Directives 2010/63/EU. Experiments were conducted with respect to the principles of the 3Rs. All the CD-1 mice used for the experiments were kept in the animal house facility of the University of Ioannina, Greece, in a room with controlled temperature (22 °C) and humidity (60%) and were fed *ad libitum*. The animal house has a license to operate a facility for breeding (EL33-BIObr01), supply (EL33-BIOsup01), and experiments (EL33BIO-exp01).

1. Preparation of reagents

1. Dilute 3-isobutyl-1-methylxanthine (IBMX) powder (see the **Table of Materials**) in dimethyl sulfoxide (DMSO) (see the **Table of Materials**) to a final concentration of 200 mM. Make 10 µL aliquots, and store at −20 °C. Use the solution within 1 month.

   NOTE: IBMX powder is kept at −20 °C.

2. Prepare all the immunofluorescence buffers, and store them at 4 °C.

   1. Prepare sterile phosphate-buffered saline (PBS) by diluting one PBS tablet (see the **Table of Materials**) in 200 mL of ddH_2O.

   2. Make PHEM buffer by adding in 80 mL of ddH_2O, 0.59575 g of HEPES, 1.81422 g of PIPES, 0.38035 g of EGTA, and 0.04066 g of $MgCl_2$ (see the **Table of Materials**) while agitating with a magnetic stirrer (see the **Table of Materials**), and simultaneously add NaOH (see the **Table of Materials**) until the pH reaches 6.9 (check using a pH/ORP meter [see the **Table of Materials**]). Then, add ddH_2O to a final volume of 100 mL.

   3. Prepare paraformaldehyde-Triton-X-100 (PFA-Tx-100) buffer by diluting PFA powder (see the **Table of Materials**) in PHEM buffer while agitating with a magnetic stirrer under heating at a final concentration of 4% PFA. Then, filter the buffer using a syringe and a 0.2 µm filter (see the **Table of Materials**), and add 0.5% Tx-100 (see the **Table of Materials**). Prepare approximately 10 mL of PFA-Tx-100 (0.4 g of PFA, 50 µL of Tx-100), which is enough for one experiment. Store it at 4 °C for 1 week maximum.

      CAUTION: Wear gloves to handle PFA, and avoid contact with skin and eyes.

   4. Prepare washing buffer by adding bovine serum albumin (final concentration: 0.5% w/v BSA) (see the **Table of Materials**) in PBS, and agitate

mechanically. Add 10% w/v NaN_3 buffer (sodium azide) at a 1:1,000 dilution to minimize the risk of fungal and bacterial contamination. Make a 10% w/v NaN_3 buffer by adding 1 g of NaN_3 powder (see the **Table of Materials**) to 10 mL of ddH_2O; store the NaN_3 buffer at room temperature.

5. Prepare blocking buffer by adding BSA (final concentration: 3% w/v) in PBS and agitating mechanically. Add 10% NaN_3 buffer at a 1:1,000 dilution.

2. Collection of GV oocytes from dissected ovaries and induction of DSBs

NOTE: All the tools and solutions should be sterile. Oocyte handling is conducted by using a mouth pipette under a stereo microscope (see the **Table of Materials**), and all the drops are covered with mineral oil (see the **Table of Materials** and **Figure 1E**).

1. Inject mice intraperitoneally with 7 international units (IU) of pregnant mare's serum gonadotropin (PMSG) (see the **Table of Materials**) 46-48 h before culling the mice by cervical dislocation.
   NOTE: All the mice used should be 8-12 weeks old.
2. Filter M2 culture medium (see the **Table of Materials**) with a syringe and a 0.2 µm filter, and add IBMX 200 mM to a final concentration 200 µM in a 14 mL round-bottom tube (see the **Table of Materials**) to keep the oocytes arrested at prophase I. Then, prepare drops of M2-IBMX medium in a plastic tissue culture dish (see the **Table of Materials**), and place it on a hot block (see the **Table of Materials**) at 37 °C for at least 30 min before oocyte isolation. Store the M2 at 4 °C.
3. Sacrifice the mice by cervical dislocation, dissect the ovaries, and place them in a 5 mL round-bottom tube (see the **Table of Materials**) with M2-IBMX.
4. Transfer the ovaries to a plastic lid containing 1.5 mL of M2-IBMX, remove any peri-ovarian adipose tissue or fallopian tube segments, and release the COCs by mechanical perforation of the ovaries with a 27 G needle (see the **Table of Materials** and **Figure 1A-C**).
5. Transfer the COCs to a culture dish with drops of M2-IBMX (approximately 25-30 µL each), and remove the cumulus cells by repeated pipetting using a narrow-bore glass Pasteur pipette (see the **Table of Materials** and **Figure 1D**).
6. Select SN GV-stage oocytes, and transfer them in a drop (25 µL) of M2-IBMX medium on a hot block at 37 °C protected from light (**Figure 1F**).
   1. Look for SN oocytes based on their bigger size and centrally placed nuclei in contrast with NSN oocytes, in which the nuclei are positioned peripherally[21]. In any case, observe the DNA configuration under a confocal microscope before taking the final decision about the type of GV oocyte (SN or NSN).
7. Induce DSBs using etoposide (see the **Table of Materials**). Place the GV-stage oocytes in drops (25 µL each) of the genotoxic agent for 1 h on the hot block at 37 °C in dark conditions.
   NOTE: Etoposide is a topoisomerase II inhibitor that introduces DSBs to the DNA[22]. Keep the etoposide in 10 µL aliquots of 20 mg/mL at room temperature protected from light. The concentrations that have been tested are 5 µg/mL, 20 µg/mL, and 50 µg/mL.

8. To keep the GV-stage oocytes arrested for a prolonged period, place the oocytes in drops of M16 culture medium (see the **Table of Materials**) supplemented with 400 µM IBMX in an incubator (see the **Table of Materials**) at 37 °C and 5% CO_2. Store the M16 at 4 °C, filter the medium with a syringe and a 0.2 µm filter, and incubate it for at least 1 h before use.

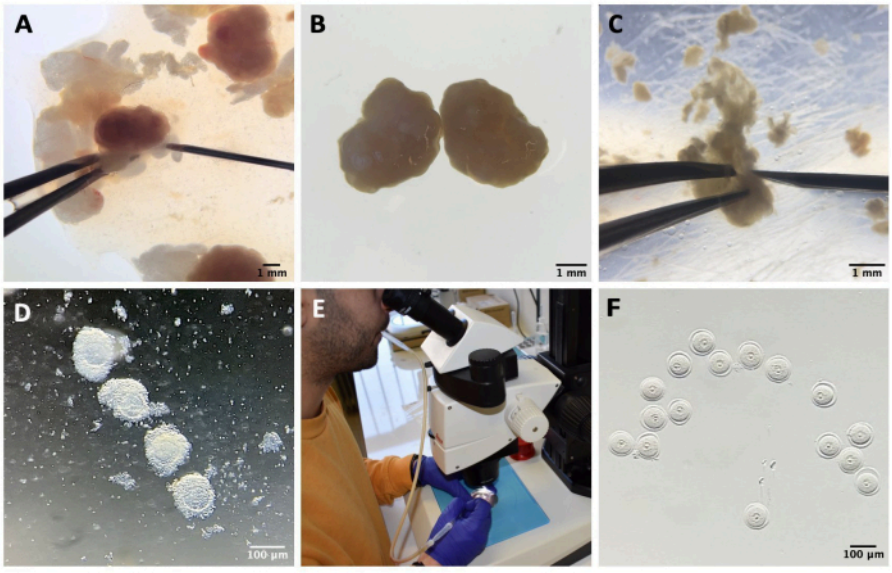

Figure 1: Oocyte isolation process. (**A**) Removal of peri-ovarian adipose tissue and leftover fallopian tube segments from ovaries in M2 medium with IBMX. Photograph obtained through the stereo microscope eyepieces. Scale bar = 1 mm. (**B**) Isolated ovaries in M2 medium with IBMX. Image obtained through the stereo microscope eyepieces. Scale bar = 1 mm. (**C**) Mechanical perforation of ovaries using a 27 G needle in M2 medium with IBMX. Image obtained through the stereo microscope eyepieces. Scale bar = 1 mm. (**D**) COCs released from ovaries after perforation in M2 medium with IBMX. Image obtained through the stereo microscope eyepieces. Scale bar = 100 µm. (**E**) Oocyte collection using a mouth pipette. (**F**) Denuded oocytes, after the removal of the surrounding cumulus cells, in M2 medium with IBMX. Image obtained through the stereo microscope eyepieces. Scale bar = 100 µm. Please click here to view a larger version of this figure.

3. Oocyte fixation and immunofluorescence

NOTE: Oocyte handling is conducted by using a mouth pipette under a stereo microscope, and all the drops are covered with mineral oil.

1. Place the control and etoposide-treated GV oocytes in different plastic tissue culture dishes with PFA-Tx-100 buffer for 40 min at room temperature.
2. Wash the oocytes in three different drops of washing buffer (50 µL each) at room temperature. Leave the oocytes for 5 min in each drop.

3. Place the oocytes in drops of blocking buffer (25 µL each) for 1 h on a hot block at 37 °C.

4. Prepare the primary antibody that recognizes γH2AX (rabbit phospho-H2AX) (Ser139) (see the **Table of Materials**) (stock solution: 1 mg/mL). Use a 1:200 dilution in blocking buffer, and place the oocytes in drops of primary antibody (15 µL each) at 4 °C overnight.
   NOTE: Phospho-H2AX (γH2AX) is a common marker for detecting DSBs in both somatic cells and GV oocytes[18,23].

5. The following day, wash the oocytes in three different drops of washing buffer (50 µL each) at room temperature. Leave the oocytes for 5 min in each drop.

6. Prepare the secondary antibody, Alexa Fluor 488-conjugated goat anti-rabbit (see the **Table of Materials**) (stock solution: 2 mg/mL). Use a 1:200 dilution in blocking buffer, and place the oocytes in drops of secondary antibody (15 µL each) for 1 h on a hot block at 37 °C protected from light.

7. Transfer the oocytes into drops of DRAQ7 (25 µL each) (stock solution: 0.3 mM; see the **Table of Materials**), which is a far-red fluorescent DNA dye that only stains DNA in permeabilized cells. Use a 1:250 dilution in washing buffer for 10 min at room temperature in dark conditions.

8. Wash the oocytes in three different drops of washing buffer (50 µL each) at room temperature. Leave them for 5 min in each drop, and then transfer them to small drops (approximately 5 µL each) of washing buffer in a 35 mm glass-bottom Petri dish (see the **Table of Materials**) for confocal microscopy (**Figure 2A**).

   NOTE: The washing of both the DNA stain and secondary antibody is performed at the same time.

4. Confocal microscopy

NOTE: Confocal microscopy should be performed immediately to avoid the reduction in the fluorescence intensity after the placement of the oocytes in glass-bottom dishes. Access to a confocal microscope (see the **Table of Materials**) with a motorized stage is required.

1. Microscope setup
   1. In the confocal system, switch on the laser controller, the lasers, the microscope controller, the lamps for the transmitted light, and the PC (**Figure 2B, D**).
   2. Open the confocal software, and choose the **40x oil lens**.
   3. Place the dish into the specimen holder, and try to focus on the oocytes by moving the stage on XY and Z axes using the joystick (**Figure 2C**).
2. Scanning of the oocytes
   1. Set the **laser power**, the **gain**, and the **pinhole size** independently for each experiment in order to minimize any saturation.
   2. For each oocyte, set the area of interest, specifically in the nucleus at the DNA area. Define the borders of the DNA area, and adjust the **z step size** to **3 µm**. Then, start the scanning.
   3. Save the images for each cell in the selected folder.
   4. When the scanning is completed, exit the software, shut down the computer, and turn off the laser controller, the lasers, the microscope controller, and the lamps for the transmitted light.

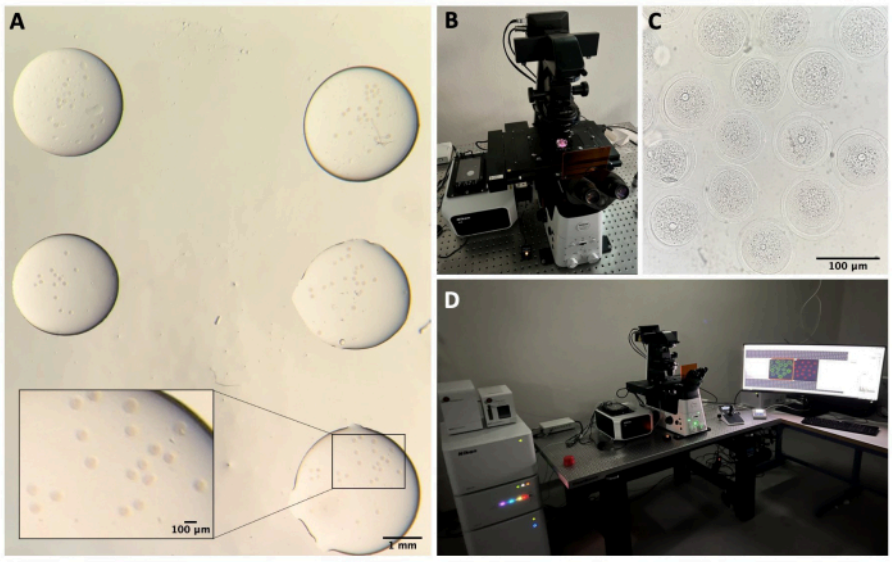

Figure 2: Confocal microscopy. (**A**) Fixed oocytes after performing the immunofluorescence protocol and DNA staining, which are in separate drops of washing buffer, covered with mineral oil, placed in a glass-bottom dish, and prepared for confocal microscopy imaging. Each drop contains a different experimental category. Image obtained through the stereo microscope eyepieces. Scale bar = 1 mm/100 µm for the zoomed-in portion. (**B**) Glass-bottom plate placed on the confocal microscope stage. (**C**) Brightfield image of oocytes obtained through confocal microscopy. Scale bar = 100 µm. (**D**) The confocal microscopy system. Please click here to view a larger version of this figure.

5. Imaging analysis

1. Download Fiji ImageJ-win64 in the browser (https://imagej.net/software/fiji/downloads), open it, and import the data as TIFF stack files.
   NOTE: Open each oocyte file separately.
2. Click on **Image | Color | Split Channels** to split all the channels.
3. Click on **LUT (Look up Table)**, and choose the preferred colors for each channel.
4. Click on **Image | Color | Merge Channels** to merge the channels for γH2AX and DNA. Leave the brightfield channel unmerged.
5. In NSN oocytes and in SN oocytes with low levels of DNA damage, γH2AX is detected as foci in the DNA region. In this case, click on the **"Multi-point" or point** command, and select every γH2AX focus that coincides with the DNA. Repeat this step for all the stacks.
6. In SN oocytes with high levels of DNA damage, the γH2AX signal is distributed throughout the whole DNA region. In this case, click on **Image | Stacks | Z project**, and with the **Freehand selections** command, select the entire DNA area.

7. To measure the γH2AX fluorescence, click on **Analyze | Measure**, and copy the measurements into a .xlsx file. Then, calculate the mean fluorescence, normalize the values, and count the number of foci before creating any graphs.
8. Click on **Analyze | Set Scale** to set the scale and then on **Analyze | Tools | Scale bar** to add a scale bar to the channels.

Representative Results

Using the procedure demonstrated here, mouse ovaries were dissected, the fat was removed, and fully grown GV-stage oocytes were collected. Then, the cumulus cells were removed by repetitive pipetting using a narrow pipette and were placed in fresh drops of M2-IBMX medium and covered with mineral oil on a hot block (37 °C) (**Figure 1A-F**). Three different etoposide concentrations were prepared (5 µg/mL, 20 µg/mL, and 50 µg/mL) by using a stock etoposide concentration of 20 mg/mL. The GV-stage oocytes were placed in three distinct etoposide concentrations for 1 h in drops covered with mineral oil and protected from light at 37 °C. The immunofluorescence protocol was then followed, as described in the protocol section in detail, and the oocytes were placed in glass-bottom dishes and observed by confocal microscopy (**Figure 2**).

In the SN GV-stage oocytes, immediately after DNA damage, the presence of γH2AX increased at all the etoposide concentrations (5 µg/mL, 20 µg/mL, and 50 µg/mL), and the γH2AX was distributed throughout the whole DNA region (**Figure 3**). DSB quantification and estimation were performed by observing the γH2AX fluorescence intensity at DNA sites. The γH2AX fluorescence intensified proportionally with increasing etoposide concentrations. Moreover, after protracted prophase arrest (20 h after etoposide treatment), the GV-stage oocytes showed the capacity to reduce the γH2AX foci number and intensity, implying the presence of active repair processes in the GV-stage-arrested oocytes (**Figure 3E**).

Unlike the SN oocytes, in which the γH2AX fluorescence was distributed through the DNA, in the NSN oocytes, γH2AX was shown in foci immediately after treatment with etoposide at 20 µg/mL. We estimated the number of foci that coincided with the DNA area, calculated the fluorescence of every focus, and presented the mean fluorescence of all the oocytes. Both the fluorescence and number of foci showed statistically significant differences between the two oocyte categories (**Figure 4**).

Confocal microscopy provides information on the number and intensity of foci in different Z stacks, thus helping to identify the presence of DNA damage and the repair dynamics at distinct time points. Galvano scanning provides precision scanning with low background and better analysis of the scanning images.

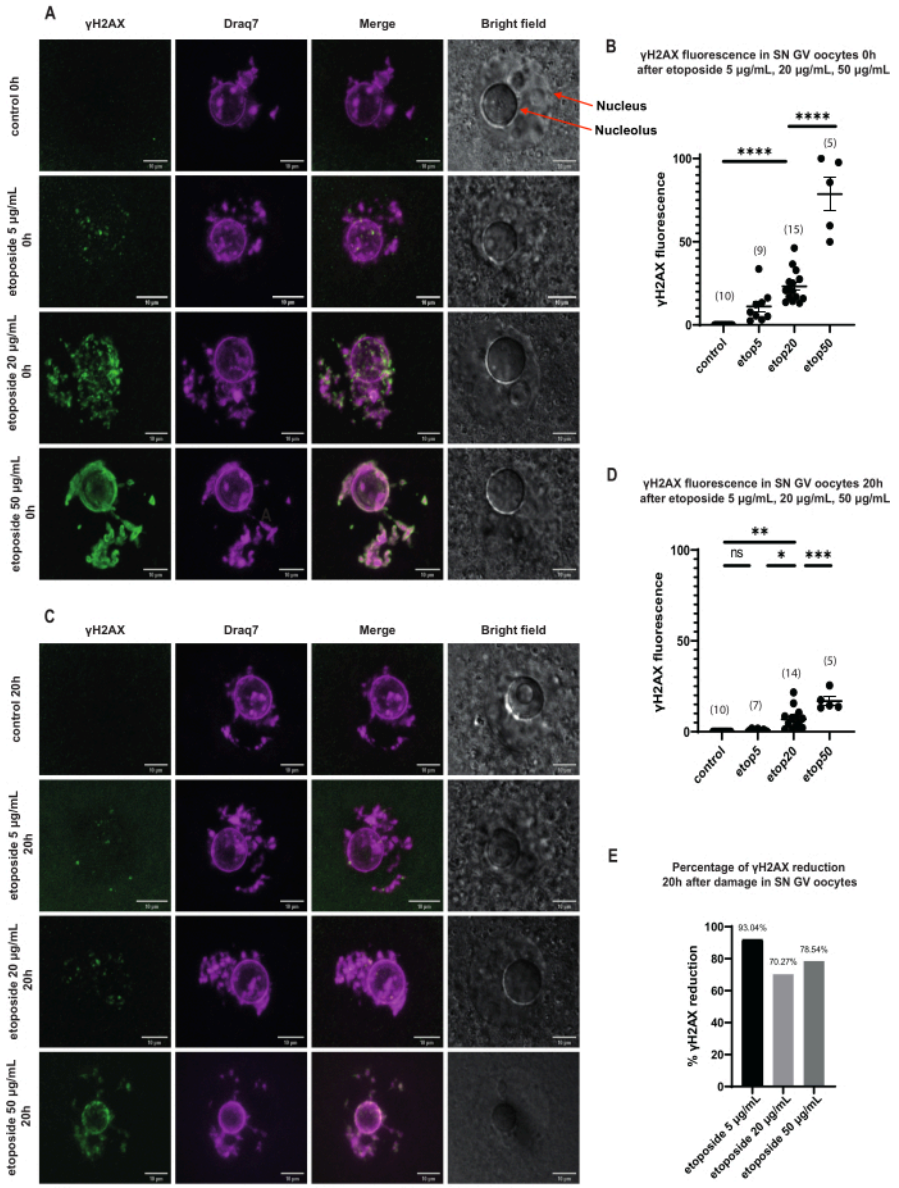

Figure 3: Reduction of γH2AX in SN GV-stage oocytes treated with three different etoposide concentrations after protracted GV arrest. (**A**) γH2AX fluorescence in SN GV-stage oocytes 0 h after etoposide treatment. The γH2AX increases immediately after exposure at all the etoposide concentrations, and the increase is concentration-dependent (green: γH2AX, magenta: DNA). The images are Z-stack projections, and the brightness/contrast have been adjusted for each channel using Fiji / ImageJ. Scale bar = 10 µm. (**B**) Graph of the γH2AX fluorescence in SN GV-stage oocytes 0 h after treatment with distinct etoposide concentrations. Data represent mean ± SEM. Each dot represents one oocyte (the number of oocytes is shown in the graph), (ns = non-significant, ** $p < 0.005$, **** $p < 0.0001$, one-way ANOVA with Tukey's multiple comparisons test). (**C**) γH2AX fluorescence in SN GV-stage oocytes 20 h after etoposide treatment. γH2AX reduces 20 h after exposure

at all the etoposide concentrations (green: γH2AX, magenta: DNA). The images are Z-stack projections, and the brightness/contrast have been adjusted for each channel using Fiji/ImageJ. Scale bar = 10 µm. (**D**) Graph of the γH2AX fluorescence in SN GV-stage oocytes 20 h after treatment with distinct etoposide concentrations. Data represent mean ± SEM. Each dot represents one oocyte (the number of oocytes is shown in the graph), (ns = non-significant, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$, one-way ANOVA with Tukey's multiple comparisons test). (**E**) Bar graph of the γH2AX fluorescence reduction in SN GV-stage oocytes after prophase arrest in etoposide-treated oocytes. The number above each column indicates the percentage decline in γH2AX fluorescence. Please click here to view a larger version of this figure.

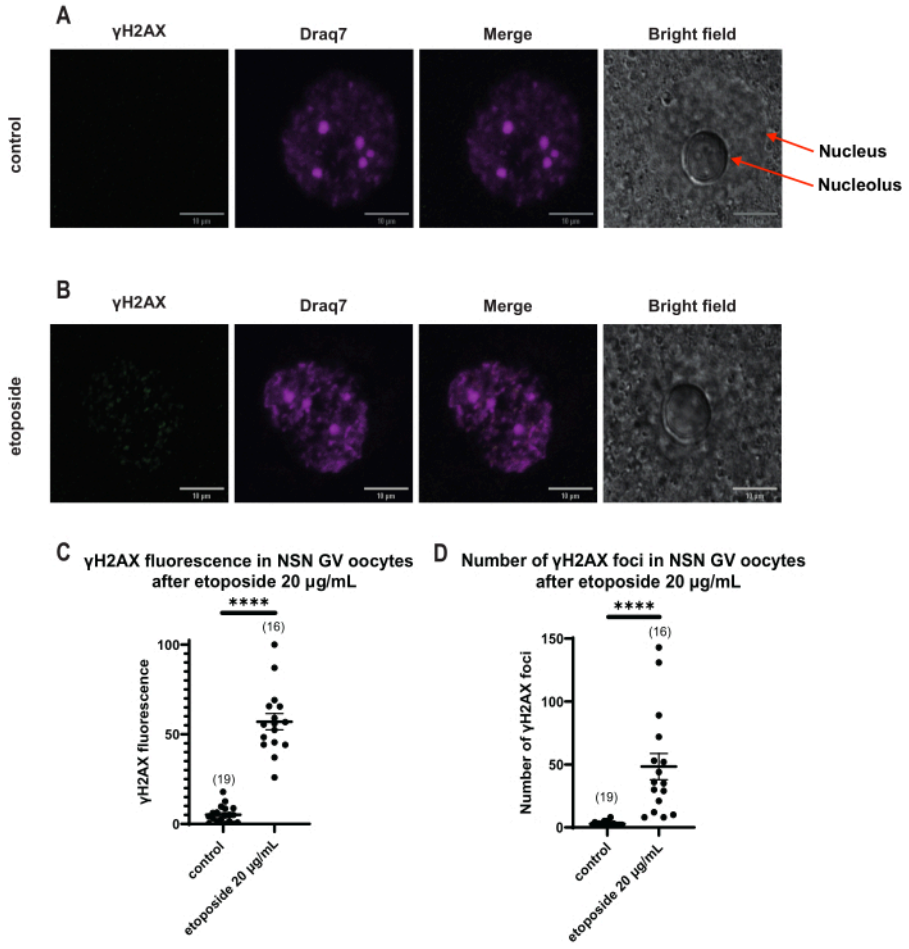

Figure 4: Phosphorylation of H2AX in NSN GV-stage oocytes after treatment with etoposide at 20 µg/mL. (**A**) Representative confocal images of one control NSN GV-stage oocyte (green: γH2AX, magenta: DNA). The images are Z-stack projections, and the brightness/contrast have been adjusted for each channel using Fiji/ImageJ. Scale bar = 10 µm. (**B**) Representative confocal images of one etoposide-treated NSN GV-stage oocyte (green: γH2AX, magenta: DNA). The oocytes were fixed 0 h after etoposide treatment. The images are Z-stack projections, and brightness/contrast have been adjusted for each channel using Fiji/ImageJ. Scale bar = 10 µm. (**C**) The normalized γH2AX fluorescence in NSN GV-stage oocytes after 20 µg/mL etoposide treatment. Data represent mean ± SEM. Each dot represents one oocyte (the number of oocytes is shown in the graph), taken from two independent experiments (**** $p < 0.0001$, unpaired non-parametric *t*-test, Mann-Whitney *U*-test). (**D**) Number of γH2AX foci in NSN GV-stage oocytes after 20 µg/mL etoposide treatment. Data represent mean ± SEM. Each dot represents one oocyte (the number of oocytes is shown in the graph), taken from two independent experiments (**** $p < 0.0001$, unpaired non-parametric *t*-test, Mann-Whitney *U*-test). Please click here to view a larger version of this figure.

Discussion

By using the method described here, we detected DSBs in mammalian oocytes. This method allows for the detection and study of the DNA repair process in oocytes. The same protocol could also be used for analyzing other proteins that participate in physiological processes in mammalian oocytes. It is important to study how oocytes respond to potential DNA damage in order to better understand the cause of female subfertility in humans.

Studying the DNA damage response in mammalian oocytes can be challenging because of the sensitivity of oocytes. Oocyte handling requires specific temperatures and CO_2 and O_2 concentrations. At the same time, the oocytes must be protected from light. All handling should be done by using glass pipettes that are not too narrow, as this could be harmful for the oocytes, but also not too wide, as this could cause the dilution of the medium and, thus, negatively affect the fixation procedure. In each step of fixation, several drops of buffers are used to minimize the dilution effect. An alternative way for observing DSBs is the Comet assay[24]. Even though this technique is more sensitive, it is more complicated. At the same time, by using the Comet assay, it is not possible to detect the exact DNA region where the damage occurs, and in cells with abundant RNA molecules, like GV-stage oocytes[25], the background could be increased, leading to a false DNA damage signal[26].

By using the immunofluorescence protocol described here, we can detect DSBs with accuracy and estimate the repair progress in GV-stage oocytes, as indicated by the reduction in γH2AX fluorescence over time. Nevertheless, one limitation of this method is that certain antibodies may present nonspecific distribution throughout the ooplasm, thus leading to images with high background fluorescence. The PFA-Tx-100 buffer is used instead of sequential PFA and Tx-100, as we have observed that it improves the fixation process by allowing the detection of less background and non-specific fluorescence. A second limitation of using γH2AX for DSB detection is that damage cannot be estimated after GVBD because of the spontaneous phosphorylation of γH2AX in meiosis[23].

In this immunofluorescence protocol, the oocytes remain in a liquid buffer and cannot be stored within slides. This fact makes it difficult to preserve the fixed cells for days after the addition of the secondary antibody. In order to attain good-quality images and not to lose signal, it is preferable to perform the imaging within a few hours after the addition of the secondary antibody. It should also be noted that the scanning of the nuclei through the Z-axis could make the signal become weaker due to overexposure. For that reason, it is preferable to lower the laser power and to increase the speed of the scanning.

Lastly, another limitation of the immunofluorescence protocol is that it can be used only for fixed/non-living cells. Therefore, we can estimate only the presence and absence of factors at specific time points without knowing if there are any fluctuations in their concentration or changes in their behavior through time. This problem could be overcome by using live-cell imaging and fluorescently tagged markers.

Disclosures

The authors have no conflicts of interest.

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