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RESEARCH ARTICLE

Detection of DNA damage in cumulus cells using a chromatin dispersion assay

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Abstract

DNA damage in cumulus cells (CCs) might be related with the developmental competence of the enclosed oocytes, however, conclusive studies are missing, partially due to the lack of a reliable, cheap, fast, and reproducible DNA damage test. We report the development of a chromatin dispersion test that allows for a fast evaluation of double strand DNA (ds-DNA) damage in CCs. The whole experiment was performed using CCs from 103 oocyte retrieval cycles evaluating the prototype D3-MAX ability (a chromatin dispersion based assay) to detect DNA breaks against *in situ* nick translation (ISNT) and a two tailed comet assay (TT-comet). Samples were collected from women younger than 35 years of age with a good response to stimulation. Pooled cumulus cells of MII oocytes were used. The chromatin dispersion assay results correlate with the double strand-DNA breaks values assessed by the TT-comet assay (Spearman Rho = 0.624; $p = 0.003$;), while the correlation was poor when compared to the single strand DNA (ss-DNA) breaks observed also with the TT-comet assay (Spearman Rho = −0.141; $p = 0.554$). ISNT showed a correspondence in the same cells between enzymatic incorporation of modified nucleotides and halos of chromatin dispersion. We conclude that D3-Max test detects mainly ds-DNA breaks in cumulus cells and is a reliable, fast, and easy reproducible assay suitable for routine clinical practices once the influence on oocyte quality has been established.

Abbreviations: CCs: cumulus cells; DNA: deoxyribonucleic acid; ds-DNA: double-strand DNA; ss-DNA: single-strand DNA; ISNT: *in situ* nick translation; MII: metaphase II; TT-comet: two tails comet; GnRH: gonadotropin-releasing hormone

Keywords

Assisted reproduction, chromatin dispersion test, cumulus cells, DNA fragmentation

History

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Introduction

The cumulus-oocyte complex (COC) is a morpho-functional unit [Su et al. 2009], and the communications between the two compartments allow for important developmental steps such as oocyte growth, meiotic maturation, and maintenance of meiotic arrest. As the oocyte reaches the final stages of maturation, the gap junctions residing at the end of the cytoplasmic extension which connect the cumulus cells, through the zona pellucida, to the oolemma, are progressively closed. As the cumulus expands, cell-cell connections are lost, and cumulus cells undergo apoptosis [Liu 2007; Luciano et al. 2004; Richards 2005]. Because of this unique relationship, cumulus cells (CCs) are thought to be a valuable proxy for assessing the quality of the oocyte they surround.

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In the last years, cumulus cells (CCs) as biomarkers of oocyte quality have been proposed, often, in the form of expression levels of a set of mRNA transcripts [Ekart et al. 2013; Huang et al. 2013; Iager et al. 2013]. However, although promising, these markers have yet to be proven valuable in differentiating levels of oocyte quality in a clinical setting. Deoxyribonucleic acid (DNA) fragmentation in mammalian cells occurs both as a programmed event, for example during meiotic recombination, or in response to both endogenous (excess reactive oxygen species) and exogenous stimuli such as chemical drugs, UV light, and ionizing radiations exposure, or cryopreservation. Both single and double strand DNA breaks carry the potential to damage the cell. Double strand DNA break (ds-DNA), if not repaired, can lead to cell cycle delay, cell cycle arrest, apoptosis, and other forms of cell death. DNA fragmentation in CCs has been shown to be adversely related to the developmental capacity of the enclosed oocyte to reach blastocyst stage in an animal model of IVF [Ikeda et al. 2003]. In human patients, CCs DNA breaks were found to be inversely correlated to the

fertilization rate by ICSI [Raman et al. 2001]. However, another report questions these results, finding no correlation between CCs apoptosis and ICSI outcomes [Abu-Hassan et al. 2006]. Part of the discrepancy in the reported results might be caused by the complexity and high inter-laboratory variability of the comet assay, which is used in most studies [Moller et al. 2010]. Although CCs DNA state represents a potentially valuable indicator of oocyte competence, a thorough investigation of its significance is missing, possibly for the lack of a simple, fast, and reliable test of DNA damage customized for this cell type, standardized among operators and laboratories, and not requiring expensive equipment. This investigation was aimed at assessing the feasibility of testing the level of DNA breaks in human cumulus cells by means of a modified chromatin dispersion assay; moreover, we set out to analyze the kind of DNA strand breaks that can be detected with this method.

Results

Morphology using the chromatin dispersion assay

CCs samples processed with the D3-Max prototype assay resulted in two main nuclei morpho-types (CCs-M) that can be distinguished under bright field (Figure 1a) or under fluorescence microscopy (Figure 1b–e). CCs-M1 nuclei displaying a small, regular, and compact halo of dispersed chromatin surrounding a regular sized core with intense staining is shown in Figure 1a arrowed and b–d. CCs-M1 nuclei were considered as those with non-fragmented DNA. CCs-M2 nuclei displaying halos of variable dimensions of dispersed chromatin with a weak staining on the halo and a visible and variable sized core are shown in Figure 1a arrow heads and b′–e′. In some of these CCs-M2 cells, the halo size tends to diminish showing faint halos of dispersed chromatin and a reduced core in the nuclei (Figure 1d′ and e′); CCs-M2 nuclei were interpreted as presenting fragmented DNA, and the dimension of the halo related to the level of fragmentation. CCs used as negative control do not exhibit any trace of chromatin dispersion. Comparative densitometry analysis of CCs-M1 (Figure 1b–e) and CCs-M2 (Figure 1b′–e′) types shows that there is a tendency to DNA loss in CCs-M2 (Figure 1b″–e″) when compared with CCs-M1 type and probably this represents more intense DNA damage as DNA available for staining is lost after protein depletion.

Indirect assessment of DNA damage: two tails comet (TT-comet) assay

The TT-comet assay revealed four distinct comet morphologies, indicating different degrees of severity of DNA damage (Figure 2). The first comet morphology visualized at the cumulus cells was revealed after the alkaline electrophoresis (Figure 2a–a′). In this case, a Y axis migrant comet (downward shift) with a consistent nuclear core and tail size, indicative of alkaline sensitive DNA stretches, is visualized in most of the cells (Figure 2a–a′); these comets were interpreted as the structural and constitutive presence of DNA motifs highly susceptible to alkali (alkali labile sites). The second morphology was revealed following application of neutral electrophoresis whereby, DNA fragments migrated on the X axis (right-hand shift), in the direction perpendicular to that of structural comets (Figure 2b–b′); these comets contain DNA fragments originating from ds-DNA breaks present at the original nuclei. The structural comets appear after the second electrophoresis (Figure 2b–b′). The third comet morphology (Figure 2c–c′) only shows enlarged comets localized on the Y axis representing DNA highly sensitive to alkaline denaturation with the absence of any comet produced after neutral electrophoresis (Figure 2c–c′). These comets indicate the presence of single-strand DNA (ss-DNA) breaks at the original nuclei. Finally, the fourth comet morphology (Figure 2d–d′) is characterized by the presence of long comets along both the X and Y axis, representing massive presence of both ds-DNA and ss-DNA breaks. Enhanced filtered comets from original images are showed in Figure 2a′–d′.

According to the criteria of classification, CCs samples obtained from 20 different patients were assessed for DNA damage using the chromatin dispersion assay and the TT-comet assay (Table 1). The results included were produced under two different experimental conditions. The first ten samples (Table 1, samples 1 to 10) were directly processed after CCs collection. The second cohort (Table 1, samples 11–20) corresponded to the control experiment where restriction endonuclease Alu-I was used to produce double strand breaks in CCs. In this case, the DNA was cleaved with the restriction enzyme at AG|TC targets. A correlation analysis to compare CCs DNA damage visualized as ds-DNA breaks at the origin with the TT-comet assay (CCs-TT3 + CCs-TT4 and represented in Table 1 as W-Comet) and that obtained after the chromatin dispersion assay, show a statistical and significant level of correlation (Figure 3a; Spearman's rho = 0.624; $p = 0.003$;). The TT-comet assay typically gave values of total DNA damage slightly higher than those observed with the chromatin dispersion test. On the contrary the correlation was very low and not significant (Spearman's rho = −0.141; $p = 0.55$; Figure 3b) when the ss-DNA breaks observed with the TT-comet assay were compared with the damage observed from the dispersion test results.

Controlled production and characterization of ds-DNA breaks

To show that the chromatin dispersion test localized native CCs chromatin ds-DNA breaks, controlled ds-DNA damage was produced using the restriction endonuclease Alu-I. Data from control and Alu-I cleavage cells are shown in Table 2. The level of detected damage is higher when the DNA was cleaved with Alu-I (Table 2; Samples 11RE to 20RE) when compared to the control experiment (Table 2; Samples 11C to 20C). When the control experiment was compared to the Alu-I cleaved ds-DNA breaks, large differences in DNA damage were observed either when assessed using a TT-comet assay (Table 2; paired samples; Wilcoxon Z = −2.803; $p = 0.005$) or with the chromatin dispersion test (Table 1; paired samples; Wilcoxon Z = −2.803; $p = 0.005$). Interestingly there is a high correlation comparing the level of controlled ds-DNA damage when measured by the TT-comet or chromatin dispersion assay (Figure 3c; Spearman's rho = 0.720; $p = 0.019$).

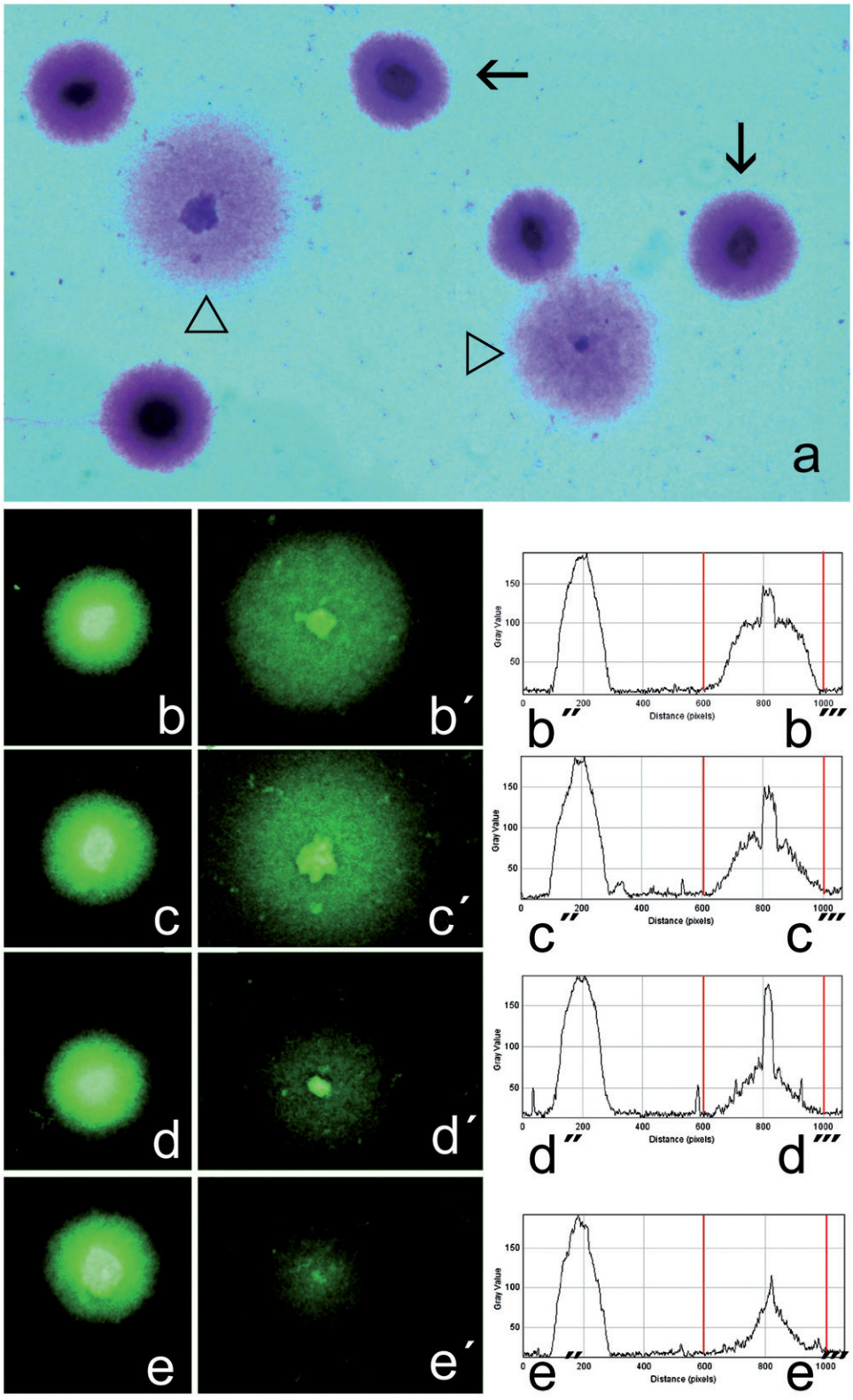

Figure 1. Visualization of cumulus cell (CC) DNA nuclei damage using the chromatin dispersion assay. (a) Bright field microscopy showing fragmented (arrow head; morphotype CC-M2) and normal nuclei (arrow; morphotype CC-M1). (b–e) Selected cumulus cells CC-M1 to be compared with CC-M2 (b′–e′) and densitometric profiles (b″– e″ and b‴– e‴) comparing both cell types.

Direct assessment of DNA damage using *in situ* nick translation (ISNT)

The optimal experimental conditions to visualize DNA labeling in order to maximize the signal as compared to noise occurred after 30 minutes of polymerase extension. Using this protocol, there was direct correspondence between enzymatic incorporation of modified nucleotides (green labeled nuclei) and those nuclei that showed chromatin dispersion halos of DNA damage. This is highlighted in Figure 4a, as those nuclei presenting a very large halo of dispersed chromatin positively labeled DNA. Using image

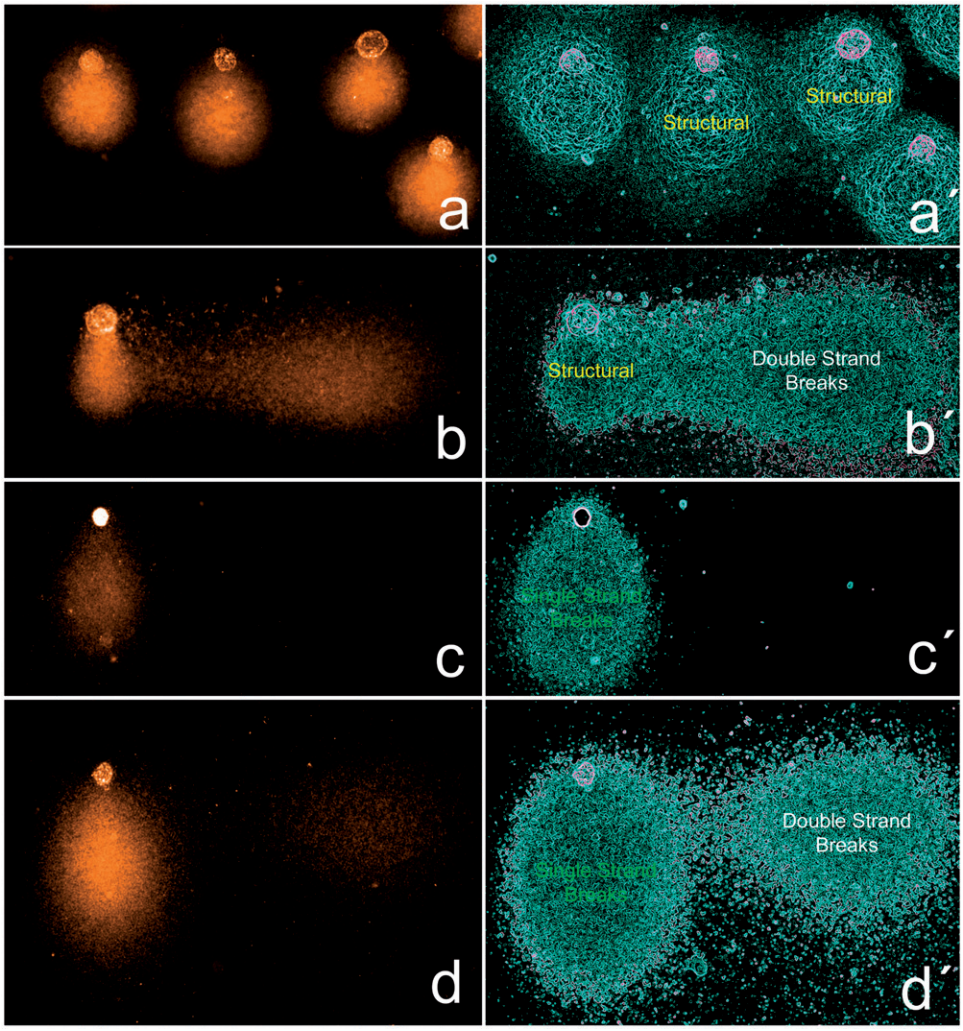

Figure 2. Visualization of cumulus cell (CC) DNA damage using a two tails comet (TT-comet) assay. The first electrophoresis (neutral) produces a right shift in the presence of double strand DNA (ds-DNA) damage. The second electrophoresis (alkaline) produces a down shift in the presence of single strand DNA (ss-DNA) damage. (a) Baseline structural comets. (b) Presence of ds-DNA damage on the X axis of the electrophoretic DNA displacement. (c) Presence of ss-DNA damage on the Y axis of the electrophoretic DNA displacement. (d) Presence of ds- and ss-DNA damage on the X and Y axis of the electrophoretic DNA displacement, respectively.

Table 1. DNA damage detection after using a TT-comet assay and the chromatin dispersion test (3D-Max).

Sample	ss-DNA	ds-DNA	ss-DNA + ds-DNb	W-Comet	3D-Max
1	15.3	0	3	3	2.3
2	16.3	0	4	4	4.3
3	11	4.6	7	11.6	10
4	15.6	1.3	6.6	8	6
5	9	3.3	0.6	4	4.3
6	9.6	1.3	2.6	4	3.3
7	11.3	6	3.6	9.6	7.3
8	7	3.3	7	10.3	13.6
9	7.3	0.6	2.6	3.3	2.3
10	13.6	0.6	5.3	6	4.3
11	6	5.3	2.3	7.6	7
12	3.3	5.3	2.3	7.6	5.3
13	13	4.3	2.6	7	8
14	11.6	2.3	9.3	11.6	4.3
15	24.6	6	2.3	8.3	6.6
16	16.6	0.3	4	4.3	3.3
17	26	5.6	3	8.6	9.6
18	21.3	4.6	10	14.6	3.6
19	14.6	1.3	5.3	6.6	2.3
20	8.6	5.6	3.3	9	6

Light gray samples (1 to 10): samples directly processed after cumulus cell (CC) collection; dark gray samples (11 to 20): Alu-I was used to produce double strand damage in CC. TT-comet: two tails comet; ds-DNA: double strand DNA breakage; ss-DNA: single strand DNA breakage. W-Comet: whole double strand DNA damage as assessed by TT-comet assay. 3D-Max: DNA damage as assessed by the chromatin dispersion test. Values represent the percentage (%) of cells analyzed that presented DNA damage in the sample.

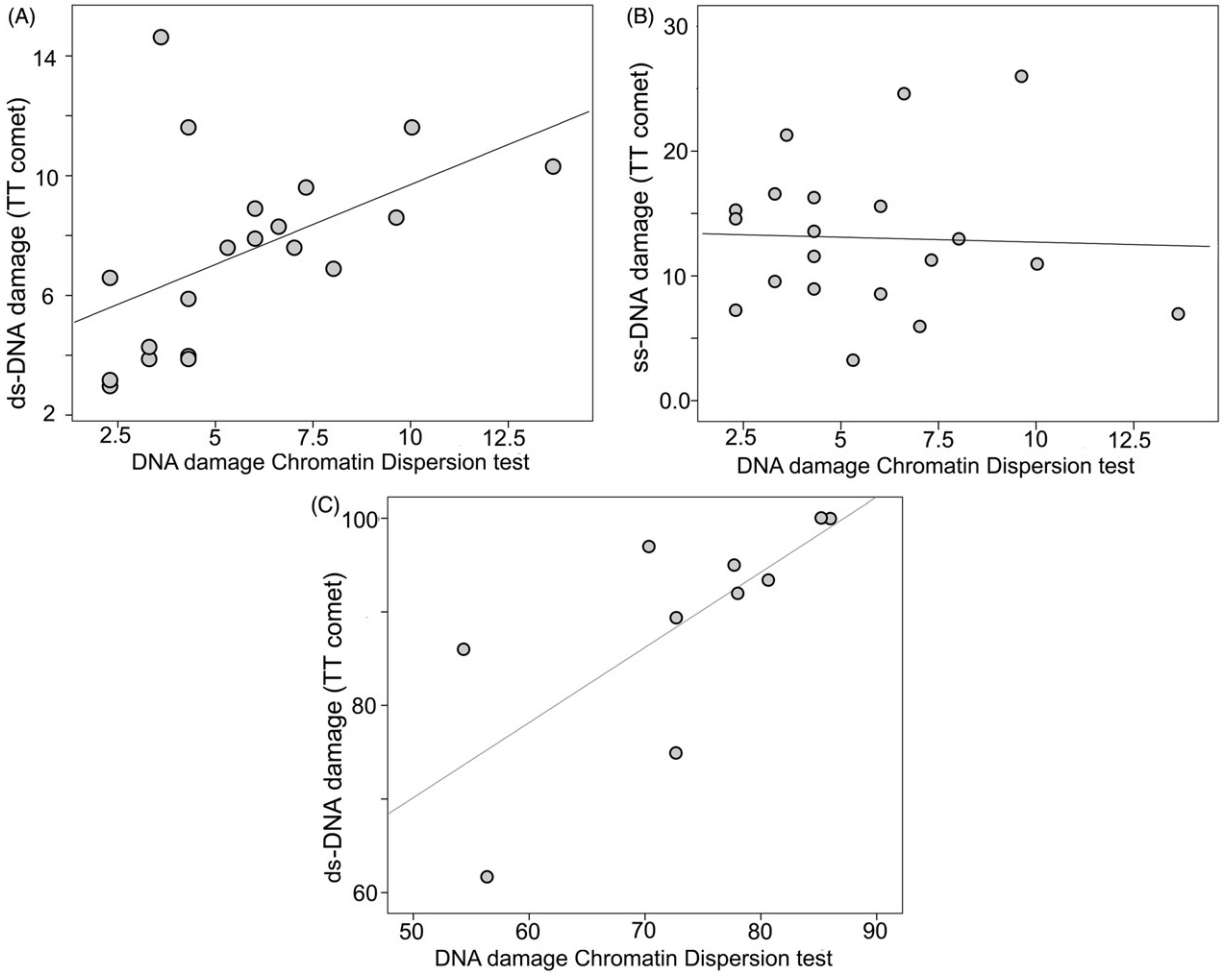

Figure 3. Graphic representation of correlation between cumulus cells (CC) DNA damage observed using a two tails comet (TT-comet) assay and the chromatin dispersion assay. (a) Double-strand DNA (ds-DNA) damage observed in native CC; X = DNA damage as assessed by chromatin dispersion test; Y = ds-DNA damage assessed by comet. (b) Single-straned DNA (ss-DNA) damage observed in native CC; X = DNA damage as assessed by chromatin dispersion test; Y = ss-DNA damage assessed by comet. (c) ds-DNA damage produced after Alu-I *in situ* digestion to produce ds-DNA cleavage; X = DNA damage as assessed by chromatin dispersion test; Y = ds-DNA damage assessed by comet. The numbers on the X and Y axis represent the percentage of cells analyzed in the sample with DNA damage.

analysis, the background green signal obtained in cells showing positive response to ISNT (CCs-M2; cells containing fragmented DNA) ranged from 5–15% higher than the values obtained in CCs-M1 (cells containing non fragmented DNA). The extension of the positive labeled chromatin after the ISNT protocol corresponds to the remnants of fragmented DNA. This was reflected by the size of the halo obtained after the chromatin dispersion test (compare positive nuclei to ISNT in Figure 4b).

Discussion

The relationship between oocyte developmental competence and CCs DNA damage is unclear, with reports indicating both a positive and a negative relationship. In this study we present a chromatin dispersion assay able to identify different degrees of DNA damage in CCs using a quick and easily implemented protocol. Additionally, the high correlation between the TT-comet assay and the presence of a halo indicates that most of the damage detected corresponds to ds-DNA breaks. By comparison with the comet assay, it seems reasonable to assume that the chromatin dispersion test does not fully reveal the extent of ss-DNA damage.

Although the reason for the presence of ss-DNA and ds-DNA breaks in cumulus cells has not been studied extensively, by extension with what are seen in other cell systems, it is likely that ds-DNA breaks correspond to the activation of apoptotic pathways [Kaina 2003; Lips and Kaina 2001] or to the presence of different isoforms of topoisomerase or DNase activities, [de Campos Nebel et al. 2002; Mandraju et al. 2008; Wang 1996]. In comparison ss-DNA breaks are considered to be a consequence of oxidative stress [Bertoncini and Meneghini 1995; Trzeciak et al. 2012], or unrepaired DNA lesions caused by failure to repopulate the genome with histones [Zhou et al. 2013], or again by exposure to toxic concentration of chemicals such as benzo(a)pyrene [Einaudi et al. 2014].

In dividing cells, ds-DNA breaks promote genomic instability, gene inactivation, and can lead to cell death [Khanna and Jackson 2001]. Different levels of CCs ds-DNA damage could reflect the developmental competence of the oocyte. Although establishing this relationship was not the

Table 2. Controlled production of double strand DNA breaks using Alu-I DNA cleavage in cumulus cells and DNA damage detection using a TT-comet assay and the chromatin dispersion assay (3D-Max).

Sample	ss-DNA	ds-DNA	ss-DNA + ds-DNA	W-Comet	3D-Max
11RE	2.6	60	35	95	77.6
12RE	27.6	41.3	20.3	61.6	56.3
13RE	6.6	69.3	24	93.3	80.6
14RE	3	73	24	97	70.3
15RE	10	64	22	86	54.3
16RE	0	57.3	42.6	100	86
17RE	1	61	31	92	78
18RE	9.3	18.6	70.6	89.3	72.6
19RE	0	90.6	9.3	100	85.3
20RE	16	39.6	35.3	75	72.6
11C	6	5.3	2.3	7.6	7
12C	3.3	5.3	2.3	7.6	5.3
13C	13	4.3	2.6	7	8
14C	11.6	2.3	9.3	11.6	4.3
15C	24.6	6	2.3	8.3	6.6
16C	16.6	0.3	4	4.3	3.3
17C	26	5.6	3	8.6	9.6
18C	21.3	4.6	10	14.6	3.6
19C	14.6	1.3	5.3	6.6	2.3
20C	8.6	5.6	3.3	9	6

TT-comet: two tails comet; ds-DNA: double strand DNA breakage; ss-DNA: single strand DNA breakage; W-Comet: whole double strand DNA damage as assessed with by TT comet assay. 3D-Max: DNA damage as assessed by the chromatin dispersion test. Values represent the percentage (%) of cells analyzed that presented DNA damage in the sample. RE: treated with restriction endonuclease C: control.

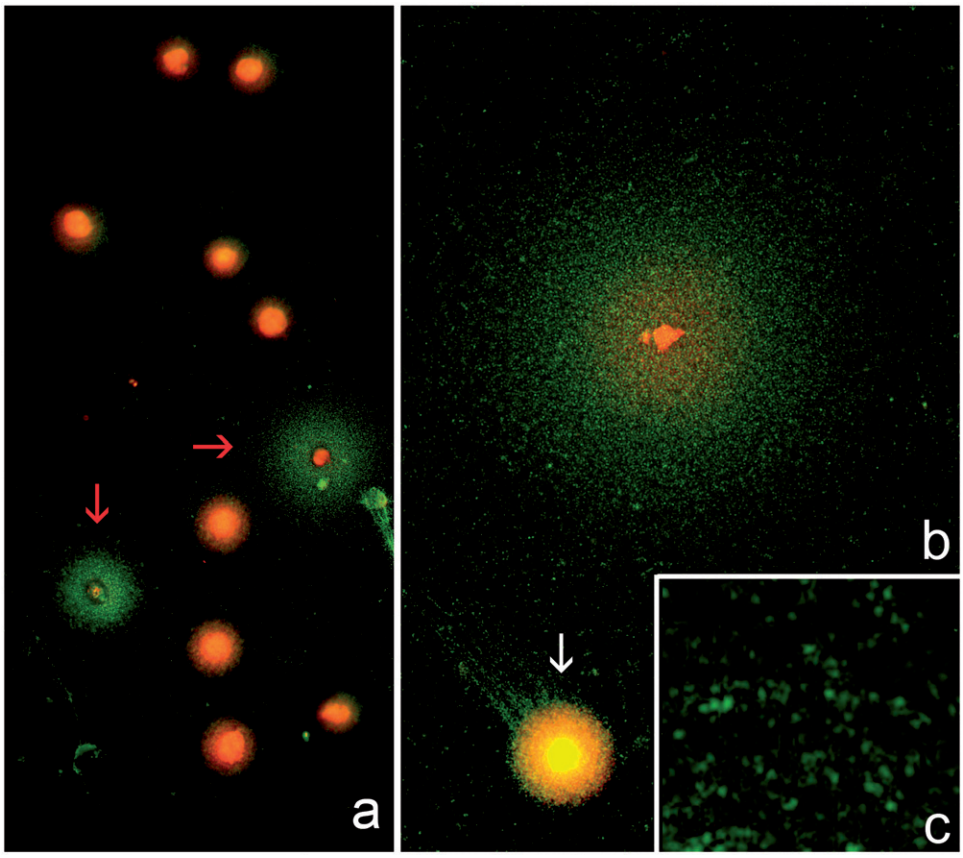

Figure 4. Direct correlation DNA damage labeling and *in situ* nick translation (ISNT) assay targeting the halo morphology produced after the chromatin dispersion and the incorporated labeled nucleotides (green fluorescence) in cumulus cells (CCs). (a) General microscope field presenting unlabeled CCs and green labeled CCs presenting large halos of dispersed chromatin (red arrows). (b) Two selected and highly magnified CCs showing a non-affected CC (white arrow) contrasting with the expansion of the DNA fragments emerging from a dismissed chromatin core and a high level of labeled nucleotide incorporation. (c) Highly magnified detail at the border of the halo of dispersed chromatin where discrete and unconnected DNA fragments can be visualized in the CCs containing a fragmented DNA molecule.

main goal of the study, in the samples we analyzed, we observed that CCs showed different levels of DNA damage, but they were not correlated with the rate of immature oocytes encountered in the pool, nor with the fertilization rate of the metaphase II (MII) (Supplementary Table 1). All women included in the study are oocyte donors of proven fertility, thus finding a relationship between CC damage and reproductive outcomes might be difficult. However, a recent report in IVF patients indicates that the level of DNA damage in the corona radiata of cumulus oocyte complexes, also assessed by a chromatin dispersion assay, can be correlated with blastulation of the embryo resulting from the enclosed oocyte [Ebner et al. 2014]. Although the authors do not analyze the nature of the DNA strand breaks they identify in CCs, the nature of the test they employ indicates that they were likely assessing ds-DNA breaks. This supports the view that this kind of DNA damage may be a potential biomarker in CCs. It remains to be determined whether there is a specific threshold of CCs ds-DNA damage which can discriminate between oocytes with high and low developmental competence. The chromatin dispersion assay is nonetheless able to discriminate between CCs containing DNA damage and those unaffected, opening the door for a semi-quantitative evaluation of CCs damage.

The test can be scaled to work with a few cells, following, for example, the experimental rationale used to assess DNA damage in selected spermatozoa after IMSI selection [Gosalvez et al. 2013]; the analysis of single CCs populations obtained after oocyte denuding can provide information on the quality of single oocytes. We found that the D3-Max protocol requires minimal training and delivers straightforward cell phenotypes based on the size of the halo and the associated densitometric profiles. Finally, no specific microscopes or laboratory equipment are required to carry out this test, which can be adapted to both bright-field and fluorescence microscopy.

Materials and Methods

Sample collection

The CCs used for this study came from 103 women who underwent a controlled ovarian stimulation cycle for oocyte donation purposes. Participants in this study were 26.6 (SD 4.9) y old on average, and their BMI was 23.3 (SD 3.8). The ovarian stimulation was carried out using gonadotropin-releasing hormone (GnRH) antagonists as hypophysis suppressors, and final oocyte maturation was elicited with a single injection of 0.3 mg of GnRH agonist. No woman was included twice in the study. The average number of COCs collected per cycle was 21 (SD 9). The CCs from all COCs available in a cycle were stripped from the oocyte by gentle pipetting in a solution of hyaluronic acid (Hy-ase 10X, Vitrolife, Göteborg, Sweden). CCs were collected, washed by centrifugation, resuspended in a freezing solution (Cryoprotect II, Nidacon International Göteborg, Sweden), and stored in liquid nitrogen until use.

Chromatin dispersion test

The degree of DNA damage in each sample was quantified using a commercial version of the Halomax test series specifically adapted to be used on CCs (D3-Max; Halotech DNA, SL Madrid, Spain). Since CCs are somatic cells, in order to produce protein depletion they do not need the reduction of strong disulfide bonds present in sperm cells and provided by the cysteine residues of the protamines. Therefore, the lysis solution has been customized to obtain optimal protein depletion in CCs.

All samples were adjusted to a concentration of 1 x 10^6 mL-1 using PMI Media 1640 (Life Technologies; Carlsbad, CA, US) after centrifugation. A volume of 25 µl of diluted sample was added to a vial containing low melting point agarose at 37°C and gently mixed. Ten µl of the agarose-cell mixture was then extended on pre-treated slides provided in the kit and covered with a glass coverslip. After gently pressing on the coverslip, each slide was placed at 4°C for 5 min to produce a microgel; once formed, the coverslip was removed from the microgel and the slide placed horizontally in 10 ml of the lysing solution provided in the kit for 5 min to achieve controlled protein depletion. The treated microgel containing partially de-proteinized CCs was then washed in dH2O for 5 min and dehydrated in a series of ethanol baths (70%, 90%, and 100%). Negative control included the standard protocol but avoided the use of the lysing solution.

For visualization under fluorescence microscopy, the slides were mounted with equal parts SYBR® Green (Sigma Aldrich, Madrid, Spain) and VectaShield® mounting medium (Vector Laboratories, Burlingame, CA, USA) to inhibit fluorochrome fading. A total of 300 CCs were counted per sample using a Leica DMRB epifluorescence microscope (Leica Microsystems, Barcelona, Spain) equipped with single-band fluorescence block filters (FITC-2024B-000; Semrock, Rochester, NY, USA). Alternatively, visualization can be performed using a standard bright field microscopy and Diff-Quick staining (Sanilabo, Valencia, Spain).

Two dimensional comet assay

A two dimensional, or two-tails, or comet assay (TT-comet), performed under neutral and alkaline conditions was used to characterize DNA damage of different origin. This approach enabled the visualization of comets that are run perpendicular to each other; mainly double strand DNA (ds-DNA) breaks when the comet is visualized in the horizontal direction (X axis) and ss-DNA breaks when the comet is localized at the vertical direction (Y axis). To interpret the results, one should be mindful that the first electrophoresis (neutral) would produce a right shift in the presence of ds-DNA damage, while the second one (alkaline) would produce a down shift in the presence of ss-DNA damage. For the neutral electrophoreses step, the slide was treated with as optimized lysis solution for five min to produce a controlled protein depletion. After this step, the slide was washed in 1 X Tris-borate-EDTA (TBE) buffer solution (0.089 M Tris, 0.089 M boric acid and 0.002 M EDTA, pH 8.2) for 5 min. The slides were then subjected to electrophoresis (8 min; 20 V) in 1 X TBE buffer to allow the DNA fragments resulting from ds-DNA breaks to migrate away from the nucleus towards the anode during application of the electrical current. At the completion of this electrophoresis, the

slide was removed and placed in 0.9% NaCl solution for two min. The slide was then transferred to a chilled (4°C) alkaline solution (0.03 M NaOH and 1 M NaCl; pH 12.5–13) for two and a half min to produce DNA denaturation in those places where ds-DNA or ss-DNA breaks were present. Finally, the slide was introduced into a fresh alkaline buffer (0.03 M NaOH; pH 12.5–13) at room temperature for the second electrophoresis (four min; 20 V) having been repositioned 90° clockwise from its original position during the first and neutral electrophoretic step. This resulted in the migration of DNA fragments arising from ds-DNA breaks towards the anode (X axis, right shift), while the DNA fragments from ss-DNA breaks migrated on the Y axis (down shift) accordingly with the level of DNA denaturation achieved. Finally, the slide was washed with neutralizing buffer (0.4 M Tris-HCL; pH 7.5) for five min, followed by two min in 1 X TBE buffer, and subsequent dehydration in a series of 70, 90, and 100% ethanol baths for two min each. Comets of fragmented DNA were visualized using GelRed® (Biotium, Hayward, CA, USA).

In a parallel experiment, 10 samples where used to produce a controlled ds-DNA breakage using the restriction endonuclease Alu-I (AG|TC; New England Biolabs, Inc, Ipswich, UK), which serves as a positive control. Fifteen IU of the restriction enzyme were prepared for *in situ* DNA digestion in 50 µL of CutSmart™ buffer which contained 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, and 100 µg/mL BSA at pH 7.9. This solution was then flooded onto the surface of the slide where the cells were included in the microgel, covered with a layer of Parafilm M (Sigma Aldrich, Madrid, Spain) and incubated for 3 h at 37°C. Selective DNA digestion was blocked using a PBS-EDTA solution by direct washing of the slide. Slides were processed using the chromatin dispersion protocol and the two tails comet assay.

In situ nick translation assay

Direct visualization of DNA damage was performed on CCs previously processed with the chromatin dispersion assay. DNA fragmentation was detected by enzymatic incorporation of labeled nucleotides using an ISNT protocol, as described [Enciso et al. 2006]; only chromatin containing DNA breaks exposing 5′ and 3′ free ends incorporates the labeled nucleotides, and the labeled cells can be directly compared with the morphology obtained after the chromatin dispersion assay. For this purpose, cells were loaded into microgels and processed as per the chromatin dispersion assay except for the dehydration step. Once protein depletion was completed, the slide was washed in PBS for 10 min; the microgel was not allowed to dry and the slide was further incubated for 5 min in the same buffer used for ISNT but without DNA polymerase. The buffer was then removed and 100 µL of the polymerase reaction buffer containing 10 units of DNA polymerase-I (New England BioLabs, Beverly, MA, USA) and biotin-16-dUTP in the nucleotide mix, was directly pipetted onto the slide, covered with a plastic coverslip, and incubated in a humidified chamber for 5, 20, and 30 min at 37°C to compare the level of DNA labeling of each CCs sample. After washing in a Tris-buffer (0.089 M Tris, 0.089 M boric acid and 0.002 M EDTA, pH 8.2) the slides were dehydrated in sequential 70% – 90% – 100% ethanol baths and air-dried. Incorporated biotin-16-dUTP was detected after incubation for 30 min with avidin conjugated with fluorescein isothiocyanate (Thermo Scientific; Rockford, IL, USA). The CCs in the microgels were counterstained using propidium iodide (2 mg/mL) in Vectashield (Vector, Burlingame, CA, USA). As a control, a designated area of the slide was incubated with the reaction buffer alone, omitting the DNA polymerase I. Control experiments were performed on the same slide by constructing a physical barrier (gel scratched off slide) between areas of the microgel exposed with and without polymerase to avoid possible diffusion of the enzyme. Following ISNT, CCs images were digitally captured using a CCD (Photometrics CoolSNAP HQ2, Tucson, AZ, USA) at 600x magnification and the proportion of labeled cells determined. The ISNT unlabeled CCs typically stained only red if the nucleotides were not incorporated but green if DNA damage was present.

Statistics and image analysis

Non parametric test (Wilcoxon Z) and correlation analysis (Spearman's rho) were performed using the SPSS 7.1 statistics package (IBM, NY, USA). For image analysis, Image J free software (NIH, Bethesda, MD, USA) was used. Digital analysis was only applied to images of comet assays (Figure 2), in this case we applied a previous routine of image enhancement [Zee et al. 2009]. Region of interests (ROIs) and threshold levels were automatically assigned by the standard algorithms incorporated into Adobe.

Ethical considerations

All the materials used in this study were discarded waste material obtained during the normal procedure of clinic ovum pick up. Permission to conduct the study was sought and obtained from the Institutional Review Board.

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Declaration of interest

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Author contributions

Data collection, manuscript preparation: PB; Manuscript revision, expert knowledge: CL-F, CG-O, AO, VV; Study design, data collection, study supervision, manuscript preparation: JG, RV.

References

Abu-Hassan, D., Koester, F., Shoepper, B., Schultze-Mosgau, S., Asimakopoulos, B., Diedrich, K., et al. (2006) Comet assay of cumulus cells and spermatozoa DNA status, and the relationship to oocyte fertilization and embryo quality following ICSI. Reproductive Biomedicine Online **12**:447–452.

Bertoncini, C.R. and Meneghini, R. (1995) DNA strand breaks produced by oxidative stress in mammalian cells exhibit 3′-phosphoglycolate termini. Nucleic Acids Res **23**:2995–3002.

de Campos Nebel, M., Larripa, I. and Gonzalez-Cid, M. (2002) Differential antigenotoxic and cytoprotective effect of amifostine in idarubicin-treated mice. Environ Mol Mutagen **39**:3–9.

Ebner, T., Shebl, O., Holzer, S., Oppelt, P., Petek, E., Schappacher-Tilp, G., et al. (2014) Viability of cumulus cells is associated with basal AMH levels in assisted reproduction. Eur J Obstet Gynecol Reprod Biol **183**:59–63.

Einaudi, L., Courbiere, B., Tassistro, V., Prevot, C., Sari-Minodier, I., Orsiere, T., et al. (2014) In vivo exposure to benzo(a)pyrene induces significant DNA damage in mouse oocytes and cumulus cells. Hum Reprod **29**:548–554.

Ekart, J., McNatty, K., Hutton, J. and Pitman, J. (2013) Ranking and selection of MII oocytes in human ICSI cycles using gene expression levels from associated cumulus cells. Hum Reprod **28**:2930–2942.

Enciso, M., López-Fernández, C., Fernández, J.L., García, P., Gosálbez, A., Gosálvez, J.. (2006) A new method to analyze boar sperm DNA fragmentation under bright-field or fluorescence microscopy. Theriogenology **65**:308–308.

Gosalvez, J., Caballero, P., Lopez-Fernandez, C., Ortega, L., Guijarro, J.A., Fernandez, J.L., et al. (2013) Can DNA fragmentation of neat or swim-up spermatozoa be used to predict pregnancy following ICSI of fertile oocyte donors? Asian J Androl **15**:812–818.

Huang, X., Hao, C., Shen, X., Liu, X., Shan, Y., Zhang, Y., et al. (2013) Differences in the transcriptional profiles of human cumulus cells isolated from MI and MII oocytes of patients with polycystic ovary syndrome. Reproduction **145**:597–608.

Iager, A.E., Kocabas, A.M., Otu, H.H., Ruppel, P., Langerveld, A., Schnarr, P., et al. (2013) Identification of a novel gene set in human cumulus cells predictive of an oocyte's pregnancy potential. Fertil Steril **99**:745–752 e746.

Ikeda, S., Imai, H. and Yamada, M. (2003) Apoptosis in cumulus cells during in vitro maturation of bovine cumulus-enclosed oocytes. Reproduction **125**:369–376.

Kaina, B. (2003) DNA damage-triggered apoptosis: critical role of DNA repair, double-strand breaks, cell proliferation and signaling. Biochem Pharmacol **66**:1547–1554.

Khanna, K.K. and Jackson, S.P. (2001) DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet **27**:247–254.

Lips, J. and Kaina, B. (2001) DNA double-strand breaks trigger apoptosis in p53-deficient fibroblasts. Carcinogenesis **22**:579–585.

Liu, Y.X. (2007) Interaction and signal transduction between oocyte and samatic cells in the ovary. Front Biosci **12**:2782–2796.

Luciano, A.M., Modina, S., Vassena, R., Milanesi, E., Lauria, A. and Gandolfi, F. (2004) Role of intracellular cyclic adenosine 3′,5′-monophosphate concentration and oocyte-cumulus cells communications on the acquisition of the developmental competence during in vitro maturation of bovine oocyte. Biol Reprod **70**:465–472.

Mandraju, R.K., Kannapiran, P. and Kondapi, A.K. (2008) Distinct roles of Topoisomerase II isoforms: DNA damage accelerating alpha, double strand break repair promoting beta. Arch Biochem Biophys **470**:27–34.

Moller, P., Moller, L., Godschalk, R.W. and Jones, G.D. (2010) Assessment and reduction of comet assay variation in relation to DNA damage: studies from the European Comet Assay Validation Group. Mutagenesis **25**:109–111.

Raman, R.S., Chan, P.J., Corselli, J.U., Patton, W.C., Jacobson, J.D., Chan, S.R., et al. (2001) Comet assay of cumulus cell DNA status and the relationship to oocyte fertilization via intracytoplasmic sperm injection. Hum Reprod **16**:831–835.

Richards, J.S. (2005) Ovulation: new factors that prepare the oocyte for fertilization. Mol Cell Endocrinol **234**:75–79.

Su, Y,Q., Sugiura, K., Eppig, J.J. (2009) Mouse oocyte control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism. Seminars in Reproductive Medicine **27**: 32–42.

Trzeciak, A.R., Mohanty, J.G., Jacob, K.D., Barnes, J., Ejiogu, N., Lohani, A., et al. (2012) Oxidative damage to DNA and single strand break repair capacity: relationship to other measures of oxidative stress in a population cohort. Mutat Res **736**:93–103.

Wang, J.C. (1996) DNA topoisomerases. Annu Rev Biochem **65**: 635–692.

Zee, Y.P., Lopez-Fernandez, C., Arroyo, F., Johnston, S.D., Holt, W.V. and Gosalvez, J. (2009) Evidence that single-stranded DNA breaks are a normal feature of koala sperm chromatin, while double-stranded DNA breaks are indicative of DNA damage. Reproduction **138**: 267–278.

Zhou, Z.X., Zhang, M.J., Peng, X., Takayama, Y., Xu, X.Y., Huang, L.Z., et al. (2013) Mapping genomic hotspots of DNA damage by a single-strand-DNA-compatible and strand-specific ChIP-seq method. Genome Res **23**:705–715.

Supplementary materials available online

Supplementary Table.