Short Communication

Prostaglandin F2α Causes Fast Degenerative Changes in Ovulated Mouse Oocytes

(DiOC6 / postovulatory aging / inflammation / prostaglandin / meiotic spindle / oocyte mitochondria)

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Abstract. The effects of prostaglandin F2α on the cytoskeleton and membrane organelles of oocytes was investigated by culturing ovulated mouse oocytes in its presence (50 or 100 ng/ml) for 3 h. Tubulin, fibrillar actin, membranes and chromatin were visualized by specific antibodies, phalloidin, lipophilic dye DiOC6 and Hoechst 33342, respectively. Control oocytes were characterized by a meiotic spindle with chromosomes aligned at its equator, and a cortical layer of microfilaments with an actin cap. Intracellular membranes were localized mostly in the central region in metaphase I and in a broader volume, but still excluding the cell periphery, in metaphase II, and were slightly concentrated around the chromosomes. In oocytes treated with 50 ng/ml prostaglandin, cortical actin staining was diminished, the membrane distribution was clustered, and chromosomes showed signs of misalignment despite the apparently preserved spindle. In cells treated with 100 ng/ml prostaglandin, both the spindle and the actin cortex had degenerated or disappeared as microscopic objects. Metaphase plates were on average broader and more disorganized than in the 50 ng/ml group, and the distribution of membrane organelles had become uniform. These effects, to our knowledge observed for the first time, did not require presence of the cumulus during the incubation. They could be regarded as acceleration of the oocyte postovulatory aging, in which cytoskeletal deterioration seemed to have a leading role.

Introduction

Mature mammalian oocytes are characterized by a relatively short life span after ovulation, limiting the time window for fertilization both *in vivo* and *in vitro*. Soon after leaving the ovary, even in optimal conditions the oocyte undergoes the so-called postovulatory aging. This process changes the cell morphology, progressively deteriorates its fertilization and developmental potential, and eventually leads to its death (Prasad et al., 2015; Miao et al., 2018). Postovulatory aging is influenced by the oocyte environment: antioxidants and free oxidant scavengers have been reported to delay it (Wang T. et al., 2017; Wang Y. et al., 2019), while damaging factors such as Fas ligand accelerate and exacerbate the degenerative changes (Zhu et al., 2015). In this respect, generators and mediators of inflammation are compounds of interest, given the known impact of inflammatory processes on female reproduction. This impact is complex and has multiple aspects, some related to the normal functioning of the female reproductive system and some associated with pathology.

On the one hand, according to recent data, local inflammatory changes in the ovary, induced by gonadotropins and at least partly mediated by prostaglandin E2, are necessary to reduce the mechanical strength of the follicle wall and allow ovulation. On the other hand, disorders disturbing oogenesis and reducing female fertility in the human, such as endometriosis and polycystic ovary syndrome, are increasingly characterized as associated with low-level inflammation that is important

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Abbreviations: BSA – bovine serum albumin, DiOC6 – 3,3′-dihexyloxacarbocyanine iodide, FITC – fluorescein isothiocyanate, IU – international units, PBS – phosphate-buffered saline, TRITC – tetra-methylrhodamine isothiocyanate.

for their negative effects on reproduction (Boots and Jungheim, 2015). Bacterial lipopolysaccharide, prostaglandin F2α and other inflammation-associated molecules, when acting upon the oocyte during its meiotic maturation, have been shown to interfere with the course of meiosis and to reduce the developmental potential of exposed oocytes (Soto et al., 2003; Bromfield and Sheldon, 2011). However, there are few data about the effects of inflammation-associated molecules upon oocytes and oocyte-cumulus complexes after ovulation, especially with regard to particular cell components. For this reason, using fluorescence microscopy we studied the changes in the visible structure and arrangement of chromosomes, tubulin and actin cytoskeleton, and intracellular membranes of ovulated mouse oocytes exposed to prostaglandin F2α, a natural mediator of inflammation.

Material and Methods

ICR female mice (8 to 10-week old) were subjected to ovarian stimulation with 7.5 IU follicle-stimulating hormone and 7.5 IU luteinizing hormone (Meriofert®, IBSA Farmaceutici Italia, Lodi, Italy). After 48 h, ovulation was induced by 10 IU of human chorionic gonadotropin (Choriomon®, IBSA Farmaceutici Italia). Mice were euthanized 16 h later, and cumulus-oocyte complexes were collected in Leibovitz medium (Sigma-Aldrich, Steinheim am Albuch, Germany) from the dissected oviducts.

Oocytes were divided into three groups. Those for studying prostaglandin-induced changes were incubated with either 50 or 100 ng/ml prostaglandin F2α tris salt (Sigma-Aldrich) in α-MEM medium (Sigma-Aldrich) for 3 h at 37 °C and 5% CO_2; the treatment was based on Soto et al. (2003). The rest of the oocytes made up two control groups: some were processed for microscopy immediately after retrieval, and others were incubated for 3 h in α-MEM without prostaglandin. The cumulus was removed by 0.5 mg/ml hyaluronidase (Sigma-Aldrich) in Leibowitz for 20 min at 37 °C. In some of the incubated oocytes, this was done before the incubation; in the rest, the cumulus was removed before fixation.

For microscopy, each oocyte group was split into two subgroups for visualization of the cytoskeleton and membranes, respectively. For observation of the cytoskeleton, oocytes were processed as described before (Nikolova et al., 2017). They were fixed in 2% paraformaldehyde (Sigma-Aldrich) and 0.04 % Triton X-100 in phosphate-buffered saline (PBS), pH 7.2, for 45 min at 37°C. Mouse anti-α-tubulin monoclonal antibody (clone DM1A; Sigma-Aldrich, Germany) was applied diluted 1 : 1000 in diluting buffer (PBS with 0.3% BSA and 0.04% Triton X-100) for 45 min at 37 °C, followed by two 10-min washes in wash buffer (PBS with 0.3 % BSA and 0.1% Tween 20). Then oocytes were treated with FITC-labelled anti-mouse IgG antibody (Sigma-Aldrich) diluted 1 : 200 in diluting buffer with 5 µg/ml Hoechst 33342 and 1 µg/ml TRITC-labelled phalloidin (Sigma-Aldrich) to stain DNA and fibrillar actin, respectively. After that, cells were washed twice in wash buffer.

For observation of membranes, oocytes were fixed as described above but without detergent. After washing twice in PBS with 0.3% bovine serum albumin (BSA) for 10 min at 37°C, the oocytes were stored in PBS with 0.3% BSA and 0.02% sodium azide at 4 °C overnight. Then they were incubated with 2.5 µM of lipophilic dye DiOC6 (3,3′-dihexyloxacarbocyanine iodide, Sigma-Aldrich) and 5 µg/ml Hoechst 33342 (Sigma-Aldrich) in PBS with 0.3% BSA for 45 min at 37 °C. After that, oocytes were washed twice in PBS with 0.3% BSA.

Stained oocytes were placed in increasing concentrations of Mowiol (Sigma-Aldrich) in PBS with 0.3% BSA and 0.02% sodium azide. Finally, the cells were mounted with 100% Mowiol on slides. They were observed by epifluorescence microscopy (Axioskop 20, Zeiss, Jena, Germany). Selected oocytes were further examined by laser-scanning confocal microscopy (Leica TCS SPE, Wetzlar, Germany).

Results and Discussion

Most oocytes (57.8 %) were in metaphase II. The majority of the rest were in metaphase I, with a small proportion (1.42 %) of immature prophase or prometaphase oocytes.

Control oocytes stained for tubulin were characterized by the presence of a well-shaped meiotic spindle. Phalloidin labelling for fibrillar actin was positive mostly in the cortical region, with an actin cap overlying the spindle (Fig. 1A). Hoechst staining visualized chromosomes aligned in a metaphase plate at the spindle equator. These findings were characteristic of both metaphase I and metaphase II oocytes. Membrane structures stained by DiOC6 were found throughout the cell. Epifluorescence observation could not reveal any details in their distribution, but confocal microscopy showed that they had a higher density in the central region of the oocyte in metaphase I and a broader distribution, though still excluding the cell periphery, in metaphase II. The labelling also showed slightly increased density around the chromosomes, which was more apparent in metaphase II (Fig. 2A, B). In addition, DiOC6 stained the zona pellucida in varying degrees.

In oocytes incubated for 3 h in the presence of 50 ng/ml prostaglandin F2α, the meiotic spindle appeared normal, but the actin localization was more diffuse than in controls, with weaker staining of the cortex and especially the cap (Fig. 1B). Metaphase plates, compared to those of the controls, were broader and showed a tendency for disorganization, from misalignment of individual chromosomes in the metaphase plate (Figs. 1B, 2C) to spreading over a large area (Fig. 2D). The distribution of membranes was overall similar to that in respective control oocytes, but the gradients were diminished, and the staining appeared clustered (Fig. 2C, D).

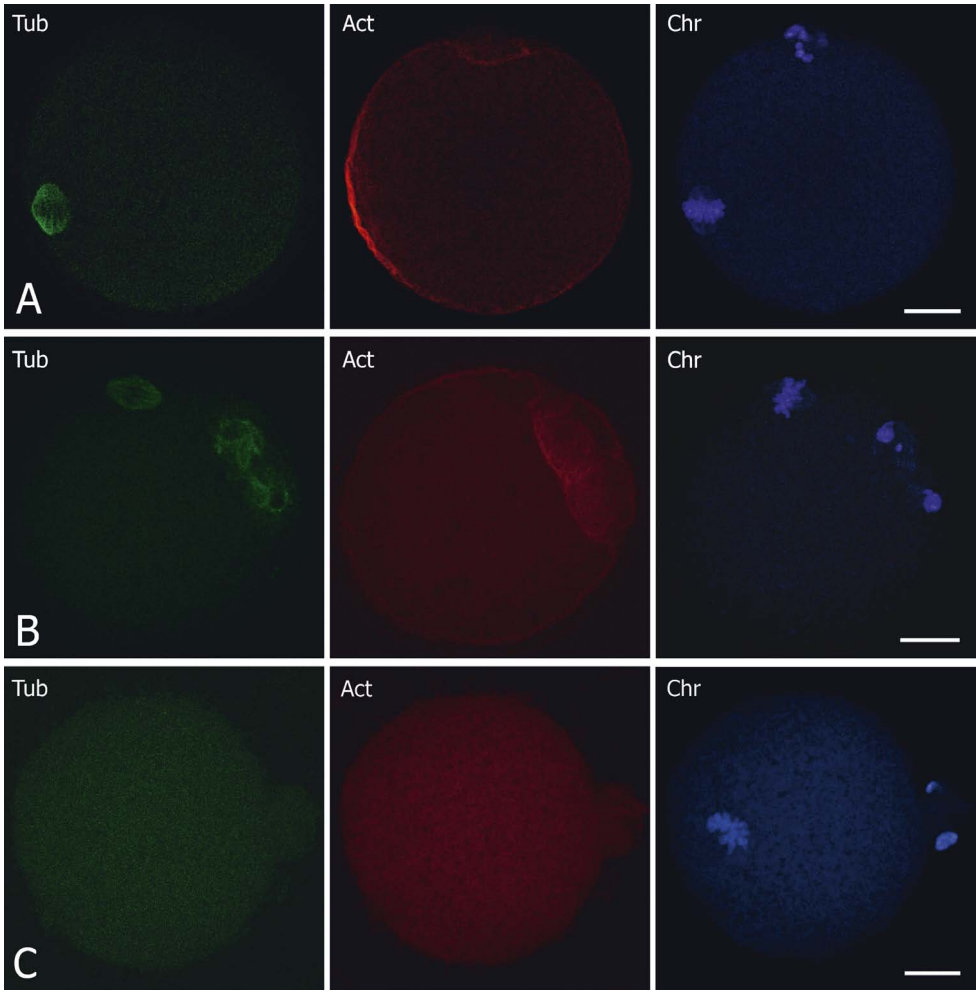

Fig. 1. Metaphase II oocytes stained for α-tubulin (Tub), fibrillar actin (Act) and chromatin (Chr), confocal microscopy. **A.** A control cell incubated for 3 h without prostaglandin and displaying a well-formed meiotic spindle, actin cortex with a cap and chromosomes aligned in a metaphase plate. The 1st polar body can be seen at the top. **B.** An oocyte incubated for 3 h with 50 ng/ml prostaglandin F2α. The spindle looks normal, but the reaction for actin in the cortex and particularly the cap is diminished, and several chromosomes are not properly aligned. **C.** An oocyte incubated for 3 h with 100 ng/ml prostaglandin F2α. Staining for tubulin and actin is diffuse, without a visible spindle or cortex, and the metaphase plate is spread and disorganized. Bars 20 μm.

In oocytes incubated with 100 ng/ml prostaglandin F2α, the meiotic spindle showed apparent degenerative changes or had disappeared as a microscopic object. Fibrillar actin had lost its cortical localization and showed diffuse staining of the cytoplasm with diminished intensity (Fig. 1C). Metaphase plates, on average, showed more pronounced spreading and disorganization of chromosomes than in cells treated with 50 ng/ml prostaglandin. Concentration of membrane structures in the central area and around the chromosomes was weak or altogether absent, resulting in uniform DiOC6 staining (Fig. 2E, F).

The above-described patterns were observed regardless of whether oocytes were incubated together with the cumulus or stripped of their cumulus before incubation.

The meiotic spindle and the cortical layer of fibrillar actin forming a cap over it, as observed in our control cells, had the appearance characteristic of normal metaphase oocytes. By contrast, in prostaglandin-treated cells, both the spindle and the cortical actin showed signs of degeneration in a dose-dependent manner, to the point of disappearing as microscopic structures in the group exposed to the higher concentration. To our knowledge, prostaglandin F2α has not been reported to have a specific effect on microtubules or microfilaments, though it has been recently found to disrupt intermediate filaments in luteal theca (Lee and Lee, 2021). It could be hypothesized that the treatment accelerated postovulatory aging of oocytes, which is known to disrupt the meiotic spindle and the alignment of chromosomes and to diminish the presence of fibrillar actin in the cell cortex (Miao et al., 2018). The observed spreading of chromosomes could be attributed to the deterioration and disassembly of the spindle. An interesting observation was that oocytes treated with the lower prostaglandin

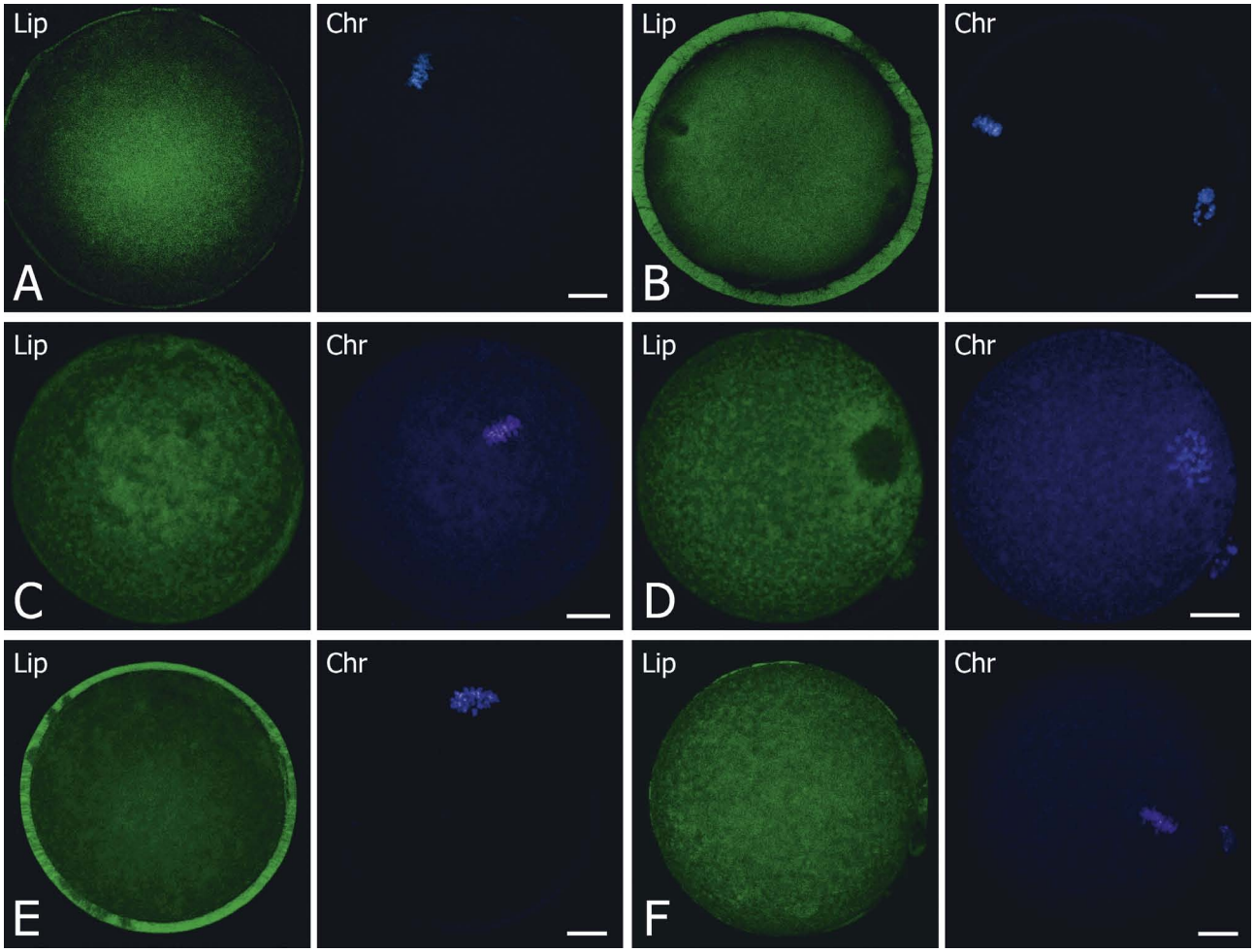

Fig. 2. Oocytes stained for membranes (Lip) and chromatin (Chr), confocal microscopy. **A, B.** Controls incubated for 3 h without prostaglandin. **C, D.** Oocytes incubated for 3 h with 50 ng/ml prostaglandin F2α. **E, F.** Oocytes incubated for 3 h with 100 ng/ml prostaglandin F2α. Cells in **A**, **C** and **E** are in metaphase I, and in **B**, **D** and **F** in metaphase II (with polar bodies at right). The metaphase I control (**A**) shows concentration of membrane structures in the central region; the metaphase II control (**B**) has membranes throughout the cytoplasm, with density diminished in the periphery and increased around the chromosomes. The prostaglandin-treated cells (**C – F**) have broader metaphase plates. The membrane distribution in oocytes treated with 50 ng/ml (**C**, **D**) has some resemblance to that in the controls but with clustered appearance, and in the cells treated with 100 ng/ml (**E**, **F**) is uniform. Bars 20 μm.

concentration (50 ng/ml) had a combination of apparently preserved meiotic spindles and imperfect alignment of chromosomes. It could be hypothesized that these spindles, despite their normal appearance, had sustained subtle damage that did not allow them to support a properly aligned metaphase plate. Another possibility is that chromosomes first detach from the still intact meiotic spindle, and microtubules collapse at a later stage after losing the stabilizing influence of kinetochores.

The lipophilic dye DiOC6 is often used to visualize mitochondria (e.g., Suzuki et al., 2005), although it stains other intracellular membranes as well (Buch et al., 2009). Studies on the distribution and dynamics of membrane organelles in oocytes have primarily addressed the mitochondria. During the normal meiotic maturation in the mouse, they have been reported to concentrate in the perinuclear region before and during metaphase I and then to disperse across a wider area with the transition to metaphase II (Van Blerkom and Runner, 1984). These changes seem to be species-specific, since different patterns have been described for other mammals; for example, Suzuki et al. (2005) report distribution of mitochondria throughout the cytoplasmic volume at metaphase I with their subsequent retraction from the cortical cytoplasm at metaphase II in hamster oocytes, while Takahashi et al. (2016) describe accumulation of mitochondria in the perinuclear region of human oocytes before germinal vesicle breakdown and their subsequent spread across the entire cytoplasm after that. Our observations on the distribution of intracellular membranes in control mouse oocytes are in accordance with the results obtained by Van Blerkom and Runner (1984) for mitochondria in the same object, except for

the slightly increased labelling around the metaphase chromosomes. The latter could correspond to the still obscure spindle-associated membrane domain described by Buch et al. (2009) in cultured mammalian cells undergoing mitosis.

In the current study, localization of intracellular membranes in prostaglandin-treated cells was characterized by a decrease and disappearance of the concentration patterns observed in controls. Since the position and distribution of membrane organelles is dependent on the cytoskeleton, their homogenous distribution in cells treated with the higher dose of prostaglandin could be secondary to the disruption of microtubules and/or microfilaments. Indeed, a similar change in the localization of mitochondria has been obtained by treating immature human oocytes with colchicine (Takahashi et al., 2016). The clustered distribution of membranes in oocytes treated with the lower prostaglandin concentration could reflect ongoing disintegration, with the supporting structures still preserved in some regions of the cytoplasm but already collapsed in other regions.

Other researchers have reported that cumulus cells accelerate oocyte aging (Zhu et al., 2015) and mediate disruption of oocyte meiosis in the presence of lipopolysaccharide (Bromfield and Sheldon, 2011), and that prostaglandin receptor genes are expressed in oocytes at low levels if at all (Rodrigues et al., 2020). In our study, however, no difference was observed between oocytes incubated before or after the removal of cumulus. This result can be explained by the use of ovulated oocytes with advanced or completed meiotic maturation, because it is known that oocyte-cumulus communications are down-regulated at this stage (e.g., Shimada, 2012). Hence, the observed effects of prostaglandin F2α, whatever their precise mechanism, must have been exercised directly on the oocyte.

In conclusion, our study was the first to show that prostaglandin F2α, when applied to ovulated mouse oocytes, causes fast degeneration of their meiotic spindle and cortical actin cytoskeleton, as well as a loss of normal chromosome alignment and intracellular membrane gradients, and these effects are dose-dependent and apparently not mediated by the cumulus.

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