DNA Fragmentation Index, pAKT And pERK1/2 In Cumulus Cells Are Related To Oocyte Competence In Patients Undergoing In Vitro Fertilization Program

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Abstract

It is known that activated pERK1/2 and pAKT are key players in supporting cell survival and proliferation pathways, and that the translocation of pERK1/2 into the nucleus, where it interacts with transcription factors and the DNA itself, is instrumental in exerting an anti-apoptotic effect. In this study, the levels of pAKT, the nuclear localization of pERK1/2 and the DNA fragmentation index (DFI) in cumulus cells of single cumulus-oocyte complexes of patients undergoing *in vitro* fertilization program were evaluated and put in correlation with the clinical outcome of the related embryos. In case of the positive clinical outcome of blastocyst development, the nuclear localization of pERK1/2 and the DFI value had a significant inverse relationship, whereas the former one and the intracellular accumulation of pAKT had a significant direct relationship. This was not observed in case of the negative clinical outcome of the arrested embryos. Moreover, the intracellular accumulation of pAKT and the DFI value had a significant inverse relationship in all the samples examined. The obtained data suggest that the intranuclear relocation of pERK1/2 along with an enhanced intracellular accumulation of pAKT may exert a survival effect and increase cell viability thereby providing a novel marker tool to facilitate the choice of the best oocyte to be fertilized and submitted to an ICSI cycle.

Keywords: Apoptosis, DFI, Molecular markers, Oocyte quality, Cell survival.

Introduction

It is widely-acknowledged that during folliculogenesis a controlled program of intercellular communication and support between the oocyte and the granulosa/cumulus cells occurs *via* the gap junctions of the zona pellucida and the release of paracrine soluble factors. Several studies on animal models have demonstrated that this "oocyte-cumulus cell bidirectional dialogue" has key reciprocal metabolic implications such as the supply of cholesterol, pyruvate or alanine from the oocyte, and the oocyte-triggered promotion of estradiol synthesis by granulosa cells. Thus, the follicle must be regarded as a single physiological unit whose compartments cooperate since the initial transition from a primary to a secondary stage, to the final event of ovulation [1,2,3,4].

Dealing with human *in vitro* fertilization (IVF), the correct realization of this bidirectional molecular transfer is required for the acquisition of oocyte competence which leads to a good quality metaphase II (MII) gamete endowed with a positive impact on embryo development, implantation and clinical outcome. Due to the still limited knowledge of the biochemical processes that are involved in the process of oogenesis, the identification of non-invasive markers that may help the selection of the gametes to be submitted to intracytoplasmic sperm injection (ICSI) procedure is a fundamental issue and a still-open field in assisted reproduction research. In this regard, cumulus and granulosa cells, which are easily available as a waste product of the manipulation procedures prior to ICSI, have been used to evaluate

the fertility potential of individual oocytes *via* non-invasive approaches. In fact, in light of the close structural and physiological connections occurring between the oocyte and the surrounding cells, the latters may reflect the quality and competence acquisition of the gamete when tested for the expression of markers of cell survival/death [5].

Previous studies [6,7] have been focused on the evaluation of the DNA fragmentation index (DFI) and the intracellular accumulation of the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and the serine/threonine kinase AKT (or protein kinase B). ERK1/2 isoforms belong to the mitogen-activated protein kinase superfamily and are involved, once activated by phosphorylation, in the control of cell proliferation and apoptosis *via* the Ras–Raf–MEK–ERK signaling cascade. Also AKT, that is one of the key components in the AKT/PI3K/PTEN signaling pathway, once phosphorylated transduce signals which promote cell survival and interfere with the onset of apoptosis [8,9,10]. From the analysis of individual cumulus-oocyte complexes (COCs), cumulus cells associated with the oocytes able to produce blastocysts were shown to possess a higher pAKT/DFI ratio than cumulus cells related to embryos arrested during the *in vitro* culture [7]. In addition, a significant direct correlation was found between pAKT and pERK1/2 accumulation, suggesting a cooperative action as survival factors in the model system under study.

The role played by ERK1/2 in apoptosis rugulation in the ovary is controversial, according to literature data [11,12].

The results presented here extend the previous observations on cumulus cell markers associated with the clinical outcome of the embryo, through the parallel immunocytochemical evaluation of the intracellular accumulation of both pAKT and pERK1/2, with a specific focus on the intranuclear localization of the latter. The data obtained on the two cytological markers were also related with the percentage of DNA fragmentation examined *via* TUNEL assay.

Materials and Methods

Study design and patients

Twenty-six women, attending the assisted reproductive treatment (ART) because of fertility problems and included in a program of blastocyst embryo transfer at the Centro di Biologia della Riproduzione (CBR, Palermo, Italy), were enrolled in the present study and signed an informed consent form to allow the use of any discarded cumulus cell for apoptosis rate assessment. The Internal Review Board of CBR considered this research ethically acceptable (June 27th 2012) as it uses cumulus/granulosa cells that are usually discarded after decoronization of the COCs prior to performing ICSI. Therefore, all procedures performed were in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments. The inclusion criteria were: normoresponder status with a minimum of 6 oocytes collected at pick-up, age ≤ 38 years (mean ± SD: 34.3 ± 3.5) , normal FSH basal level (<12 UI/mL), and body mass index (BMI) <28 kg/m^2 (mean ± SD: 22 ± 3.18). Azoospermia or severe oligoasthenospermia (motile sperms < 0.5 millions/ml) were considered as an exclusion criterion.

Ovarian stimulation

All patients were subjected to administration with the GnRH agonist Buserelin (Suprefact, Sanofi-Aventis, Italy, 0.2 ml/day) starting on day 21 of the previous cycle. Administration of 150 IU r-FSH/day (Gonal-f, Merck, Rome, Italy) was started at day 8 after Buserelin treatment and the follicular growth was monitored every two days using ultrasound and serum estradiol E2 levels, starting on day 6 of stimulation, modifying the dose of r-FSH as a consequence. The dose of 10,000 IU of hCG (Ovitrelle; Merck, Rome, Italy) was administered when at least 3 follicles showed a diameter $\geq$ 18 mm.

Preparation of cumulus cells

Sample preparation was performed as reported by Bosco et al. [13]. Essentially, after hyaluronidase treatment of the COCs, cumulus cells were fixed in 3.7% paraformaldehyde for 60 min, harvested by centrifugation at 2,000 rpm for 7 min and resuspended in phosphate-buffered saline (PBS). Slides were prepared through cytospin centrifugation at 1,000 rpm for 5 min on polylysine-coated glass slides. Cell permeabilization was performed at 4°C in 0.1% Triton X-100 plus 0.1% sodium-citrate in PBS prior to immunostaining and TUNEL assay. Oocytes were transferred to fertilization medium (SAGE IVF; Irvine Scientific, California, USA) and incubated at 37°C and 6% CO_2 until ICSI.

Fluorescent in situ TUNEL and immunodetection assays

The microscopic analyses were performed as reported by Bosco et al.[7]. In order to assess the extent of apoptotic DNA fragmentation, cumulus cells were submitted to TUNEL assay using the DeadEndFluorometric TUNEL System (Promega Italia, Milan, Italy), according to the manufacturer's instructions, in parallel with a positive and a negative control. The reaction was blocked with saline sodium citrate (SSC), and after exhaustive washing in PBS the cumulus cells were counterstained with propidium iodide (1µg/ml).

For the in *situ* immunofluorescence assays, the cell preparations were blocked with 3% bovine serum albumin (BSA)/PBS and co-incubated at 4°C overnight with anti-pAKT polyclonal antibody from rabbit (p-Akt1/2/3 (Ser 473)-R, sc-7985-R, working dilution 1:50,Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-pERK1/2 antibody from mouse (p-ERK (E-4), sc-7383, working dilution 1:50, Santa Cruz Biotechnology) in 3% BSA/PBS. The primary antibody was omitted in the negative controls. The samples were then co-exposed for 1 h to the secondary antibodies, i.e. anti-rabbit IgG (whole molecule)–FITC conjugate (F0382, working dilution 1:50, Sigma, St. Louis , MO, USA) for anti-pAKT and anti-mouse IgG (whole molecule) TRITC conjugate (T5393, working dilution 1:50, Sigma) for anti-pERK1/2, counterstained for 10 min. with Hoechst 33342 (Invitrogen) and mounted in 10 µl DABCO solution.

All samples were observed under an Olympus BX 50 microscope equipped with a reflected light fluorescent attachment (Olympus), and a 20×/0.40 objective. The densitometric analysis of fluorescent signals was carried out using NIS-Elements BR 3.10 image analyzer software (Nikon) as reported by Choi et al. [14].

Statistics

The accumulation levels of pAKT and pERK1/2 and the DFI of oocytes was summarized by means of median and interquartile range. The statistical significance of the difference between the accumulation levels of pAKT and pERK1/2 and the DFI of cumulus cells resulting in transferred *vs* arrested blastocysts was checked through the non-parametric Kruskall-Wallis test. The Spearman's coefficient correlation was calculated between paired data of pAKT, pERK1/2 and DFI of cumulus cells. Statistical significance of the Spearman's coefficient was assessed though the Student's t test. A p-value < 0.05 was considered statistically significant. Statistical analysis was performed through Stata/SE 14.0.

Results and Discussion

In total, 91 MII oocytes were fertilized by ICSI, and the derived embryos had the following evolution: 53 developed to 5-6 days old blastocysts and were transferred "in utero", 8 were arrested at different cleavage stages during the 7 days of *in vitro* culture and were discarded, and the remaining 30 were cryopreserved.

In a first set of assays, cumulus cells of the corresponding COCs were submitted to immunofluorescence microscopy and densitometric analysis of the images for the assessment of the accumulation levels of both total pAKT and intranuclear pERK1/2. A representative microscopic field of cumulus cells, immunolocalized and counterstained with Hoechst 33342 for total nuclei, and the resulting merged image is shown in the panel of Figure 1. In parallel, the DFI was evaluated *via* TUNEL assay. A representative microscopic field of cumulus cells showing DNA fragmentation and counterstained with propidium iodide for total nuclei, and the resulting merged image is shown in the panel of Figure 2. For each COC, at least 60 (for immunostaining) or 450 cells (for DFI) were analyzed.

The accumulation levels of pAKT and pERK1/2 and the DFI of cumulus cells do not show any statistically significant difference between transferred and arrested embryos (Table 1). The statistical correlation among the immune-quantitation, the DFI rate and the clinical outcome of the embryos is shown in Table 2. In case of a positive clinical outcome, we found that in cumulus cells of the corresponding COCs the nuclear localization of pERK1/2 showed a significant inverse correlation with the DFI value, the latter being an apoptosis hallmark, (r_s = -0.39, p = 0.007), and a significant direct correlation with the intracellular accumulation of pAKT (r_s = 0.477, p < 0.001). These results were not obtained with cumulus cells of COCs related to a negative clinical outcome of the arrested embryos. In addition, a significant inverse correlation between the intracellular accumulation of pAKT and the DFI value (r_s = -0.37, p < 0.001) was observed in all cumulus cells examined.

Previous results by Bosco et al. [7] had already validated the apoptosis rate and the inverse correlation between DFI and pAKT accumulation in cumulus cells as molecular markers of oocyte competence, suggesting their prognostic

meaning with regard to blastocyst formation. In the present study, we extend the previous observations to the co-localization analysis of pAKT and pERK1/2 in cumulus cells of single COCs, correlating the results obtained with the DFI and the evolution of embryo development. It is known that unphosphorylated ERK is localized in the cytoplasm and on the surface of organelles where it binds anchoring and scaffold proteins thereby controlling cellular activities such as cell-cell and cell-matrix adhesion, intracellular trafficking and resistance to apoptosis [15]. Upon phosphorylation, ERK is allowed to translocate into the nucleus via an importin 7-mediated mechanism. Interestingly, ERK activation and nuclear localization was proven to regulate the activity of cell cycle factors further promoting cell viability, but also to trigger cell death *via* apoptosis promotion thus being unpredictive of the subsequent specific cellular response [9,16,17]. Concerning the COC model system, Du et al. [18] reported that ERK1/2 activity in mouse cumulus cells is essential for their gonadotropin-induced expansion, whereas Xu et al. [19] demonstrated that the miRNA-mediated downregulation of ERK1 in human granulosa cells may impair their proliferation and differentiation as well as the production of steroid hormones and the follicular development. Our data represent first evidence of i) a potential cell survival effect played by intranuclear pERK1/2 in human cumulus cells that adds to the upregulation of intracellular pAKT, and ii) a significant correlation of these histochemical signatures with a positive clinical outcome of the embryos.

Taking literature data and our results together, we therefore suggest that high levels of nuclear pERK1/2 accumulation coupled with an increase of pAKT concentration and a low DFI rate value in the cumulus cells of the corresponding COCs may be considered as a marker of oocyte competence leading to the development of embryos of presumed good quality.

Competing Interests

The authors have declared that no competing interest exists.

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Figure legends

Figure 1 Panel of fluorescence micrographs showing the immunolocalization of pAKT, in green (A) and pERK1/2, in red (B) and total nuclei counterstain with Hoechst 33342, in blue (C) in a representative cumulus cell preparation. The merged image is shown in (D) and the arrows in the enlarged view (E) point to pERK1/2 localization in the nucleus, in fuchsia, due to the merging of the blue fluorescence of Hoechst 33342 and the red fluorescence of pERK1/2 (n) and in the cytoplasm, in orange, due to the merging of the green fluorescence of pAKT and the red fluorescence of pERK1/2 (c). Bar = 30 μM

Figure 2 Panel of fluorescence micrographs showing the DNA fragmentation detection by TUNEL assay (A), and total nuclei counterstain with propidium iodide (B) in a representative cumulus cell preparation. The merged image is shown in (C). Bar = 30 μM