

Rate of Cumulus Cell Apoptosis from Fertilized and Unfertilized Oocytes and Acid Phosphatase Levels in Follicular Fluids after Intracytoplasmic Sperm Injection

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ABSTRACT

The main role of cumulus cells (CC) is to provide nutritious materials for developing oocytes. Follicular fluid (FF) contains the enzyme acid phosphatase, which plays a role in ovulation and fertilization of oocyte. The objective of this prospective study was to evaluate the levels of acid phosphatase in FF and the rate of apoptosis in CC of fertilized and unfertilized oocytes in intracytoplasmic sperm injection (ICSI) program with male factor infertility. Following ovarian hyperstimulation, 101 mature oocytes were prepared for ICSI. Fertilized (n = 51) and unfertilized (n = 50) oocytes were evaluated for apoptosis in CC. CC of each oocyte were stained with Hoechst 33258 for immunofluorescent microscopy. For evaluation of acid phosphatase, FF was centrifuged and the enzymes levels were measured with spectrophotometer. Nuclei of apoptotic cells were fragmented, the chromatins condensed, and the apoptotic bodies were observed in some cells. Rates of apoptotic CC in fertilized and unfertilized oocytes were 15.83% and 13.34%, respectively ($P > 0.05$, $r = 0.520$). The acid phosphatase level was reduced as the rate of apoptosis increased ($P < 0.05$, $r = -0.520$). Also, the concentration of the enzyme increased when the percentage of normal CC increased $P < 0.05$. Our results confirm that neither the evaluation of CC apoptosis, nor the level of acid phosphatase have a prognostic value in the outcome of ICSI. However, the FF level of acid phosphatase is directly related to the quality of retrieved MII oocyte. *Iran. Biomed. J. 9(4): 163-167, 2005*

Keywords: Apoptosis, Acid phosphatase, Cumulus cells (CC), Intracytoplasmic sperm injection (ICSI)

INTRODUCTION

Cumulus cells (CC) form a multilayered cell mass that surround the oocyte during maturation process within the follicle as well as ovulation. These cells not only protect the oocytes but also provide nutrients to the oocyte through gap junctions [1]. Due to this direct association between the oocyte and CC, the quality of these cells may influence the development of the oocyte [2]. The development of cumulus-oocyte complex is influenced by some factors including the apoptosis. Apoptosis is a genetically controlled form of cell death that plays a major role in homeostasis, and is closely associated with most of the reproductive processes. A high level of apoptosis in human CC may cause poor

development of oocytes as well as embryos because the supportive role of the CC would be altered [3]. There have been several studies concerning the role of CC apoptosis in assisted reproductive techniques (ART). Several authors have reported that the incidence of apoptosis in granulosa or CC may be used as a predictive factor in the outcome of ART cycles [4-7]. They have found that the incidence of apoptosis in the aforementioned cells can influence the oocyte maturation, oocyte quality as well as fertilization and embryo development. It has been postulated that oocytes with good morphological feature is an important factor for successful fertilization and embryo implantation.

In addition, the lysosomal enzyme of acid phosphatase is secreted in many different cells including granulosa cells. The changes in acid

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phosphatase activity are under the influence of pituitary released hormones [8]. Luteinizing hormone surge or hCG injections for induction of ovulation will cause the rupture of lysosomal membrane which will release the content into follicular fluid (FF). The acid phosphatase in FF will cause degeneration of follicles right before ovulation. Therefore, the presence of acid phosphatase in FF can be used as a factor for determination of ovulation time as well as oocyte maturation [9]. It has been suggested that acid phosphatase has a direct effect on zona pellucida of oocyte in enhancing the sperm penetration and subsequent fertilization [10].

The objective of this study was to evaluate the rate of CC apoptosis in fertilized and unfertilized MII oocytes, also the FF levels of acid phosphatase after intracytoplasmic sperm injection (ICSI) cycles in patients with male factor infertility. According to our knowledge, this is the first study on CC apoptosis and FF acid phosphatase in ICSI program.

MATERIALS AND METHODS

Patient selections. The study population consisted of 17 young couples with male factor infertility that underwent ICSI program. The ages of the male patients varied between 29 and 40 years (median 34.2 ± 3.4), and of the females were between 20 and 37 years (median 29.4 ± 4.4). Controlled ovarian hyperstimulation with hMG/hCG was performed as described before [11]. Transvaginal ultrasound-guided oocyte retrieval was performed approximately 36 h post hCG injection.

ICSI procedure. ICSI protocol was done as previously described [11, 12]. Briefly, oocytes were incubated for 4 h and then denuded from CC using 80 IU hyaluronidase/ml (Sigma Chemical Co., USA) along with mechanical aid of Pasteur pipettes. Following incubation, each mature oocyte was injected with a mechanically immobilized spermatozoan. Immature oocytes were excluded from this study. The injected oocytes were then rinsed and paced individually in fresh droplets covered with washed mineral oil. Fertilization was checked 15-18 h after microinjection which was confirmed by the presence of two pronuclei. Approximately 48 h after microinjection, adequate numbers of embryos were transferred to respective patients according to the guidelines of the American Society for Reproductive Medicine.

Assessment of apoptosis. A total of 101 MII oocyte-cumulus complexes were denuded separately. Each cumulus mass was collected and washed twice in PBS solution. The CC pellet was then pipette onto albumin-coated microscope slide and a smear was prepared. The slides were air-dried and then fixed in 4% neutral buffered formalin for 15 min. Each slide was washed in PBS twice and then the cells were stained with 0.5 mg/ml of Hoechst 33258 (Sigma Chemical Co., USA). A fluorescence microscope (Zeiss Co., Germany) was used for observation of apoptotic cells. These cells contained fragments of condensed chromatin or the cytoplasmic fragments containing condensed chromatin. The apoptotic cells were identified and counted among 100 CC at $\times 400$ magnifications.

Acid phosphatase assay. Each sample of FF was first filtered, then centrifuged at $500 \times g$ for 10 min and stored at -20°C for acid phosphatase assay. After slow thawing at room temperature, each sample was appropriately diluted and processed following the manufacturer's instructions. The assay method was done using final concentrations of 0.012 M disodium p-nitrophenyl phosphate as substrate and acid sodium citrate buffer (pH 4.8). The FF samples (0.25 ml) were incubated with substrate solution (0.5 ml) and acid sodium citrate buffer (0.5 ml) at 37°C for 30 min. Following incubation, 5.0 ml of NaOH 0.1 was added and the absorbance of the solutions was read at 405 nm with spectrophotometer (Milton Ray Co., Belgium). Acid phosphatase activity is expressed as micromoles of p-nitrophenyl formed/min/liter of FF at 37°C and pH 4.8. Each Bess Lowry Unit corresponding to 1 micromol of phenol thus formed.

Statistical analysis. Statistical packet for social sciences for windows (SPSS version 10, USA) was used for data analysis. For statistical analysis, Student's *t*-test and Pearson rank correlation coefficient test were used. Statistical significance was set at $P < 0.05$. The data were presented as the mean \pm S.D.

RESULTS

Out of 101 mature oocytes that were evaluated, 51 oocytes were fertilized 15-18 h post microinjection. The incidence of apoptosis in CC from fertilized oocytes was $15.83 \pm 8.4\%$ (min. 50.58% and max. 100%) that was insignificantly higher compared to unfertilized oocytes ($13.34 \pm 10.1\%$; $P > 0.05$, min.

29.0% and max. 40.14%). The nuclei of CC with normal morphology were round and homogenous; while, the nuclei of apoptotic cells were fragmented, the chromatins were condensed, and the apoptotic bodies were observed in some cells (Figs. 1-3). Apoptotic CC were observed in all samples; however, it varied from 5.5% in some cells to 100% in others. The incidence of CC apoptosis in three oocytes was 100%. Also, the rates of fertilization in three and one ICSI cycles were 100% and 0%, respectively.

The results also showed that the level of acid phosphatase in FF samples was reduced as the rate of apoptotic CC increased ($P < 0.05$; $r = -0.520$). Also, the concentration of the enzyme increased when the percentage of normal CC increased ($P < 0.05$). There was an insignificant correlation between the level of acid phosphatase and fertilization rates ($P > 0.05$; $r = 0.005$). The mean numbers of cleaving embryo formation and embryo transfer into uterine cavity were 3.33 ± 0.4 and 3.08 ± 0.9 , respectively.

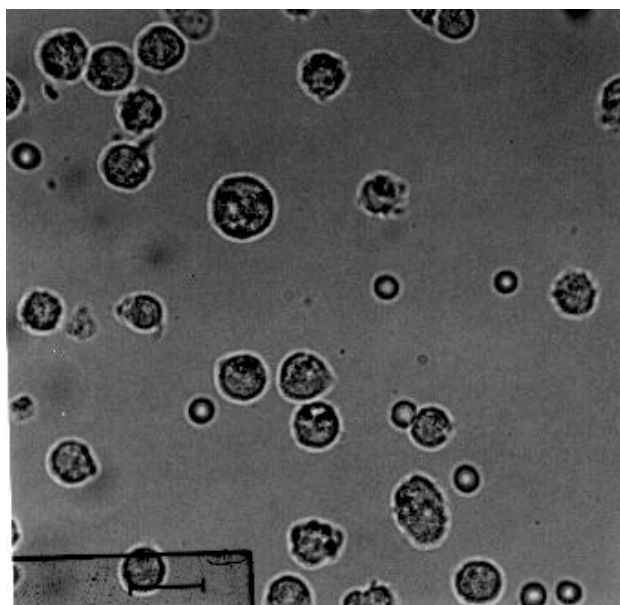


Fig. 1. Unstained cumulus cells collected from one mature oocyte before ICSI ($\times 1000$).

DISCUSSION

Cell apoptosis is a form of suicide that is accompanied by biochemical and morphological changes. It involves DNA fragmentation, cytoplasmic shrinkage, formation of membrane-bound structures that contain pieces of organelles called apoptotic bodies, and activation of lysosomal enzymes. Apoptosis plays an important role during

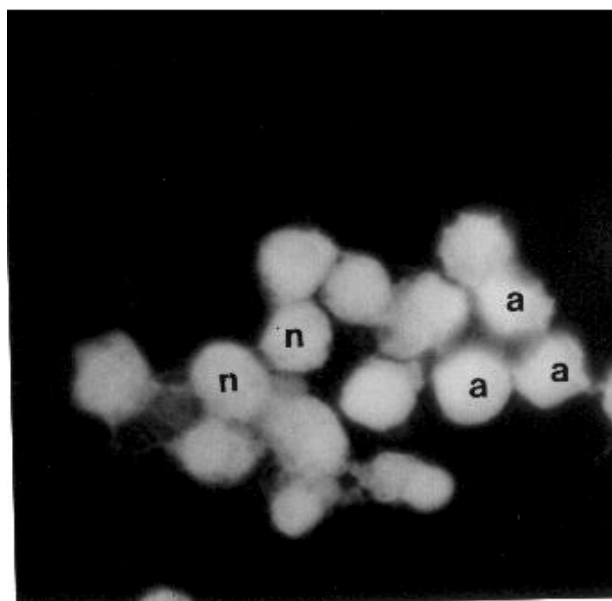


Fig. 2. Immunofluorescent microscopic detection of apoptosis in cumulus cells stained with Hoechst 33258 ($\times 1000$). (n), normal cumulus cells; (a), apoptotic cumulus cells with chromatin condensation.

development [13]. Therefore, it is important to evaluate the role of apoptosis in CC surrounding the human oocytes. In this study, there was an insignificant rate of apoptosis between fertilized and unfertilized oocytes. This indicates that apoptosis does not play any role in fertilization process in ICSI setting. However, it should be emphasized that all the cases were male infertiles whom their wives had normal menstrual cycles and uterine walls. It is

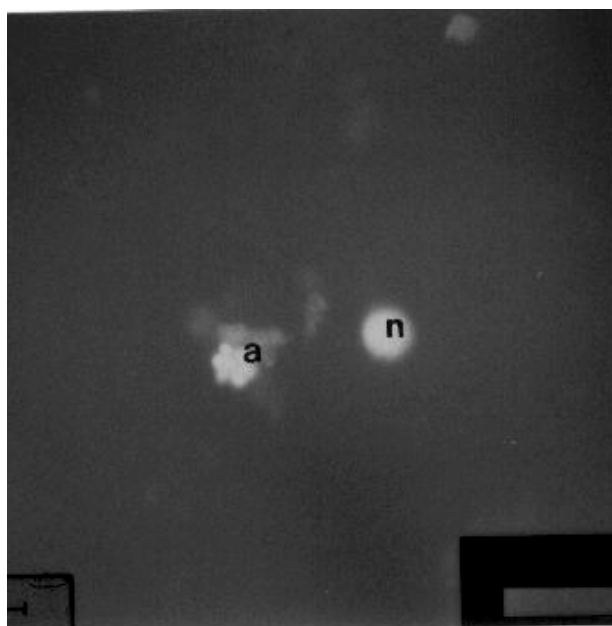


Fig. 3. One normal cumulus cell (n), and one apoptotic cell with fragmented chromatin (a). ($\times 1000$).

possible that different results would be generated if our cases were with female factor infertility. This has been confirmed by Lee *et al.* [5] who studied the rate of CC apoptosis in IVF. CC masses from 91 MII oocytes were collected and the incidence of apoptosis was assessed using apoptosis detection kit fluorescein. In contrast to our results, they observed that the patient's age influence the incidence of CC apoptosis. Also, increased incidence of CC apoptosis was noticed in unfertilized oocytes. Also, Kaneko *et al.* [14] observed that high incidence of apoptosis is usually associated with lower oocyte quality, poor fertilization and embryo development. Therefore, the incidence of apoptotic CC is a sensitive indicator for estimation of oocyte quality as well as fertilization in IVF program.

Recently, Alisch *et al.* [4] evaluated the apoptotic activity of over 240 cumulus-oocyte complexes to the outcome of ICSI cycles. Their results showed that high quality oocytes directly correlated with low rate of apoptosis. However, this was not related with pregnancy outcome their results are in agreement with our data. In addition, Clavero *et al.* [15] did not observe any significant correlations between apoptosis in granulosa cells and oocyte maturity or fertilization in ICSI with male infertility. Therefore, it seems that evaluation of CC apoptosis does not have a prognostic value in the outcome of ICSI cases with male factor infertility. It can only be used for oocyte maturity/quality in ICSI program. However, in contrast to our findings, Suh *et al.* [16] observed a correlation between apoptosis and pregnancy outcome in IVF with female infertility cases. They proposed that apoptosis analysis could be used as a prognostic tool for IVF success. In a recent study by Idil *et al.* [17], the incidence of apoptosis was found to be higher in cases with unexplained infertility than in cases with tubal infertility ($P < 0.05$). It was suggested that granulosa cells apoptosis might have a role in the etiology of unexplained infertility.

Acid phosphatase is the marker enzyme for lysosomes and has been used to monitor cell death and lysis [8]. Our results also demonstrated that the level of acid phosphatase in FF samples is high, which is related to CC quality. It was observed that high level of acid phosphatase was directly related to the normal morphology of CC. Concurrent with this, in the presence of apoptosis, lower levels of acid phosphatase were present in FF samples.

However, acid phosphatase concentration did not show any correlation with fertilization rate. Therefore, this enzyme plays an important role during the early process of reproductive cycles.

Gonzalez *et al.* [9] also investigated the acid phosphatase levels in relation to oocyte maturity in patients who underwent laparoscopy or laparotomy. Their results showed that acid phosphates levels in FF containing mature oocytes were significantly higher than those of follicles with immature oocytes. They also suggested that acid phosphatase levels might be used as an indicator for follicular maturity and to choose the optimal time for follicular puncture in ART program. In addition, Kleinman *et al.* [10] evaluated the acid phosphatase levels in follicles of 52 patients following ovulation induction in IVF setting. Their results indicated that FF acid phosphatase levels were significantly higher in women whose ova were fertilized and cleaved than those with no fertilization. This, however, does not agree with our results which may be related to the type of infertility treatment employed. We believe that FF levels of acid phosphatase can only serve as an indicator for proper timing of follicular puncture and oocyte quality following ovulation induction in ICSI.

In an interesting study, the effects of different ovarian hyperstimulation protocols on the incidence of apoptotic granulosa cells were investigated [14]. Their results showed that in GnRHa + hMG + hCG as well as hMG + hCG groups, the total number of mature oocytes retrieved were significantly higher than those in natural cycle. Moreover, the incidence of apoptotic cells in hMG + hCG cycles was significantly lower than that in natural and GnRHa + hMG + hCG cycles. Therefore, it was suggested that hMG + hCG is the most appropriate ovarian hyperstimulation protocol for retrieval of good quality oocytes. We also used the hMG + hCG protocol for our cases, and successfully retrieved 101 mature oocytes from 17 ICSI cycles.

Although our results are not definitive, it would appear that detection of CC apoptosis and FF acid phosphatase may not be applicable as prognostic tool for the outcome of ICSI procedures in cases with male factor infertility. However, evaluation of acid phosphatase levels can be used to predict the quality of oocytes following controlled ovarian hyperstimulation. In addition, further studies in cases with unexplained infertility may present a different picture on the roles of CC apoptosis and acid phosphatase in ICSI setting.

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