Effect of GnRH down-regulation on cumulus cell viability and apoptosis as measured by fluorescence-activated cell sorting

Pericles Assad Hassun Filho •
Mariana Antunes Ribeiro • André Monteiro da Rocha •
José Roberto Allegrati • Paulo Cesar Serafini •
Eduardo Leme Alves da Motta

Received: 31 March 2008 / Accepted: 29 September 2008 / Published online: 21 October 2008
© Springer Science + Business Media, LLC 2008

Abstract

Objective To determine whether gonadotropin releasing hormone (GnRH)-agonist or -antagonist induces higher percentages of cumulus cell apoptosis and if the use of either is detrimental to ART outcomes.

Patients Women in a private facility under treatment for IVF had their cumulus cells isolated and analyzed by flow cytometry. Viable, apoptotic, and dead cumulus cell rates related to ovarian stimulation by GnRH-agonist or -antagonist were measured and compared with fertilization and implantation rates.

Results Treatment with GnRH-agonist produced a greater number of follicles than treatment with GnRH-antagonist. No differences in implantation and pregnancy rates were found. While cumulus cell (CC) apoptosis was positively correlated with estradiol on the day of hCG administration, no significant difference in the percentage of apoptotic cells between treatments was detectable. Additionally, implantation rate and the average follicular estradiol production on the day of hCG administration were no different between treatments.

Conclusions GnRH-agonist or -antagonist treatment protocols induce similar levels of apoptosis in CCs and are not detrimental to ART outcomes.

Keywords ART · Cumulus cells · Apoptosis · GnRH · Ovarian stimulation · Flow cytometry

Introduction

Controlled ovarian stimulation (COS), used for development of multiple ovarian follicles per cycle, is widely implemented with Assisted Reproduction Technology (ART). Specifically, COS is achieved by administering exogenous FSH to develop supra-numerary follicles until they reach the proper size for final maturation. One risk of this process is unintended ovulation in response to the endogenous hormonal environment. Administration of GnRH-agonist or — antagonist can eliminate this risk and avoid premature ovulation [1]. It has been speculated that administration of exogenous gonadotropin results in supra-numerary follicles by overriding a natural process: selection of a dominant follicle and subsequent follicular atresia of subordinate follicles [2], which occurs through apoptosis initiated in the granulosa cells [3–6]. Granulosa cells (GCs), and their specialized oocyte-communicating counterpart cumulus cells (CCs), play an important role in essential oocyte maturation processes [7]. GC apoptosis and/or necrosis may influence oocyte quality and future embryo development. Studies have reported a positive correlation of GC number with poor IVF/ICSI results [8–13].

Conflicting information exists regarding the effects of endogenous gonadotropin down-regulation and GC apo-

Capsule abstract Mode of GnRH down regulation does not influence cumulus cell survival.

P. A. Hassun Filho (✉)
Genesis Genetics Brazil,
Rua Mato-Grosso, 306, CJ.506 CEP.,
01239-040 São Paulo, São Paulo, Brazil
e-mail: pericles.hassun@gmail.com

M. A. Ribeiro • A. M. da Rocha • J. R. Allegrati • P. C. Serafini •
E. L. A. da Motta
Huntington - Reproductive Medicine,
Sao Paulo, Brazil

ptosis. Assessment of GC death, suspended in the follicular fluid, revealed that the mechanism of GnRH-induced down-regulation did not influence GC survival [2]. However, studies assessing mural GC apoptosis revealed that programmed cell death is more common in treatments with GnRH agonist + human menopausal gonadotropin (hMG) + hCG than in hMG + hCG or spontaneous cycles [8]. Cultured luteinized granulosa cells exposed to a GnRH-agonist, Leuprolide acetate, suffered DNA synthesis inhibition and were more susceptible to apoptosis than those exposed to a GnRH-antagonist [9]. Interestingly, Corn et al. evaluated apoptosis in cumulus cells in relation to blastocyst development without considering the mode of GnRH down-regulation [14]. Therefore, we sought to verify whether controlled ovarian stimulation with GnRH-agonist or -antagonist induces apoptosis in cumulus cells, and if the percentage of apoptosis has significant detrimental impacts on fertilization or implantation rates.

Materials and methods

This prospective observational study was composed of 35 women undergoing controlled ovarian stimulation as follows:

1. Delivery of recombinant-hFSH beginning on either day 2 or 3 of the menstrual cycle, continuing with a full dose until ≥ 2 codominant follicles reached 18 mm in the largest diameter as measured by transvaginal ultrasonography. When two codominant follicles reached 13–14 mm or the patient reached day 6 of stimulation, a daily subcutaneous SC injection of 0.25 mg Cetrotide (CTD) was initiated. From the first day of CTD treatment until the day of maturational hCG administration, the patient returned to the clinic at least every two days for ultrasound imaging and blood sampling. [15].
2. Daily SC injections of Leuprolide acetate (0.5 mg; Reliser®, Serono Laboratories, Inc.) were administered in the midluteal phase of the previous menstrual cycle to cause pituitary desensitization, after which recombinant hFSH was initiated. The criteria for pituitary desensitization were met when women began menstrual flow accompanied by serum E_2 levels < 60 pg/mL in the absence of ovarian cysts (> 10 mm) and exhibited an endometrial thickness < 3 mm by transvaginal sonography [15].

Administration of either the GnRH antagonist or the GnRH agonist was extended until the day of final follicle maturation (defined by ultrasonic detection of ≥ 2 follicles > 18 mm in diameter). Subsequently, a maturational dose of 10,000 IU of urinary hCG (Profasi®, Serono Laboratories, Inc.) was injected to accommodate oocyte retrieval within 35–36 h [15]. Patients were assigned to yield a ratio of 1:2 follicles produced by agonist:antagonist. Information was

collected regarding the patient's age, basal FSH, estradiol and progesterone concentrations (basal and on hCG day), total FSH dose, and number of follicles.

Retrieved oocyte cumulus complexes (OCCs) were cultured for 4 h prior to removal of the cumulus cells. Mature oocyte cumulus complexes were individualized in 250 μ L G1 (VitroLife, Sweden) droplets and were transferred to denudation medium containing 80 IU/mL of hyaluronidase. Pasteur pipettes were used to gently manipulate oocyte cumulus complexes and remove cumulus cells. Single cell suspensions and hyaluronidase wash-out were obtained by three washes in PBS [16]. Cumulus cells were dispersed in a dilution buffer to a concentration $< 1.10^5$ cells/mL, and an unstained aliquot was subjected to flow cytometry for equipment calibration. Fifty microliters of cell suspensions were incubated with 450 μ L ViaCount reagent (Guava Technologies Inc., California, USA) for 5 min. After staining, samples were loaded into sample tubes and subjected to flow cytometry (Guava PC, Guava Technologies Inc., California, USA) until all cells were counted or a maximum of 10,000 cells was reached. Analysis for individualized oocyte cumulus complexes was performed with Cytosoft in ViaCount mode (Guava Technologies Inc., California, USA). Cytograms discriminating live and dead cells were also obtained.

Oocytes were evaluated, and mature metaphase II oocytes were inseminated by sperm injection. Fertilized zygotes and subsequent embryos were cultured within individual microdrops until morphologic selection [17] of one to four embryos per patient. Fluorescence-activated cell sorting analysis of viable, apoptotic and dead cells for each OCC was done after cell staining with Guava ViaCount Reagent following the manufacturer's instructions and using a Guava PCA flow cytometer. The percentages of viable, apoptotic and dead cells per OCC were calculated with Cytosoft software (version 2.1.4, Guava Technologies, Inc, California, USA). Pearson's correlation coefficients were calculated for the percentages of viable, apoptotic, and dead CCs from the oocytes resulting in transferred embryos in relation to the patient's basal FSH, estradiol and progesterone concentrations (basal and on hCG day), total FSH dose and implantation rate [18]. These data sets were split into GnRH-agonist and GnRH-antagonist groups for comparison. All data were analyzed using a Kolmogorov-Smirnov normality test, F-test for variance homogeneity and Student's t test for differences in means. Differences were considered significant at $p < 0.05$ [18].

Results

Patients assigned to treatment with GnRH-agonist (9 patients; 76 OCCs) and GnRH-antagonist (26 patients; 162 OCCs) protocols were of similar ages at the time of

Table 1 Mean and standard deviation of various characteristics compared between the GnRH agonist and GnRH-antagonist groups

Groups		Ovarian stimulation protocol	
		GnRH-agonist	GnRH-antagonist
Patient age		37±1.6	37±0.8
Estradiol (pg/ml)	Basal	24.9±15	38.5±23.5
	On hCG day	2,107±1,377	1,115±769
Progesterone (ng/ml)	Basal	0.589±0.402	1.13±2.44
	On hCG day	10.2±22	7.1±18
FSH(UI)	Basal	6.57±2.65	7.14±2.76
	Total rFSH	2,332±816	1,926±894
Number of follicles on hCG day		15.25±5.44 ^a	8.35±5.21 ^b
E ₂ /follicle		132.9±76.3	140.4±71.5
Number of transferred embryos		3±1	3±1
Viable (%)		24.8±14.5	25.4±15.1
Apoptotic (%)		16.87±9.72	12.5±9.65
Dead (%)		58.7±15.5	62.1±17.1
Fertilization rate		0.527±0.303	0.649±0.358
Implantation rate		0.188±0.372	0.102±0.295
Pregnancy rates		55%	41%

Data presented as mean ± SEM. Different letters indicate statistical significance ($p < 0.05$)

their treatment (37±1.6 vs. 37±0.8 years), and no differences in their basal hormonal profiles were observed. Differences in the total FSH doses administered per patient were not significant between groups. Serum concentrations of estradiol and progesterone on the day of hCG administration were not significantly altered, despite the higher number of follicles in the agonist group. Fertilization, number of transferred embryos, implantation and pregnancy rates were similar between the two groups, and gonadotropin-releasing hormone modality produced no differences in the percentage of viable, apoptotic and dead CCs (Table 1).

Estradiol concentration on hCG day and estradiol per follicle were positively correlated with the percentage of apoptotic CCs. Fertilization rates correlated with percentages of viable and dead cells, and implantation rates were

positively and negatively correlated to percentages of apoptotic and dead cells, respectively. Pearson's correlation coefficient values are shown in Table 2.

Discussion

We found that treatment with GnRH-agonist or -antagonist did not significantly increase the apoptotic population of CCs associated with oocytes. These results are similar to those of Giampietro and colleagues [2] who reported similar levels of apoptosis in granulosa cells suspended in follicular fluid using an Annexin V/PI flow cytometry assay. However, experimental differences exist between these two studies. Cells examined in the previous study were

Table 2 Pearson's correlation coefficient for viable, apoptotic, and dead cumulus cells and hormonal profiles and rFSH treatment

Parameters			Pearson's coefficient	Viable cells	Apoptotic cells	Dead cells
Parameters						
Estradiol	Basal	r		-0.328*	0.018	0.288*
	On hCG day	r		0.086	0.310*	-0.197
Progesterone	Basal	r		0.146	0.115	-0.118
	On hCG day	r		0.044	0.076	-0.079
FSH	Basal	r		0.000	-0.062	0.026
	Total dose (rFSH)	r		-0.021	0.154	-0.046
Number of follicles on hCG day		r		0.043	0.153	-0.096
Fertilization rate		r		-0.479*	0.050	0.392*
Implantation rate		r		0.133	0.536*	-0.332*

The number of follicles, fertilization rate, and implantation rate are also reported

*statistical significance ($p < 0.05$)

from follicular fluid, while our study examined detached cells directly from OCCs.

Greater than 99.9% of the follicles present at birth are destined to die via apoptosis during reproductive life [19]. Typically, cytoplasmic blebbing and catalytic cleavage events featured during apoptosis commonly lead to loss of function. However, in the cells of the inner layer of the ovarian follicle, the steroidogenic function is preserved through actin migration and a cytoplasmic compartmentalization that segregates and protects steroidogenic machinery from proteosomes activated in the executioner pathways of apoptosis [20–23]. We found a subtle positive correlation between estradiol and apoptosis, as was reported in a study showing that cultured granulosa cells had an increment in steroidogenesis during programmed cell death [21]. However, we did not find a correlation between progesterone levels on hCG day and the percentage of apoptotic cells. The shift in steroid production probably relies on the dose of FSH used during stimulation, which influences GC estrogenic pathways [24].

Previous studies have investigated the incidence of GC apoptosis and its relation to IVF/ICSI results [8–13]. However, it is important to recognize that the majority of these studies assessed apoptosis of cells suspended in follicular aspirates. Those values represent an evaluation of apoptosis within the mural granulosa cells that detached from the follicular wall during follicle aspiration [9–13]. Mural granulosa cells are protected from apoptosis by bFGF—acting as a survival factor—sequestered in the basal lamina [19]. Thus, apoptosis of mural cells obtained from an individual follicle can be viewed as an impairment factor. Since apoptosis takes place even with the protective effect of bFGF, this might indicate that the follicle is undergoing atresia. In natural cycles, apoptosis occurs in cells from large follicles in an asynchronous pattern, probably due to differences in cell cycle stages. However, in cycles under controlled ovarian stimulation, evaluation of granulosa cell cycle revealed that the majority of cells were in G0/G1 stages, indicating a synchronization among the cells during a stage in which they are more susceptible to programmed cell death [1].

Our results led us to hypothesized that apoptosis in CCs from follicles under controlled ovarian stimulation were a sign of maturity and differentiation of the follicle rather than poor developmental potential of oocytes. The observed percentage of apoptotic cells correlated with the implantation rate supports our hypothesis. However, in this study, we based embryo selection on standard morphology features since using apoptosis in cumulus cells as a marker of oocyte developmental potential remains under debate. In conclusion, GnRH-agonist or antagonist treatment protocols induce similar levels of apoptosis in CCs, and this cell death is not detrimental to ART outcomes.

Financial support None

References

- Muasher SJ, Abdallah RT, Hubayter ZR. Optimal stimulation protocols for in vitro fertilization. *Fertil Steril*. 2006;86:267–73. doi:10.1016/j.fertnstert.2005.09.067.
- Giampietro F, Sancilio S, Tiboni GM, Rana RA, Di Pietro R. Levels of apoptosis in human granulosa cells seem to be comparable after therapy with a gonadotropin-releasing hormone agonist or antagonist. *Fertil Steril*. 2006;85:412–9. doi:10.1016/j.fertnstert.2005.08.020.
- Palumbo A, Yeh Y. Apoptosis as a basic mechanism in the ovarian cycle: follicular atresia and luteal regression. *J Soc Gynecol Investig*. 1995;2:565–73. doi:10.1016/1071-5576(94)00062-6.
- Tilly JL, Kowalski KIN, Johnson AL, Such AD. Involvement of apoptosis in ovarian follicles atresia and postovulatory regression. *Endocrinology*. 1991;129:2799–801.
- Billig H, Furuta I, Hsueh AJ. Gonadotropin-releasing hormone directly induces apoptotic cell death in the rat ovary: biochemical and in situ detection of deoxyribonucleic acid fragmentation in granulosa cells. *Endocrinology*. 1994;134:245–52. doi:10.1210/en.134.1.245.
- Piquette GN, Tilly JL, Prichard LE, Simon C, Polan ML. Detection of apoptosis in human and rat ovarian follicles. *J Soc Gynecol Investig*. 1994;1:297–301.
- Kaneko T, Saito H, Takahashi T, Ohta N, Saito T, Hiroi M. Effects of controlled ovarian hyperstimulation on oocyte quality in terms of the incidence of apoptotic granulosa cells. *J Assist Reprod Genet*. 2000;17:580–5. doi:10.1023/A:1026439409584.
- Thoma FH, Vanderhyden BC. Oocyte granulosa cell interactions during mouse follicular development: regulation of kit ligand expression and its role in oocyte growth. *Reprod Biol Endocrinol*. 4:19. (<http://www.rbej.com/content/4/1/19>).
- Nakahara K, Saito H, Saito T, Ito M, Ohta N, Takahashi T, et al. The incidence of apoptotic bodies in membrane granulosa can predict prognosis of ova from patients participating in in vitro fertilization programs. *Fertil Steril*. 1997;68:312–7. doi:10.1016/S0015-0282(97)81521-X.
- Sifer C, Bénifla JL, Bringuier AF, Porcher R, Blanc-Layrac G, Madélénat P, et al. Could induced apoptosis of granulosa cells predict in vitro fertilization-embryo transfer? A preliminary study of 25 women. *Eur J Obstet Gynecol Reprod Biol*. 2002;103:150–3. doi:10.1016/S0301-2115(02)00043-X.
- Bencomo E, Pérez R, Arteaga MF, Acosta E, Pena O, Lopez L, et al. Apoptosis of cultured granulosa-lutein cells is reduced by insulin-like growth factor I and may correlate with embryo fragmentation and pregnancy rate. *Fertil Steril*. 2006;85:474–80. doi:10.1016/j.fertnstert.2005.08.014.
- Suh CS, Jee BC, Choi YM, Kim JG, Lee JY, Moon SY, et al. Prognosis implication of apoptosis in human luteinized granulosa cells during IVF-ET. *J Assist Reprod Genet*. 2002;19:209–14. doi:10.1023/A:1015319617598.
- Oosterhuis GJ, Michgelsen HW, Lambalk CB, Schoemaker J, Vermes I. Apoptotic cell death in human granulosa lutein cells: a possible indicator of in vitro fertilization outcome. *Fertil Steril*. 1998;70:747–9. doi:10.1016/S0015-0282(98)00266-0.
- Corn CM, Hauser-Kronberger C, Moser M, Tews G, Ebner T. Predictive value of cumulus cell apoptosis with regard to blastocyst development of corresponding gametes. *Fertil Steril*. 2005;84:627–33. doi:10.1016/j.fertnstert.2005.03.061.
- Serafini P, Yadid I, Motta ELA, Alegretti JR, Fioravanti J, Coslovsky M. Ovarian stimulation with daily late follicular phase

- administration of low-dose human chorionic gonadotropin for in vitro fertilization: a prospective, randomized trial. *Fertil Steril*. 2006;86:830–8. doi:[10.1016/j.fertnstert.2006.02.110](https://doi.org/10.1016/j.fertnstert.2006.02.110).
16. Ruvolo G, Bosco L, Pane A, Morici G, Cittadini E, Roccheri MC. Lower apoptosis rate in human cumulus cells after administration of recombinant luteinizing hormone to women undergoing ovarian stimulation for in vitro fertilization procedures. *Fertil Steril*. 2007;87:542–6. doi:[10.1016/j.fertnstert.2006.06.059](https://doi.org/10.1016/j.fertnstert.2006.06.059).
 17. Veeck L. Atlas of the human Oocyte and Early Conceptus, vol 2. Baltimore: Williams & Wilkins; 1991.
 18. Zar JH. Biostatistical analysis. New Jersey: Prentice Hall; 1999.
 19. Hsueh AJW, Billing H, Tsafiri A. Ovarian Follicle Atresia: a hormonally controlled apoptotic process. *Endocr Rev*. 1994;15:707–24. doi:[10.1210/er.15.6.707](https://doi.org/10.1210/er.15.6.707).
 20. Amsterdam A, Keren-Tal I, Aharoni D. Cross-talk between cAMP and p53-generated signals in induction of differentiation and apoptosis in steroidogenic granulosa cells. *Steroids*. 1996;61:252–6. doi:[10.1016/0039-128X\(96\)00031-1](https://doi.org/10.1016/0039-128X(96)00031-1).
 21. Amsterdam A, Dantes A, Selvaraj N, Aharoni D. Apoptosis in steroidogenic cells: structure-function analysis. *Steroids*. 1997;62:207–11. doi:[10.1016/S0039-128X\(96\)00182-1](https://doi.org/10.1016/S0039-128X(96)00182-1).
 22. Amsterdam A, Dantes A, Hosokawa K, Schere-Levy CP, Kotsuji F, Aharoni D. Steroid regulation during apoptosis of ovarian follicular cells. *Steroids*. 1998;63:314–8. doi:[10.1016/S0039-128X\(98\)00016-6](https://doi.org/10.1016/S0039-128X(98)00016-6).
 23. Amsterdam A, Keren-Tal I, Aharoni D, Dantes A, Land-Bracha A, Rimón E, et al. Steroidogenesis and apoptosis in the mammalian ovary. *Steroids*. 2003;68:861–7. doi:[10.1016/j.steroids.2003.09.003](https://doi.org/10.1016/j.steroids.2003.09.003).
 24. Chian RC, Ao A, Clarke HJ, Tulandi T, Tan SL. Production of steroids from human cumulus cells treated with different concentrations of gonadotropins during culture in vitro. *Fertil Steril*. 1999;71:61–6. doi:[10.1016/S0015-0282\(98\)00416-6](https://doi.org/10.1016/S0015-0282(98)00416-6).