Effect of gonadotropin-releasing hormone agonist and antagonist on proliferation and apoptosis of human luteinized granulosa cells

The GnRH agonist leuprolide acetate (LA) inhibited DNA synthesis in epidermal growth factor–stimulated human granulosa luteinized cell cultures. This effect was blocked by the prior addition of a GnRH antagonist antide (ANT), and this compound per se was able to produce a stimulatory effect of DNA synthesis on basal conditions. Leuprolide acetate produced an increase in the percentage of apoptotic cells, and when these two factors were co-incubated, ANT blocked the apoptotic effect produced by LA. (Fertil Steril® 2006; 85:1064–7. ©2006 by American Society for Reproductive Medicine.)

Patients undergoing assisted reproductive techniques (ART) receive GnRH agonist (GnRH-a) or GnRH antagonist to suppress endogenous gonadotropin secretion and control ovarian cycles. This suppression of gonadotropin secretion can be achieved by two pathways. The first, chronic administration of GnRH-a, leads to pituitary desensitization by reducing the number of GnRH receptors on the cell membrane. The second one, administration of GnRH antagonist, rapidly inhibits gonadotropin secretion by competitive blockage of the GnRH receptor. Over the last few years, GnRH antagonist has been used more frequently in ART, offering the advantages of the absence of flare-up (an initial LH and FSH hypersecretion seen with the GnRH-a) and a reduction in the number of days using gonadotropins (1, 2).

In addition to the main pituitary GnRH function of regulating synthesis and secretion of gonadotropins, there has been increasing evidence that GnRH is an intraovarian regulatory factor with autocrine–paracrine actions on follicular development and steroidogenesis (3–5). This is supported by the fact that a GnRH-like peptide, GnRH receptors, as well as their mRNA, have been found in ovarian tissues from rats and humans (4–7). Several studies performed on rats have described the antigonadal effects of GnRH agonists, both in vivo and in vitro (8–11). We have demonstrated in rat follicular cultures that some of these effects are mediated by an increase in follicular apoptosis through interference of FSH, cyclic adenosine 3′:5′ monophosphate, and/or growth factor pathways and by changes in the expression of bcl-2–related genes (3, 12–14). In addition, we also have detected a direct inhibitory action of a GnRH-a, leuprolide acetate (LA), on LH receptor expression and aromatase activity in human granulosa luteinized cells (hGLC; 15). However, there still is some controversy about the physiological function of GnRH-like peptides in the human ovary.

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These observations led us to study and compare the direct effect of a GnRH-a, LA (Lupron; Abbott Labs, Buenos Aires, Argentina) and a GnRH antagonist, antide (ANT; Sigma Chemical Co., St. Louis, MO) on growth, apoptosis, and steroidogenesis of hGLC that was obtained from patients undergoing ART. For this study, approved by our institutional ethical review board, hGLCs were obtained from the follicular fluid of 10 women undergoing ART (15) and were isolated as described elsewhere (15). One hundred thousand cells were plated in collagen-coated multiwells and cultured at 37°C in 5% $CO_2$for 48 hours in Dulbecco's Modified Eagle's Medium (DMEM)–Ham's F-12 (1:1), HCO_3^- (2.2 g/L), and 10% fetal bovine serum culture medium. The cell viability was not modified during this incubation period. Then, cells were washed and cultured for 48 hours more for the proliferation assay, in serum-free medium, together with different factors (epidermal growth factor [EGF]: 10 ng/mL; insulin: 1, 10, and 100 ng/mL; insulin-like growth factor-1: 1, 10, and 100 ng/mL; LA: 10 and 100 ng/mL; and/or ANT: 10^{-7} M). Serum-free medium was chosen to avoid possible interferences from factors present in serum. One microcurie of ^{3}H-thymidine (^{3}H-T; Nen, Dupont, Boston, MA) then was added to each microwell, and DNA synthesis was assessed 24 hours later by ^{3}H-T incorporation (16). In the case of cells treated with LA+ANT, LA was added 3 hours after ANT.

Figure 1A shows the effect of LA, ANT, or the combination of these two factors on basal DNA synthesis. Leuprolide acetate had no effect on cell proliferation, whereas interestingly, ANT alone produced a significant stimulatory effect of 350% ($P<.05$ vs. basal) on ^{3}H-T incorporation. In addition, in those cells incubated with the combination of both factors (LA+ANT), we observed only the increased proliferation effect of ANT. Given that basal ^{3}H-T incor-

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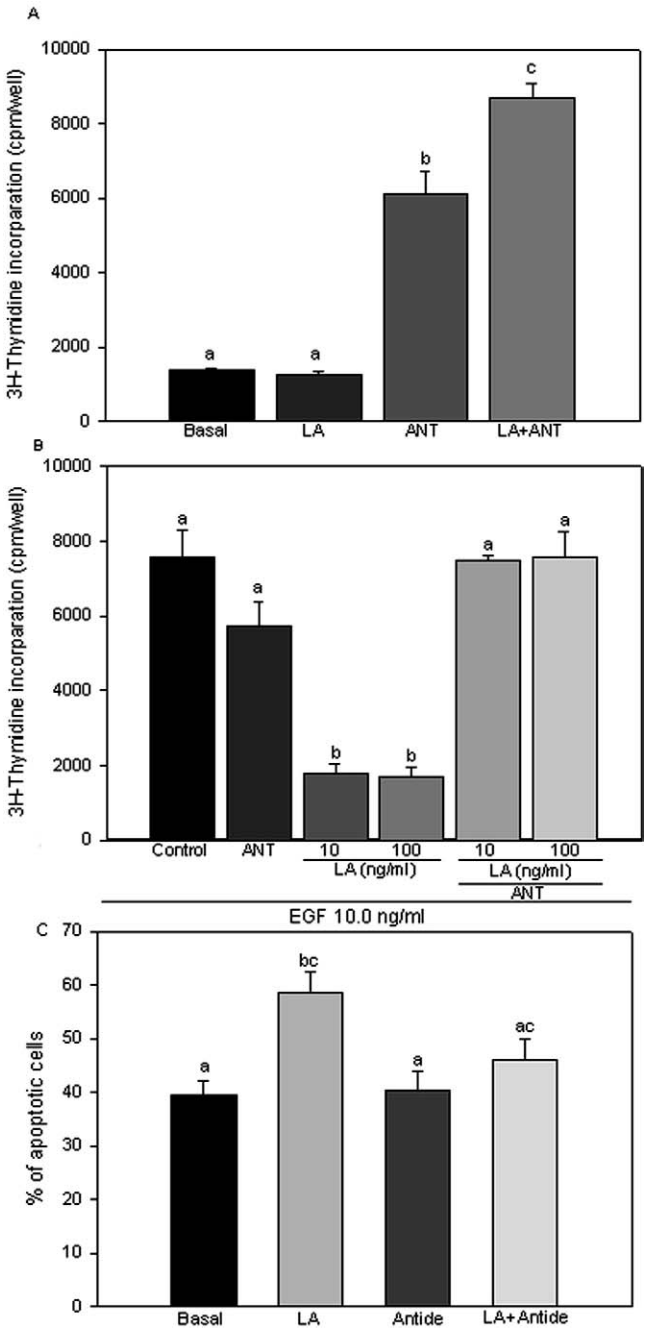
FIGURE 1

(**A**) Effect of LA and ANT on DNA synthesis measured by 3H-T incorporation at basal conditions in human luteinized granulosa cells stimulated with LA (100 ng/mL), ANT (10_{-7} M), or the combination (LA+ANT). (**B**) Effect of LA and ANT on DNA synthesis measured by 3H-T incorporation on EGF-stimulated cells (control: EGF, 10 ng/mL). Luteinized granulosa cells were incubated with LA (10 and 100 ng/mL), ANT (10_{-7} M), or the combination (LA+ANT). (**C**) Effect of LA and ANT on cellular apoptosis. Apoptosis was analyzed by the acridine orange–ethidium bromide technique at basal conditions in cells stimulated with LA (100 ng/mL), ANT (10_{-7} M), or the combination (LA+ANT). In all panels, different letters indicate significance ($P<.05$).



Vitale. GnRH analogues action on human luteinized granulosa cells. Fertil Steril 2006.

poration in hGLC cells is low, growth factors were added to the culture medium in order to increase cellular proliferation and therefore detect possible inhibition by the factors under study (13, 17). The addition of 10 ng/mL of EGF caused a significant increase in the percentage of ^{3}H-T incorporation. On the contrary, the addition of insulin or insulin-like growth factor-1 did not produce changes in ^{3}H-T incorporation (data not shown).

We then added LA to EGF (10 ng/mL)-stimulated cell cultures (Fig. 1B), in which EGF was considered to be the control. Under these conditions, ^{3}H-T uptake was downregulated by LA ($P<.05$), suggesting that the lack of LA inhibitory effect on basal DNA synthesis (Fig. 1A) could be caused by the low proliferation capability of luteinized granulosa cells in culture. In addition, when ANT was added to EGF-stimulated cell cultures, no effect was observed. However, the inhibitory effect exerted by LA was completely blocked by prior addition of ANT to the culture medium ($P<.05$; Fig. 1B).

To assess whether this inhibition of DNA synthesis described in the previous two paragraphs was caused by an increase in the rate of apoptosis, hGLC were cultured in the presence of LA and/or ANT, and DNA fragmentation was quantified by using the acridine orange–ethidium bromide technique (18). Human granulosa luteinized cells were cultured as described in medium with 10% FBS (fetal bovine serum). Then, cells were cultured for another 24 hours in serum-free medium containing LA, ANT, or a combination of these two factors, with LA added 3 hours after the addition of ANT. Culture media were collected to determine P levels by RIA as described elsewhere (14), and acridine orange (1 mg/L)–ethidium bromide (250 mg/L) mix was added to the cells. The number of apoptotic cells was counted with a fluorescence microscope and expressed as a percentage of apoptotic cells; data are given as mean ± SE from quadruplicate cultures. One-way analysis of variance followed by Tukey test was used to compare the mean values among treatments.

Treatment with LA showed a significant increase in the level of apoptosis, from 39.6% ± 2.5% at basal conditions to 58.6% ± 4% ($P<.05$). The percentage of apoptotic cells detected under basal conditions is likely to be a consequence of the incubation in serum-free medium. Furthermore, ANT alone had no effect on basal apoptosis, whereas the increase in the percentage of apoptotic cells produced by LA was blocked by ANT (LA+ANT: 46.1% ± 4% vs. LA, $P>.05$; Fig. 1C).

Finally, no significant differences ($P>.05$) were found in the P concentrations among the different groups studied (basal: 996 ± 75 ng/mL; LA: 1,023 ± 82 ng/mL; ANT: 998 ± 71 ng/mL; LA+ANT: 1,154 ± 248 ng/mL). Cells stimulated with LH were used as a positive control for P production, and a significant stimulation after LH treatment was detected (74% ± 14% stimulation, $P<.05$), indicating that the capability of these cells to produce steroids is intact.

Over the last 2 decades, GnRH antagonists have become an important therapeutic agents for synchronizing follicle growth during gonadotropin stimulation for IVF.

Although it is controversial that GnRH receptors in human ovaries are functional, few studies have been performed to test the effects of GnRH antagonist on them (19). In this study, we described that GnRH-a LA alone had no effect on basal DNA synthesis in hGLC cultures. However, when LA was added to EGF-stimulated cell cultures, a significant inhibition of ^{3}H-T incorporation was observed. In addition, the inhibitory LA effect was blocked completely by the prior addition of ANT to the culture medium, suggesting that LA action is mediated mainly by an ovarian GnRH receptor. Furthermore, the GnRH antagonist ANT per se was able to produce a stimulatory effect of ^{3}H-T incorporation on basal conditions. These results support previous results from our laboratory showing that ANT injection in superovulated prepuberal rats produced an increase in the number of preovulatory follicles, as well as an increase in the ovarian weight. In addition, LA decreased the number of preovulatory follicles, whereas ANT interfered with this inhibitory effect of LA (20). Collectively, these findings support the hypothesis that ANT improves follicular development, likely by a blockage at the GnRH receptor level, on the inhibitory action of an endogenous ovarian GnRH peptide.

Taking into account that follicular atresia is mediated by apoptosis (21, 22), we have studied the effects of the two analogues on apoptosis. Leuprolide acetate produced a significant increase in the percentage of apoptotic cells, whereas ANT had no effect on this culture. In addition, when these two factors were co-incubated, ANT blocked the apoptotic effect produced by LA. These findings are closely consistent with the stimulatory effects of GnRH-a on granulosa cell apoptosis that were described elsewhere (3, 13, 23). Finally, there is uncertainty about the steroidogenic effect exerted by GnRH analogues in hGLC in vitro (24). Our results are in agreement with those of other investigators who have shown that GnRH-a (25, 26) and antagonists (19, 27, 28) have no direct effect on steroidogenesis.

In summary, our findings suggest that a GnRH-like peptide acts as an intraovarian modulating factor able to interfere with the proliferation of hGLC, causing the death of those cells by apoptosis.

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REFERENCES

1. Borm G, Mannaerts B. Treatment with the gonadotrophin-releasing hormone antagonist ganirelix in women undergoing ovarian stimulation with recombinant follicle stimulating hormone is effective, safe and convenient: results of a controlled, randomized, multicentre trial. The European Orgalutran Study Group. Hum Reprod 2000;15:1490–8.
2. Shapiro DB. An overview of GnRH antagonists in infertility treatments. Introduction Fertil Steril 2003;80 Suppl 1:S1–7.
3. Andreu C, Parborell F, Vanzulli S, Chemes H, Tesone M. Regulation of follicular luteinization by a gonadotropin-releasing hormone agonist: relationship between steroidogenesis and apoptosis. Mol Reprod Dev 1998;51:287–94.
4. Stojilkovic SS, Reinhart J, Catt KJ. Gonadotropin-releasing hormone receptors: structure and signal transduction pathways. Endocr Rev 1994;15:462–99.
5. Hsueh AJ, Jones PB. Extrapituitary actions of gonadotropin-releasing hormone. Endocr Rev 1981;2:437–61.
6. Behrman HR, Preston SL, Hall AK. Cellular mechanism of the antigonadotropic action of luteinizing hormone-releasing hormone in the corpus luteum. Endocrinology 1980;107:656–64.
7. Peng C, Fan N, Ligier M, Vaananen J, Leung PC. Expression and regulation of gonadotropin-releasing hormone (GnRH) receptor messenger ribonucleic acids in human granulosa-luteal cells. Endocrinology 1994;135:1740–6.
8. Clayton RN, Harwood JP, Catt KJ. Gonadotropin-releasing hormone analogue binds to luteal cells and inhibits progesterone production. Nature 1979;282:90–2.
9. Sridaran R, Philip GH, Li H, Culty M, Liu Z, Stocco DM, et al. GnRH agonist treatment decreases progesterone synthesis, luteal peripheral benzodiazepine receptor mRNA, ligand binding and steroidogenic acute regulatory protein expression during pregnancy. J Mol Endocrinol 1999;22:45–54.
10. Hsueh AJ, Adashi EY, Jones PB, Welsh TH Jr. Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocr Rev 1984;5:76–127.
11. Kitajima Y, Endo T, Manase K, Nishikawa A, Shibuya M, Kudo R. Gonadotropin-releasing hormone agonist administration reduced vascular endothelial growth factor (VEGF), VEGF receptors, and vascular permeability of the ovaries of hyperstimulated rats. Fertil Steril 2004;81 Suppl 1:842–9.
12. Parborell F, Dain L, Tesone M. Gonadotropin-releasing hormone agonist affects rat ovarian follicle development by interfering with FSH and growth factors on the prevention of apoptosis. Mol Reprod Dev 2001;60:241–7.
13. Parborell F, Pecci A, Gonzalez O, Vitale A, Tesone M. Effects of a gonadotropin-releasing hormone agonist on rat ovarian follicle apoptosis: regulation by EGF and the expression of Bcl-2-related genes. Biol Reprod 2002;67:481–6.
14. Irusta G, Parborell F, Peluffo M, Manna PR, Gonzalez-Calvar SI, Calandra R, et al. Steroidogenic acute regulatory protein in ovarian follicles of gonadotropin-stimulated rats is regulated by a gonadotropin-releasing hormone agonist. Biol Reprod 2003;68:1577–83.
15. Guerrero HE, Stein P, Asch RH, de Fried EP, Tesone M. Effect of a gonadotropin-releasing hormone agonist on luteinizing hormone receptors and steroidogenesis in ovarian cells. Fertil Steril 1993;59:803–8.
16. Dain LB, Bley MA, Baranao JL, Tesone M. Evidence for the production of a growth-inhibitory factor by human granulosa-luteal cells. Mol Reprod Dev 1993;36:159–63.
17. Tilly JL, Billig H, Kowalski KI, Hsueh AJ. Epidermal growth factor and basic fibroblast growth factor suppress the spontaneous onset of apoptosis in cultured rat ovarian granulosa cells and follicles by a tyrosine kinase-dependent mechanism. Mol Endocrinol 1992;6:1942–50.
18. Abrams JM, White K, Fessler LI, Steller H. Programmed cell death during Drosophila embryogenesis. Development 1993;117:29–43.
19. Ortmann O, Weiss JM, Diedrich K. Embryo implantation and GnRH antagonists: ovarian actions of GnRH antagonists. Hum Reprod 2001;16:608–11.
20. Parborell F, Irusta G, Vitale AM, Gonzalez O, Pecci A, Tesone M. GnRH antagonist antide inhibits apoptosis of preovulatory follicle cells in rat ovary. Biol Reprod 2005;72:659–66.
21. Chun SY, Eisenhauer KM, Minami S, Billig H, Perlas E, Hsueh JW. Hormonal regulation of apoptosis in early antral follicles: follicle-stimulating hormone as a major survival factor. Endocrinology 1996;137:1447–56.
22. Tilly JL. Cell death and species propagation: molecular and genetic aspects of apoptosis in the vertebrate female gonad. In: Lockshin RA, Zakeri Z, Tilly JL, eds. When cells die: A comprehensive evaluation of apoptosis and programmed cell death. New York: Wiley-Liss. 1998:431–52.
23. Birnbaumer L, Shahabi N, Rivier J, Vale W. Evidence for a physiological role of gonadotropin-releasing hormone (GnRH) or GnRH-like material in the ovary. Endocrinology 1985;116:1367–70.
24. Ortmann O, Diedrich K. Pituitary and extrapituitary actions of gonadotrophin-releasing hormone and its analogues. Hum Reprod 1999;14 Suppl 1:194–206.
25. Casper RF, Erickson GF, Rebar RW, Yen SS. The effect of luteinizing hormone-releasing factor and its agonist on cultured human granulosa cells. Fertil Steril 1982;37:406–9.
26. Lanzone A, Panetta V, Di Simone N, Arno E, Fulghesu AM, Caruso A, et al. Effect of gonadotrophin-releasing hormone and related analogue on human luteal cell function in vitro. Hum Reprod 1989;4:906–9.
27. Minaretzis D, Jakubowski M, Mortola JF, Pavlou SN. Gonadotropin-releasing hormone receptor gene expression in human ovary and granulosa-lutein cells. J Clin Endocrinol Metab 1995;80:430–4.
28. Lin Y, Kahn JA, Hillensjo T. Is there a difference in the function of granulosa-luteal cells in patients undergoing in-vitro fertilization either with gonadotrophin-releasing hormone agonist or gonadotrophin-releasing hormone antagonist? Hum Reprod 1999;14:885–8.