Original Article

DOI: 10.5582/bst.2011.v5.3.120

# Gonadotropin-releasing hormone-agonist induces apoptosis of human granulosa-luteal cells *via* caspase-8, -9 and -3, and poly-(ADP-ribose)-polymerase cleavage

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**Summary** **Gonadotropin-releasing hormone-agonist (GnRH-Ag) used in controlled ovarian hyperstimulation (COH) for *in vitro* fertilization and embryo transfer (IVF-ET) has been known to directly affect apoptosis of human ovarian cells, but its mechanism is not clearly understood. Therefore, the purpose of the present study was to investigate whether caspase-8, -9, and -3 activation and poly-(ADP-ribose)-polymerase (PARP) cleavage are involved in the mechanism by which GnRH-Ag induces apoptosis in human granulosa-luteal cells. The prospective study was conducted in the research institute and clinical fertility center of university hospital. Human granulosa-luteal cells collected from IVF-ET patients were cultured and treated with $10^{-6}$ M GnRH-Ag or saline as a control. To access apoptosis in the cells, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end-labeling (TUNEL) assay and DNA fragmentation analysis were preformed 24 h after treatment. Activity of caspase-8, -9, and -3 in the cells was examined using a fluorogenic substrate. Caspase-8, -9, and -3 activation and PARP cleavage were analyzed by Western blot. DNA fragmentation in the cells increased at concentrations over $10^{-6}$ M GnRH-Ag. In TUNEL assays, the rate of apoptotic cell formation in GnRH-Ag treatment increased significantly compared with that of saline treatment ($p < 0.05$). The activity of caspase-8, -9 and, -3 investigated using a fluorogenic substrate increased only in the apoptotic cells. In Western blot analysis, cells treated with GnRH-Ag revealed an increase in active forms of caspase-8, -9, and -3 compared with saline treatment. In addition, cleavage of PARP also increased in cells treated with GnRH-Ag. These results suggest that activation of caspase-8, -9, and -3 and cleavage of PARP might be involved in apoptosis of human granulosa-luteal cells induced by GnRH-Ag.**

***Keywords:*** Apoptosis, caspase, human granulosa-luteal cells, gonadotropin-releasing hormone-agonist, poly-(ADP-ribose)-polymerase

## 1. Introduction

Gonadotropin-releasing hormone (GnRH) is a 10-amino acid protein synthesized in the hypothalamus that stimulates luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion *via* a connection with a receptor in the pituitary gland. This gonadotropin affects the gonads and associated organs (*1*). GnRH affects the ovary directly without involving the pituitary gland; specifically, GnRH influences physiologic changes in ovarian granulosa cells, ovarian growth, and ovulation (*2*,*3*). Because the half-life of GnRH secreted in hypothalamus is very short and GnRH is not detected in serum, the action of GnRH in the ovary was thought to be caused by GnRH synthesized within the ovary (*4*). Indeed, after the receptor for GnRH was identified in the human ovary, there have been studies focusing on the direct action of GnRH on the ovary (*5*,*6*).

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Specifically, there have also been active advances regarding the apoptotic action of GnRH on the ovary, as GnRH has been shown to directly induce apoptosis of human ovarian cells (*7*,*8*). GnRH has been reported to increase the expression of the Bax gene associated with apoptosis in luteal cells, but decrease the expression of the Bcl-xL gene associated with growth. Local inhibition of vascular endothelial growth factor (VEGF)-A activity appears to produce an increase in ovarian apoptosis through an imbalance among the BCL2 family members, thus leading a larger number of follicles to atresia (*9*,*10*). In addition, GnRH is known to be associated with a decrease in nitric oxide (NO), a known growth factor in luteal cells, but the signal transduction mechanism has not been clearly elucidated (*11*).

Generally, the mechanism of apoptosis involves the signal transduction process by which intracellular protein is degraded by a proteinase (caspase) and many kinds of caspases have been reported to be associated with apoptosis (*12*). Caspase-8 is activated by cell apoptosis induction materials, such as tumor necrosis factor (TNF)-α or Fas ligand, and activated caspase-8 activates other caspases which results in cellular apoptosis (*13*,*14*). During apoptosis, cytochrome c is released through a channel which exists in the mitochondrial membrane and is regulated by Bcl-2 proteins making up the channel. The released cytochrome c connects with Apaf-1, caspase-9, and dATP, and then activates caspase-9 (*15*,*16*). Activated caspase-9 activates caspase-3 which results in cellular apoptosis (*17*). As a result, activated caspase-3 activates poly (ADP-ribose) polymerase (PARP), which results in cellular apoptosis by inducing DNA fragmentation and nuclear condensation (*18*).

The purpose of the current study was to determine if apoptosis of human granulosa-luteal cells by GnRH-agonist (Ag) is caused by the above mechanism and to generate basic data needed for clinical use of GnRH-Ag. For this, cultured human granulosa-luteal cells were treated with different doses of GnRH-Ag and apoptosis was detected by DNA fragmentation and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end-labeling (TUNEL) method. In addition, to determine which caspases this apoptosis is associated with, we evaluated intracellular activity using a fluorescent substrate for caspases-8, -9, and -3 (*19*) and confirmed activated protein caspases-8, -9, and -3 and PARP by Western blot. As a result, the objective of this study was to elucidate the process of cellular apoptosis in human granulosa-luteal cells by GnRH-Ag.

## 2. Materials and Methods

### 2.1. *Obtaining human granulosa-luteal cells*

Human granulosa-luteal cells were obtained during ovum aspiration of patients undergoing *in vitro* fertilization (IVF). The use of human granulose-luteal cells was approved by the Clinical Committee of Life Science Institute in the Department of Obstetrics and Gynecology of Eulji Medical Center of Eulji University. All patients gave informed consent. All patients underwent the same ovarian stimulation protocol. Ovarian stimulation was achieved with a combination of pure FSH (Metrodin; Serono, Aubonne, Switzerland) and human menopausal gonadotropin (hMG) (Merional; IBSA, Lugano, Switzerland) in a step down fashion without a GnRH-Ag long protocol. Human chorionic gonadotrophin (hCG) (Profasi, 10,000 IU; Serono, Aubonne, Switzerland) was administered IM when at least 2 follicles reached an average diameter of 17 mm. Transvaginal oocyte retrieval (Medison 128; Medison Co., Seoul, Korea) was performed 35-36 h after hCG administration. The aspirated follicular fluid was transferred to culture dishes. The oocytes obtained under microscopy were analyzed and transferred to culture media. The granulosa-luteal cells existing within follicular fluid were obtained and transferred to culture media. To remove red blood cells, cultured fluid (1 mL) containing granulosa-luteal cells was layered on a 3 mL Percoll gradient and centrifuged for 20 min at 300 × g. After centrifugation, the granulosa-luteal cells were within the middle layer and the red blood cells had sedimented to the bottom.

The granulosa-luteal cells were washed 3 times with culture media and transferred to culture media with 0.1% collagenase (Sigma, St. Louis, MO, USA). After 30 min in culture media at 37°C, the cell clusters were separated into single cells by repetitive aspiration and expulsion through a 28G needle. The cells were counted on a hemocytometer. To determine viability, cells were stained with trypan blue. We used cells with a viability > 70% for culture at 100,000 cells per mL.

### 2.2. *Culture of granulosa-luteal cells*

Granulosa-luteal cells were placed in 24-well culture plates (Nunc, Roskilde, Denmark) at 100,000 cells per well; the cells were cultured in medium at 37°C with 95% air + 5% $CO_2$ in 100% humidity. The culture medium was Dulbecco's modified Eagle medium (dMEM; GIBCO BRL, Roskiville, NY, USA), 10% fetal bovine serum (FBS; GIBCO BRL), 2 mM L-glutamine (GIBCO BRL), 100 U/mL penicillin (GIBCO BRL), and 100 μg/mL of streptomycin (GIBCO BRL) were added.

Granulosa-luteal cells were transferred to 24-well culture plates and cultured for 24 h. The cells attached to the bottom of the plate were confirmed and the culture medium was changed. Cultured granulosa-luteal cells were treated with $10^{-6}$ M GnRH-Ag (Sigma), or normal saline as a control. After culture for 24 h, the cells were analyzed for cellular apoptosis and apoptosis-associated proteins.

2.3. *Analysis of DNA fragmentation to identify apoptosis*

Grind buffer (0.2 mL) was added to the granulosa-luteal cells, they were ground with a tissue grinder, and added to 12.5 μL of 10% SDS at 65°C. After 30 min, 35 μL of 8 M potassium acetate was added and mixed. Over a 60 min time interval, proteins were precipitated in ice at 4°C, and centrifuged at 5,000 × g for 10 min. The upper layer was transferred to a microcentrifuge test tube. After an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) was added, DNA was obtained. The same amount of chloroform/isoamyl alcohol (24:1, v/v) was added, and the DNA was re-obtained.

The upper layer fluid was transferred to a microcentrifuge test tube and 100% ethanol (2.5× volume) at 0°C was added, and then precipitated at −70°C in an ultra-low temperature refrigerator for > 1 h. DNA was obtained after centrifugation at 14,000 × g for 30 min at 4°C. The sediment was dissolved in 50 μL of 1× TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0); 1 μL of DNase-free RNase (500 μg/mL; Boehringer-Mannheim, Indianapolis, IN, USA) was added for 60 min at 37°C.

The DNA was extracted with the same amount of phenol/chloroform/isoamyl alcohol. After extraction, the DNA was re-extracted with the same amount of chloroform/isoamyl alcohol. After collecting the upper layer fluid, DNA was placed in 3 M sodium acetate (0.1 × volume) and 100% ethanol (2.5× volume) at 0°C, then at −70°C in a low-temperature freezer for > 60 min. The suspension was centrifuged at 14,000 × g for 30 min at 4°C, and was washed and dried in 80% ethanol (0.2 mL) at 0°C. We dissolved a compression determinant in distilled water (25 μL) at −20°C after measuring the quantity of DNA at 260 nm. A 5% agarose gel was loaded with DNA (5 μg) with TBE solution in running buffer, and stained with ethidium bromide after 3 h electrophoresis at 50 V, and observed in an ultraviolet ray transilluminator.

2.4. *TUNEL dyeing for determining cellular apoptosis*

Twenty-four hours after culture, apoptosis was detected using an *in situ* apoptosis detection kit (ApopTag; Intergen Co., Purchase, NY, USA) after fixation by TUNEL in the cultured granulosa-luteal cells. Granulosa-luteal cells were attached to a 24-well culture plate. Four percent neutral buffered formalin (500 μL) was placed in each well and the cells were fixed for 10 min and washed in Tris buffer. First, an equilibration buffer in an ApopTag kit was used for 5 min and after terminal deoxynucleotidyl transferase (TdT) enzyme was added, the response was observed for 90 min at 37°C. To stop the response, stop buffer was added for 10 min at room temperature, and samples were washed three times with Tris buffer. Anti-digoxigenin antibody with Texas red was applied for 30 min at 37°C, samples were washed with Tris buffer, and dyed secondarily with Hoechst 33258. After mounting with fluorescence mounting solution, we evaluated apoptosis in the cultured human granulosa-luteal cells using fluorescence microscopy. After the cells stained by TUNEL assay were counted, the ratio of the total cells was analyzed by staining with Hoechst 33258. Using an original magnification of 200×, the total number of cell nuclei and the number of TUNEL-positive cell nuclei were counted 5 times in randomly chosen fields on each treated or control coverslip. For quantitative analysis of apoptosis, we counted 300-500 cells for each slide. These counts were repeated five times in other experiments.

2.5. *Caspase-8 , -9, and -3 activity assay using fluorescent substrates*

Caspase activity in the cultured granulosa cells was assessed using the fluorescent substrate for each caspase (*19*). The cultured cells were washed in PBS containing the fluorescent substrates for caspases and incubated for 3 h at 37°C. After dyeing nuclei with Hoechst 33258, the cells were observed under fluorescent microscopy. Fluorescent substrates for activated caspase-8, -9, and -3 exhibited green fluorescence using fluorescent microscopy.

2.6. *Western blot analysis of caspase-8, -9, and -3 and PARP*

Granulosa cells were broken in homogenization buffer containing 50 mM Tris-base (pH 7.4), 150 mM NaCl, 10 mM EDTA, 0.1% Tween-20, and protease inhibitors (0.1 mM phenyl methyl-sulfonylfluoride, 5 g/mL aprotinin, and 5 g/mL leupeptin), then centrifuged at 12,000 × g for 30 min. Protein concentration was measured using a DC protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), following the manufacturer's protocol and electrophoresis was performed with the same sample amount in 10% SDS-PAGE (polyacrylamide gel electrophoresis) under reducing conditions, as previous described (*20*). The proteins separated by electrophoresis were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech), based on the procedures of Towbin *et al*. (*21*) and immersed in Tris-buffered saline (TTBS; 10 mM Tris-HCl, pH 7.6, containing 150 mM NaCl, and 0.1% Tween-20) containing 5% non-fat dry milk as a blotting method to prevent non-specific bonding. The blocked membrane was treated for 1 h in TTBS with rabbit polyclonal anti-human caspase-8, -9, and -3 and PARP antibodies at a 1:1,000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After treating 3 times for 1 min, the membranes were treated with anti-rabbit horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology) at a dilution

of 1:1,000 in TTBS for 40 min at room temperature. The signal of these membranes was visualized using a chemiluminescence solution (ECL kit; Amersham Life Science, Buckinghamshire, UK) and observed with X-ray film (Hyperfilm, Amersham Life Science).

### 2.7. *Statistical analysis*

Student's *t*-test was used for statistical comparisons. Statistical significance was defined as $p < 0.05$.

## 3. Results

### 3.1. *Checking apoptosis by the TUNEL method*

DNA fragmentation was analyzed to assess apoptosis of the cultured human granulosa-luteal cells after treatment with GnRH-Ag at different doses. We confirmed that DNA fragmentation in the cultured human granulosa-luteal cells increased at a GnRH-Ag concentration > $10^{-6}$ M (Figure 1). On the basis of this result, GnRH-Ag at a concentration of $10^{-6}$ M GnRH-Ag was used in each experimental group.

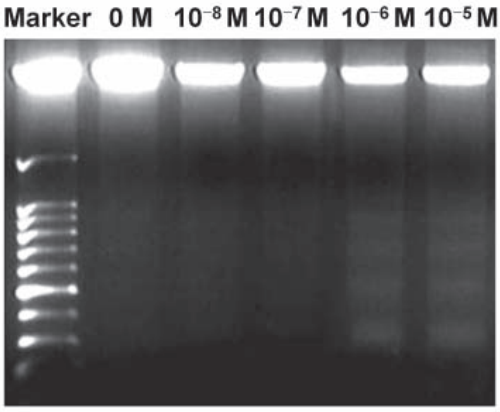


**Figure 1. Analysis of DNA fragmentation in cultured human granulosa-luteal cells after GnRH-Ag treatment in a dose dependent manner.** DNA fragmentation increased in concentrations over $10^{-6}$ M GnRH-Ag.

The TUNEL method was used to evaluate cellular death induced by apoptosis in cultured human granulosa-luteal cells. Some cellular condensation was observed using a phase contrast microscope; nuclei in these cells was condensed and fragmented. After treatment of cultured human granulosa-luteal cells with normal saline or $10^{-6}$ M GnRH-Ag, cellular apoptosis increased in the group of cells treated with GnRH-Ag (Figure 2). Quantitative analysis of apoptotic cells in the human granulosa-luteal cells cultured with saline or GnRH-Ag was conducted. The percent of apoptotic cells in saline compared to GnRH-Ag was 8.02 ± 5.73 *vs*. 36.83 ± 12.34. The rate of apoptosis following GnRH-Ag treatment (36.83 ± 12.34) increased significantly compared to saline treatment (8.02 ± 5.73; $p < 0.05$) (Figure 3).

### 3.2. *Caspase-8 -9, and -3 activity in cultured human granulosa-luteal cells*

Caspase-8, -9, and -3 activity associated with apoptosis was assessed using each fluorogenic substrate for caspase-8, -9, and -3, as described in the Methods section (*19*). Apoptotic cells displaying condensed nuclei showed intense caspase-8, -9, and -3 activity. As a result, we confirmed that the fluorogenic substrates for caspase-8, -9, and -3 activated in apoptotic cells was dissolved and green fluorescence was produced. In contrast, green fluorescence was not produced in normal cells (Figure 4).

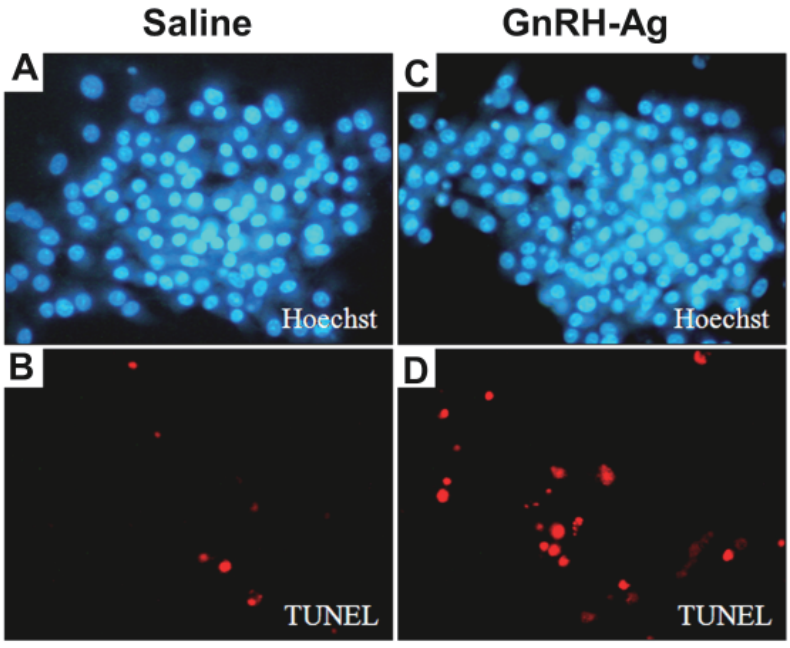


**Figure 2. Evaluation of apoptosis in cultured human granulosa-luteal cells using the TUNEL method.** Twenty-four hours after culture, apoptosis was detected using an *in situ* apoptosis detection kit after fixation. Nuclei were counterstained with Hoechst 33258 and observed under a fluorescence microscope **(A** and **B)**. Cells with red-colored nuclei were considered apoptotic. The number of apoptotic cells increased with GnRH-Ag **(D)** compared with saline treatment **(B)**. Original magnification, ×400.

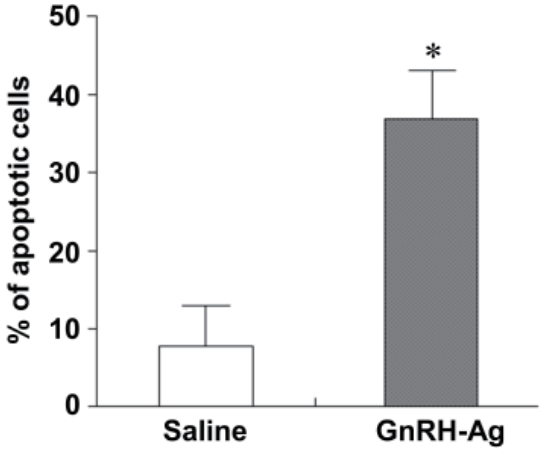


**Figure 3. Quantitative analysis of apoptotic cells in human granulosa-luteal cells cultured with saline or GnRH-Ag.** For the quantitative analysis of apoptosis, apoptotic cells and total cells 300 to 500 cells were counted for each slide. The rate of apoptotic cells in GnRH-Ag treatment increased significantly compared with that of saline treatment. Data points represent the mean ± S.E.M. of five independent experiments. * $p < 0.05$ compared to the corresponding saline group.

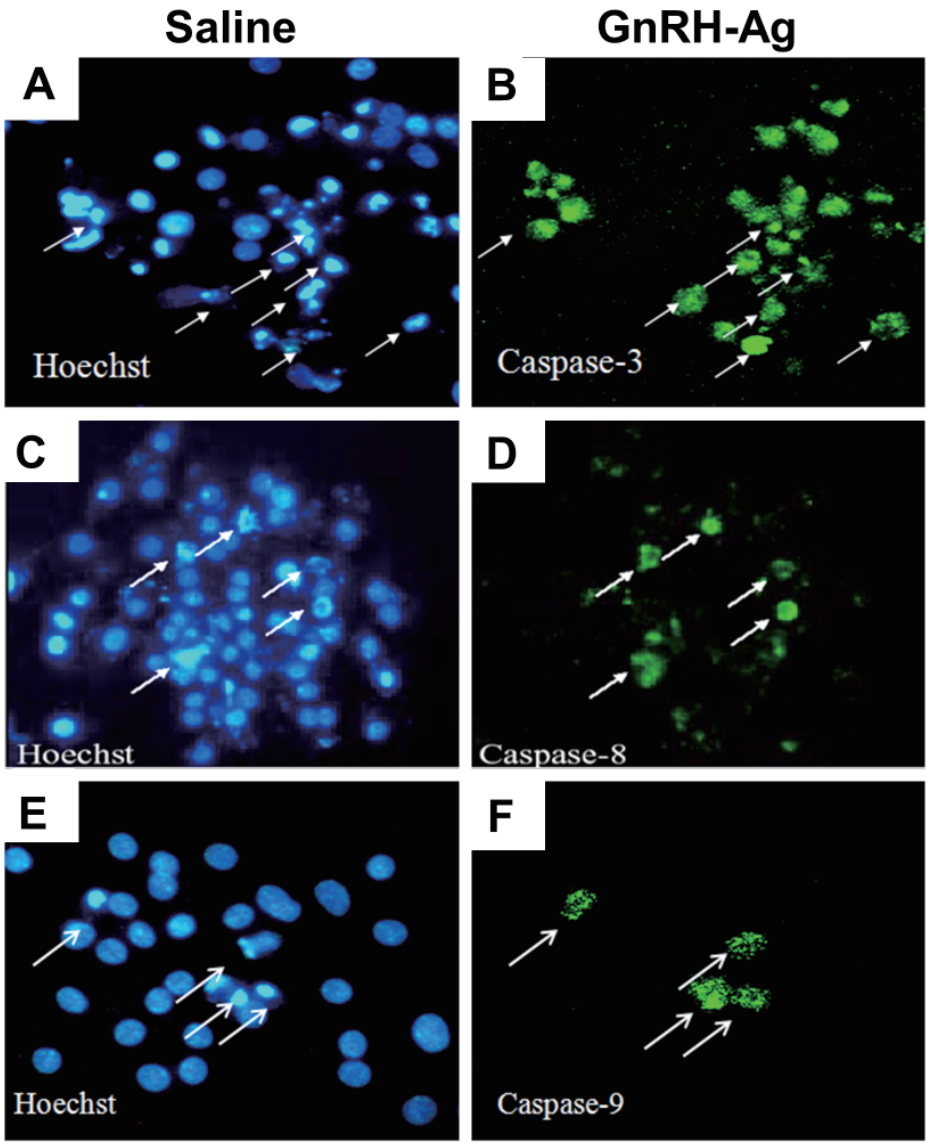


**Figure 4. Caspase-8, -9, and -3 activity in cultured human granulosa-luteal cells.** Caspase-8, -9, and -3 activity in the cells was assessed using a fluorogenic substrate for caspase-8, -9, and -3 as described in Methods. Apoptotic cells displaying the condensed and fragmented nucleus show the intensity of caspase-3 **(A** and **B)**, -8 **(C** and **D)**, and -9 **(E** and **F)**. Arrows indicate the cells showing activation of caspase-8, -9, and -3 stained with fluorescent substrates of caspases as a control to compare with GnRH-Ag. Original magnification, ×400.

3.3. *Increase in caspase-8, -9, and -3 and PARP activation in cultured human granulosa-luteal cells by GnRH-Ag*

Western blot analysis was used to determine whether or not apoptosis of human granulosa-luteal cells induced by GnRH-Ag was caused by activation of caspase protein. Initially, a 32 kDa inactivated pro-form of caspase-3 decreased, but the 17 kDa and 12 kDa cleaved form of caspase-3 increased following GnRH-Ag treatment (Figure 5A). A 55 kDa pro-form of caspase-8 was present in both lysates. A 40 kDa cleaved form of caspase-8 increased in the GnRH-Ag treatment (Figure 5B).

Also, a 47kDa pro-form of caspase-9 decreased, but a 37 kDa cleaved form of caspase-9 increased in the GnRH-Ag treatment group (Figure 5C). Analysis of PARP activity by Western blot, such as the final stage of apoptosis, showed that the 86 kDa cleaved form of PARP increased similar to the results of the caspases (Figure 5D).

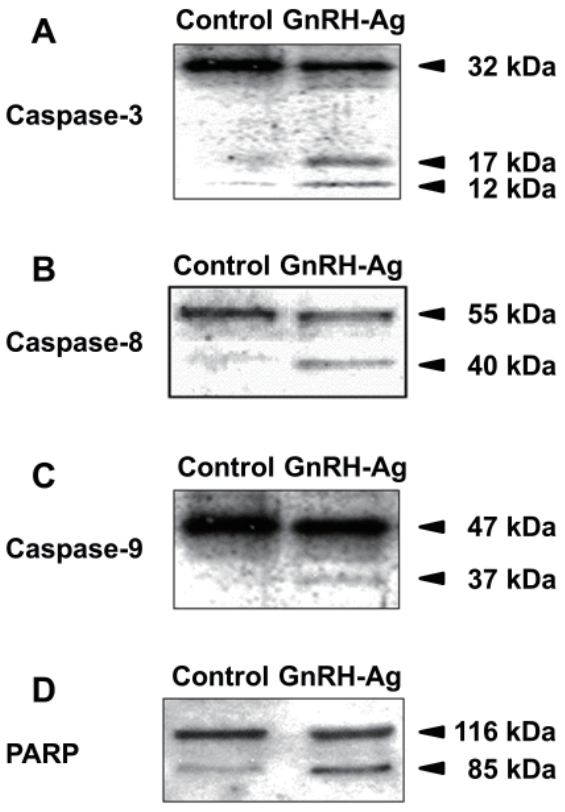


**Figure 5. Western blot analysis of caspase-8, -9, and -3 and PARP in cultured human granulosa-luteal cells after GnRH-Ag treatment.** Lysates from human granulosa-luteal cells treated with saline or GnRH-Ag were probed for caspase-8, -9, and -3 and PARP using anti-caspase-8, -9, and -3 and PARP antibodies. **(A)** A 32 kDa pro-form of caspase-3 was present in both lysates. A 17 kDa cleaved form of caspase-3 increased in the GnRH-Ag treatment. **(B)** A 55 kDa pro-form of caspase-8 was present in both lysates. A 40kDa cleaved form of caspase-8 increased in the GnRH-Ag treatment. **(C)** A 47 kDa pro-form of caspase-9 was present in both lysates. A 37 kDa cleaved form of caspase-9 increased in the GnRH-Ag treatment. **(D)** A 116 kDa pro-form of PARP was present in both lysates. A 86 kDa cleaved form of PARP increased in the GnRH-Ag treatment.

## 4. Discussion

GnRH has been shown to control the function of the ovary by direct action, as well as indirect action through the pituitary gland (*2*,*3*). Generally, the direct effect of GnRH has been shown to inhibit and promote ovarian function simultaneously; this GnRH effect on the ovary has been reported to act directly on the ovary through the GnRH receptor, which exists in ovarian granulosa-luteal cells (*22*).

It has been shown that GnRH may inhibit follicular growth and steroidogenesis in the ovary (*1*). It has also been reported that GnRH affects the cellular physiologic changes in the ovary, and oocyte growth and ovulation (*2*,*3*). This GnRH effect on ovarian function is associated with apoptosis in the ovary (*7*,*8*), but the mechanism has not yet been defined. To ascertain apoptosis in the ovary after treatment of granulosa-luteal cells with GnRH-Ag, we studied apoptosis and the associated protein changes.

After demonstrating induction of DNA fragmentation in cultured human granulosa-luteal cells by GnRH-Ag in a dose-dependent manner, DNA was extracted, and electrophoresis was performed. We confirmed DNA fragmentation increased as the concentration of GnRH-Ag in the cultured human granulosa-luteal cells increased. DNA fragmentation is a phenomenon specifically expressed in apoptotic cells and has been used as an indicator of apoptosis (*23*). DNA fragmentation in the cultured human granulosa-luteal cells increased at a GnRH-Ag concentration > $10^{-6}$ M (Figure 1). On the basis of this result, GnRH-Ag at a concentration of $10^{-6}$ M GnRH-Ag was used in each experimental group.

This dosage of $10^{-6}$ M GnRH-Ag that induced apoptosis is the physiological concentration. In recent research, it has been reported that GnRH-Ag is related to comparative effects on the proliferation, apoptosis, and differentiated function of cultured porcine granulosa cells from varying follicular stages (*24*). One downregulates granulosa cell proliferation in immature follicles as well as steroidogenesis in mature follicles, and the other upregulates apoptosis of granulosa cells regardless of the stage of follicular growth. Also, it has been reported that GnRH agonist increases in a dose dependent manner in the incidence of apoptotic human luteinized granulosa cells. This research suggests that clinical use of GnRH-Ag in IVF and embryo transfer (IVF-ET) should perhaps be reconsidered in the context of its apoptosis-inducing effect (*3*).

During apoptosis, nuclear changes, such as nuclear fragmentation and chromatin condensation with DNA fragmentation is known to accompany apoptosis (*25*).

Also, cellular morphologic changes following apoptosis are a phenomenon in which the cell shrinks and is split into some fragments, thus forming an apoptotic body with chromatin condensation. This apoptotic body was confirmed in our experiments, and TUNEL staining showed that cells with apoptotic bodies were stained.

The mechanism of intracellular apoptosis of the ovary by GnRH has been postulated to be induced by the pathway of protein kinase C through GnRH, thus increasing the amount of intracellular calcium and changing phosphatidylinositol (*26*,*27*). Recently, when the GnRH, buserelin, was given to white rats, it was reported to decrease the expression of inducible nitric oxide synthase (iNOS) mRNA and promote cytostasis in ovarian follicular development (*28*). Also, it has been reported that apoptosis is directly suppressed after *S*-nitroso-*N*-acetyl-D,L-penicillamine, a NO inducible product, was used to treat cultured granulosa cells (*29*). Apoptosis was suppressed after sodium nitroprusside, another NO inducible product, was used to treat rat ovary (*30*). These results suggested that an increase in apoptosis of granulosa-luteal cells by GnRH-Ag may be induced by decreasing the intracellular NO concentration, but the signal transduction mechanism has not been clearly elucidated.

Recently, the understanding of apoptosis has increased, and a variety of intracellular signal transduction materials have been implicated. It has been reported that interleukin-1b converting enzyme (ICE) family cystein protease among apoptosis signal transduction materials plays an important role in apoptosis (*31*). Fifteen additional caspases have been investigated in several organisms (*32*,*33*). Generally, caspases exist within a cell in inactive forms and become active through break-down by proteolytic enzymes. Caspase-8, -9, and -3 are among these caspases that play a central role in apoptosis.

Caspase-8 is activated in response to cellular apoptosis induction materials, such as TNF-α or Fas ligand, and becomes active in a complex associated with cytoplasmic death receptor (*34*,*35*). Caspase-9 is activated by material inducing extraction of cytochrome c from mitochondria, and requires dATP, apoptotic protease activating factor 1 (APAF-1), and connection with extracted cytochrome c for activation (*36*). Caspase-3 may expand the signals of caspase-8 and caspase-9 to result in apoptosis (*37*,*38*). We do not fully understand which role each caspase plays in the apoptosis process, but it has been shown that owing to massive research on caspase-3, that this enzyme dissolves substrates, such as PARP (*39*,*40*), inhibitor of caspase activated deoxyribonuclease (ICAD) (*41*), and gelsolin (*42*). These materials are needed to compose the intracellular skeleton, or preserve intranuclear DNA. If these materials are dissolved or activated, they induce apoptosis through cellular condensation and DNA fragmentation.

We have demonstrated that the activities of caspase-8, -9, and -3 increase within granulosa-luteal cells with nuclear condensation, a characteristic of apoptosis. We confirmed the protein of activated caspase-8, -9, and -3, by Western blot , increased during apoptosis of cultured human granulosa-luteal cells by treatment with GnRH-Ag.

As a result of our experiments, the expression of proteins of activated caspase -8, -9, and -3 increased. Similarly, activated PARP protein increased in the group of cells treated with GnRH-Ag. Taken together, we concluded that the mechanism of apoptosis of human granulosa-luteal cells involves signal transduction induced by activation of caspase-8, -9, and -3, and finally activation of PARP, thus inducing intranuclear DNA fragmentation.

Considering the function of each caspase-8, -9, and -3 as previously mentioned, we expected that sequential activation of caspase-8, -9, and -3 and cleavage of PARP may induce the apoptosis of human granulosa-luteal cells by GnRH-Ag, which warrants further investigation.

Our results are important data showing the direct induction of apoptosis in the cells of the ovary by GnRH-Ag. The apoptosis signaling transduction pathway by GnRH, delineated in part, will promote a better understanding of gynecologic endocrine disease. Through our research on GnRH-Ag used in IVF-ET procedures which directly affect apoptosis of human granulosa-luteal cells in the ovary as well as downregulation through the pituitary gland, we hope to obtain the basic data needed to develop new ovarian hyperstimulation regimens.

Moreover, our *in vitro* experimental results suggest potential mechanisms responsible for follicular atresia through regulation of apoptosis of granulosa cells by GnRH, which warrants further investigation.

**Acknowledgements**

We would like to thank the great help of the Life Science Institute and Infertility Clinics at Eulji Medical Center of Eulji University. Also, the authors appreciate the instruction of professor Byung Moon Kang of Asan Medical Center of Ulsan University.

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*(Received February 22, 2011; Revised April 18, 2011; Accepted April 26, 2011)*