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Short Communication

Evaluation of Mn^{2+} Stimulated and Zn^{2+} Inhibited Apoptosis in Rat Corpus Luteal Cells by Flow Cytometry and Fluorochromes Staining

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Abstract

We had reported that Mn^{2+} is the most effective divalent ion in stimulating apoptosis and Zn^{2+} inhibites apoptosis in corpus luteal (CL) cells of rat, which evidenced by the appearance of internucleosoma DNA fragmentation in electrophoretic gel. To further understand morphological character of apoptosis undergone in CL cells, we performed fluorochromes staining and flow cytometric studies on Mn^{2+} and Zn^{2+} treated CL cells. Cells dispersed by collegenase-DNase from PMSG-hCG primed pseudopregnant rats were incubated in KRBGAL solution for 4 h with 5 mM Mn^{2+} or Zn^{2+}. Staining of nuclei with the DNA binding fluorescent dyes, acridine orange-ethidium bromide (AO-EB), showed an increased green fluorescence of AO in Mn^{2+} treated cells as compared with control or Zn^{2+} group. DNA content analysis showed that Mn^{2+} caused an absolute shift from right to left side in the scatter profile by Flow Cytometry. The consistency of above results supported that Mn^{2+} is the most effective divalent ions in stimulating apoptosis in CL cells of rat, and provided a morphological charactor of apoptosis undergo in CL cells. The results also suggest that fluorochromes staining and flow cytometry are effective and convenient methods in study of apoptosis in CL cells.

Key Words: apoptosis, Mn^{2+}, corpus luteal cells, flow cytometry, fluorochromes staining

Introduction

The methods of flow cytometry and fluorochromes staining were widely applied to study of apoptosis triggered in the human leukemic HL-60 cell line and in rat thymocytes (1-4). To our knowledge, it has not been reported that these two methods were applied in the study of apoptosis in corpus luteal cells.

We have reported that Mn^{2+} significantly stimulated the occurrence of internucleosoma DNA fragmentation by gel electrophoresis in both cultured corpus luteal cells and nuclei incubation experiments; on the other hand, Zn^{2+} inhibited the spontaneous occurrence of apoptosis (5, 6). The results that Mn^{2+} is the most effective divalent ion in stimulating apoptosis and Zn^{2+} is much effective in inhibiting apoptosis in CL cells of rats were evidenced by the appearance of internucleosoma DNA fragmentation in electrophoretic gel. To further understand the morphological character of apoptosis in rat CL cells and ascertain the actions of Mn^{2+} and Zn^{2+} on apoptosis of CL cells, we re-evaluation of Mn^{2+} stimulated and Zn^{2+} inhibited apoptosis in rat corpus luteal cells by

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application of the methods of flow cytometry and fluorochromes staining. We also observed the effects of human inhibin α N-terminal Tyr-1-32 fragment (P^{33}) on apoptosis which has been demonstrated that could strongly inhibited progesterone secretion by cultured corpus luteal cells of rat (7, 8).

Materials and Methods

Regents and Materials

Ethidium bromide (EB), acridine orange (AO), propidium iodide (PI), RNase A, collagenase-II, bovine pancreatic DNase-I, and hCG were purchased from Sigma Chemical Company. $MnCl_2$, $ZnCl_2$ were purchased from Beijing No.1 Reagent Plant. Human inhibin α subunit N-terminal fragment (Tyr)-1-32 (P^{33}) was synthesized by Ying Sun and Professor Gui-Cheng Lu, Institute of National Materia Medica, Academia Sinica, Beijing.

Animal and Tissue Collection

Immature (25-28 day old) female Sprague Dawley rats (Animal Center of Institute of National Family Planing, Beijing) were given subcutaneously 65 IU of Pregnant Mares Serum Gonadotropin (PMSG, Tianjun Biological Products Institute), followed by another subcutaneous injection of 50 IU hCG 48 hr later. The animals were maintained under controlled conditions of temperature (25°C) and lighting (lights on 0600-1800 h) and provided with food and water ad *libitum*.

CL Cell Dispersion and Incubation

Seven days after hCG injection, the rats were sacrificed by decapitation and the ovaries were excised and cleaned of surrounding connective tissue. Corpus luteal cells were obtained by the method as described previously (7, 8). Briefly, tissues from the animals ovary were subjected to digestion with Ca^{2+} and Mg^{2+} free KRBG solution containing collagenase-II, DNase-I saturated with 95%O_2 and 5% CO_2, and were incubated for 1 h at 37°C. After incubation, the contents were filtered through nylon mesh, dispersed cells were washed by centrifugation (250 × g; 5 min) and resuspended in 1 ~ 5 ml KRBGAL solution, counted in a hemocytometer, the viability was about 90% using trypan blue test. The concentration of luteal cells for incubation was 10^6 cells per ml. The cell suspension was distributed into 50 ml culture tubes (13 × 10^6 cells/tube) and a period of 60 min incubation at 37°C at an atmosphere of 95%O_2-5% CO_2 was allowed in order to stabilize the culture condition, followed by incubation for another 4 h with tubes contained either a blank consisting of medium alone, or zinc chloride 5 mM, P^{33} 1 μg /ml or manganese chloride 5 mM.

All incubations were replicated three times with three separated tissue preparations (n=10). After incubation, cells were then collected by centrifugation for flow cytometry and fluorochromes staining analysis, respectively.

Fluorescent Microscopic Examination of Nuclei Staining

The effects of Mn^{2+}, Zn^{2+} and P^{33} on apoptosis were determined morphologically by fluorescent microscopy after labeling with acrodine orange and ethidium bromide as described by Piazza G. A (9). Cells were collected and washed 3 times in PBS. One ml aliquots of 1 × 10^6 cells were centrifuged (300 × g). The pellet was resuspended in 25 μl of media and 1 μl of dye mixture containing 100 μg/ml of acridine orange and 100 μg /ml of ethidium bromide prepared in PBS and mixed gently. Ten μl of mixture was placed on a microscope slide and covered with a 22-mm^2 coverslip and examined under 40 × dry objectives with the use of epillumination and filter combination. Live and dead apoptotic cells were identified by nuclear condensation of chromatin stained by the acridine orange or ethidium bromide, respectively. Necrotic cells were identified by uniform labeling of the cells with ethidium bromide.

Determination of Apoptosis by Flow Cytometric Examination of DNA Content of PI-labeled Cells

Cells (5 × 10^5) were harvested immediately after 4 h in vitro culture, fixed in cold 70% ethanol. The fixed samples were kept at 4°C. Before analysis, the samples were centrifuged for 5 min at 200 × g. The supernatant was discarded, and the samples were washed twice with PBS, then permeabilized with 0.1% Triton-X-100, treated with 5 μg /ml RNase, and incubated at 37°C for 15 min before staining with 50 μg/ml PI for 60 min at 4°C, and then passed through the FACSTAR for analysis (10). Parameter : FL2, total events : 5000, gated events : 5000.

Results

Fluorescent Microscopic Examination of Nuclei Staining

Figure 1 depicts the nuclei staining feature after 4 h incubation of corpus luteal cells in the presence of Zn^{2+}, Mn^{2+} and P^{33}, respectively. Obvious morphological differences were observed between Mn^{2+} group and control or Mn^{2+} group and Zn^{2+} treated cells.

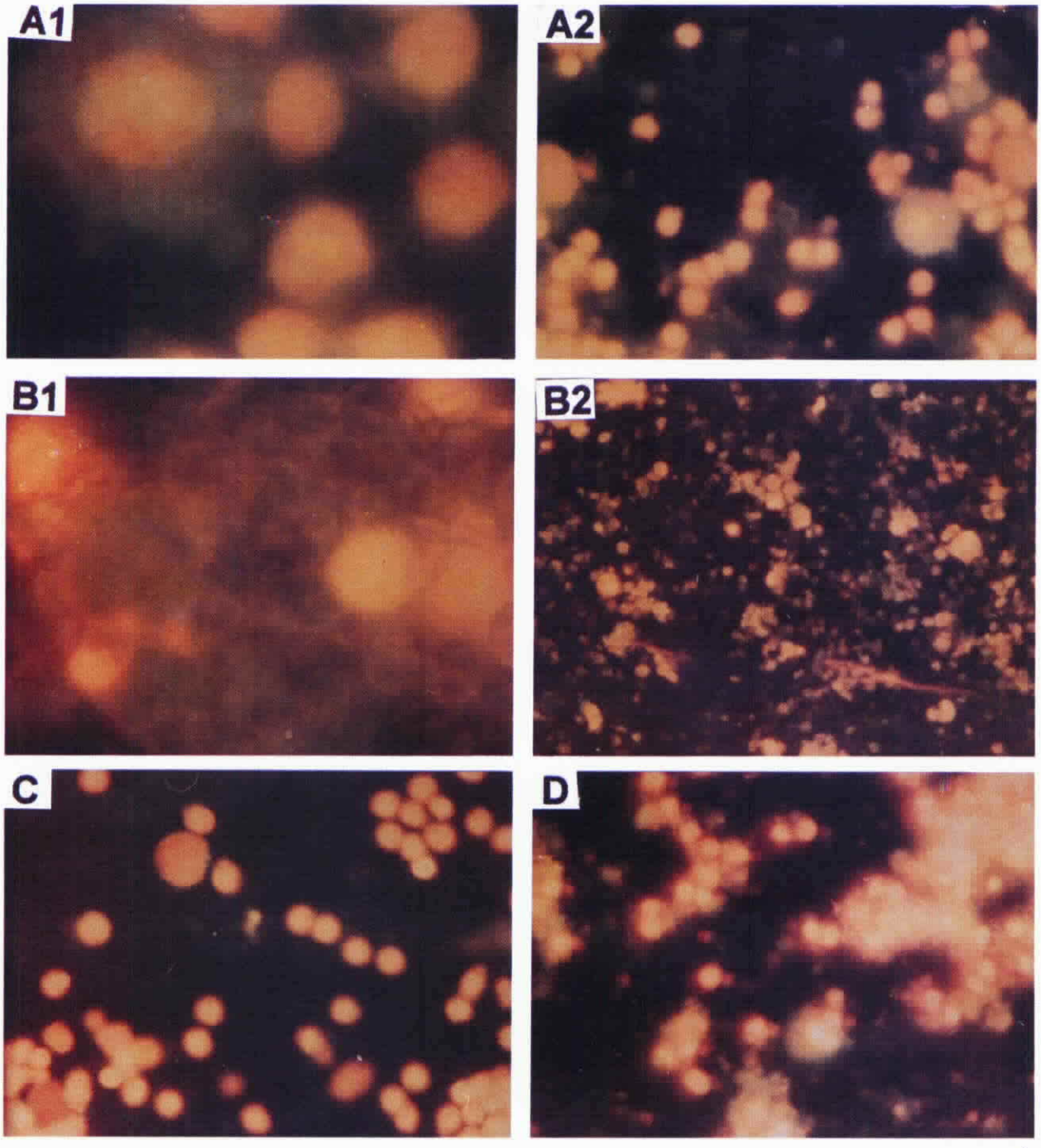

Fig. 1. Feature of acridine orange/ethidium bromide staining rat corpus luteal cells. After 4 hr incubation of corpus luteal cells in the presence of Zn^{2+}, Mn^{2+}, P^{33} respectively, the cells were collected and stain with AO-EB and observed with fluorescence microscopy (Magnification, A: control, A1 400×, A2 100×; B: Mn^{2+}, B1 400×, B2 100×; C: Zn^{2+} × 100, D: P^{33} × 100). Live and dead apoptotic cells were identified by nuclear condensation of chromatin stained by the acridine orange (green fluorescence) or ethidium bromide (red fluorescence), respectively. Necrotic cells were identified by uniform labeling of the cells with ethidium bromide (red fluorescence). AO intercalates into double stranded DNA fluoresces green. The green fluorescence in B and D indicated that DNA double-strand breaks (apoptotic bodies). Normal nuclei fluoresce bright yellow (A and C).

Obviously increased green fluorescence in Mn^{2+} treated corpus luteal cells was observed when compared to control or Zn^{2+} groups. The amount of green fluorescence in P^{33} group is less than that in Mn^{2+} group, but more than that in control.

Determination of Apoptosis by Flow Cytometric Examination of DNA Content of PI-labeled Cells

Figure 2 A and B depict the flow cytometric feature of cells treated with Zn^{2+}, Mn^{2+} or P^{33}. DNA

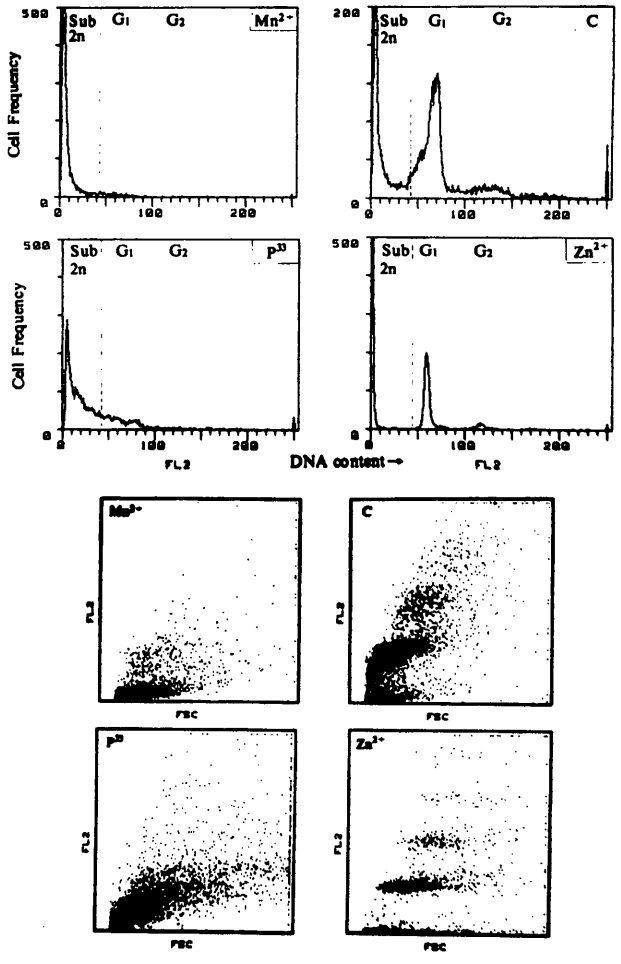

Fig. 2. Size distribution of DNA from cultured rat corpus luteal cells. Corpus luteal cells obtained from rat ovaries after treatment with Zn^{2+}, Mn^{2+} and P^{33} respectively in KRBGAL solution (contain 5 mM Ca^{2+} and 5 mM Mg^{2+}) for 4 h and then collected for Flow Cytometry. The profiles were generated by accumulating the passage of 5,000 events through the flow cytometer. **A**: The *ordinate* indicates the number of cells in each size category; the *abscissa* is an arbitrary scale of cell size. **B**: The dotplot histograms show the dotplots of total cells. Each dot represents a single event. Representative profiles from a single experiment are shown. Similar profiles were obtained for three additional experiments.

content in cells of Mn^{2+} group was shift from large to small (from right to left side of the figure) absolutely. Quantification of the subdiploid apoptotic fraction by flow cytometry showed the increase in degradation of DNA in Mn^{2+} and P^{33} groups. Flow cytometer dotplot histograms showed that the forward scatters in P^{33} and Mn^{2+} treated cells were less than untreated or Zn^{2+} treated cells.

Discussion

As only a little work has been done in vitro about apoptosis in corpus luteum (11), the precise role of apoptosis in luteal regression remains to be established. Although recent biochemical evidences have demonstrated the occurrence of apoptotic DNA fragmentation during luteal regression in cattle (12), rabbit (13) and ovine (14) in vivo, the evidence of apoptosis in rat corpus luteal cells had not been well demonstrated. So, in this work, the first and important step is to trial to get stronger evidences of apoptosis in rat corpus luteal cells.

The methods of flow cytometry and fluorochromes staining were widely applied to the study of apoptosis triggered in the human leukemic HL-60 cell line and in rat thymocytes (1-4). We have not seen the reports about the application of these two methods in the study of apoptosis in corpus luteal cells by now.

According to the biological feature of apoptosis, gel electrophoretic analysis had been widely used to reveal the evidence of apoptosis. Our prevous work have analyzed low mol wt DNA from cultured rat CL cells by gel electrophoresis for detection of oligonucleosomal DNA fragmentation (biological feature), and demonstrated that typical ladder of DNA in Mn^{2+} treated CL cells. In this work, fluorescent microscopic examination of nuclei staining and flow cytometric examination of DNA content of PI-labeled cells were conducted for morphological evidences.

As background, necrosis and apoptosis are two types of cell death that display different morphological and functional characteristics. Necrosis involves the disruption of plasma membrane integrity, whereas apoptosis is considered to be a physiological process where plasma membrane integrity is maintained. To reveal the morphologic feature of Mn^{2+} induced apoptosis and /or necrosis, we used the acridine orange/ ethidium bromide assay, which allowed for simultaneous measurement of both types of cell death based on chromatin structure, as well as membrane permeability (9). The metachromatic dye AO can differentially stain double stranded and denatured DNA in cells or cell nuclei. The dye intercalates into double stranded DNA and, when bound in this form, fluoresces green. AO can also condense the denatured sections of DNA and in the condensed form AO luminesces red (15). Fluorescent microscopic examination of nuclei staining showed that obviously increased green fluorescence in Mn^{2+} and P^{33} treated corpus luteal cells as compared with control or Zn^{2+} groups. The green fluorescence in Mn^{2+} and P^{33} treated cells indicated that DNA double-strand breaks occur and the double-strand fragmentation of DNA must be membrane-bound fragments (deduced from no EB staining), which corresponds to apoptotic body, a typical morphological feature in the late stage of apoptosis.

Examination of the subdiploid apoptotic fraction by flow cytometry clearly showed the increases in

degradation of DNA in Mn^{2+} and P^{33} groups. Our previous work have shown that the DNA extracted from corpus luteal cells of Mn^{2+} treated group displayed the typical ladder-like DNA fragmentation profile, indicative of apoptosis (5, 6). Hence, the shift of DNA content in CL cells from large to small after Mn^{2+} and P^{33} treatment could be due to CL cell apoptosis and the development of apoptotic body.

Above discussion suggested that our previous results obtained by visual observation of DNA fragmentation in gel electrophosesis were further confirmed by flow cytometry and fluorescent microscopic examination. A consistent feature from the different methods in our experiments was that the large proportion of corpus luteal cells from Mn^{2+} and P^{33} treated groups displayed lower DNA content than control (i.e. hypochromicity). All of these indicate the cell death is via apoptosis.

Inhibin is composed of an α-subunit and one of two β-subunits (βA or βB). It is a glycoprotein hormone produced by the gonads that feeds back to the pituitary gland to selectively inhibit release of FSH. While , recently increasing evidences raised intriguing possibilities about the role of inhibin α subunit or its precursor as a local regulator in the pituitary-gonadal axis: the secretion of free α subunit by rat granulosa cells (16), the considerable excess expression of α-inhibin mRNA in the ovary of rat (17), the conduction of gonadal tumors in mice by deletion of the α-inhibin gene (18) etc. These all suggest that the α-inhibin is a physiologically relevant protein, independent of bioactive inhibin dimer. To ascertain this hypothesis, we synthesized four inhibin α subunit fragments and observed their effects on ovarian function, and demonstrated that P^{33} was most effective in inhibition progesterone production in cultured rat corpus luteal cells (7). In the present study, we further demonstrated the inhibitory effect of P^{33} on luteal apoptosis, and the degree of apoptosis in observed four groups was $Mn^{2+}>P^{33}>C>Zn^{2+}$ and Mn^{2+} is a good positive control for the study of apoptosis in corpus luteal cells.

In summary, the applications of both flow cytometry and fluorescent microscopic examination for quantification the population of corpus luteal cells and characterization (observation) of the difference within sample aliquots provided rapid, repeatable information. These methods are suitable not only for the study of apoptosis triggered in the human leukemic HL-60 cell line and rat thymocytes, but also for the research of molecular and biochemical mechanisms of apoptosis in corpus luteal cells. And Mn^{2+} and Zn^{2+} is a good pair of positive and negative control groups for the study of apoptosis in corpus luteal cells.

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References

1. Compton, M.M., J.S. Haskill, and J.A Cidlowski. Analysis of glucocorticoid actions on rat thymocyte decoxyribonucleic acid by fluorescence-activated flow cytometry. *Endocrinology* 122: 2158-2164, 1988.
2. Bruno, S., P. Lassota, W. Giaretti, and Z. Darzynkiewicz. Apoptosis of rat thymocytes by prednisolone, camptothecin or teniposide is selective to G0 cells and is prevented by inhibitors of proteases. *Oncology Res.* 4: 29-35, 1992.
3. Del Bino, G., J.S.Skierski, and Z.Darzynkiewicz. The concentration- dependent diversity of effects of DNA topoisomerase I and II inhibitors on the cell cycle of HL-60 cells. *Exp. Cell Res.* 195: 485-491, 1991.
4. Hotz, M.A., G. Del Bino, P. Lassota, F. Traganos, and Z. Darzynkiewicz. Cytostatic and cytotoxic effects of fostriecin on human promyclocytic HL-60 cell and lymphocytic MOLT-4 cells. Cancer Res. 52: 1530-1535, 1992.
5. Zhang, J.H., X.D. Zhao, R.M. Liu, W.X. Li, and C.P. Cheng. Stimulation by Mn^{2+}, Ca^{2+}, Mg^{2+} and Inhibition by Zn^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} Ions of Nuclease Responsible for DNA Fragmentation in Rat Corpus Luteal Cells (Abstract). The 3rd International Conference on Reproductive Endocrinology, Beijing, China, 1997, AB-18.
6. Zhang, J.H., W.X. Li, Y.W. Wu, S.D. Zong, and C.P. Cheng., Apoptosis in Rat Luteal Tissue Associated with Luteolysis: in vivi and in vitro Analysis. 97-International Symposium for Yong Chinese Reproductive Biologists, Beijing, China, 1997, p. 74.
7. Zhang, J.H., Qi-Fa Wang and Chi-Ping Cheng. The effect of 4 synthetic human inhibin alpha fragments on progesterone production by rat corpus luteal cells and its the relation to protein kinase A system. *J. Repro. Med.* 5:25-29,1996.
8. Zhang, J.H., J.Ni, C.P.Cheng, Y.Sun, and G.C.Lu. Effect of human inhibin ñf ragments and monoaminergic neurotranamitters on progesterone (P_4) production by corpus luteum (CL) of rat in vitro. *Acta Phisiologica Sinic*a 48: 284-292, 1996.
9. Piazza, G.A., A. L. Kulchak Rahm, M. Krutzsch, et al. Antineoplastic drugs sulindac sulfide and sulfone inhibit cell growth by inducing apoptosis. *Cancer Res.* 55: 3110-3116, 1995.
10. Belloc, F., P. Bumain, M. R. Boisseau, et al. A flow cytometric method using hoechst 33342 and propidium iodide for simultaneous cell cycle analysis and apoptosis determination in unfixed cells. *Cytometry* 17: 59-65, 1994.
11. Kenny, N, R.E. Williams and L.B. Kelm. Spontaneous apoptosis of cells prepared from the nonregressing corpus luteum. *Biochem. Cell Biol.* 72: 531-536, 1994.
12. Juengel, J.L., H.A.Garrerick, A.L. Johnson, R.S. Youngquist, and M.F. Smith. Apoptosis during luteal regression in cattle. *Endocrinology* 132: 249-254,1993.
13. Nicosia, S.V., J. Diaz, R.F. Nicosia, B.O. Saunders, and C. Muro-Cacho. Cell proliferation and apoptosis during development and aging of the rabbit corpus luteum. *Ann. Clin. Lab. Sci.*; 25: 143-157, 1995.
14. Rueda, B.R., J.A. Weger, S.L. Marion, D.D. Wahlen, and P.B. Hoyer. Internucleosomal DNA fragmentation in ovine luteal tis-

sues associated with luteolysis: in vivo and in vitro analyses. *Biol. Reprod.* 52: 305-312, 1995.

15. Darzynkiewicz, Z., S. Bruno, G.D. Bino, W. Gorczyca, M.A. Hotz, P. Lassota, and F. Traganos. Feature of apoptotic cells meatured by flow cytometry. *Cytometry* 13: 795-808, 1992.
16. Bicsak, T.A., Cajander, S.B., Vale, W., Hsueh, A.J.W., Inhibin studies of stored and secreted forms by biosynthetic labelling and immunodetection in cultured rat granulosa cells, *Endocrinology*, 122 (1988) 741-748.
17. Matzuk, M.M., Finegold, M.J., Su J-G.J., Hseuh, A.J.W., Bradley, A., α Inhibin is a tumor-suppressor gene with gonadal specificity in mice, *Nature*, 360 (1992) 313-319.
18. Meunier, H., Rivier, C., Ecans, R.M., Vale, W., Gonadal and extragonadal expression of inhibin α, βA, and βB subunits in various tissues predicts diverse functions, *Proc. Natl. Acad. Sci. USA*, 85 (1988) 247-251.