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Luteolytic effects of DL111-IT in pregnant rats

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Abstract

The present studies were conducted to evaluate the effects of DL111-IT [3-(2-ethyl phenyl)-5-(3-methoxy phenyl)-1*H*-1, 2, 4 triazol] on ovaries of pregnant rats. Pregnant rats were i.m. treated with DL111-IT 2.5 mg kg^{-1} day^{-1} or camellia oleum (vehicle control) 0.2 ml/day from day 6 of pregnancy for 1, 3 or 5 days. Blood and ovaries were collected 24 h after the last injection. Ovarian fresh weight and protein contents, activities of the 3β-hydroxysteroid dehydrogenase (3β-HSD) and 20α-hydroxysteroid dehydrogenase (20α-HSD) in ovaries, and cell apoptosis of corpus luteum (including hematoxylin–eosine stain, in situ 3′-end labeling and nucleosomal banding) were estimated. Compared with that in the control group, ovarian fresh weight declined 11% and 22% after DL111-IT-3 days and -5 days; protein content dropped 29% after 5-day administration. DL111-IT for 3 days provoked a marked decrease of serum progesterone, by 31% of the control; the activity of 3β-HSD decreased 34.4% after i.m. DL111-IT for 5 days, while that of 20α-HSD increased dramatically after only one injection of DL111-IT ($P < 0.01$). Histological analysis and in situ 3′-end DNA labeling indicated that DL111-IT induced the pyknosis of cells and the formations of apoptotic bodies and intense oligonucleosomes in luteal cells of pregnant rats. The cell apoptosis induced by DL111-IT was further confirmed by evaluation of nucleosomal DNA fragmentation by agarose gel electrophoresis in cultured luteal cells exposed to DL111-IT for 24 h. In conclusion, all results, including shrunken luteal cells, decreased concentration of protein content and serum progesterone, changed activities of 3β-HSD and 20α-HSD and formation of DNA fragments in luteal cells, showed the luteolytic effect of DL111-IT in pregnant rats.

Keywords: DL111-IT; Luteal cell; Apoptosis; Luteolysis; 3β-HSD (3β-hydroxysteroid dehydrogenase); 20α-HSD (20α-hydroxysteroid dehydrogenase)

1. Introduction

Corpus luteum is one of the fastest growing tissues in the adult female and is one of the few mature tissues that exhibit periodic growth and regression (Jablonka et al., 1993; Reynolds et al., 1994). When pregnancy occurs, human chorionic gonadotropin secreted by the placenta maintains the structure and physiological function of the corpus luteum. Corpus luteum produces and secretes progesterone, which is of great importance during the whole normal pregnancy. Progesterone stimulates the decidualization and mitotic activity of endometrium to establish appropriate surroundings for normal growth and development of fertilized eggs. It has been proposed (Kuhn and Briley, 1970) that serum progesterone concentration is related to two enzymes in ovaries: 3β-hydroxysteroid dehydrogenase (3β-HSD) which is responsible for the conversion of pregnenolone to progesterone, and 20α-hydroxysteroid dehydrogenase (20α-HSD) which converts progesterone into 20α-dihydroprogesterone. If pregnancy does not occur, functional and structural regression of the corpus luteum will start spontaneously. Functional regression includes a decrease of progesterone secretion, while the structural regression involves a decline of cell count and size, and an increase in DNA fragmentation (oligonucleosomes), which is the hallmark of apoptosis and indicates that the regressing corpus luteum is undergoing apoptosis (Juengel et al., 1993).

DL111-IT [3-(2-ethyl phenyl)-5-(3-methoxy phenyl)-1*H*-1, 2, 4 triazol], a nonhormonal compound, has high contragestational activity in rodents, dogs and primates (Galliani et al., 1981; Galliani et al., 1982; Galliani et al., 1986; Yang and Fang, 1996). Histological examination revealed that the targets of its action were the ectoplacenta and decidual cells (Galliani et al., 1986). It also triggered pregnancy arrest by interfering with the chain of events by which progesterone regulates the mitotic activity of decid-

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ual cells and trophoblastic cells (Galliani et al., 1986). So, if DL111-IT changes the progesterone level, it will play a key role in the termination of early pregnancy. The aims of the present study, therefore, were to assess the effects of DL111-IT on the structure and function of corpus luteum in pregnant rats. The observations included: (1) fresh weight, protein content of ovaries and activities of 3β-HSD and 20α-HSD in ovaries, and serum progesterone content; (2) luteal cell degeneration (by morphologic examination); (3) apoptosis of luteal cells (by detection of nucleosomal fragmentation of DNA by agarose gel electrophoresis and also by 3′ end labeling in tissue sections).

2. Material and methods

2.1. Drugs and reagents

DL111-IT and camellia oleum (vehicle for injection) were manufactured by Zhengjiang Xianju Pharmaceutical

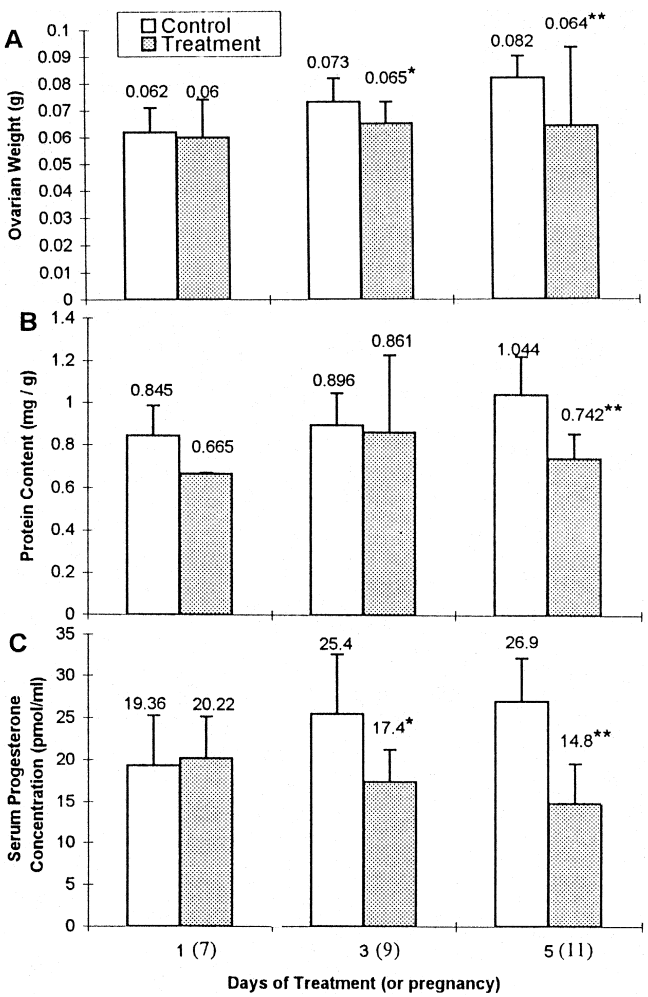

Fig. 1. Effects of DL111-IT on ovarian fresh weight (A), protein content (B) and serum progesterone concentration (C) of pregnant rats. Rats (from day 6 of pregnancy) i.m. DL111-IT 2.5 mg kg^{-1} day^{-1} or camellia oleum (vehicle control) 0.2 ml/day for 1, 3 or 5 days. $n = 6 \sim 8$, $\bar{x} \pm s$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

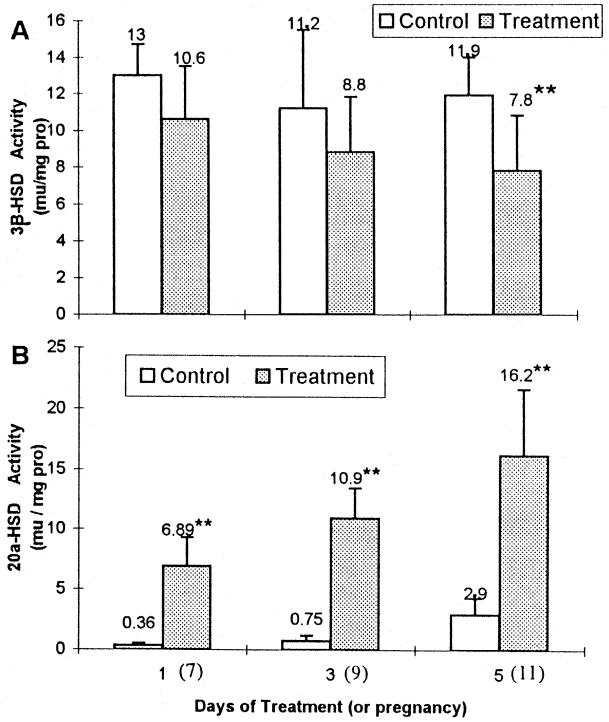

Fig. 2. Changes in the activity of 3β-HSD (A) and 20α-HSD (B) of pregnant rats. Rats (from day 6 of pregnancy) i.m. DL111-IT 2.5 mg kg^{-1} day^{-1} or camellia oleum (vehicle control) 0.2 ml/day for 1, 3 or 5 days. $n = 8$, $\bar{x} \pm s$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

(Lot No 9506002). Collagenase (type II), pregnenolone, 20α-hydroxypregn-4-en-3-one, McCoy's 5A medium, $NADP^{+}$ and NAD^{+} were purchased from Sigma. Progesterone radioimmunoassay kits were obtained from the Research Center for RIA Reagents, Shanghai Institute of Biological Products. Programmed Cell Death Assay kits, PCR Marks, Proteinase K and DNase-free RNase were obtained from Sino-American Biotechnology.

2.2. Animals

Sprague–Dawley rats (♀, 210–250 g, $n = 150$; ♂, 300–340 g, $n = 20$; ♀, 22–25 days, $n = 40$), provided by the Shanghai Experimental Animal Center, were housed with a 14 h light–10 h dark period, 22°C, and 60% humidity. They were mated and pregnancy was timed from the occurrence of sperm in the vaginal smear (day 1). On day 6, pregnant rats were randomly divided into 6 groups: i.m. camellia oleum (vehicle control) 0.2 ml/day × 1 day (A), × 3 days (C), or × 5 days (E); i.m. DL111-IT 2.5 mg kg^{-1} day^{-1} × 1 day (B), × 3 days (D), or × 5 days (F).

Venous blood samples were taken from hearts 24 h after the last injection to determine serum progesterone. All rats were killed by decapitation 24 h after treatment. The ovaries were enucleated and weighed to measure 3β-HSD and 20α-HSD activities, or to undergo histological examination and apoptotic detection.

2.3. Protein concentration and activities of 3β-HSD and 20α-HSD

The ovaries were weighed and homogenized in 0.25 M sucrose at a concentration of 10 mg wet weight/ml at 0°C. The homogenates were centrifuged at 105,000 × *g* for 60 min. The supernatant fluids were used for the assay of 20α-HSD activity. The precipitate was re-homogenized and centrifuged at 800 × *g* for 5 min. The supernatant was used for the assay of 3β-HSD activity. The assay mixture of 3β-HSD contained 40 μmol glycine–NaOH (pH 9.4), 0.9 mg bovine serum albumin, 0.5 μmol NAD^+ and enzyme solution (0.05 ml). That of 20α-HSD contained 50 μmol Tris–HCl (pH 7.5), 0.9 mg bovine serum albumin, 0.5 μmol $NADP^+$, and enzyme solution (0.05 ml). The assay mixture was preincubated for 5 min at 37°C, and the reaction was started by the addition of the 3β-HSD or the 20α-HSD substrates in a final volume of 0.8 ml. The substrate for 3β-HSD was 0.005 mg pregnenolone (0.1 mg/ml in ethanol). That for 20α-HSD was 0.015 mg 20α-hydroxypregn-4-en-3-one (0.5 mg/ml in ethanol). The 3β-HSD or 20α-HSD activities were assayed spectrophotometrically in units/mg protein. The amount of enzyme producing 1 μmol NADH or NADHP was defined as 1 unit of enzyme activity (Telleria et al., 1995). The method of Lowry was used for the protein determination with bovine serum albumin as the standard.

2.4. Progesterone determination

Serum progesterone was measured using radioimmunoassay kits. The inter- and intra-assay coefficients of variation were 10% and 5%, respectively.

2.5. In vitro cell culture

Sprague–Dawley rats (22–25 days) were injected s.c. with pregnant mare serum gonadotropin 65 IU, and 65 h later, s.c. human chorionic gonadotropin 35 IU. The animals were killed by cervical dislocation on day 5 after s.c. human chorionic gonadotropin for collection of luteal cells and the ovaries were excised from the pregnant rats. The corpus luteum was dissected out under a microscope and seeded at a density of $2–3 \times 10^5$ cells/well in 0.5 ml McCoy's 5A medium supplemented with 10% neonatal bovine serum, benzylpenicillin potassium 25 IU/l and

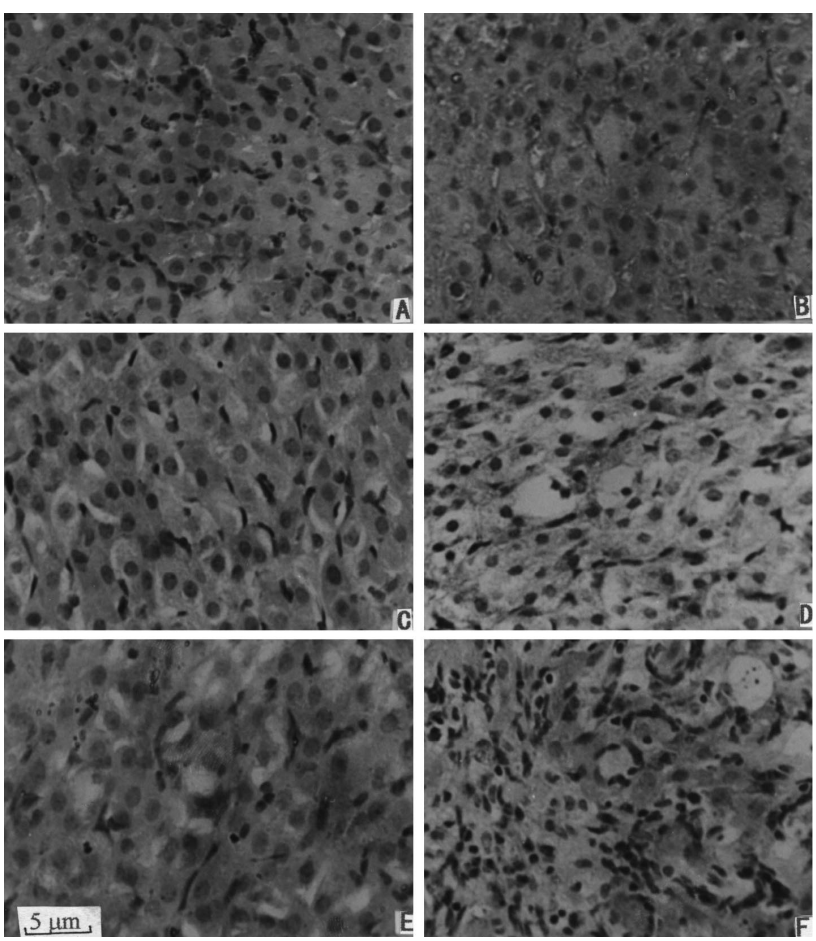

Fig. 3. Luteal cells of rats 24 h after i.m. camellia oleum (solvent control) 0.2 ml/day × 1 day (A), × 3 days (C), or × 5 days (E) and i.m. DL111-IT 2.5 mg kg^{-1} day^{-1} × 1 day (B), × 3 days (D) or × 5 days (F). No obvious morphological changes appeared in B; pyknosis appeared in D; apoptotic bodies appeared in F. Hematoxylin–eosine stain.

streptomycin 0.1 g/l (Wiltbank et al., 1989). After 24-h incubation, DL111-IT was added at a final concentration of 1.5, 3.0 or 6.0 mg/l. Four wells were used for each dosage. Besides, 4 wells received media only or vehicle (0.2% and 0.4% ethanol) as control groups. Cells were cultured at 37°C in 5% CO_2 for 24 h. At the end of each treatment, the cells were collected and stored in liquid nitrogen for nucleosomal banding assay. Cell viability was assessed by trypan blue dye exclusion.

2.6. Apoptosis assays

2.6.1. Histological examination and apoptosis in situ

Enucleated ovaries were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (5 μm), and mounted onto glass slides, then divided randomly into two groups. One was stained with hematoxylin-eosine and observed under light microscopy. The other group was treated with proteinase K 20 μg/ml for 15 min and treated with 0.3% H_2O_2 for 20 min. Sections then were incubated in buffer (30 mM Tris, 140 mM cacodylate, 1 mM cobalt chloride, pH 7.2) containing terminal deoxynucleotidyl transferase 0.5 u/μl and biotin-11-deoxyuridine triphosphate (dUTP, 5 μM) for 1 h at 37°C. Nuclei with fragmentation of DNA were visualized with 3′,3-diaminobenzidine 0.5 mg/ml containing H_2O_2 for 10 min (Jolly et al., 1997).

2.6.2. Nucleosomal banding

Genomic DNA was isolated from frozen luteal cells as described by Fraser with modifications (Fraser et al., 1995). Briefly, 0.3 ml PBS and 0.3 ml DNA isolated buffer (10 mM Tris–HCl pH 8.0, 0.1 M EDTA pH 8.0, 20 μg/ml DNase-free RNase 20 μg/ml, 0.5% sodium dodecyl sulfate) were added to each sample, and the sample then was incubated at 37°C for 1 h. After incubation, 3 μl proteinase K (20 mg/ml in 10 mM Tris–HCl and 5 mM EDTA pH 8.0) was added, and the mixture was incubated at 55°C overnight. Each sample then was extracted twice with 0.6 ml of ice-cold phenol (pH 8.0)/chloroform/isoamyl alcohol (25: 24: 1, v: v: v). The aqueous phase was recovered, and DNA was precipitated with −20° 100%

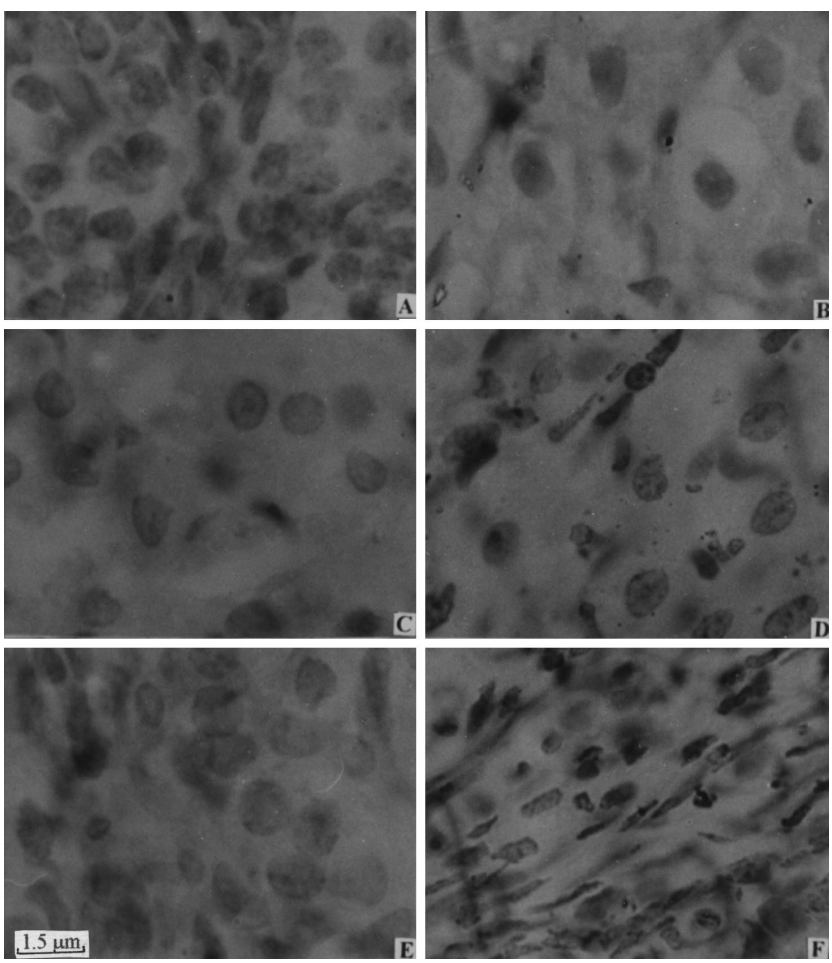

Fig. 4. In situ 3′ end-labeling of apoptotic luteal cells counterstained with hematoxylin. Groups i.m. camellia oleum 0.2 ml/day × 1 day(A), × 3 days (C) or × 5 days (E) and i.m. DL111-IT 2.5 mg kg^{-1} day^{-1} × 1 day (B) indicated apoptosis-negative nuclei. Groups i.m. DL111-IT 2.5 mg kg^{-1} day^{-1} × 3 days (D) and × 5 days (F) were undergoing severe apoptosis (brown coloration) with shrunken nuclei.

ethanol overnight. After two rinses with ice-cold 75% ethanol, each DNA sample was dried 15 min at room temperature, and then was dissolved in Tris–EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). DNA content was measured with a spectrophotometer (A260/A280) and only samples with a ratio ≥ 1.8 were used. Isolated DNA (2.0–4.0 μg/well) from each sample was electrophoretically separated by size in 2% agarose gels in Tris–acetic acid–EDTA (0.04 M Tris–acetic acid, 0.1 mM EDTA, pH 8.0) buffer at 40 v for 4 h. The gels were stained with ethidium bromide (0.5/ml) and photographed by UV transillumination. For each gel, a marker DNA ladder and HL 60 apoptotic cells (HL60 cells were exposed to camptothecin 1 μM for 4 h) were used to estimate the relative size of fragmented DNA and as the positive control.

2.7. Statistics

Results were expressed as means ± S.E.M. and the difference between treatment vs. control was evaluated with Student's *t*-test.

3. Results

3.1. Ovary weight and protein contents, and serum progesterone concentration

Ovary fresh weight and protein contents and serum progesterone concentration of control groups showed a remarkable and progressive rise with the increase in pregnancy days, while those of the treatment groups exhibited a continuing decrease. The values of ovary fresh weight did not change after i.m. DL111-IT-1 day. The ovarian weight with DL111-IT treatment for 3 days and 5 days dropped 11.0% ($P < 0.05$) and 78.0% ($P < 0.01$), respectively, when compared with that of corresponding control groups. A significant difference ($P < 0.01$) in protein contents only appeared after i.m. DL111-IT-5 days and it was 71.1% of control groups (Fig. 1A,B).

Serum progesterone concentration decreased 31.5% ($P < 0.05$) after i.m. DL111-IT-3 days and declined further ($P < 0.01$) after treatment with DL111-IT-5 days to reach 55.0% of that of control groups (Fig. 1C).

3.2. Luteal 3β-HSD and 20α-HSD activities

Treatment with DL111-IT-1 day did not modify 3β-HSD activity with respect to the control. 3β-HSD activity declined progressively after administration of DL111-IT for 3–5 days, and a 35.5% decrease appeared after i.m. DL111-IT-5 days (Fig. 2A).

A low activity of 20α-HSD was observed in all control groups. One injection of DL111-IT provoked a sharp increase in 20α-HSD activity by 19.1-fold ($P < 0.01$). With an increase in treatment days, 20α-HSD activity increased progressively and markedly (Fig. 2B).

3.3. Histological examination

Hematoxylin–eosine stain showed that treatment with DL111-IT for 1 day did not induce degeneration of luteal cells. Treatment with DL111-IT for 3 days and 5 days caused a low ratio of cytoplasm to nucleus, condensed nuclei with deep-stained in luteal cells, and enlarged intercellular space. Control groups exhibited larger luteal cells with circular and regular nuclei. Apoptotic bodies could been observed in 5-day treatment groups (Fig. 3).

3.4. Analysis of DNA for oligonucleosome formation

Examination of DNA with the sensitive 3′ end labeling technique confirmed the existence of oligonucleosome formation in apoptosis-positive cells of 3-day and 5-day treatment groups. The degeneration of cells with shrunken nuclei increased with increasing treatment days. The difference in staining intensity between control groups and the 1-day treatment group was negligible (Fig. 4). Consistent with these results, evaluation of genomic DNA, extracted from luteal cells cultured in vitro, to assess apoptosis using agarose gel electrophoresis revealed nucleosomal fragmentation of DNA in the treatment groups (luteal cells

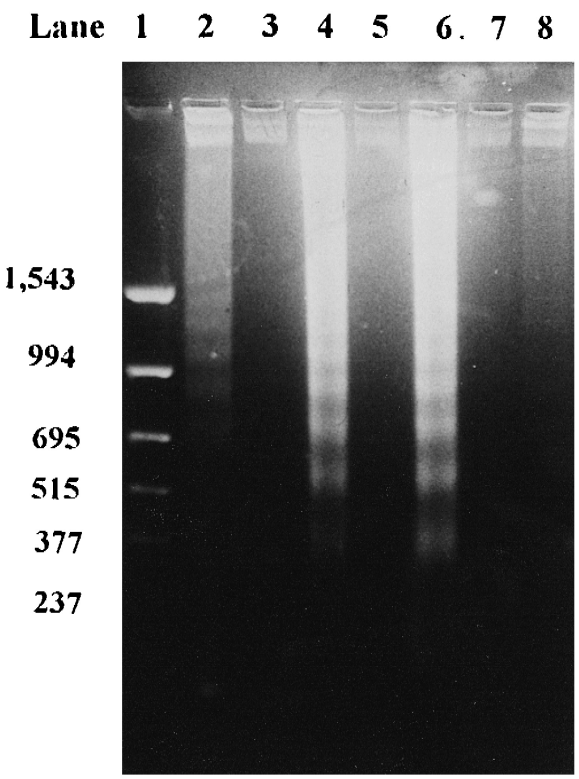

Fig. 5. Ethidium bromide staining of agarose gel (2%) electrophoresis of DNA isolated from cultured rat luteal cells incubated with DL111-IT 6.0 (lane 4), 3.0 (lane 6) and 1.5 (lane 8) mg/l for 24 h or without DL111-IT (lane 3: only media; lane 5: with 0.2% ethanol and lane 7: with 0.4% ethanol). DNA marks and positive control (HL60 cells were exposed to camptothecin 1 μM for 4 h) indicated on the left (lanes 1 and 2). DNA fragmentation was detectable in all treatment groups.

exposed to DL111-IT 1.5, 3.0 or 6.0 μg/ml for 24 h). Nucleosomal fragmentation was not detectable in the control groups, including media, 0.2% and 0.4% ethanol (vehicle) (Fig. 5).

4. Discussion

In the present study, pregnant rats treated with DL111-IT exhibited a rapid and progressive decrease in fresh weight and protein concentration of ovaries, and progesterone serum concentration. Decline of fresh weight and protein content are indexes of decrease of cell count or/and size, while the marked decrease in serum progesterone means the regression of steroid synthesis (Aheng et al., 1994). These data showed that DL111-IT had effects on morphology and function of corpus luteum. This interpretation was supported by the changes in 3β-HSD and 20α-HSD activities. These two enzymes are located in the corpus luteum. The key function of 3β-HSD modulated by hormones at the level of gene expression is responsible for the conversion of pregnenolone to progesterone (Hankins et al., 1993; Telleria et al., 1995). The increase of 20α-HSD activity is the important index of luteolysis (Matsuda et al., 1990). 20α-HSD catalyses the conversion of progesterone to 20α-hydroxypregn-4-en-3-one, which has no progesterone activity. The activity of 20α-HSD is low and that of 3β-HSD is elevated in pregnant ovaries, with a strong ability to produce and secrete progesterone (Kawano et al., 1988). 3β-HSD activity is modulated by progesterone and prostaglandin $F_{2\alpha}$ level (Tanaka et al., 1993). A high level of progesterone could raise the activity of 3β-HSD, while an increase of prostaglandin $F_{2\alpha}$ concentration could stimulate protein kinase C to reduce the expression of 3β-HSD mRNA, and finally provoke the inactivation of 3β-HSD (McGuire et al., 1994). The present results indicated that i.m. DL111-IT-5 days caused a marked decrease of 3β-HSD activity, and a significant decline of progesterone concentration appeared after administration of DL111-IT for 3 days. This suggested that the decrease in progesterone concentration induces a reduction of 3β-HSD activity, which could further induce a loss of biosynthesis of progesterone. Comparing the effect of DL111-IT on the activity of 3β-HSD with that of 20α-HSD, DL111-IT had a stronger influence on 20α-HSD. The data also showed 20α-HSD activity increased immediately and dramatically after one injection of DL111-IT, and a great and progressive rise was observed with increasing treatment days. Other experiments in our laboratory showed a marked increase of serum prostaglandin $F_{2\alpha}$ after DL111-IT injection. Since 20α-HSD activity is regulated by prostaglandin $F_{2\alpha}$ concentration, this indicated that DL111-IT stimulated prostaglandin $F_{2\alpha}$ secretion to activate 20α-HSD, which is the marker of luteolysis. Consequently, DL111-IT inhibited 3β-HSD activity to reduce progesterone production, raised 20α-HSD activity to enhance the metabolism of progesterone, and finally resulted in a rapid and notable decrease in the serum progesterone level which indicated a luteolytic effect of DL111-IT in pregnant rats and disturbed pregnancy. It seemed that the change in 20α-HSD activity plays an important role in causing a decrease of serum progesterone level. The evidence that DL111-IT had an indirect inhibitory effect on 20α-HSD through raising the level of prostaglandin $F_{2\alpha}$ should be confirmed by further experiments.

The control group given vehicle for 1-5 days showed a decrease of 3β-HSD activity, without significant difference, and a drastic increase in 20α-HSD activity. These changes did not cause a reduction in the serum progesterone concentration. On the contrary, it increased by 39%. These contradictory results might be explained as follows. First, though the mean value for 3β-HSD activity decreased a little, it was still at a high level. 20α-HSD activity increased 8.1-fold from day 7 to day 11 of pregnancy, but was not as high as that of 3β-HSD. Thus, the activity of 3β-HSD which involves progesterone synthesis was still higher than that of 20α-HSD which evokes the catabolism of progesterone. Therefore, the serum progesterone of control groups showed a persistent rise. The measurement of enzyme activity mimics the physiological catalytic reaction of the enzyme in vivo. The substrates and product in vitro and in vivo are the same, and the results of enzyme activities obtained through this measurement could reflect the physiological state of these enzymes (Kuhn and Briley, 1970). Second, there are other enzymes in the process of progesterone production, such as hydroxylase and lyase which catalyze the conversion of cholesterol to pregnenolone (the substrate of progesterone). Although 3β-HSD and 20α-HSD are important to synthesize progesterone, other enzymes are necessary. All enzymes that contribute to progesterone production are physiologically relevant. So, the changed activities of hydroxylase and lyase during pregnancy could alter the content of pregnenolone, which could further regulate progesterone synthesis (Yang et al., 1997). Third, the serum progesterone of mid-pregnancy is contributed by two tissues: corpus luteum and trophoblast. The normal developing trophoblast could secrete progesterone directly into the blood circulation to elevate serum progesterone concentration. DL111-IT could damaged ectoplacenta (Galliani et al., 1986), so the ability of trophoblast to produce and secrete progesterone might be inhibited, which would decrease the serum progesterone concentration of treatment groups. This report was only focused on progesterone production by the corpus luteum, especially on the activities of 3β-HSD and 20α-HSD. So, further research on DL111-IT will be extended to the trophoblast and other enzymes related to progesterone synthesis.

Apoptosis, also known as programmed cell death, plays a complementary but opposing role to that of mitosis in regulating normal and neoplastic tissue growth and regression (Kerr et al., 1972; Gerschenson and Rotello, 1991;

Zheng et al., 1994), and has been reported to occur during normal as well as prostaglandin $F_{2\alpha}$-induced luteal regression (Sawyer et al., 1990). Apoptosis is characterized biochemically by the cleavage of DNA into fragments which are multiples of approximately 185 bp in length (Williams and Smith, 1993). When separated by agarose gel electrophoresis, these DNA fragments form a ladder pattern, which is the hallmark of apoptosis (Gavrieli et al., 1992). In the present study, the presence of DNA ladder in agarose gel could be detected in cultured luteal cells exposed to DL111-IT 1.5, 3.0 and 6.0 mg/l. It indicated that DL111-IT induced apoptosis of luteal cells in vitro or in vivo. Hematoxylin–eosine stain showed pyknotic luteal cells with deep stain, loss of cytoplasm and apoptotic bodies in i.m. DL111-IT 3 days or 5 days groups, respectively. In situ 3′-end labeling also showed that a rapid increase of DNA fragmentation accompanied by shrunken nuclei was induced by 3-day and 5-day treatment. These results, in vivo or in vitro, all suggested that DL111-IT induces apoptosis to cause a luteolytic effect in pregnant rats. Since the elevation of prostaglandin $F_{2\alpha}$ concentration (unpublished observation) and the occurrence of luteolysis were almost simultaneous after DL111-IT injection, and prostaglandin $F_{2\alpha}$ is the primary physiological luteolytic factor, we could not distinguish whether DL111-IT induced luteolysis directly or through prostaglandin $F_{2\alpha}$.

In summary, the results of present study demonstrated that DL111-IT induced: (1) apoptosis to decrease ovarian weight and protein content (belongs to the structural corpus luteum regression); (2) inactivation of 3β-HSD, inactivation of 20α-HSD, and lowering of progesterone concentration (belongs to the functional corpus luteum regression). All results indicated that DL111-IT induced luteolysis in pregnant rats. Since DL111-IT has anti-fertility activities, including luteolysis and the effects on decidua and ectoplacenta (Galliani et al., 1986), it could be used as a potential drug to terminate early pregnancy.

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