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Impacts of Dexamethazone on Progesterone, Calcitonin, and Progesterone Receptors in Domestic Pregnant Rabbit (*Oryctolagus cuniculus*)

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Abstract--Dexamethasone (DEX) is used to treat several diseases and medical conditions in both animals and humans. But the administration of synthetic glucocorticoids, including dexamethasone, as a treatment, brings about endocrine misbalance. Such a disequilibrium underlies pregnancy complications. The current study investigates effects of High Dose of DEX (HD) (1.125 mg) and Low Dose of DEX (LD) (0.562 mg) on Progesterone (P), Progesterone Receptors (PR) and Calcitonin (C) in domestic pregnant does (*Oryctolagus cuniculus*). Sixty pregnant does were caged in the animal house and divided into six groups; A, B, C, D, E and F (n=10). These groups are treated as follows: Groups A and B (as an experimental groups) were daily injected by (1ml) from day 5 to 9 of gestation (dG) with HD and LD, respectively; whereas group C was injected by (1 ml) of sodium chloride (0.9%) (as a control group). Then, all the groups (A, B and C) were dissected at 10 and 28 dG. In addition, groups D and E (as an experimental groups) were daily injected by (1ml) from 10 to 17 dG with HD and LD, respectively; whereas group F was injected by (1 ml) of sodium chloride (0.9%) (as a control group). Then, groups D, E and F were dissected at 18 and 28 dG. Blood sera were collected for ELISA-hormonal assays, and fetal-maternal placental biopsies were harvested for gene expression of PR using quantitative reverse transcription Real-Time-PCR (qRT-PCR). The results showed that the

progesterone was significantly decreasing ($p < 0.05$) in all treated groups compared to control groups. In contrast, calcitonin concentration was significantly increasing ($p < 0.05$) in all treated groups compared to control groups. PR gene expression in the uterus had increased significantly ($p < 0.05$) in all treated groups compared to control groups. Moreover, there were significant variations in PR among treated groups in terms of doses and timing of dissection where; it increases significantly in the HD as compared to LD injected groups and it significantly decreases in the 28th day of dissection as compared to 10 and 18. In conclusion, this study observed that (DEX) has extreme effects on progesterone, calcitonin, and PR during early, mid, and late pregnancy.

Keywords---Dexamethasone, Progesterone, Calcitonin and Progesterone Receptors, Domestic Pregnant Rabbits (*Oryctolagus cuniculus*).

Introduction

Glucocorticoids are steroid hormones synthesized and released by the adrenal cortex and regulated by the hypothalamic-pituitary-adrenal (HPA) axis¹. Basal glucocorticoid is important for the establishment (as in patients who undergoing premature ovarian failure and oligospermia)², maintenance (as in women with a history of recurrent miscarriage), and improve (as women undergoing assisted conception -using a medical intervention to help getting conception)³ of fertility. Studies showed the importance of glucocorticoid for the initiation of labor by stimulating the production of cyclooxygenase-2(COX-2) which in turns contributing to the synthesis of prostaglandins⁴. Prostaglandins stimulate myometrial contractions, cervical ripening, and induce fetal membrane rupture⁵. But exceed glucocorticoid exposure, either by stress or by exogenous treatment, including dexamethazone, leads to serious reproductive dysfunction⁶.

Progesterone is a steroid hormone produced by corpus luteum (CL) and placenta during pregnancy⁷. Human Chorionic Gonadotropin (hCG), has luteinizing hormone (LH) like activity, stimulates the corpus luteum to produce progesterone and protects it from regression. The corpus luteum continues in progesterone production until around mid-gestation when placenta begins to produce considerable amount of production⁸. Progesterone has intrinsic role in the maintenance of pregnancy by its immunomodulatory functions⁹ and supporting uterine receptivity and quiescence¹⁰. However, decreasing of progesterone concentration or its receptor (PR) expression promotes parturition or abortion⁸. Recent studies suggest that progesterone action may be come from inhibiting of contractile genes expression of the uterus and cervix and preventing the chemokines production that stimulates chemotaxis of immune cells¹¹.

Progesterone receptors (PR) affect cellular function through genomic pathway and nongenomic pathway. Genomic pathway: Progesterone interaction with nuclear PRs (nPRs) induces nPRs dissociation from the nPR-chaperone complex in the cytoplasm. The progesterone-nPR complex then affects gene expression by direct

and indirect (extranuclear pathway) pathways. The direct pathway, progesterone-nPR undergoes a conformational change to form dimer that translocate to the nucleus where it binds to progesterone response element in the promoter region of target gene and recruit cell specific coregulator to form a functional complex that modulates gene transcription. For the indirect pathway, progesterone-nPR interacts with cytoplasmic signaling molecule which in turn activates cascades of pathways leading to act on specific transcription factors within the nucleus which then affect gene expression. Nongenomic pathway: Progesterone interacts with membrane PRs (mPRs) to activate intracellular signaling cascades. recent study observed that hormone receptors could be functional by their own ligand (homologous regulation) and by other specific molecules (heterologous regulation)¹¹.

Calcitonin is, a peptide hormone, synthesized and secreted by the parafollicular C cells of the thyroid gland. It's function to lower blood calcium by inhibiting osteoclast activity¹². Also, this hormone is present in other tissues such as lung, intestine, central nervous system, and uterus. It's distribution throughout the body, including no-bony skeleton animals, suggests that calcitonin has other effects in addition to its action on lower blood calcium. The common effect of calcitonin in various physiological actions could be calcium fluxion across the cell membranes¹³. Recent study revealed that calcitonin is synthesized in glandular epithelial cells of the pregnant rat and human uterus and it peaks on the day before implantation and regulated by progesterone¹⁴. The mechanism of action may involve the calcitonin-induced increase of intracellular calcium that decreases endometrial cell expression of E-cadherin, a glycoprotein that mediates cell-cell adhesion among epithelial cells, consequently dissolving of gap junctions between cells. These act to increase permeability to facilitate blastocyst implantation¹⁵.

Materials and Methods

Animals

In this study, sixty of domestic does (*Oryctolagus cuniculus*) were used. Does age were between 1-1.5 year and weighed about 1500 mg. They were managed in the animal house of Babylon University\ Science College\ Biology Department, and adjusted four weeks before the commencement of the experiment. To obtain pregnancies, one female was caged overnight with two males for ensure to get pregnancy. The presence of a vaginal plug was considered as day 1 of pregnancy.

Treatment

To know the effects of (DEX) on pregnancy, dexamethasone acetate was administered by subcutaneous injections (1ml) of high dose (HD) (1.125 mg\ml)¹⁶ and low dose (LD) (0.562 mg\ml). The animals were divided into six groups (n=10): three groups (A, B, C) were daily injected from 5 to 9 dG with (HD) and (LD) of (DEX) as experimental groups and an equivalent volume of sodium chloride (0.9%) as a control group. Another three groups (D, E, F) were daily injected from 10 to 17 dG with (HD) and (LD) of (DEX) as experimental groups and an equivalent volume of sodium chloride (0.9%) as a control group. From each (A,

B, C) group, seven pregnant rabbits were scarified after anesthetized with chloroform on 10 dG, and the other three ones were scarified on 28 dG (except control group continued in gestation until term). From each (D, E, F) group, seven pregnant rabbits were sacrificed on 18 dG and, the other three ones were scarified on 28 dG (except control group continued in gestation until term).

Blood Samples Collection and Analysis

Five ml of blood samples were drawn and allowed to clot for 10-20 minutes at room temperature. Sera were centrifugated at 2000-3000 RPM for 20 minutes and stored at -20 until assayed for progesterone and calcitonin using Rabbit Progesterone and Calcitonin ELISA Kits (My BioSource, San Diego, CA, USA). For progesterone assay, the minimal detectable concentration (Sensitivity) was 0.03ng/ml. For calcitonin assay, the minimal detectable concentration was 5 pg/ml.

Tissues collection and qRT-PCR for PR Gene Expression analysis

100-250 mg of fetal-maternal placental tissues were excised and dissected then put in (3 ml) RNA Later solution for 24 hours at room temperature. All tissue samples were stored at -20 C until used for (PR) estimation using quantitative RT-PCR.

Total uterine RNA was extracted using GENEzol™ TriRNA Pure Kit (Geneaide - New Taipei, Taiwan) as the manufacturer's instructions. The primers and housekeeping genes [glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene] designed by Humanizing Genomics macrogen (Biotechnology companies, South Korea). PR forward and reverse primers sequences are 5'-AGACCTCCAGAAAAGGACAGC-3' and 5'-CAACACCCCTTTG GTAGCG-3', respectively. GAPDH forward and reverse housekeeping genes sequences are 5'-TGGTGAAGGTCGGAGTGAAC-3' and 5'-ATGTAGTGGAGGTCAATGAATGG-3', respectively. Gene expression levels of (PR) were performed by qRT-PCR using Trans Script® Green One-Step qRT-PCR SuperMix kit (TRANS-Beijing, China) according to the manufacture's manual. The qRT-PCR reactions amplified (PR) gene and (GAPDH) gene (used as an internal control) were run in a Rotor-Gene-Q thermal cycler (Qiagen- USA). Briefly, these reactions were carried out in a 20µl volume that consists of: RNA template (1µl), forward primer or housekeeping gene (10µM, 0.5µl), reverse primer or housekeeping gene (10µM, 0.5µl), green one step qPCR supermix (10µl), green one step enzyme mix (0.5µl), and RNase-free water (7.5µl). The amplification conditions are: hold 45 °C 10min., hold 94 °C 30sec., denaturation 95 °C 10sec., and annealing and elongation 60 °C 30sec. for 40 cycles, the total time is 114min, Figure (1) shows amplification curve. The specificity of PCR reaction was confirmed using melting curve analysis. Melting curve thermal profile is: ramp from 60 degrees to 99 degrees, rising by 1 degree each step, waiting for 90 sec. of pre-melt, waiting for 5 sec. for each step, Figure (2) shows melting curve.

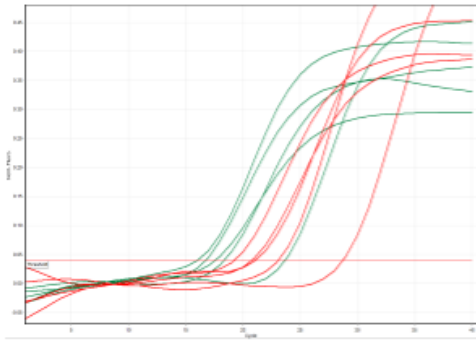


Figure (1): qRT-PCR amplification curve

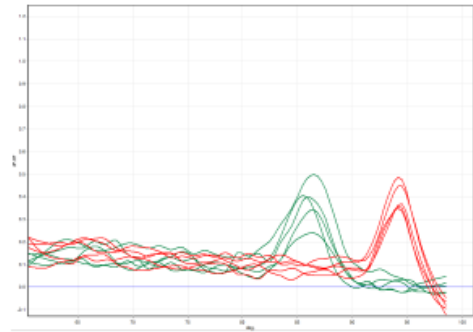


Figure (2): qRT-PCR melting curve

Statistical analyses

Data collected were expressed as means \pm standard deviations (SD). Statistical evaluations of the data were performed using one-way analysis of variance (ANOVA), followed by a Post Hoc-Duncan test to determine the significant differences for all comparisons. Significant differences were accepted at $p < 0.05$. Statistical analysis was carried out using IBM® SPSS® Statistics version 24 predictive analytics software.

Results

The changes in serum progesterone concentrations in pregnant rabbit following the administration (HD) and (LD) of (DEX) from 5 to 9 dG and sacrificed at 10 and 28 dG are presented in Table (1) and Figure (1), and the administration (HD) and (LD) of (DEX) from 10 to 17 dG and sacrificed at 18 and 28 dG are presented in Table (2) and Figure (2). Progesterone concentration decreased significantly ($p < 0.05$) in all the treated groups compared to control groups.

Table (1)
Serum progesterone concentration ng/ml. DEX administration from 5 to 9 dG

Group	N	dG	Mean \pm SD
Control	7	10	12.231 ^a \pm 3.426
High dose	7	10	1.187 ^b \pm 0.161
Low dose	7	10	1.882 ^b \pm 0.690
High dose	3	28	0.956 ^b \pm 0.105
Low dose	3	28	1.328 ^b \pm 0.234

N= number of repeated

dG= day of gestation

SD= standard deviation

a, b= significant variation ($p < 0.05$)

Figure (1) Serum progesterone concentration ng\ml. DEX administration from 5 to 9 dG

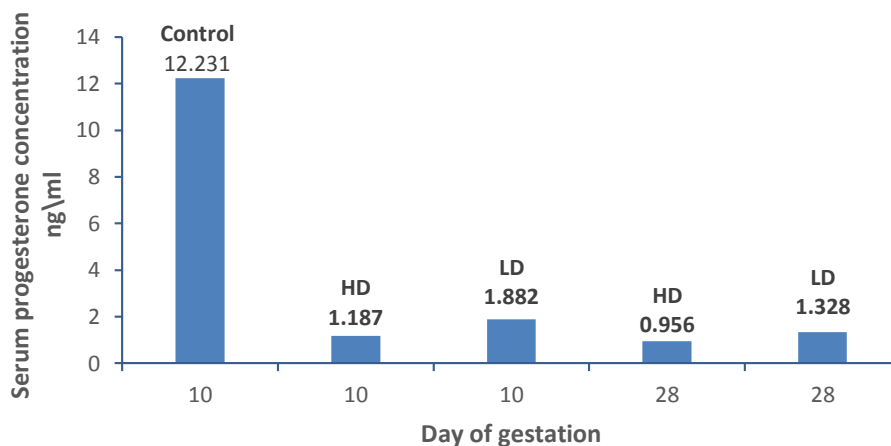


Table (2) Serum progesterone concentration ng\ml. DEX administration from 10 to 17 dG

Group name	N	dG	Mean \pm SD
Control	7	18	10.441 ^a \pm 2.395
High dose	7	18	1.492 ^b \pm 0.623
Low dose	7	18	2.080 ^b \pm 0.828
High dose	3	28	2.252 ^b \pm 0.206
Low dose	3	28	1.668 ^b \pm 0.211

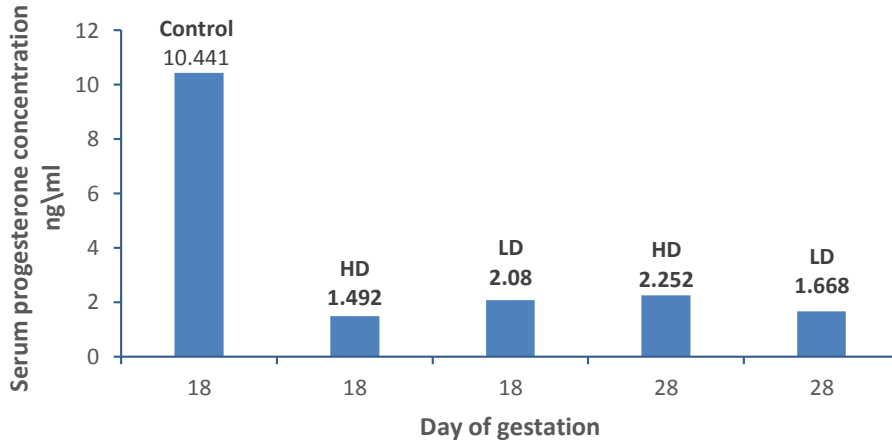
N= number of repeated

dG= day of gestation

SD= standard deviation

a, b= significant variation ($p < 0.05$)

Figure (2) Serum progesterone concentration ng\ml. DEX administration from 10 to 17 dG



qRT-PCR was performed using total RNA isolated from fetal-maternal placenta after pregnant rabbits administrated with (HD) and (LD) of (DEX) from 5 to 9 dG and sacrificed at 10 and 28 dG are presented in Table (3) and Figure (3), and the administration (HD) and (LD) of (DEX) from 10 to 17 dG and sacrificed at 18 and 28 dG are presented in Table (4) and Figure (4), PR gene expression was corrected for that of GAPDH. Gene expression of PR in the uterus increased significantly ($p < 0.05$) in all groups compared to control group. Moreover, there were significant variations in PR among treated groups in terms of doses and timing of dissection where; it increases significantly in the HD as compared to LD injected groups and it significantly decreases in the 28th day of dissection as compared to 10 and 18.

Table (3)
Relative expression PR\ GAPDH. DEX administration from 5 to 9 dG

Group	N	dG	Mean \pm SD
Control	5	10	0.995 ^a \pm 0.123
High dose	5	10	9.117 ^b \pm 2.251
Low dose	5	10	4.836 ^c \pm 1.056
High dose	3	28	4.740 ^c \pm 1.227
Low dose	3	28	2.916 ^d \pm 0.760

N= number of repeated

dG= day of gestation

SD= standard deviation

a, b, c, d= significant variation ($p < 0.05$)

Figure (3) Relative expression PR\ GAPDH. DEX administration from 5 to 9 dG

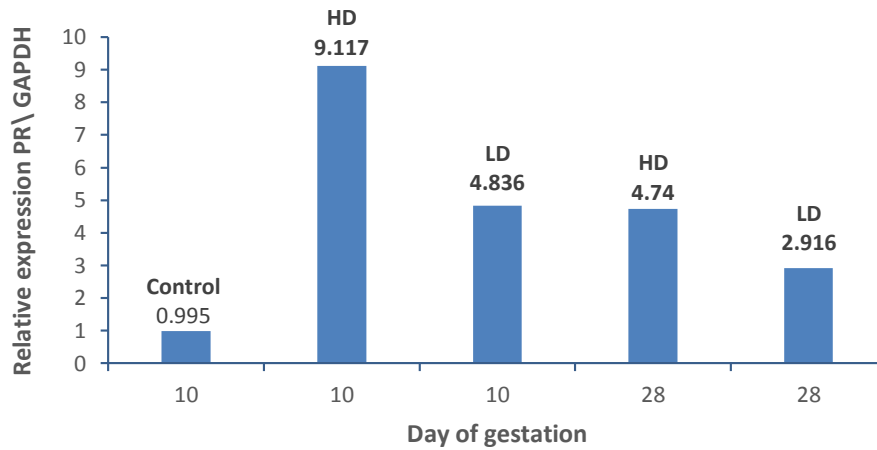


Table (4)

Relative expression PR\ GAPDH. DEX administration from 10 to 17 dG

Group	N	dG	Mean \pm SD
Control	5	18	0.988 ^a \pm 0.121
High dose	5	18	10.514 ^b \pm 3.780
Low dose	5	18	4.140 ^c \pm 0.740
High dose	3	28	6.139 ^d \pm 2.105
Low dose	3	28	1.851 ^e \pm 0.710

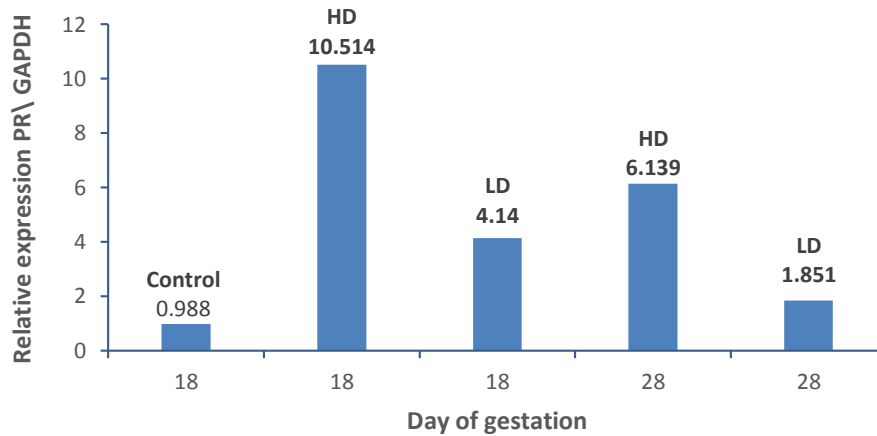
N= number of repeated

dG= day of gestation

SD= standard deviation

a, b, c, d, e= significant variation ($p < 0.05$)

Figure (4) Relative expression PR\ GAPDH. DEX administration from 10 to 17 dG



On another hand, serum calcitonin concentrations in pregnant rabbit following the administration (HD) and (LD) of (DEX) from 5 to 9 dG and sacrificed at 10 and 28 dG are presented in Table (5) and Figure (5), and the administration (HD) and (LD) of (DEX) from 10 to 17 dG and sacrificed at 18 and 28 dG are presented in Table (6) and Figure (6). Calcitonin concentration increased significantly ($p < 0.05$) in all the treated groups compared to control groups.

Table (5)
Serum calcitonin concentration pg\ml. DEX administration from 5 to 9 dG

Group	N	dG	Mean \pm SD
Control	7	10	152.329 ^a \pm 12.012
High dose	7	10	329.216 ^b \pm 22.605
Low dose	7	10	322.725 ^b \pm 35.058
High dose	3	28	315.726 ^b \pm 26.820
Low dose	3	28	324.083 ^b \pm 39.652

N= number of repeated

dG= day of gestation

SD= standard deviation

a, b= significant variation ($p < 0.05$)

Figure (5) Serum calcitonin concentration pg/ml. DEX administration from 5 to 9 dG

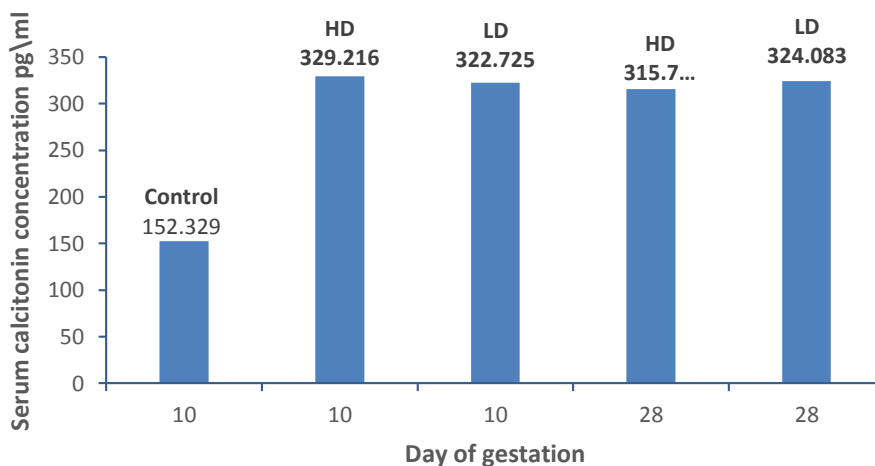


Table (6)

Serum calcitonin concentration pg/ml. DEX administration from 10 to 17 dG

Group	N	dG	Mean \pm SD
Control	7	18	262.067 ^a \pm 25.261
High dose	7	18	342.654 ^b \pm 13.569
Low dose	7	18	320.830 ^b \pm 43.932
High dose	3	28	340.577 ^b \pm 17.279
Low dose	3	28	329.145 ^b \pm 16.155

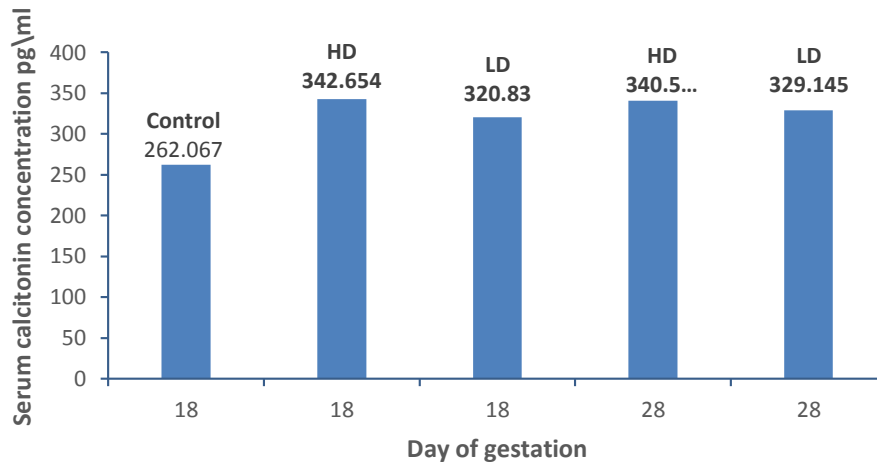
N= number of repeated

dG= day of gestation

SD= standard deviation

a, b= significant variation ($p < 0.05$)

Figure (6) Serum calcitonin concentration pg/ml. DEX administration from 10 to 18 dG



It is noteworthy, the results showed that the three pregnant animals of each control group continued until term gave birth at 30-33 dG.

Discussion

Progesterone concentration increases progressively as the pregnancy is in advance in order to maintain the uterus quiescent during gestation⁸. The hostile effects of dexamethasone may be related to changing in the normal concentrations of progesterone and its receptors during pregnancy¹¹.

The lower progesterone concentration of current study in treated groups compared to control groups could be attributed to DEX suppressed the secretion of LH and FSH through a direct effect on the anterior pituitary¹⁷. Also, two neuropeptides, kisspeptin (KISS1) and gonadotropin-inhibitory hormone (GnIH), are responsive to high levels of glucocorticoids as DEX. KISS1 has stimulatory effects on GnRH secretion. KISS1 neurons of the hypothalamus express GR. In mice, corticosterone administration reduced hypothalamic expression of KISS1 neurons and suppressed the hypothalamic- pituitary gonadal (HPG) axis. The second neuropeptide, GnIH inhibits the activity of GnRH neurons and KISS1 neurons^{18,1}. Many studies showed that dexamethasone caused significantly decreased progesterone concentrations in sheep⁸, mice¹⁷, and rats while progesterone receptors (PR) were upregulated¹⁹. Glucocorticoids and progesterone are potent activators of GR and PR, respectively. Relative binding affinity of PR is progesterone 100% ²⁰, corticosterone 2.6% ²¹, and dexamethasone 0.2% ²². It's expression in immune cells is limited to specific cell but in uterus is too high²³. With respect to GR, the relative binding affinity is progesterone 1-6% ²⁰, corticosterone 85% ²¹, and dexamethasone 100% ²². It's expression in immune cells is too high^{24,25} but in uterus is lesser²⁶. That means PR and GR have exchangeable binding ability for each other but in limit manner. In this study, PR upregulation in treated groups may a compensatory mechanism to enhance progesterone sensitivity. The mechanism is accomplished through increasing of PR mRNA levels and gene transcription⁸. Moreover, our data showed that

significant increase of PR between (HD) compared to (LD) in all treated groups. These observations demonstrated that the effects of glucocorticoids are dependent on dose concentration. For example, early gestational exposure to high level of glucocorticoids inhibits endometrial receptivity, reduces placental weight and fetal size¹. As well as, there was significant decrease in PR at 28 dG compared to other groups of the same dose. It is probably that the PR started recovery because the dexamethazone effects may be reversible after DEX withdrawal²⁷. Also, current study results showed that increase in calcitonin concentration between controls groups at 18 dG compared to 10 dG. Similarly, Cote and Gage²⁷ found that the calcitonin increases with pregnancy progressing. Silva and et. al.,²⁸ observed that parathyroid hormone (PTH) concentration was increased in women during pregnancy. Also, they found that intestinal absorption of calcium increases in pregnancy, it could be attributed to increase in PTH. The hypercalcemia serves to protect the maternal skeleton against demineralization²⁷ and the fetus calcium needs are met²⁸. However, our results observed that significant increase of calcitonin in all treated groups compared to control groups. Muszynski and et. al.,²⁹ found that the dexamethasone administration caused increase in calcitonin between 4 and 6 days of treatment, and demonstrated that dexamethazone or corticosterone treated cells were undergone morphological changes from flattened ovoid bipolar and triangular cells with extended processes to rounded cells with more distinct borders. Thus, DEX stimulated production and secretion the calcitonin by acting on gene transcription²⁷.

In conclusion, current research data demonstrated that dexamethazone is very effective on progesterone concentration, PR, and calcitonin during early, mid, and late pregnancy. It is noticeable that the dose and timing of administration affects gestation.

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