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Effects of Dexamethasone on Trophoblastic, IDO, and Macrophage Cells in Domestic Pregnant Rabbits (*Oryctolagus cuniculus*)

Amal Faisal Alabedi
Department of Animal Production, College of Agriculture, Al-Qasim Green University
Email: jakhahnoaman@agre.uoqasim.edu.iq

Alaa Jawad Hassan
Department of Biology, College of Science, University of Babylon
Email: alnajaralaa71@gmail.com

Akram Yousif Yasear
Department of Dentistry, Hilla University College
Email: yakram@yahoo.com

***Abstract*---**The healthy pregnancy requires a healthy fetal-maternal placental development that depends not only on invasion of fetal trophoblast but also on the presence of immune cells, such as macrophages. Dexamethasone (DEX) is used to treat several diseases, but exceed exposure to exogenous DEX may impair placental and fetal development and subsequent fetal loss. The current study investigates the effects of high dose of DEX (HD) (1.125 mg) and low dose of DEX (LD) (0.562 mg) on trophoblastic (T), IDO, andCD68 macrophage (M) cells in domestic pregnant rabbits (*Oryctolagus cuniculus*). Forty-eight pregnant does were caged in the animal house and divided into six groups, eight for each. 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. Fetal-maternal placental biopsies were harvested for immunohistochemical assays of T, IDO, and M cells by using anti-cytokeratin7 (Ck7), anti-IDO, anti-CD68 antibody kits, respectively.

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***Corresponding author**: Alabedi, A.F.; Email: jakhahnoaman@agre.uoqasim.edu.iq*
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The results showed that all of three parameters were significantly decreasing ($p < 0.05$) in all treated groups compared to control groups. In conclusion, this study observed that (DEX) has extreme effects on T, IDO, and M cells during early, mid, and late pregnancy.

***Keywords*---**Dexamethasone, Trophoblasts, IDO, Macrophage, 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) axis[1]. 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, but exceed glucocorticoid exposure, either by stress or by exogenous treatment, including dexamethasone, leads to serious reproductive dysfunction[4].

Trophoblast cells have vital role in both placental and fetal development, and their proliferation, migration and invasion are essential for the establishment and maintenance of a successful pregnancy. Defects in trophoblast function brings about pregnancy complications such as recurrent spontaneous abortion, intrauterine growth retardation, and preeclampsia. Trophoblasts are reported to express IDO[5], and IDO-positive tumor cell has a higher ability for proliferation and metastasis than IDO-negative tumor cell[6,7]. Therefore, it suggests that trophoblast is similar to tumor cells in regard to IDO may play an important role in trophoblast proliferation and migration[5]. However, trophoblast secretes many factors such as transforming growth factor TGF-β which induces monocyte differentiation into M2 macrophages phenotype and enhance the phagocytosis capacity[8,9].

Macrophages are present in all tissues and derived from monocytes [10,11]. Placental macrophages represent 20%–30% of the total body macrophages[12], so that estrogens cause macrophage chemotaxis to the endometrium indirectly [13]. It stimulates fibroblasts to produce cytokines which attract macrophages, also it stimulates macrophage proliferation, enhances the phagocytic ability[14], and polarization to M2 phenotype[15]. The main function of macrophages is immune tolerance to protect the semi-allogeneic fetus from recognizing by maternal immune system[16]. Macrophages are divided into M1 (Pro-inflammatory phenotype) and M2 (Anti-inflammatory phenotype) macrophages, and during the peri-implantation period, when the uterus is exposed to seminal fluid, the number of M1 macrophage is more abundant that is important for successful implantation[17,18], by clearance of pathogens and sperms[12]. As pregnancy progresses, M2 macrophages become more plentiful in order to establish and maintain tolerance to the fetus[19,9]. Macrophage phenotypes are governed by microenvironment milieu[12]. Lipopolysaccharide and T helper1 cytokines such as IFNɣ activate macrophages into M1 phenotype which releases cytokines and chemokines such as TNFα and IL-1β resulting in pro-inflammatory cells. On the

other hand, IL-4 and IL-13 activate macrophages into M2 phenotype which releases cytokines such as IL-10 and TGF-β[9].

Indoleamine 2,3-dioxygenase (IDO) is a tryptophan catabolizing enzyme[20] that found in many tissues such as intestine and placenta, and expressed by different cell types as trophoblast[5], antigen presenting cells[21,22]. It inhibits proliferation of many pathogens, including tumors, parasites[21] and T-cells[23,24] by tryptophan depletion. Also, IDO manifests immunosuppressive properties that play a vital role in prevention of allogeneic fetus rejection by producing metabolites resulted from tryptophan depletion. The tryptophan depletion-produced metabolites generate Treg cells which in turn secrete pro-inflammatory cytokines such as IL-10 and TGF-β to suppress T cells activation and effector cells of allergic inflammation such as basophils and eosinophils[25].

Materials and Methods

Animals

In this study, forty-eight of domestic does (*Oryctolagus cuniculus*) were used. Does age was between 1-1.5 year and weighed about 1500 mg. They were managed in the animal house of University of Babylon / College of Science / 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

Dexamethasone acetate was administered by subcutaneous injections (1ml) of high dose (HD) (1.125 mg\ml) [26] and low dose (LD) (0.562 mg\ml). The animals were divided into six groups (n=8): 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, five 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, five 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).

Protocol

Tissues preparation was done according to the manual procedure of Suvarna and et. al., and Bolon [27,28].

Immunohistochemistry Technique

IHC procedure for T, IDO, and M cells using anti cytokeratin7 (Ck7), IDO, CD68 antibody kits, respectively, was done depending on manufacturer (My BioSource, Inc, San Diego, CA. USA).

Image Analysis

Data analysis was done by Image J software[29]. The immunohistochemical staining was assessed by two observers. For each sample, 4 fields were evaluated at magnification 400x. In case of CD68 marker, all stained cells were counted in the field microscope, then reacted cells to total cells were presented to mm^2.

The expression levels of the proteins (CK7 and IDO) were scored as follows: the total scores come from the sum of the percentage of stained cells and the staining intensity. The percentage of stained cells was scored as (0) when <5%, (1) when 5–30%, (2) when 30–70%, and (3) when >70% of stained cells. The staining intensity was scored as (0) when no staining, (1) when weakly stained, (2) when moderately stained, and (3) when strongly stained. The final scores were (0) when the sum of the percentage of stained cells score and the staining intensity score was 0–1, (1+) when the sum was 2–3, (2+) when the sum was 4–5, and (3+) when the sum was 6 [30,31].

Statistical analyses

Data collected were expressed as means ± 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 Ck7 expression level (Trophoblasts) in pregnant rabbit's placenta 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 the administration (HD) and (LD) of (DEX) from 10 to 17 dG and sacrificed at 18 and 28 dG are showed in Table (2). Ck7 protein level decreased significantly ($p < 0.05$) in all the treated groups compared to control groups (table 3). The immunohistochemical images of Ck7 in the placenta of pregnant rabbits at different gestational periods and different DEX doses are showed in figure (1).

Table (1)
Ck7 expression level %/mm^2. DEX administration from 5 to 9 dG

Group	N	dG	Mean ± SD
Control	5	10	40.18^a ± 5.546
HD	5	10	15.45^b ± 3.943
LD	5	10	17.49^b ± 3.021

HD	3	28	$19.26^{b} \pm 1.476$
LD	3	28	$20.18^{b} \pm 2.768$

N= number of repeated
dG= day of gestation
SD= standard deviation
a, b= significant variation ($p < 0.05$)

Table (2)
Ck7 expression level %/mm^2. DEX administration from 10 to 17 dG

Group name	N	dG	Mean ± SD
Control	5	18	$38.27^{a} \pm 8.342$
HD	5	18	$18.62^{b} \pm 2.664$
LD	5	18	$21.24^{b} \pm 4.112$
HD	3	28	$15.27^{b} \pm 3.952$
LD	3	28	$18.94^{b} \pm 2.338$

N= number of repeated
dG= day of gestation
SD= standard deviation
a, b= significant variation ($p < 0.05$

Table (3): Ck7 expression level scores of the percent positivity of stained cells and the staining intensity

Group name	N×4	dG	Score		
			0	1+	2+ 3+
Control	5(20)	10	0 5	3	12
HD	5(20)	10	4 0	15	1
LD	5(20)	10	3 0	17	0
HD	3(12)	28	5 0	14	1
LD	3(12)	28	3 1	16	0
Control	5(20)	18	0 2	2	16
HD	5(20)	18	4 1	14	1
LD	5(20)	18	5 1	13	1
HD	3(12)	28	4 0	14	2
LD	3(12)	28	3 1	15	1

0= Negative, 1+= Weak, 2+= Moderate, 3+= Strong

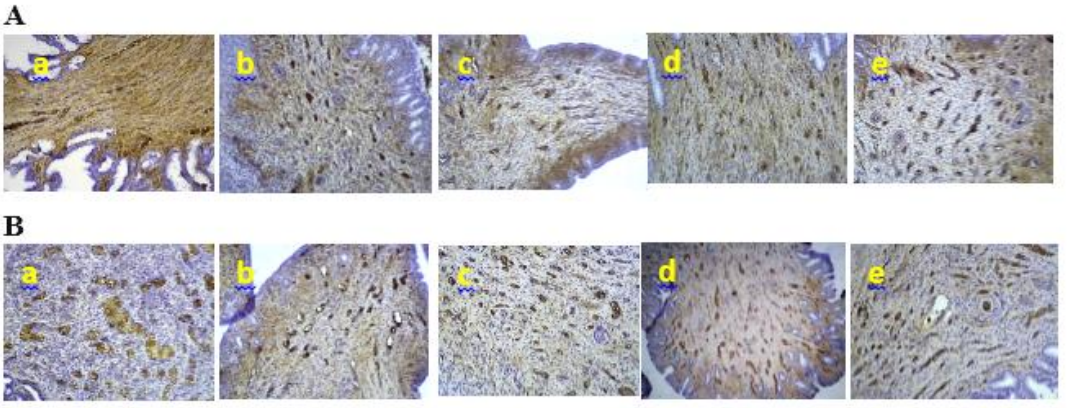

Figure (1): Immunohistochemical staining images of Ck7 (brown color) in the placenta of pregnant rabbits at different gestational periods and different DEX doses. A: (a) control at 10 dG. (b) HD at 10 dG. (c) LD at 10 dG. (d) HD at 28 dG. (e) LD at 28 dG. B: (a) control at 18 dG. (b) HD at 18 dG. (c) LD at 18 dG. (d) HD at 28 dG. (e) LD at 28 dG. (Magnification: 100 ×).

CD68 expression level (Macrophages) in pregnant rabbits' 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 (4), and the administration (HD) and (LD) of (DEX) from 10 to 17 dG and sacrificed at 18 and 28 dG are showed in table (5). CD68 protein level decreased significantly ($p < 0.05$) in all the treated groups compared to control groups. Figure (2) shows immunohistochemical images of CD68 in the placenta of pregnant rabbits at different gestational periods and different DEX doses.

Table (4)
CD68 cells number/mm^2. DEX administration from 5 to 9 dG

Group	N	dG	Mean ± SD
Control	5	10	$6748^a \pm 139$
HD	5	10	$2681^b \pm 374$
LD	5	10	$3057^b \pm 125$
HD	3	28	$2928^b \pm 289$
LD	3	28	$2785^b \pm 311$

N= number of repeated
dG= day of gestation
SD= standard deviation
a, b= significant variation ($p < 0.05$)

Table (5)
CD68 cells number/mm^2. DEX administration from 10 to 17 dG

Group name	N	dG	Mean ± SD
Control	5	18	$5927^a \pm 553$
HD	5	18	$2148^b \pm 266$
LD	5	18	$1927^b \pm 218$

HD	3	28	$1989^{b} \pm 333$
LD	3	28	$2025^{b} \pm 229$

N= number of repeated
dG= day of gestation
SD= standard deviation
a, b= significant variation ($p < 0.05$)

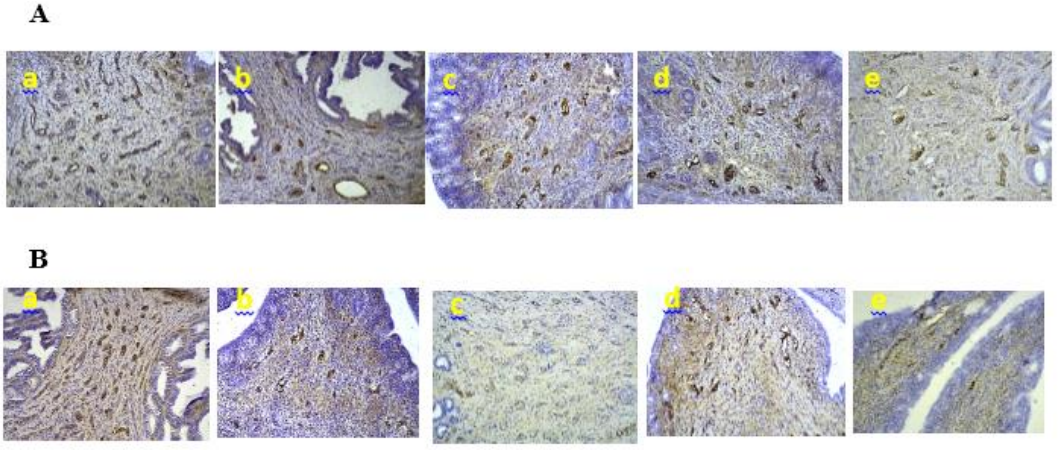

Figure (2): Immunohistochemical staining images of CD68 (brown color) in the placenta of pregnant rabbits at different gestational periods and different DEX doses. A: (a) control at 10 dG. (b) HD at 10 dG. (c) LD at 10 dG. (d) HD at 28 dG. (e) LD at 28 dG. B: (a) control at 18 dG. (b) HD at 18 dG. (c) LD at 18 dG. (d) HD at 28 dG. (e) LD at 28 dG. (Magnification: 100 ×).

IDO expression level in pregnant rabbits following the administration (HD) and (LD) of (DEX) from 5 to 9 dG and sacrificed at 10 and 28 dG are presented in table (6), and the administration (HD) and (LD) of (DEX) from 10 to 17 dG and sacrificed at 18 and 28 dG are illustrated in table (7). IDO protein level decreased significantly ($p < 0.05$) in all the treated groups compared to control groups (table 8), in addition figure (3) shows immunohistochemical images of IDO in the placenta of pregnant rabbits at different gestational periods and different DEX doses.

Table (6)
IDO expression level %\mm^2. DEX administration from 5 to 9 dG

Group	N	dG	Mean ± SD
Control	5	10	$36.86^{a} \pm 8.571$
HD	5	10	$10.88^{b} \pm 2.382$
LD	5	10	$13.34^{b} \pm 1.021$
HD	3	28	$14.05^{b} \pm 1.934$
LD	3	28	$13.17^{b} \pm 4.721$

N= number of repeated
dG= day of gestation
SD= standard deviation
a, b= significant variation ($p < 0.05$)

Table (7)
IDO expression level %/mm². DEX administration from 10 to 17 dG

Group name	N	dG	Mean ± SD
Control	5	18	$37.44^{a} \pm 2.044$
HD	5	18	$12.47^{b} \pm 3.621$
LD	5	18	$12.35^{b} \pm 3.883$
HD	3	28	$15.32^{b} \pm 3.366$
LD	3	28	$13.08^{b} \pm 2.914$

N= number of repeated
dG= day of gestation
SD= standard deviation
a, b= significant variation ($p < 0.05$)

Table (8)
IDO expression level scores of the percent positivity of stained cells and the staining intensity

Group name	N×4	dG	Score		
			0	1+	2+ 3+
Control	5(20)	10	0	1	16 3
HD	5(20)	10	3	14	2 0
LD	5(20)	10	1	18	0 1
HD	3(12)	28	3	15	2 0
LD	3(12)	28	4	15	1 0
Control	5(20)	18	0	0	15 5
HD	5(20)	18	2	16	2 0
LD	5(20)	18	3	14	3 0
HD	3(12)	28	1	18	1 0
LD	3(12)	28	1	17	1 1

0= Negative, 1+= Weak, 2+= Moderate, 3+= Strong

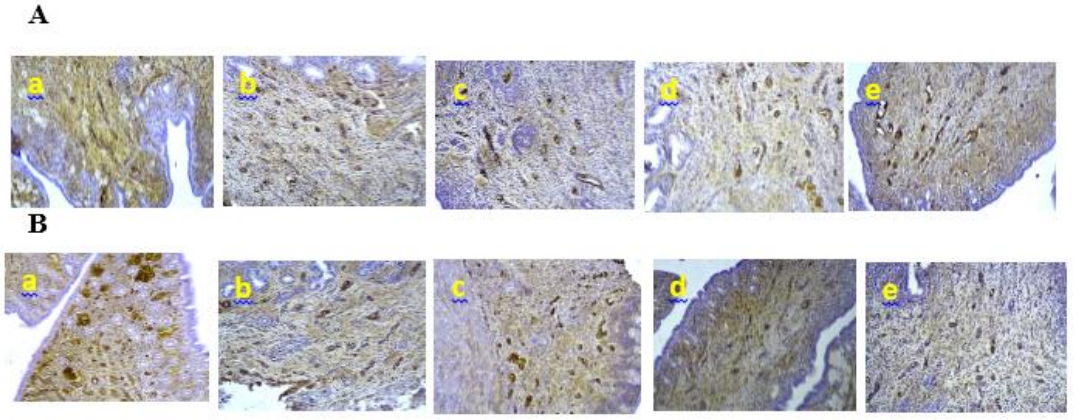

Figure (3): Immunohistochemical staining images of IDO (brown color) in the placenta of pregnant rabbits at different gestational periods and different DEX doses. A: (a) control at 10 dG. (b) HD at 10 dG. (c) LD at 10 dG. (d) HD at 28 dG. (e) LD at 28 dG. B: (a) control at 18 dG. (b) HD at 18 dG. (c) LD at 18 dG. (d) HD at 28 dG. (e) LD at 28 dG. (Magnification: 100 ×).

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

Discussion

The data of current study showed that there are significantly decreases in all parameters, including trophoblasts, macrophages, and IDO of treated groups compared to controls groups.

First of all, Progesterone and estrogen are essential hormones for successful gestation. Both of them have many functions such as: estrogen acts indirectly to attract macrophages (chemotactic) to the endometrium[13]. It stimulates fibroblasts to produce cytokines which in turn draw macrophages inward, also it stimulates macrophage proliferation[14]. Progesterone promotes an immunotolerance in maternal-fetal interface by inducing differentiation of macrophages into M2 phenotype[32], prevents dendritic cells maturation [33], and consequently generating Treg cells and suppressing of CD8+ T cell cytotoxicity during pregnancy[34,35]. Also, progesterone has effects on hemostasis and activity of trophoblasts such as stimulation of trophoblast through progesterone receptors to produce VEGF [36,37].

Previous study confirmed that DEX were associated with decreased progesterone and 17β-estradiol levels, and reduced uterine macrophages in pregnant BALB/c mice[38]. Moreover, differentiated Trophoblastic cells have capacity to produce cytokines, growth factors, and hormones such as placental prolactin (PRL) which is considered as a monitor for trophoblasts activities. Maternal dexamethasone treatment results in disturbance in trophoblasts development then dysregulation of PRL gene expression by inhibition of Akt/protein kinase B pathway thereafter decreasing phosphorylation of Akt and the pro-apoptotic protein BAD finally increasing poly (ADP-ribose) polymerase (PARP) cleavage, an indicator of apoptosis[5]. Besides, IDO is expressed by different cell types as antigen presenting

cells such as macrophages[22,20], and trophoblasts[5]. IDO manifests immunosuppressive properties that play a vital role in prevention of allogeneic fetus rejection by producing metabolites resulted from tryptophan depletion. The tryptophan depletion-produced metabolites generate Treg cells which in turn induce IDO expression in macrophages[25]. In addition to, IDO promotes trophoblast proliferation and migration by decreasing STAT3 phosphorylation led to MMP9 expression[39,40]. From above information, the lowering in macrophages, trophoblasts, and IDO could be attributed to those reasons.

In conclusion, DEX has highest degree immunosuppressive effects on placental growth parameters. It is very effective on trophoblasts, macrophages, and IDO during early, mid, and late pregnancy.

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