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Trophoblastic Migration in Dexamethasone Treated Pregnant Does

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بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ

﴿ قَالُواْ سُبْحَٰنَكَ لَا عِلْمَ لَنَآ إِلَّا مَا عَلَّمْتَنَآ إِنَّكَ أَنتَ ٱلْعَلِيمُ ٱلْحَكِيمُ ﴾

صدق الله العليّ العظيم

سورة البقرة-آية 32

Supervisors Certificate

We certify that this dissertation entitled '' **Trophoblastic Migration in Dexamethazone Treated Pregnant Does**" was carried under our supervision at the Department of Biology/College of Science/University of Babylon as a partial requirement for the Degree of Doctorate of Philosophy in Biology / Zoology.

Accordingly, we recommended this study for discussion.

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DEDICATION

I would like to dedicate this humble work to the Prophet **Mohammed** and his family "**Allah** blessings and mercy on all of them" and to all our martyrs.

Amal

2022

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Summary

Dexamethasone (DEX) is a drug used to treat several 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 the effects of high dose of DEX (HD) (1.125 mg) and low dose of DEX (LD) (0.562 mg) on progesterone, progesterone receptors (PR), calcitonin, the histology of the fetal-maternal uterine tissues, trophoblasts migration, macrophage cells, indolamine 2,3- dioxygenase (IDO), and the spiral arteries remodeling in domestic pregnant does (*Oryctolagus cuniculus*). This study was carried out during the period from January (2021) to October (2021) in the Department of Biology, College of Sciences, University of Babylon. Sixty pregnant does were caged in the animal house and divided into six groups (10 rabbits for each group). These groups are treated as follow: Group (1) (as a control group) was daily subcutaneous injected by (1ml) from 5 to 9 dG with normal saline (0.9%). Then, seven does were sacrificed at 10 dG, and three does were continued in gestation until term. Group (2) was daily subcutaneous injected by (1ml) from day 5 to 9 day of gestation (dG) with HD. Then, seven does were sacrificed at 10 dG, and three does were sacrificed at 28 dG. Group (3) was daily subcutaneous injected by (1ml) from 5 to 9 dG with LD. Then, seven does were sacrificed at 10 dG, and three does were sacrificed at 28 dG. Group (4) (as a control group) was daily subcutaneous injected by (1ml) from 10 to 17 dG with normal saline (0.9%). Then, seven does were sacrificed at 18 dG, and three does were continued in gestation until term. Group (5) was daily subcutaneous injected by (1ml) from 10 to 17 dG with HD. Then, seven does were sacrificed at 18 dG, and three does were sacrificed at 28 dG. Group (6) was daily subcutaneous injected by (1ml) from 10 to 17 dG with LD. Then, seven does were sacrificed at 18 dG, and three does were sacrificed at 28 dG. Blood samples were collected and sera were

prepared for hormonal assays of progesterone and calcitonin concentrations. Fetal-maternal placental biopsies were harvested for; gene expression of PR, histological study, immunohistochemical detection of trophoblasts, IDO, and macrophage cells**,** in addition to spiral arteries remodeling**.**

The results have showed that sera progesterone level, trophoblasts migration, IDO, and macrophage cells were significantly decreased ($p \leq 0.05$) in all treated groups compared to control groups. In contrast, the level of calcitonin was significantly increased ($p \leq 0.05$) in all treated groups compared to control groups. PR gene expression in the fetal-maternal placental had increased significantly ($p \leq 0.05$) 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 gestation where; it was increased significantly in the HD as compared to LD of treated groups, but it was significantly decreased in the 28^{th} day of gestation as compared to 10 and 18. Besides, the results observed that there was failure of implantation in treated group administrated with DEX from 5-9 dG, and resorption of embryos in treated group administrated with DEX from 10-17 dG, compared to control groups. Also, there was unremodeling in spiral arteries of treated group administrated with DEX from 5-9 dG, and early remolding in spiral arteries of treated group administrated with DEX from 10-17 dG, compared to control groups. Each of the effects (unremodeling and early remodeling) continued until 28 dG. The results also have observed that the three pregnant animals of each control group continued until term gave birth at 30-33 dG**.**

In conclusion, the current study confirmed that DEX has profound effects on all parameters; progesterone sera level, calcitonin sera level, PR, trophoblasts, IDO, macrophage cells, implantation and resorption of embryo, and on the spiral arteries remodeling during early, mid, and late gestation. Thus, its effects were continued throughout the gestation.

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List of Abbreviation

Abbreviation	*Description*
ACTH	**Adrenocorticotrophic Hormone**
Am	**Antimesometrial Side**
BAD	**BCL2 Associated Agonist**
CCL14	**C-C Motif Chemokine Ligand 14**
CCT	**Cell Column Trophoblast**
CD8+	**Cluster of Differentiation 8+**
CD68	**Cluster of Differentiation 68**
CHMP	**Committee for Medicinal Products for Human**
Ck7	**Cytokeratin7**
CRH	**Corticotrophin-Releasing Hormone**
CTBs	**Cytotrophoblasts**
CXCL6	**C-X-C Motif Chemokine Ligand 6**
CXCL8	**C-X-C Motif Chemokine Ligand 8**
D	**Decidua**
DEX	**Dexamethasone**
dG	**day of Gestation**
E	**Embryo**
ELISA	**Enzyme-Linked Immunosorbent Assay**
En	**Endometrium**
EVT	**Extravillous Trophoblast**
F	**Fibrinoid**
Fig	**Figure**
FSH	**Follicle-Stimulating Hormone**

G	Group
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GAPDHF	Glyceraldehyde 3-Phosphate Dehydrogenase Forward Gene
GAPDHR	Glyceraldehyde 3-Dhosphate dehydrogenase Reverse Gene
GC	Glucocorticoid
GC-GR	Glucocorticoid-Glucocorticoid Receptor
GLIZ	Gliotoxins Regulator
GR	Glucocorticoid Receptor
GnIH	Gonadotropins Inhibitory Hormone
GnRH	Gonadotropins Releasing Hormone
HAT	Histone Acetyltransferase
HD	High Dose
HDAC2	Histone Deacetylase 2
HCG	Human Chorionic Gonadotropin
HLA	Human Leukocyte Antigen
HPA	Hypothalamic-Pituitary-Adrenal Axis
HPG	Hypothalamic-Pituitary-Gonadal Axis
11β-HSD2	11β-Hydroxysteroid Dehydrogenase Type 2 Enzyme
IDO	Indolamine 2,3- Dioxygenase
IFNɤ	Interferon-ɤ
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-13	Interleukin-13
IUGR	Intrauterine Growth Restriction

KISS	**kisspeptin**
KISSR	**Kisspeptin Receptor**
L	**Lumen**
LD	**Low Dose**
LH	**Luteinizing Hormone**
M	**Mesometrial Side**
Me	**Mesometrium**
MHC	**Major Histocompatibility Complex**
MKP-1	**Mitogen-Activated Protein Kinase Phosphatase 1**
mTOR	**mammalian Target of Rapamycin**
My	**Myometrium**
N	**Number of Animals**
OD	**Optical Density**
P	**Perimetrium**
PARP	**Poly-ADP-Ribose Polymerase**
PC5/6	**Proprotein Convertases**
PDC	**Placental-Decidual co-Culture**
PIGF	**Placental Growth Factor**
Pl	**Placenta**
PR	**Progesterone Receptor**
PRf	**Progesterone Receptor Forward Primer**
PRL	**Placental Prolactin**
PRr	**Progesterone Receptor Reverse Primer**
PTH	**Parathyroid Hormone**
qPCR	**quantitative Polymerase Chain Reaction**

Qrt,RT-PCR	**Quantitative reverse transcription Real-Time-Polymerase Chain Reaction**
RDS	**Respiratory Distress Syndrome**
Rm	**Reichert Membrane**
SD	**Standard Deviation**
SLPI	**Secretory Leukocyte Protease Inhibitor**
Sm	**Smooth Muscle**
STAT3	**Signal Transducer and Activator of Transcription 3**
STB	**Syncytiotrophoblast**
T	**Trophoblast**
TGF- β	**Transforming Growth Factor-β**
TNFα	**Tumor Necrosis Factor-α**
TUNEL	**Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling**
Ul	**Uterine Lumen**
uNK	**Uterine Natural Killer**
VSMC	**Vascular Smooth Muscle Cell**
VEGF	**Vascular Endothelial Growth Factor**
VEGFR	**Vascular Endothelial Growth Factor Receptor**

Chapter One
Introduction

1: Introduction

Pregnant women with obstetric complications may intake synthetic glucocorticoids (GCs), such as dexamethasone (DEX) during pregnancy. Dexamethasone is used during pregnancy for prophylaxis against nausea and vomiting associated with pregnancy (Heitmann *et. al.,* 2015), prevention of preterm labor, the treatment of asthma, autoimmune diseases, and improvement of pregnancy outcomes in women with a history of recurrent miscarriage. Women at risk of preterm birth before 34 weeks of delivery are routinely given a course of antenatal DEX because there is good evidence that treatment reduces neonatal death, respiratory distress syndrome (RDS) and intraventricular hemorrhage (IVH) (Miracle *et. al.,* 2008). Similarly, to all drugs, DEX has negative side effects. Thus, it is risker when used at large doses and over long treatment periods. Previous study has shown that long-term DEX use to be associated with immunosuppression and increased risks of infection, hyperglycemia, hypertension, Cushing syndrome, and osteoporosis (Shaikh *et. al.,* 2012). Another data found that high doses of DEX were associated with an increased abortion rate, enhancement of the immunosuppressive effect of the decidua, decreased progesterone and 17β-estradiol levels, and reduced macrophages and uterine natural killer (uNK) cells (Ahmadabad *et. al.,* 2016). Furthermore, the increased exposure of fetuses to DEX in mid to late pregnancy may result in intrauterine growth restriction, postnatal hypertension, increased postnatal activity of the hypothalamus-pituitary-adrenal axis, and an increased risk of preterm labor (Michael and Papageorghiou, 2008).

In early pregnancy, various events occur in the uterus, especially the endometrium, leading to a receptive uterus. The receptive uterus provides a hospitable environment for blastocyst implantation and the establishment and

maintenance of pregnancy (Wu *et. al.,* 2021). The main events leading to the formation of a receptive uterus of embryo are the elevation of estrogen and progesterone levels, the proliferation and differentiation of endometrial cells, the increase of glands and blood vessels in the endometrium, the up-regulation of the expression of cell adhesion molecules on the surface of endometrial epithelial cells, and the secretion of cytokines and chemokines in the endometrium (Singh and Aplin, 2015). Despite extensive studies on the consequences of increased DEX exposure in mid to late pregnancy, relatively little is known regarding the significance of DEX in early pregnancy. The existing literature indicates that the use of DEX in early pregnancy is controversial. Previous study has demonstrated that DEX exerts actions that could be both negatively and positively influence on early pregnancy (Boomsma *et. al.,* 2012). Moreover, DEX can exert a range of positive effects that promote the establishment of early pregnancy such as suppressing uNK cells, stimulating human chorionic gonadotropin secretion, and promoting trophoblast proliferation and invasion. However, DEX can also exert a range of negative effects that impede the pregnancy such as inducing placental and/or decidual apoptosis, and impairing placental nutrient transport (Ahmadabad *et. al.,* 2016).

The Aim of Study

The purpose of the present study is to evaluate the effect of high and low dose of DEX on pregnancy outcomes in domestic does (*Oryctolagus cuniculus*) during early, mid, and late pregnancy using some hormonal, molecular, apoptotic, immunohistochemical, and histological techniques. It has been achieved through the following objectives:

1- Determination the concentration of maternal hormones (progesterone and calcitonin) by using Enzyme-Linked Immunosorbent Assay (ELISA) technique
2- Study of gene expression of uterine progesterone receptors (PR) by using Quantitative reverse transcription Real-Time-polymerase chain reaction (Qrt,RT-PCR) technique .
3- Detection of apoptosis in the cell wall of uterine spiral arteries by using Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) technique.
4- Identification and quantification the of placental cytokeratin7 trophoblastic cells, CD68 Macrophages, and indolamine 2-3, dioxygenase (IDO) by using immunohistochemical technique.
5- Characterization the level of implantation.

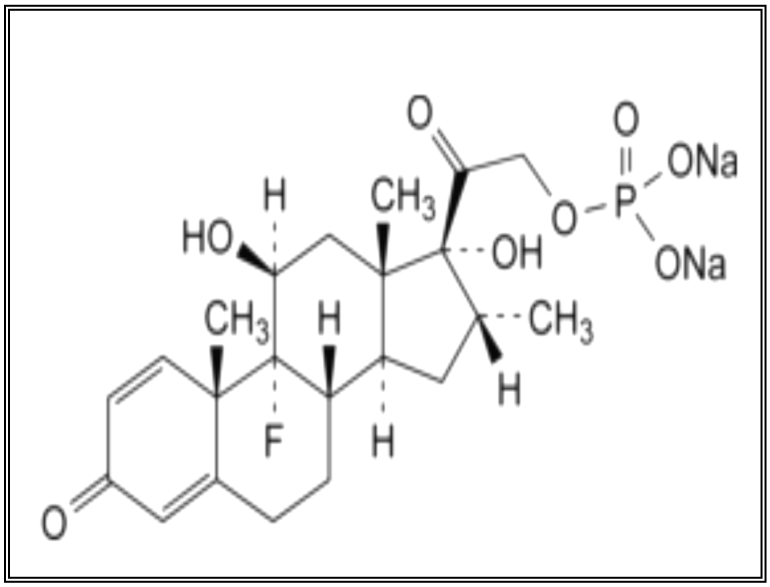
Chapter Two
Literature Review

2: Literature Review

2.1: Definition of Dexamethasone

Dexamethasone (DEX), as synthetic corticosteroid, is a potent anti-inflammatory and immunosuppressive medication that are frequently used during pregnancy for a variety of fetal and maternal indications. Pregnant women who are at risk of preterm delivery routinely receive corticosteroids at a late stage of gestation to stimulate fetal lung maturation and reduce neonatal complications associated with preterm delivery (Audette *et. al.,* 2011; Alsaad *et. al.,* 2019). Furthermore, maternal medical conditions including autoimmune diseases, allergies, asthma and dermatological conditions may necessitate the use of DEX throughout pregnancy (Tegethoff *et. al.,* 2009; Bartholomew *et. al.,* 2014). The Committee for Medicinal Products for Human Use (CHMP), (2020) has mentioned that DEX is also being used as prophylaxis and treatment of vomiting as part of anti- emetic medication. DEX is a mono-fluorinated glucocorticoid with anti-allergic, anti-inflammatory, immunosuppressive, and anti-proliferative effects. It is approximately 7.5 times more potent than prednisolone, and 25-30 times more potent than hydrocortisone.In addition, DEX is widely distributed in the organism with a degree of plasma protein binding of 70%, and it is distributed into several tissues, including the eyes, breast milk and crosses the placental barrier. Its effects are maintained for up to 24 hours, and detected in urine and feces, about 9 to 10% of the dose is excreted as unchanged dexamethasone in the urine. It is mainly metabolized in liver to 6-beta-hydroxydexamethasone, and other metabolites are also formed, e.g., 6-beta-hydroxy-20-dihydrodexamethasone. After short-term period administration of dexamethasone (less than 14 days) the hypothalamic-pituitary-adrenal axis (HPA) is

not suppressed. Therefore, treatment may be stopped immediately without having an adverse effect (Szabo and Winkler, 1995). Dexamethasone sodium phosphate molecular formula: C22H28FNa2O8P, and the molecular weight for DEX is 516.4. The chemical structure for DEX is shown in Fig. (2.1) (CHMP, 2020).



(Fig. 2.1): Chemical structure of DEX (CHMP, 2020)

2.2.: Pharmacological Properties of DEX

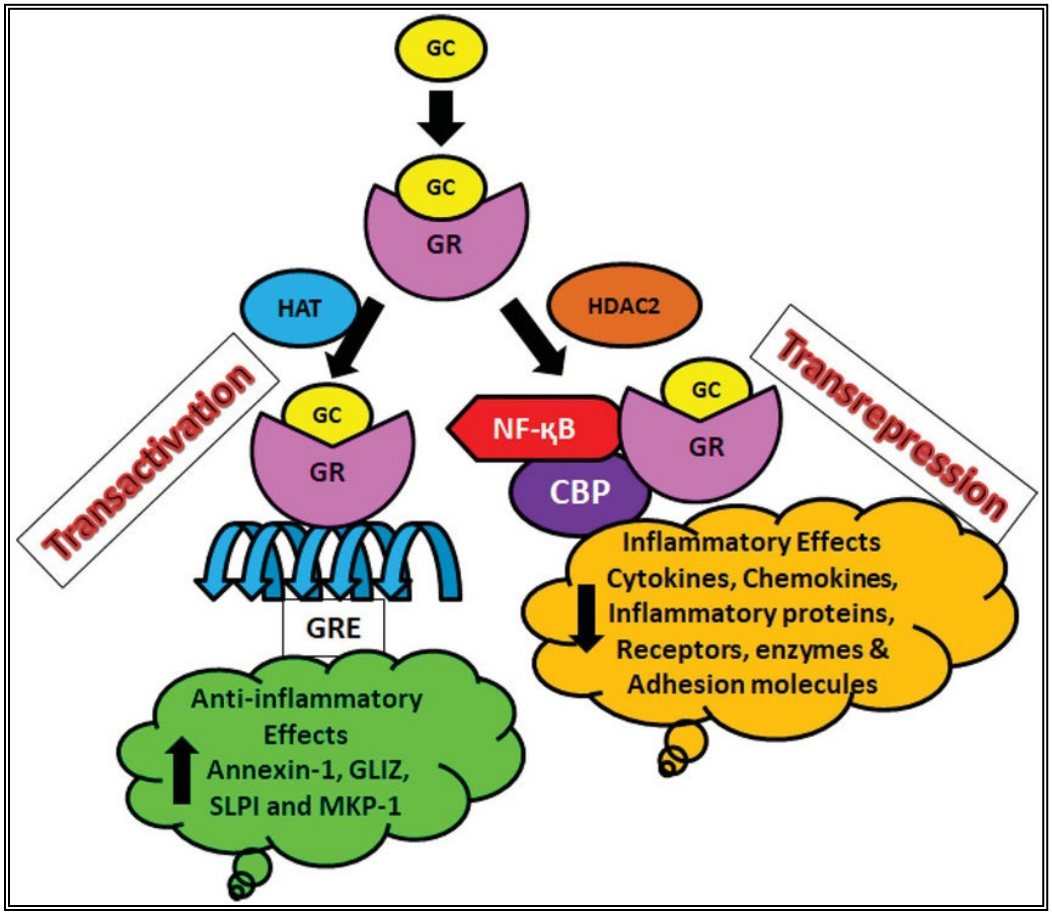
The plasma half-life refers to the time required for the initial concentration of a drug in the body to be reduced by half. An average plasma half-life of DEX is 4 hours following oral administration, and 4.6 hours following parenteral administration. Biological half-life represents the duration of influence on the target tissue, which is longer than the plasma half-life. Betamethasone and dexamethasone drugs are long-acting corticosteroids with biological half-lives ranging between 36 and 54 hours (Tegethoff *et. al.,* 2009; Alsaad *et. al.,* 2019). The degree and selectivity of protein binding corticosteroids affect its biological activity. Only the

non-protein-bound fraction is biologically active. Plasma binding of DEX is 75% which is constant across a wide concentration range (Alsaad *et. al.,* 2019). In addition, maternal plasma volume expansion leads to decreased albumin serum concentrations with gestation, consequently, protein-binding capacity reduces. For synthetic corticosteroids such as dexamethasone which binds to albumin specifically, the unbound biologically active proportion subsequently increases. As only the unbound fractions pass through the placenta, greater amounts reach the fetus as the pregnancy proceeds. In contrast, the fetal concentration of albumin increases to be equal to or even higher than concentrations of maternal albumin at term. Accordingly, the amount of corticosteroid bound to fetal albumin increases, making it less biologically active (Olufemi *et. al.,* 1991; Matata and Elahi, 2011).

2.3: Mechanism of Action of Dexamethasone

Dexamethasone has anti-inflammatory and immunosuppressive effects (Selvaraj *et. al.,* 2020), in general, attributed to the suppression of multiple mechanisms and pathways involved in the inflammatory response, leading to a decrease in pro-inflammatory chemokines and cytokines levels at injury site (Menezes-Carneiro *et al.,* 2014). Dexamethasone's immunosuppressive action results from its ability to inhibit normal protein synthesis and stabilization of cell membranes, which leads to inhibition of cell activation and leukocyte degranulation (Sauvage and Levy, 2013). The glucocorticoids molecule diffuses across cell membranes and binds to glucocorticoid receptors, which causes a conformational change in the receptor (Derendorf *et al.,* 2006; Williams, 2018). The receptor-glucocorticoid complex is able to move into the cell nucleus, where it dimerizes and binds to glucocorticoid response elements, furthermore, glucocorticoid response elements are associated with genes that either suppress or stimulate transcription,

these effects are called transrepression or transactivation, respectively (Raissy *et al.,* 2013). Ultimately, these agents inhibit transcription factors that control synthesis of pro-inflammatory cytokines and chemokines (Ye *et al.,* 2017). Another important effect is inhibition of phospholipase A2, which is responsible for production of numerous inflammatory mediators (Ericson-Neilsen and Kaye, 2014). In contrast, corticosteroids initiate upregulation of anti-inflammatory proteins such as annexin A1, a protein that reduces neutrophil migration to inflammatory sites, secretory leukocyte protease inhibitor (SLPI), an inhibitor of serine proteases, Mitogen-Activated Protein Kinase Phosphatase 1 (MKP-1), an inhibitor of macrophage differentiation, and Gliotoxins regulator (GLIZ), a suppresser of macrophage immune function, as shown in Fig. (2.2). Because corticosteroid action occurs intracellularly, its effects persist even when detection in the plasma is absent (Ingawale and Mandlik, 2020).



(Fig. 2.2): Mechanism of action of glucocorticoids: glucocorticoid after binding with glucocorticoid receptor forms GC–GR complex that exhibits two types of mechanism such as transactivation through its acetylation by HAT (histone acetyltransferase) and transrepression; through its deacetylation by HDAC2 (histone deacetylase 2). The transactivation mechanism it shows various anti-inflammatory effects whereas; transrepression mechanism exhibits numerous inflammatory effects (Ingawale and Mandlik, 2020).

2.4: Endogenous Corticosteroids Production in Pregnancy

Glucocorticoids, such as cortisol, are hormones involved in successful implantation of the embryo and appropriate growth and development of the fetus and placenta (CHMP, 2020). Endogenous cortisol production by the adrenal gland is controlled by the hypothalamic-pituitary- adrenal (HPA) axis. Corticotrophin-

releasing hormone (CRH) is released from the hypothalamus and acts on the anterior pituitary to release adrenocorticotrophic hormone (ACTH) which stimulates cortisol production and release from the adrenal gland (Whirledge and Cidlowski, 2017). Under non-stressed conditions, cortisol production is approximately 20 mg daily in adults. In addition to the normal production and control of cortisol secretion, physical or psychological stress (including infection, major trauma, and diseases) also is associated with increased levels of cortisol. There is clinical evidence that the daily production can increase to 150–200 mg during physical or mental stress (Gupta and Bhatia, 2008). Cortisol is produced in the adrenal gland through cholesterol metabolism, also a variety of other hormones, including mineralocorticoid (aldosterone) and androgens, are produced through the common pathway of cholesterol metabolism. The adrenal gland cortex consists of 3 functional zones. Cortisol is the product of cholesterol metabolism in the zona fasciculata. The mineralocorticoid is produced in the zona glomerulosa, whereas androgens and sex hormones, including progesterone, estrogens, and testosterone, are produced in the zona reticularis. A functioning and intact HPA axis is important for maintaining health and metabolic functions (Williams, 2018). The maternal HPA axis undergoes phenomenal regulatory changes during pregnancy, and one such change is a three-fold rise in cortisol levels compared to non-pregnant periods (Lindsay and Nieman, 2005). This is important in the development and maturation of fetal organs. Cortisol levels are at their highest during the third trimester corresponding to maximal fetal organ maturation (Fowden, 1995). The mechanisms that cause this increase in cortisol levels are: 1) Estrogen stimulation of corticosteroid binding globulin 2) Placental secretion of large amounts of CRH which, in turn, stimulates the maternal pituitary gland, thus elevating levels of ACTH and cortisol. In response to the above

mechanisms, maternal cortisol increases the placental synthesis of CRH, and a positive feedback is initiated (Fowden, 1995; Lindsay and Nieman, 2005). Moreover, as pregnancy progresses into the later trimesters, the response of the HPA axis to physical and mental stress is increased. The fetus is protected by the placental 11β-hydroxysteroid dehydrogenase type 2 enzyme (11β-HSD2), which is able to metabolize substantial amounts of cortisol. This placental barrier is functional throughout pregnancy, but factors such as maternal anxiety, infection and inflammation may compromise this protective mechanism (Alsaad *et. al.,* 2019).

2.5: Exogenous Corticosteroids

There are certain structural variations that exist between exogenous corticosteroids and their endogenous equivalents. Introduction of a double bond at the first and second carbon position of endogenous corticosteroids such as cortisone and hydrocortisone has produced prednisone and prednisolone. Similarly, structural modifications among exogenous corticosteroids have led to the production of more potent corticosteroids such as dexamethasone and betamethasone, which increase glucocorticoid activity and reduce mineralocorticoid activity. In obstetric practice, prednisolone, dexamethasone and betamethasone are the most commonly synthetic corticosteroids (Kemp *et. al.,* 2016).

2.6: Fetoplacental Transfer of DEX

The properties of the drug, placental characteristics and other maternal and fetal-related factors affect the permeability of drugs through the fetoplacental unit (Rubinchik-Stern and Eyal, 2012; Zhang *et. al.,* 2015). Placental 11β-HSD2 enzyme is a potent barrier that controls the passage of maternal glucocorticoids. It catalyzes the rapid conversion of bioactive glucocorticoids to their inactive metabolites.

However, this barrier is incomplete, and some maternal corticosteroids are able to cross the placenta to the fetus (Seckl, 2004). The metabolism profile of 11β-HSD2 enzyme and its transplacental passage differ considerably between endogenous and synthetic glucocorticoids (Braun *et. al.,* 2013). As poor substrates for 11β-HSD2, dexamethasone readily pass the placenta. The fetal plasma concentration of dexamethasone is 0.3–0.5 times that found in maternal plasma (Kurtoğlu *et. al.,* 2011). Placental enzyme saturation has been suggested, where the conversion capacity of 11β-HSD2 decreases with increasing corticosteroid concentrations (Staud *et. al.,* 2006). Maternal use of high doses of corticosteroids, or corticosteroid use for a prolonged period of time, may result in greater amounts of corticosteroids crossing the placental barrier, thus increasing fetal exposure (Alsaad *et. al.,* 2019).

2.7: Effect of DEX on Placental Development and Fetal Growth Restriction

The prenatal administration of DEX on dGs 7.5, 8.5, and 9.5 in pregnant mice negatively affected placental development and efficiency (Lee *et. al.,* 2012). Also, Newnham *et. al.,* (1999) showed that the infusion of DEX into ewes in late pregnancy resulted in decreased placental size. In another study, Ain *et. al.,* (2005) demonstrated that pregnant rats treated with DEX in the second half of gestation exhibited decreased placental weight. These data suggest that the administration of DEX during pregnancy can interfere with placental development.

Administration of synthetic glucocorticoids for threatened preterm labor has become standard obstetrical practice. Treatment with DEX promotes fetal lung maturation and decreases the incidence of respiratory distress syndrome in the neonate. Although DEX treatment has clear neonatal benefit, exogenous GC exposure may also adversely affect the growth of the fetus. Controlled clinical trials

have shown that multiple courses of DEX treatment are associated with decreased fetal weight, height, and head circumference (Audette *et. al.,* 2011). Also, a lower treatment burden (i.e., one or two courses) results in fetal growth restriction than greater treatment burden (i.e., four or more courses) (Vesce *et. al.,* 2014). Exposure to DEX retards also fetal growth in animal models. In rats, maternally administered DEX decreases birth weight and placental weight (Gluckman, 2001; Sugden and Langdown, 2001; McDonald *et. al.,* 2003). Low-dose of DEX given during pregnancy reduces birth weight and causes later hypertension, hyperglycemia, and hyperinsulinemia, in addition, Similar effects of DEX have been reported in sheep, pigs, and guinea pigs (Braun *et. al.,* 2009).

2.8: Effect of DEX on Progesterone

A normal pattern of progesterone and estradiol secretion is necessary for the establishment and maintenance of pregnancy. Progesterone not only plays multiple immunomodulatory functions, but also it supports uterine receptivity and quiescence (Lédée *et. al.,* 2016; Griesinger *et. al.,* 2019). The impairment of progesterone production is a risk factor for pregnancy loss. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) regulate levels of 17β-estradiol and progesterone during pregnancy. DEX modulate the hypothalamic-pituitary-gonadal (HPG) axis by directly inhibiting the release of gonadotropins releasing hormone (GnRH) from the hypothalamus and the synthesis and release of gonadotropins from the pituitary. Two neuropeptides, kisspeptin (KISS1) and gonadotropin-inhibitory hormone (GnIH), have opposing effects on GnRH release from the hypothalamus and are responsive to high levels of DEX. KISS1 exerts stimulatory effects on GnRH secretion through its receptor (KISS1R). KISS1 neurons in the preoptic area of the hypothalamus express glucocorticoid receptor (GR), suggesting that DEX can act

directly on the neurons (Takumi *et. al.,* 2012). Impairment of KISS1neurons represents a newly discovered mechanism by which DEX are able to suppress the HPG axis. Regarding GnIH, it inhibits the activity of GnRH neurons and KISS1 neurons (Ubuka *et. al.,* 2009; Whirledge and Cidlowski, 2017). Ahmadabad *et. al.,* (2016) observed that injection the pregnant BALB/c mice with DEX (5 mg/kg body weight per injection) on gestational days 0.5 to 4.5, and were sacrificed on 13.5 dG, results in decreased progesterone and 17β-estradiol levels, and an increased abortion rate. In vivo study, (Sowers *et. al.,* 1979) showed that the short-term administration of DEX suppressed the secretion of LH and FSH via a direct effect on the anterior pituitary. It has been well demonstrated that endogenous or exogenous excessed GCs, such as DEX, lead to the development of acquired hypogonadotropic hypogonadism. This complication is characterized by the decreased production of estrogen and progesterone by female ovarian follicular cells (Skalba and Guz, 2011). In addition, there was a progressive fall in the plasma concentrations of human chorionic gonadotropin (HCG) in pregnant following dexamethasone therapy (Ogueh *et. al.,* 1999). Maintenance of the corpus luteum is regulated by the HCG. This hormone has LH-like activity that protects the corpus luteum from regression and stimulates its production of progesterone (Przygrodzka *et. al.,* 2021).

2.9: Effect of DEX on Progesterone Receptor

In general, hormone receptors are regulated both by their own ligand (homologous regulation) and by other regulatory molecules (heterologous regulation). Endogenous glucocorticoids are known to be involved in the heterologous upregulation of several hormone receptors. DEX might have played an essential role in the upregulation of PR (Yahi *et. al.,* 2017a). Yahi *et. al.,* (2017b) found that DEX administered at (0.25mg/kg body weight) was caused significantly

decreased progesterone concentrations and caused abortion, while PR were upregulated in Yankasa sheep. The abortion could probably be due to decreased progesterone concentrations as a consequence of the adverse effects on placenta. The PR upregulation may be a compensatory mechanism to increase progesterone sensitivity. Another study observed similar phenomenon in rats (McDonald *et. al.,* 2003). The mechanism is probably through regulation of receptor mRNA levels by influencing increase in PR mRNA levels and gene transcription as reported by Kraus and Katzenellenbogen, (1993) in rats and Leavitt *et. al.,* (1977) in humans.

2.10: Effect of DEX on Calcitonin

Calcitonin is, a peptide hormone, synthesized and secreted by the parafollicular C cells of the thyroid gland. It's function to lower blood calcium (Khan and Farhana, 2021). In response to hypercalcemia, the C cells release calcitonin rapidly, which, in turn, lowers blood calcium by inhibiting osteoclast activity and thereby reducing bone resorption and remodeling (Zhu *et. al.,* 1998). Several investigators have found that serum calcitonin may be increased during gestation (Silva *et. al.,* 1981; Felsenfeld and Levine, 2015). It is known that parathyroid hormone (PTH) levels are increased in women during pregnancy. In this regard, intestinal absorption of calcium doubled in pregnancy, a fact they attributed, in part, to increased PTH (Silva *et. al.,* 1981). The increases in calcitonin may be important in the transfer of maternal calcium to the fetus and in the prevention of maternal bone loss. Calcitonin has an immediate effect on decreasing osteoclast activity and has been used for treatment of hypercalcemia (Felsenfeld and Levine, 2015; Schiffer *et. al.,* 2020). Furthermore, Wimalawansa, (2010) observed that DEX stimulates the production and secretion of calcitonin by enhancing the transcribed mRNA of calcitonin.

2.11: Effect of DEX on Implantation

The process of implantation consists basically of three stages: i) Apposition of the blastocyst to the uterine luminal epithelium ii) Stable adhesion to the epithelium iii) Penetration through the epithelium and basal lamina and invasion into the stromal vasculature. This phenomenon occurs during the window of implantation, a limited period of endometrial receptivity (Chimote *et. al.,* 2010). In human, the uterus becomes receptive during the midsecretory phase of the menstrual cycle (days 19 to 23) (Gnainsky *et al.,* 2010), but in rabbit, the window of implantation occurs at day 7-8 of gestation. In rabbit, on day 6 of gestation, blastocysts had fixed their position in the uterine lumen for implantation, but they had not yet attached to the uterine mucosa. On day 7 of gestation, implantation sites were macroscopically recognized as protrusions of the uterine wall. Histological examination revealed that ob-placental implantation occurs in the antimesometrial region on days 7 and 8, while placental implantation occurs in the mesometrial region on day 9 of gestation (Nishimura, 2001).

A large number of molecules mediate the implantation including adhesion molecules, cytokines, growth factors (Chimote *et. al.,* 2010). It is believed that cytokines may play an important role in the successful establishment of pregnancy not only by facilitating the attachment reaction of adhesion molecules but also by regulating the activity of invasive proteinases through the signaling cascades. It is hypothesized that implantation failure may be caused by abnormal cytokine expression by embryos and endometrium. While initial exposure to pro-inflammatory cytokines is necessary to stimulate invasion of the blastocyst and formation of new blood vessels at the time of implantation, prolonged exposure of pro-inflammatory cytokines to the pregnancy is detrimental. Thus, for pregnancy to

be successful a change in balance of secretion of cytokines from pro-inflammatory to anti-inflammatory cytokines must occur. Human endometrium is an active site of cytokine production and action and the embryo is able to communicate with the endometrium using the same cytokine receptor language (Tabibzadeh *et al.,* 1995; Chimote *et. al.,* 2010). DEX can suppress cytokines and the expression of cellular adhesion molecules such as integrins from trophoblast which is one of the most important of cellular adhesion molecules (Jeklova *et. al.,* 2008). Thus, DEX caused abortions in some species of animals like cattle, sheep and dog (Yahi *et. al.,* 2017a and Yahi *et. al.,* 2017b).

2.12: Embryo Resorption

High percentage of blastocysts are lost before or after implantation by resorption, because the resorption is very fast, therefor the embryo resorbed is unpredictable. It is initiated by embryo endogenous apoptosis without maternal interference (Drews *et. al.,* 2020). The dying cells express caspase 3 and expose signals, the phosphatidylserine flip, on their surface which in turn attract embryonic immune cells (Hochreiter-Hufford and Ravichandran, 2013). By sterile inflammation, the degeneration of embryonic lacunar trophoblast and maternal decidua capsularis (breaking down the fetal-maternal border) are occurred. Subsequently, the maternal neutrophils invade it to form purulent liquification which come in into the uterine lumen (Drews *et. al.,* 2020) and rapidly resorbed by macrophages that present in the muscular layer. The macrophages transform gradually into foam cells characterized by densely packed intracytoplasmic vacuoles (Abrahams *et. al.,* 2004).

2.13: Effect of DEX on Spiral Artery Remodeling

After blastocyst implantation, extravillous trophoblasts arisen from placental cytotrophoblast invade the maternal endometrium (decidua) and participate in spiral arteries remodeling ensuring that nutrients and oxygen meet fetal needs. During the remodeling, maternal spiral arteries are transformed from narrow, small, and high resistance vessels into wide, large, and low-resistance vessels leading to around 5-10-fold dilatation of the vessel for a considerable increase in blood supply to the fetus (Duan *et. al.,* 2016; Schiffer *et. al.,* 2020). Inadequate remodeling of the spiral arteries affects the development of the placenta as high velocity blood flow could cause mechanical stress on the tissue (Saghian *et. al.,* 2017). Absence of spiral arterial remodeling is a crucial factor in hypertension in pregnancy and low birth weight babies (Nobis *et. al.,* 2020). Failures in this process have been noticed in different pregnancy complications such as preeclampsia, intrauterine growth restriction, stillbirth, or recurrent abortion (Pollheimer *et. al.,* 2018). Spiral artery remodeling is carried out in two steps. In the first step, IL-6 and CXCL8 secreted by extravillous trophoblasts induce endothelial cell to express CCL14 and CXCL6, in which the CCL14 is a chemotaxis of both uNK cells and decidual macrophages, whereas CXCL6 is a chemotaxis of uNK cell (Choudhury *et. al.,* 2017). Both macrophages and uNK cells express angiogenic factors; vascular endothelial growth factor (VEGF), placental growth factor (PIGF), and angiopoetin-2 (Hazan *et. al.,* 2010). Also, both of them produce matrix metalloprotease 7 and 9 (MMP-7, 9) which are important in degradation of the endometrial extracellular matrix (Pollheimer *et. al.,* 2018) and trophoblast invasion (Cohen *et. al.,* 2006). Besides, MMP-9 of leukocytes destructs vascular extracellular matrix and basement membrane allowing more infiltration of leukocytes and dispersal of vascular smooth muscle cells

(VSMC) (Whitley and Cartwright, 2009). Loss of cellular anchoring to the extracellular matrix triggers a form of apoptotic cell death known as anoikis (Ingber, 2002). Therefore, disruption and apoptosis of VSMC layer and extracellular matrix transform the tightly coiled decidual spiral arteries into dilated sinusoids capable of increasing uterine blood volume (Smith *et. al.,* 2016). This step takes place in the presence of uNK cells and macrophages and in the absence of trophoblast cells (Robson *et. al.,* 2012). In the second step, the fetal trophoblasts invade the arteries, replace the endothelial cells, and eventually reline the vessels (Hazan *et. al.,* 2010; Faas and De Vos, 2017). Failure of spiral arteries remodeling is associated with intrauterine growth restriction, miscarriage, and preeclampsia (Choudhury *et. al.,* 2017). Long *et. al.,* (2013), and Kweider *et. al.,* (2014) observed that fetal exposure to high exogenous glucocorticoid levels produces intrauterine growth restriction (IUGR) and preeclampsia in multiple species, including sheep and non-human primates, by preventing the normal rise of VEGF (Hewitt *et. al.,* 2006; Ozmena *et. al.,* 2015). Previous studies suggest that DEX induced restriction of fetal and placental growth is mediated, in part, via inhibition of placental VEGF expression and an associated reduction in placental vascularization (Smink *et. al.,* 2003; Alagappan *et. al.,* 2005). Ain *et. al.,* (2005) mentioned that DEX administration led to a decrease in VEGF, VEGFR. and PIGF expression during pregnancy. Growth retarded fetuses seen in DEX treated pregnancies, may be a result of altered angiogenic factor expression of the placenta.

2.14: Effect of DEX on Trophoblasts

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. Retardation in trophoblast function brings about pregnancy complications such as recurrent spontaneous abortion, intrauterine growth retardation, and preeclampsia (Zong *et. al.,* 2016). After implantation the trophectoderm, the outermost cell layer of the blastocyst, gives rise to mononuclear cytotrophoblasts (CTBs) proliferating to form primary placental villi. Later, primary villi, consisting of proliferative CTBs, transform into secondary villi and mature tertiary villi (Pollheimer *et. al.,* 2018). These tree-like structures of the placenta display a surface area completely covered with multinuclear syncytiotrophoblasts (STBs). STBs are generated by cell fusion of villous CTBs and fulfill a vast range of functions such as production of pregnancy hormones, transport of oxygen and nutrients from the maternal blood stream to the growing fetus and clearance of fetal waste products (Aplin, 2010). However, early placental development and fetal growth occurs in the absence of maternal blood and oxygen and are likely supported by growth factors and proteins secreted from endometrial glands (Burton *et. al.,* 2010). Upon attachment of villi to the maternal decidua, the endometrium of the pregnant uterus, proliferative proximal cell column trophoblasts (CCTs), also called anchoring villi, differentiate into distal CCTs. Invasive extravillous trophoblast (EVTs) are formed upon detachment from the distal cell column. These cells deeply migrate into the maternal uterine mucosa (called “decidua” during pregnancy), and the first third of the underlying myometrium. Two types of EVTs can be discerned within the maternal uterine compartment, interstitial EVTs, colonizing the decidua, and endovascular EVTs, penetrating the maternal spiral arteries (Rango, 2008;

Pollheimer *et. al.,* 2018). Trophoblast invasion ensures the anchoring of the placenta within the uterine wall and the access of the fetus to the maternal vascular system to assure the supply of oxygen and nutrients. Matrix-type fibrinoid is a secretory product of invasive extravillous trophoblast cells, however, it is secreted in a polar fashion, embedding the secreting cells. Like basement membranes, it contains laminins, collagen IV, and heparan sulfate. In addition, oncofetal fibronectins, vitronectin, and i-glycosylated molecules but no collagens I, III and VII can be found. As a kind of "glue", it anchors the placenta to the uterine wall and seems to play an important role in maternal-fetal immune interactions at this particular site (Kaufmann *et. al.,* 1996). The invasive capacity of the EVT is strongest during the first trimester and declines afterwards (Schaaps *et. al.,* 2005). During pregnancy, the trophoblasts carrying the paternal HLA gene (the human leukocyte antigen system is a gene complex encoding the major histocompatibility complex (MHC) proteins) are excluded recognition by both cytotoxic T and NK cells, so it will be regarded as a homograft (Szekeres-Bartho, 2018). In addition, trophoblasts are reported to express many factors such as indoleamine 2,3- dioxygenase (IDO) that plays an important role in trophoblast proliferation and migration (Zong *et. al.,* 2016). However, trophoblast secretes transforming growth factor-beta (TGF-β) which induces monocyte differentiation into macrophages and enhance the phagocytosis capacity (Aldo *et. al.,* 2014; Yao *et. al.,* 2019). DEX administered during early placentation inhibits the proliferation and invasion of trophoblasts, and manifests reduced placental and fetal size (Szekeres-Bartho, 2018). On the other hand, DEX can exert a positive effect that would be expected to promote the establishment of early pregnancy such as promoting trophoblast proliferation and invasion (Michael and Papageorghiou, 2008; Boomsma *et. al.,* 2012).

2.15: Effect of DEX on Macrophages

It is critical that a balance between maintaining tolerance to the semi-allogenic fetus and upholding immune function for protection against infection is established to ensure a healthy pregnancy (Chambers *et. al.,* 2021). Macrophages are present in all tissues and derived from monocytes (Lash *et. al.,* 2016; Gordon and Plüddemann, 2017). Placental macrophages represent 20%–30% of the total body macrophages (Chambers *et. al.,* 2021). Estrogens cause macrophage chemotaxis to the endometrium indirectly (Vishnyakova *et. al.,* 2019); it stimulates fibroblasts to produce cytokines which attract macrophages, in addition, estrogen stimulates macrophage proliferation, enhances the phagocytic ability (Pepe *et. al.,* 2018), and polarization to M2 phenotype (Villa *et. al.,* 2015). Macrophages have multiple functions: spiral arteries remodeling by secreting angiogenic factors such as fibroblast growth factor and vascular endothelial growth factor (Lash *et. al.,* 2016), uterus tissues remodeling by synthesizing remodeling factors such as matrix metalloproteinases (Lash *et. al.,* 2016; Vishnyakova *et. al.,* 2019), and it has high phagocytic activity for up taking of debris of apoptotic cells resulting from remodeling of spiral arteries and decidua (Vishnyakova *et. al.,* 2019). The main function of macrophages is immune tolerance to protect the semi-allogeneic fetus from recognizing by maternal immune system (Vargas *et. al.,* 2016). 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 (Brown *et. al.,* 2014; Yang *et. al.,* 2017), by clearance of pathogens and sperms (Chambers *et. al.,* 2021). As pregnancy progresses, M2 macrophages become more plentiful in order to establish and

maintain tolerance to the fetus (Faas and De Vos, 2017; Yao *et. al.,* 2019). Macrophage phenotypes are governed by microenvironment milieu (Chambers *et. al.,* 2021). Lipopolysaccharide and T helper1 cytokines such as IFNɤ 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 (Yao *et. al.,* 2019). A wealth of data highlights that high levels of progesterone are critically required to switch the maternal immune responses toward tolerance (Shah *et. al.,* 2019). Progesterone promotes a tolerogenic profile on innate immune cell subsets, such as macrophages and dendritic cells, which is essential for successful uterine tissue remodeling and pregnancy maintenance (Solano, 2019). For example, *in vitro* stimulation with progestogens induces maturation of macrophages with M2 profile (Tsai *et. al.,* 2017), and prevents the differentiation of dendritic cells toward a mature phenotype (Xiu *et. al.,* 2016). High dose of DEX (5 mg/kg) was associated with enhancement of the immunosuppressive effect of the decidua, and a reduced frequency of macrophages and uNK cells in mice (Ahmadabad *et. al.,* 2016). Sugimoto *et. al.,* (2003) found that the anti-inflammatory effects of GCs such as DEX were mediated by the inhibition of leukocyte migration

2.16: Indoleamine 2,3-Dioxygenase (IDO)

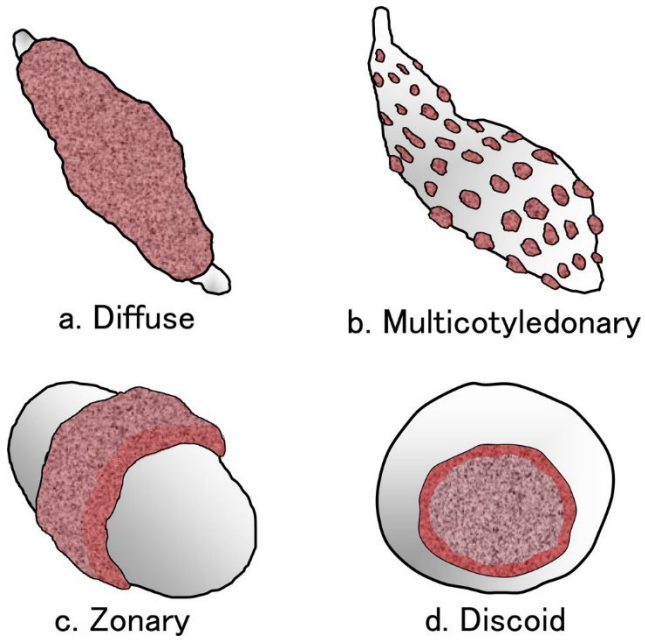
IDO is a tryptophan catabolizing enzyme that found in many tissues such as intestine and placenta, and expressed by different cell types as trophoblast (Zong *et. al.,* 2016; Krupa and Kowalska, 2021) antigen presenting cells (Hemmer *et. al.,* 2015; Schön, 2019). It inhibits proliferation of many cells including tumors, parasites (Hemmer *et. al.,* 2015) and T-cells (Antonelli *et. al.,* 2015; Betterle *et. al.,* 2019) by tryptophan depletion. Moreover, it prevents rejection of the allogeneic

fetus by depleting tryptophan and producing tryptophan metabolites which causes the apoptosis of maternal T cells and natural killer cells that are extremely sensitive to tryptophan shortage (Wu *et. al.,* 2018). Also, the tryptophan depletion-produced metabolites manifest immunosuppressive properties that play a vital role in prevention of allogeneic fetus rejection. It generates T regulatory (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 (YU e*t. al.,* 2017). However, Treg cells can induce IDO expression (Nishizawa *et. al.,* 2011). In pregnancy, IDO is highly expressed in the placenta, and the level of IDO expression may be associated with trophoblast cell proliferation and migration via the STAT3 signaling pathway (Zong *et. al.,* 2016).

2.17: Placentae Types

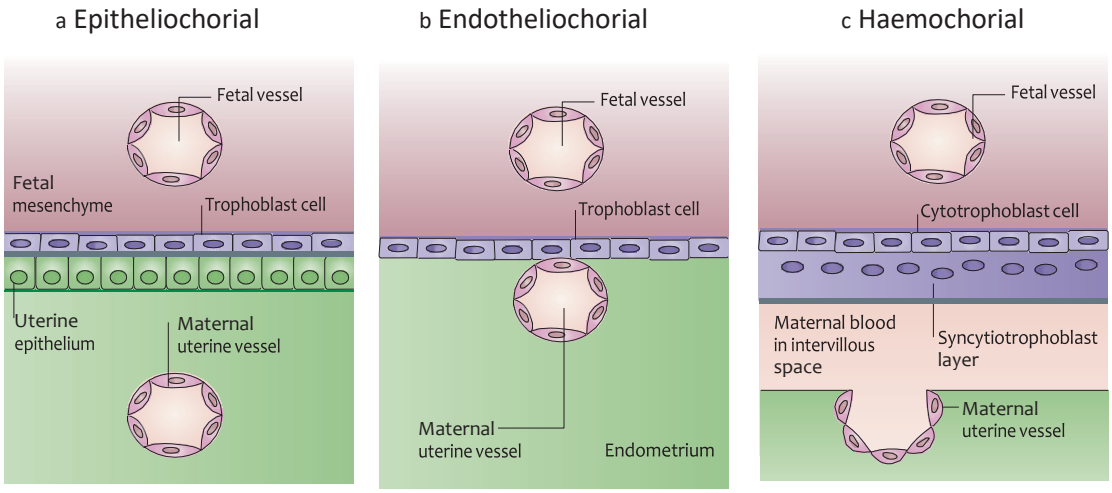
The placenta is one of the important organs that is the interface between the dam and developing embryos/fetuses. It is multifaceted organ that performs a number of important functions throughout gestation, and these functions include anchoring the developing fetus to the uterine wall, mediating maternal immune tolerance, O2/CO2 exchange, providing nutrients for the fetus and removing waste products during embryonic development. It also protects the embryo/fetus as a barrier against xenobiotics and releases a variety of steroids, hormones and cytokines. However, there is a diversity of placental morphologies in different animal species (Enders and Blankenship, 1999; Furukawa *et. al.,* 2014). Four main types are recognized according to the gross morphology of the placenta, Fig. (2.3). The basis of the classification is whether maternal-fetal exchange area is found over all the available surface of the chorionic sac or whether it is restricted. (a) Diffuse: this type of placenta occurs over the entire surface of the uterine luminal epithelium

with formation of folds/villi and is found in horses and pigs. (b) Multicotyledonary: this type of placenta is characterized by many spot-like placental regions of the endometrium known as caruncles (from 100 to 120 caruncles in sheep and 4 caruncles in deer). Intervening areas of the chorion are smooth and relatively avascular, and this type of placenta is found in ruminants. (c) Zonary: this type of placenta shows an intimate interdigitating contact zone that forms a belt around the chorionic sac, and this type of placenta is found in carnivores. (d) Discoid/bidiscoid: this type of placenta is characterized by a single (discoid) or double disc (bidiscoid), and interaction is confined to a roughly circular area. This type of placenta is found in primates, rodents and rabbits (Furukawa *et. al.,* 2014).



(Fig. 2.3): Classification of placental according to anatomical features (Furukawa et. al., 2014).

Classification based on histological structure, three main types are recognized according to the histologic relationship established between the chorion and uterine wall, as shown in Fig. (2.4). (a) Epitheliochorial type: this type is the most superficial placenta and lacks significant invasion of the uterine lining. Pockets of columnar trophoblasts are loosely applied to the maternal endometrial epithelium. No destruction or invasion of the maternal tissues occurs and no layers are removed. The epitheliochorial type is found in horses, pigs and ruminants (Carter and Martin, 2010). (b) Endotheliochorial type: the maternal uterine epithelium and connective tissue disappear after implantation, and the trophoblasts come into direct contact with the endothelial cells of maternal uterine blood vessels. The endotheliochorial type is found in carnivores (Enders and Carter, 2012). (c) Haemochorial type: this type is the most invasive placenta. All maternal tissue layers disappear through erosion, leading to direct connection between the chorion and maternal blood. There are haemomonochorial (primates), haemodichorial (rabbits), and haemotrichorial (rats and mice) placentas, with one, two and three trophoblast layers, respectively (Furukawa *et. al.,* 2014).



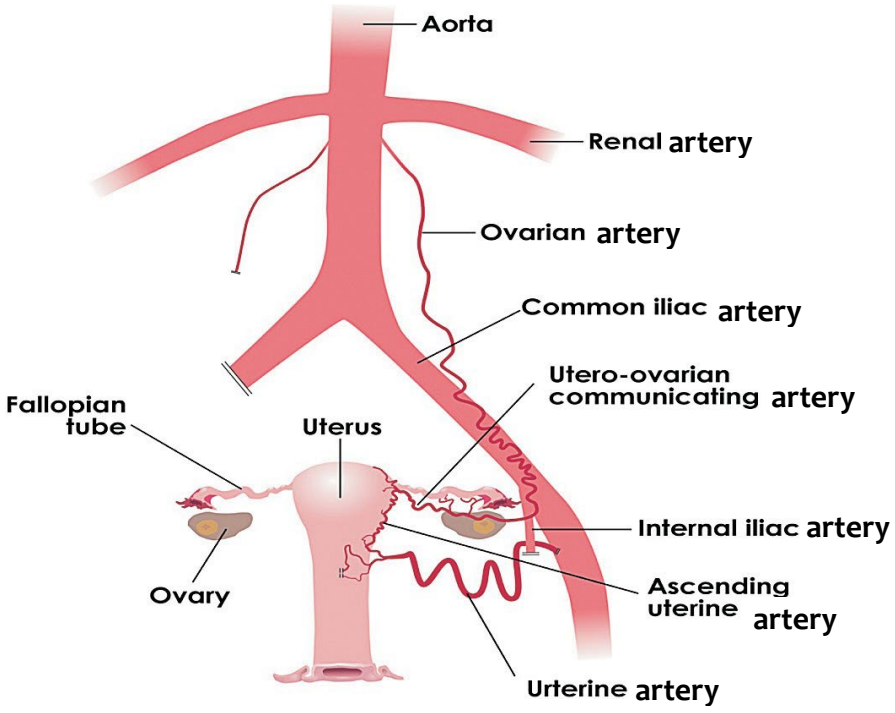
(Fig. 2.4): Classification based on histological structure. Three main types of placentation, showing the relationship between the fetal trophoblast cells and maternal blood. (a) Epitheliochorial: Trophoblast cells of the placenta are in direct apposition with the surface epithelial cells of the uterus but there is no trophoblast-cell invasion beyond this layer. (b) Endotheliochorial: The uterine epithelium is breached and trophoblast cells are in direct contact with endothelial cells of maternal uterine blood vessels. (c) | Haemochorial: Maternal uterine blood vessels are infiltrated by trophoblast cells causing rupture and release of blood into the intervillous space (Moffett and Loke, 2006).

2.18: Blood Supply of Uterus

Blood reaches the uterus primarily from the right and left uterine arteries, as shown in Fig. (2.5). Each uterine artery arises from the internal iliac artery or from one of its major branches or divisions (Gomez-Jorge *et. al.,* 2003). It joins the lateral borders of the uterus and grows rapidly in diameter in response to increasing blood flow during pregnancy, but do not appear to have the capacity to grow rapidly in length (Burbank, 2009). The ovarian arteries most commonly arise directly from the abdominal aorta but can originate as branches of the right or left renal arteries, from adrenal, or iliac arteries (Pelage *et. al.,* 2005).

The blood supply of the uterus is provided by the uterine and ovarian arteries. After entering the myometrium, the uterine arteries give rise to the arcuate arteries

which branch into the radial arteries. The radial arteries divide into the basal arteries which supply the basal portion of the endometrium (critical for endometrial regeneration after menstruation) and the spiral arteries which continue toward the endometrial surface. The term ‘‘spiral arteries’’ reflects the coiled appearance of these vessels, whose role is to supply blood to the upper functional layer of the endometrium. Each spiral artery supplies approximately 4–9 mm^2 of endometrial surface. Blood from the intervillous space is drained through the uteroplacental veins, whose openings are on the floor of the intervillous space. The uteroplacental veins undergo less dramatic changes than the arteries. Trophoblast may or may not invade the venous wall, even though free endoluminal trophoblast has been found in these vessels (Espinoza *et. al.,* 2006).



(Fig. 2.5): Shows the extrinsic arteries of the left side of the uterus, including the aorta and renal and ovarian arteries arising from the abdominal aorta, the uterine artery arising from the internal iliac artery, and the utero-ovarian communicating artery. Symmetrical arteries are present on the right side but are not shown (Burbank, 2009).

Chapter Three
Materials and Methods

3: Materials and Methods

3.1: Materials

3.1.1: Equipment and Apparatus

The instruments and their suppliers that used in the current study are listed in table (3.1).

Table (3.1): Instruments and their suppliers.

No.	Equipment and tools	Suppliers
1	Centrifuge	Gemmy- Taiwan
2	Collection Tube	IBI Scientific- USA
3	Digital Camera	Genex- Germany
4	Disposable Tip	Indiamart- India
5	Dissecting Set	Elphor- Germany
6	Drill	SCM- China
7	Electrical Oven	Memmert- Germany
8	Electronic Micropipette	Slamed- Germany
9	Eppendorf Tube	Firatmed- Turkey
10	Petri Dish	Sun Trine- China
11	Gel and Activator Tube	Lassco- India
12	Histology Slide	Happy Science- China
13	Humidity Chamber	Histo-Line- Italy
14	Incubator	Memmert- Germany
15	Latex Glove	HiGeen- Gordan
16	Light Microscope	Genex- USA
17	Medical Syringe	Shengguang- China

18	Microplate Reader	Biotek- USA
19	Microtome	RWD- China
20	Multi-Channel Dispenser Micropipette	Slamed- Germany
21	Plain Tube	Vacumed- Italy
22	Positively Charged Slide	Huida- China
23	RB Column	IBI Scientific- USA
24	Real-Time PCR Cycler	Qiagen- USA
25	Refrigerator	Vestel- Turkey
26	Surgical Blade	Aspen Surgical- USA
27	Vortex Mixer	Gemmy- Taiwan
28	Water Path	Raymond- England

3.1.2: Chemicals

The chemicals and their manufacturers used in this study are listed in Table (3.2).

Table (3.2): Chemicals and their manufacturers used in this study.

No.	Chemicals	Manufacturers
1	Absolute Ethanol	Scharlau- Spain
2	Apoptosis Assay (TUNEL) Kit	MyBioSource- USA
3	Chloroform	BDH- England
4	Dexamethasone Acetate	Arpimed- Ukraine
5	Di-sodium Hydrogen Phosphate (Na2HPO4)	Merck- Germany
6	Distyrene Plasticizer Xylene (DPX)	CellPath- England

7	Eosin	BDH- England
8	Formaldehyde (37%)	Merck- Germany
9	GENEzol^{TM} TriRNA Pure Kit	Geneaide- Taiwan
10	Goat Serum	Thermo Fisher- USA
11	Hematoxylin	BDH- England
12	Hydrogen Peroxide (H2O2)	Sigma-Aldrich-Germany
13	Methanol (100%)	Scharlau- Spain
14	Mouse/Rabbit PolyVue Plus^{TM} HRP/DAB Detection System	Diagnostic-BioSystems-Netherlands
15	Normal Saline	Pioneer- Iraq
16	Paraffin Wax	GCC- England
17	Phosphate Buffer Saline (PBS)	Merck- Germany
18	Positively Charged Slides	PathnSitu- USA
19	Primers and Housekeeping Genes	Macrogen- South Korea
20	Rabbit Anti-CD68 Antibody Kit	MyBioSource- USA
21	Rabbit Anti-Cytokeratin7 Antibody Kit	MyBioSource- USA
22	Rabbit Anti-IDO Antibody Kit	MyBioSource- USA
23	Rabbit Progesterone and Calcitonin ELISA Kits	MyBioSource- USA
24	RNA Later Solution	SigmaAldrich- Germany
25	Sodium Di-hydrogen Phosphate	Merck- Germany
26	Trans Script® Green One-Step qRT-PCR Super Mix kit	Trans- China
27	Tris Buffered Saline 10X (TBS)	Merck- Germany
28	Xylene	Scharlau- Spain

3.2: Laboratory Animals

Sixty domestic does (*Oryctolagus cuniculus*) (female rabbit) were used in the present study. Does age were ranged between 1-1.5 year and weigh about 1500 g. They were managed in the animal house of University of Babylon / College of Science / Biology Department under normal conditions:12 hours of daylight and 12 hours of darkness, and at temperature 22-27˚ C. Animals were adjusted for four weeks before the commencement of the experiment. To obtain pregnancies, one female was caged overnight with one male for a successful pregnancy. The presence of a vaginal plug was considered as 1$^{st.}$ day of pregnancy.

3.3: Animal Groups

The animals that used in the present study were divided into six groups (see Fig. 3.1):

Group 1 (G1): Ten does (as a control group) were daily subcutaneous injected by (1ml) from 5 to 9 dG with normal saline (N.S.) (0.9%). Then, seven does were sacrificed at 10 dG, and three does were continued in gestation until term.

Group 2 (G2): Ten does were daily subcutaneous injected by (1ml) from 5 to 9 day of gestation (dG) with high dose (HD) of DEX (1.125 mg), then, seven does were sacrificed at 10 dG, and three does were sacrificed at 28 dG.

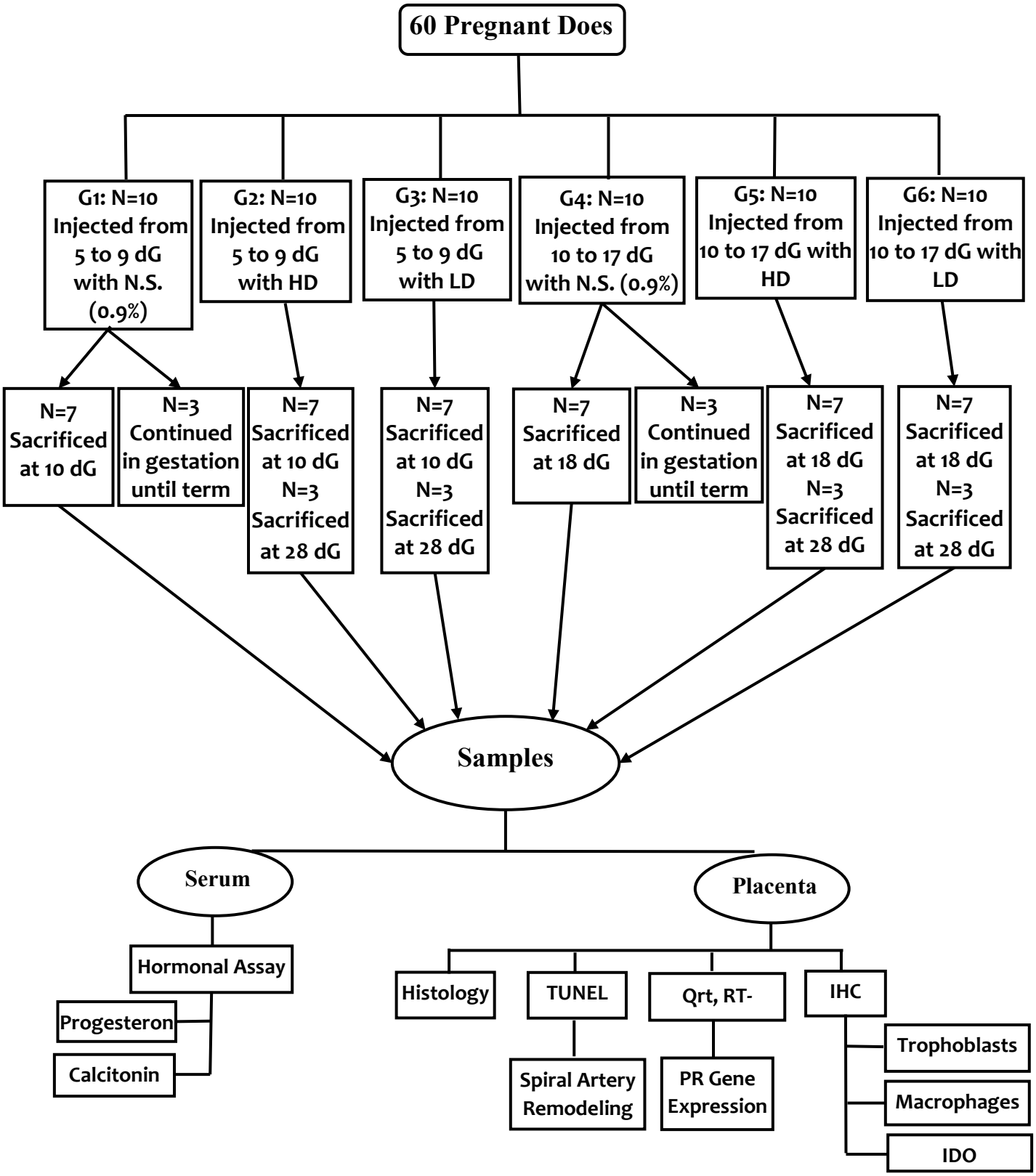
Group 3 (G3): Ten does were daily subcutaneous injected by (1ml) from 5 to 9 dG with low dose (LD) of DEX (0.562 mg). Then, seven does were sacrificed at 10 dG, and three does were sacrificed at 28 dG.

Group 4 (G4): Ten does (as a control group) were daily subcutaneous injected by (1ml) from 10 to 17 dG with N.S. (0.9%). Then, seven does were sacrificed at 18 dG, and three does were continued in gestation until term.

Group 5 (G5): Ten does were daily subcutaneous injected by (1ml) from 10 to 17 dG with HD of DEX (1.125 mg), after that, seven does were sacrificed at 18 dG, and three does were sacrificed at 28 dG.

Group 6 (G6): Ten does were daily subcutaneous injected by (1ml) from 10 to 17 dG with LD of DEX (0.562 mg). Then, seven does were sacrificed at 18 dG, and three does were sacrificed at 28 dG.

Finally, five ml of blood samples were drawn by heart puncture and allowed to clot for 15 minutes at room temperature. Sera were collected by centrifugation at 3000 rpm for 10 minutes. Sera were used for hormonal assays (Progesterone and Calcitonin) using Enzyme-Linked Immunosorbent Assay (ELISA) technique. Fetal-maternal placental biopsies were harvested for; gene expression of PR using Quantitative reverse transcription Real-Time-polymerase chain reaction (Qrt, RT-PCR) technique, histological study, immunohistochemical (IHC) assays of trophoblasts, IDO, and macrophages using anti-cytokeratin7 (Ck7), anti-IDO, and anti-CD68 antibody kits, respectively**,** in addition spiral arteries remodeling using Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) technique**.**



(Fig. 3.1): Experimental design of the study.

3.4: Preparation of DEX Doses

The high dose (HD) (1.125 mg\ml) (Hoffman *et.al.,* 1984) and low dose (LD) (0.562 mg\ml) were prepared as shown in Equation (1):

$$HD = \frac{\text{animal weigh (Kg)} \times \text{dose of Hoffman (mg)}}{\text{animal weigh of Hoffman (Kg)}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$HD = \frac{1.5 \times 3}{4} = 1.125\ mg$$

$$LD = \frac{1.125}{2} = 0.5625\ mg$$

Each ampule of DEX Sodium phosphate has 8mg\2ml.

To obtain 1.125 mg\ml and 0.5625 mg\ml, Equation (2) (Skoog *et. al.,* 2013) has been used:

V1 (ml) × C1 (mg\ml) = V2 (ml) × C2 (mg\ml) …….... (2)

2 ml × 8\2 mg\ml = X × 1.125 mg\ml or 0.5625 mg\ml

X = 7.1 ml or 14.22 ml

Finally, added 7.1 ml or 14.22 ml of normal saline per ampule, now, each ml has 1.125 mg or 0.5625 mg of DEX, respectively.

3.5: Hormonal assay: Enzyme-Linked Immunosorbent Assay (ELISA)

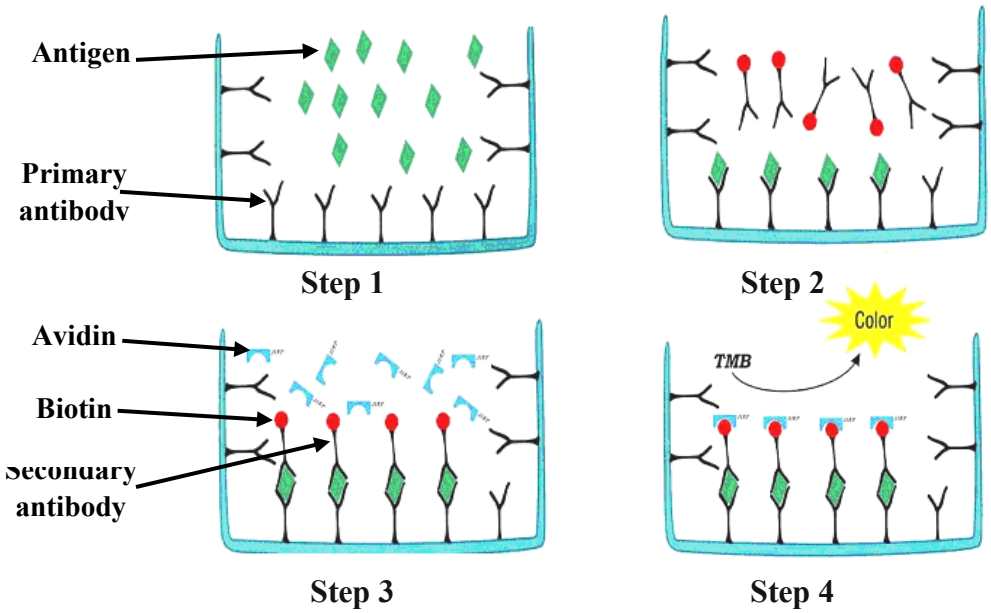
3.5.1: Blood Samples Collection

Five ml of blood samples were drawn gel and activator tube and allowed to clot for 15 minutes at room temperature. Sera were collected by centrifugation at 3000 rpm for 10 minutes and stored at -20 until assayed for progesterone and calcitonin using Rabbit Progesterone (PROG) and Calcitonin ELISA Kits (My

BioSource, San Diego, CA, USA) as the manufacture's instruction. For progesterone assay, the minimal detectable concentration (Sensitivity) was 0.03ng/ml, while for calcitonin assay, the minimal detectable concentration was 5 pg/ml.

3.5.2: Assay Principle

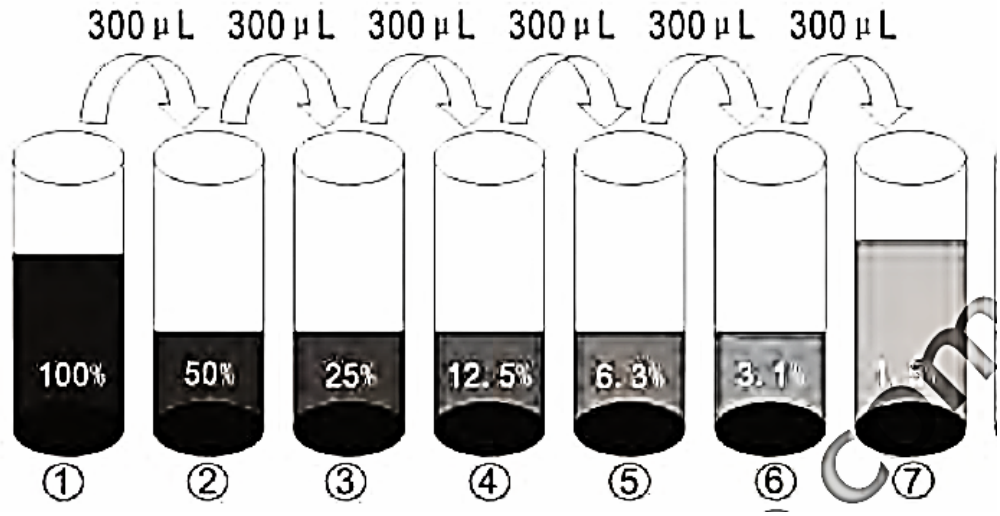
These kits, Enzyme-Linked Immunosorbent Assay (ELISA), employ Double Antibody Sandwich Technique. The principle of Double Antibody Sandwich is based on characteristics of the tested antigen with two valances which can identify the coated antibody and the detection antibody at same time. The plate has been pre-coated with Rabbit PROG or calcitonin antibody (monoclonal antibody). PROG or calcitonin present in the sample is added and binds to antibody coated on the wells, then biotinylated Rabbit PROG or calcitonin antibody (polyclonal antibody) is added and binds to PROG or calcitonin in the sample. Then avidin-horseradish peroxidase (avidin-HRP) is added and binds to the biotinylated PROG or calcitonin antibody. After incubation unbound avidin-HRP is washed away during a washing step. Color reagent TMB (chromogen substrate) is then added and blue color develops in proportion to the amount of Rabbit PROG or calcitonin. The reaction is terminated by addition of acidic stop solution (the blue color turns into yellow under the action of acid) and absorbance is measured at 450 nm, as noted in Fig. (3.2).



(Fig. 3.2): Schematic diagram of the Rabbit PROG and calcitonin ELISA kits.

3.5.3: Reagent Preparation

1. **Standard:** 1.0 ml of standard diluent was added into Rabbit PROG or calcitonin standard. The standard was allowed to sit for 30 min. prior to making dilutions. After the standard completely dissolved, it was mixed slightly and marked label on the tube with (1). Duplicate standard diluents were prepared by serially diluting the standard stock solution (1) with standard diluent to produce (100, 50, 25, 12.5, 6.25, 3.12, 1.56 ng/ml), with respect to progesterone, and (1000, 500, 250, 125, 62.5, 31.2, 15.6 pg/ml), with respect to calcitonin, To prepare standard dilutions: seven clean tubes were taken and labeled with (2), (3), (4), (5), (6), (7). 300µl of standard diluent was added into each tube. 300µl was pipetted out from tube (1) to tube (2) and mixed well. 300µl was pipetted out from tube (2) to tube (3), and mixed well. Steps above were repeated up to tube (7), as shown in Fig. (3.3).



(Fig. 3.3): Standard dilutions.

2.**Wash Buffer(1x):** The concentrated washing solution was diluted with double distilled water in a ratio of (1:25).

3. **Biotinylated Antibody:** The antibody diluent was added to the concentrated biotinylated antibody in a ratio of (1:100).

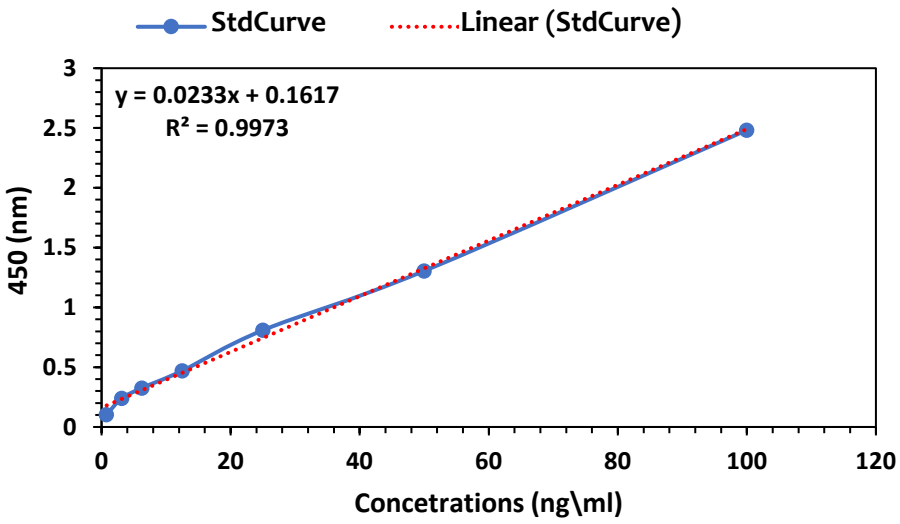
4. **Enzyme-Conjugate:** The concentrated enzyme-conjugate was added to the enzyme-conjugate diluent in a ratio of (1:100).

5. **Color Reagent:** The color reagent A was added to the color reagent B in a ratio of (9:1).

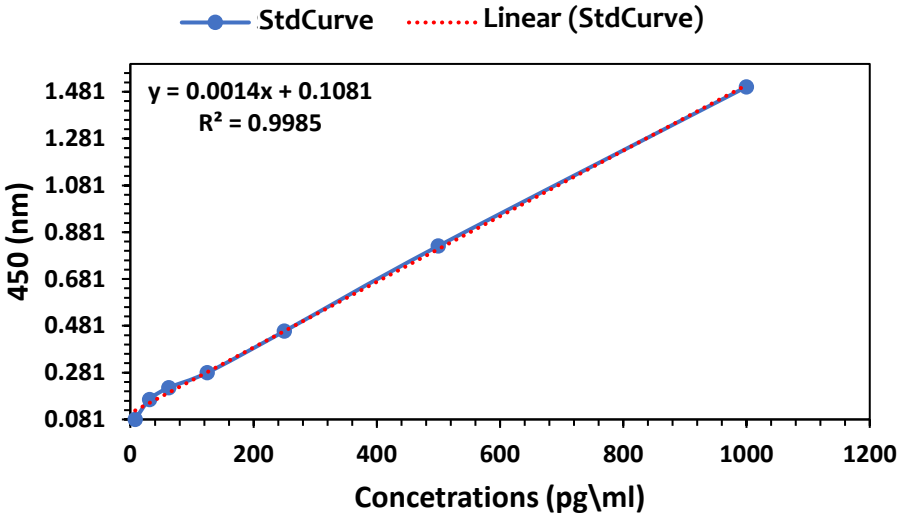
3.5.4: Procedure

1. Samples were brought to room temperature before 20 min. of starting the assay.
2. The samples were Centrifuged before use.
3. One hundred µl of standard solutions were added to standard wells, and 100µl of samples were added to sample wells. It was incubated at 37°C for 90 min.

4. The plate was washed 2 times for 30 sec. with wash buffer.
5. One hundred µl of the biotinylated PROG or Calcitonin antibody was added to sample wells, and incubated at 37℃ for 60 min.
6. The plate was washed 3 times for 30 sec. with wash buffer.
7. One hundred µl of enzyme-conjugate was added to sample wells and standard wells, mixed well, and incubated for 30 min. at 37°C.
8. The plate was washed 5 times for 1 min. with wash buffer.
9. One hundred µl of color reagent was added to each well. Then, the plate covered with a sealer was incubated at 37°C. When the color for high concentration of standard became darker and color gradient appeared, 100µl of color reagent C (stop solution) was added to each well, the blue color was changed into yellow immediately.
10. The optical density (OD value) of each well was determined immediately using a microplate reader set to 450 nm within 10 mins. after adding the stop solution, as shown in Fig. (3.4) and (3.5).



(Fig. 3.4): OD standard curve of progesterone.



(Fig. 3.5): OD standard curve of calcitonin.

3.6: PR Gene Expression Assay

3.6.1: Tissues Collection

Fetal-maternal placental tissues (100-250 mg) were excised and dissected, then placed in 3 ml RNA later solution (Sigma-Aldrich, Merck, Darmstadt, Germany) to stabilize and protect the RNA for 24 hours at room temperature. All tissues samples were stored at -20 C until used for PR estimation by Qrt-RT-PCR as the manufacturer's instructions.

3.6.2: Solution Preparation

The DNase I Solution**:** (5 µl) of DNase I was added to (45 µl) of DNase I Reaction Buffer.

3.6.3: RNA Purification Protocol Procedure

3.6.3.1: Tissues Homogenization and Lysis

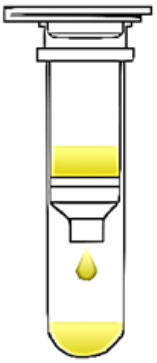
Total fetal-maternal placental RNA was extracted using GENEzol^{TM} TriRNA Pure Kit (Geneaide -New Taipei, Taiwan) as the manufacturer's instructions.

1. The tissues were removed from RNA later solution.
2. The tissues were washed with phosphate buffer solution and centrifuged at 10000 rpm for 1 min., 3 times.
3. The tissues and 700 µl of GENEzol™ Reagent were transferred to Eppendorf tubes, and grinded with drill until the tissue homogenized.
4. The homogenized tissues were incubated for 5 min. at room temperature.

3.6.3.2: RNA Binding

1. The samples were centrifuged at 10000 rpm for 1 min. to remove cell debris, then, the clear supernatants were transferred to new Eppendorf tubes.
2. One volume of absolute ethanol was added directly to 1 volume of sample mixture with GENEzol™ Reagent.

3. It was mixed well by vortex mixer, then, an RB column was placed in a collection tube, Fig. (3.6).
4. Seven hundred µl of the sample mixture was transferred to the RB column. It was centrifuged at 10000 rpm for 1 min., then, discarded the flow-through.



(Fig. 3.6): RB Column in a Collection Tube.

3.6.3.3: DNA Digestion

1. Four hundred µl of wash buffer was added to the RB column, then, centrifuged at 10000 rpm for 1 min.
2. The flow-through was discarded, and the RB column was placed back in the collection tube.
3. The DNase I solution was gently pipetted, then, 50µl was added into the RB column.
4. The column was incubated for 15 min. at room temperature.

3.6.3.4: RNA Wash

1. Four hundred µl of Pre-Wash Buffer was added to the RB column, then, centrifuged at 10000 rpm for 1 min.
2. The flow-through was discarded, then, the RB column was placed back in the collection tube.

3. Six hundred µl of Wash Buffer was added and centrifuged at 10000 rpm three times for 1 min., each time.
4. The flow-through was discarded, then, the RB column was placed back in the collection tube.
5. It was centrifuged at 10000 rpm for 3 min. to dry the column matrix.

3.6.3.5: RNA Elution

1. The dry RB column was placed in a clean Eppendorf tube.
2. One hundred µl of RNase-free Water was added into the column matrix.
3. It was let stand for 10 min. to ensure the RNase-free Water is completely absorbed by the matrix.
4. It was centrifuged at 10000 rpm for 1 min. to elute the purified RNA.
5. The samples were stored at (-20) until estimated for PR gene expression using Qrt, RT-PCR.

3.6.4: PR Gene Expression Assay: Quantitative Reverse Transcription Real-Time Polymerase Chain Reaction Qrt, RT-PCR

3.6.4.1: Assay Principle

Gene expression levels of PR were performed by Qrt, RT-PCR using Trans Script® Green One-Step qRT-PCR SuperMix kit (TRANS-Beijing, China) according to the manufacture's manual and Real-time PCR handbook, (2012). This kit firstly synthesizes first-strand cDNA with RNA as templates using reverse gene-specific primers, and then performs qPCR with the synthesized cDNA as templates using both forward and reverse gene-specific primers to achieve one step from reverse transcription to qPCR in a single tube.

3.6.4.2: Primers and Housekeeping Genes Design

The primers and housekeeping genes (an internal control) [Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene] designed by Humanizing Genomics macrogen (Biotechnology companies, South Korea). PR forward (PRf) and reverse (PRr) primers sequences are 5'-AGACCTCCAGAAAAGGACAGC-3' and 5'-CAACACCCCTTTGGTAGCG-3', respectively. GAPDHF forward (GAPDHF) and reverse (GAPDHR) genes sequences are 5'-TGGTGAAGGTCGGAGTGAAC-3' and 5'-ATGTAGTGGAGGTCAATGAATGG-3', respectively.

Stock solution prepared by 300 µl of distilled water was added for each primer and GAPDH gene to obtain 100 pmol / µl.

3.6.4.3: Procedure

1. Five µl was taken from each PRf and PRr primer, and from each GAPDHf and GAPDHr housekeeping gene. Then, it was diluted with (90µl) of distilled water to obtain 10 pmol\ µl for each primer and housekeeping gene.
2. The Qrt, RT-PCR reactions amplified PR gene and GAPDH gene were run in a Real-Time PCR cycler (Qiagen- USA). these reactions were carried out in a 20µl volume that consists of:

   (1µl) of RNA template

   (1µl) of diluted primer or housekeeping gene

   (10µl) of Green One-step qPCR SuperMix (SYBR® Green dye)

   (0.5µl) of One-Step RT/RI Enzyme Mix (Taq Polymerase)

   (7.5µl) of RNase-free water

The amplification conditions of Qrt, RT-PCR are illustrated in Table (3.3).

Table (3.3): The amplification conditions of Qrt, RT-PCR.

conditions	Reasons
45 ˚C for 10 min.	converting RNA to cDNA
94 ˚C for 30 sec.	Deactivation of reverse transcriptase
95 ˚C for 10 sec.	Denaturation
60 ˚C for 30 sec.	Annealing and elongation
40 cycles	Cycles number
114 min.,	Total time

3. 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. and Fig. (3.8) has shown melting curve.

3.6.5. Quantification

Expression Fold differences were calculated as shown in Equation (1), (2) and (3):

Target concentration of sample (PR gene) = Cts

Target concentration of housekeeping gene = Cth

$\Delta Cts = Cts - Cth$……. (1)

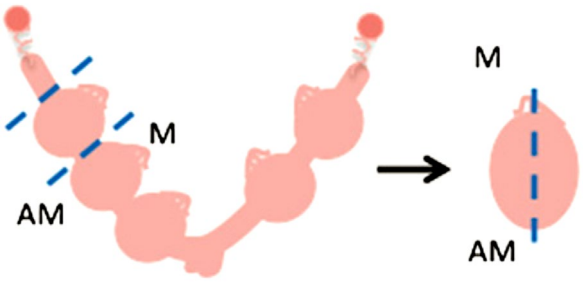
$\Delta\Delta Cts = \Delta Cts$ - mean of ΔCts of control group……. (2)

Expression Fold difference = $2^{-\Delta\Delta Cts}$…….…..…..… (3)

3.7: Histological Study

3.7.1: Tissue Collection and Processing

Does were anaesthetized with chloroform and the abdominal contents were exposed by a mid-line incision. Fetal-maternal placental tissues were excised and cross section was done as shown in Fig. (3.7).



(Fig. 3.7): Shows how fetal-maternal placental tissues were excised and cross section was done, M= Mesometrial side, AM= Anti-Mesometrial side (Felker and Croy, 2016).

3.7.2: Solution Preparation

Neutral Buffered Formalin 10% (NBF) (1 liter): Double distilled water (900 ml), disodium hydrogen phosphate (Na2HPO4) (6.5 grams), sodium dihydrogen phosphate (NaH2PO4.H2O) (4 grams), and formaldehyde 37% (100 ml). The pH was adjusted to be 7 with 1N NaOH or 1M HCl.

3.7.3: Histological Processing and Staining

The histological study was done according to the Suvarna *et. al.,* (2013), Bolon (2014), and Mescher (2018).

3.7.3.1: Histological Processing

1. Fixation: To preserve tissue structure and prevent degradation by enzymes released from the cells or microorganisms, tissues were placed after removal from

the body in stabilizing or cross-linking solution called neutral buffered formalin 10% (NBF) for 24 hours.

2. Dehydration: The tissues were placed in a series of ascending grades of alcohol solutions (70% ethanol 1 h., 80% ethanol 1 h., 90% ethanol 1 h., 100% ethanol 1 h., 100% ethanol 1 h.) which remove all water.
3. Clearing: Xylene was used as a clearing agent in order to remove of dehydrating solutions and to make the tissue miscible with the embedding media. Two changes of xylene were used for 1.5 h. each time.
4. Infiltration: The fully cleared tissue was then placed in three changes of melted paraffin at 60°C, for 1 h., each time.
5. Embedding: The paraffin-infiltrated samples were immersed in a small mold with melted paraffin at room temperature and allowed to harden.
6. Sectioning: The blocks were cut by microtome at 4 µm thickness. The tissue sections were floated on warm water at 55°C to be flattened, then taken by slides.

3.7.3.2: Histological Staining

1. Dewaxing: Slides were heated in oven at 60°C for 15 min., then put in xylene for 10 min.
2. Rehydration: The tissues were transferred to 100% ethanol two times for 5 min., each time.
3. Staining: The cells nuclei of tissues were stained with hematoxylin for 5 min., then, washed with tap water for 2 min. Thereafter, the extracellular matrices and cytoplasm were stained with Eosin for 30 sec., then, washed with tap water for 2 min.
4. Dehydration: The slides were washed in (90% ethanol for 1 min., 95% ethanol for 1 min., two times of 100% ethanol for 1 min.).

5. Clearing: The slides were placed in xylene for 2 min.
6. Mounting: Drop of distyrene plasticizer xylene (DPX) was put on the slide and applied the coverslip.

3.8: Immunohistochemical Assay (IHC)

3.8.1: Assay Principle

Immunohistochemistry technique is based on the antigen-antibody reaction, in which primary antibody binds to specific antigen, then the antibody-antigen complex binds with an enzyme conjugated secondary antibody. With presence of coloring substrate such as chromogen, the enzyme catalyzes to generate colored deposits at the sites of antibody-antigen binding (Suvarna *et. al.,* 2013).

3.8.2: Reagent Preparation

1. **Tris Buffered Saline (TBS) (1 L):** 100 mL of 10X TBS was added to 900 mL of distilled water.

2. **Hydrogen Peroxide-Methanol Blocking Buffer:** 3% H2O2 was mixed with 100% methanol in a ratio of (1: 9) to block endogenous peroxidase.

3. **10% Goat Serum Blocking Buffer:** 100 ml of goat serum was added to 900 ml phosphate buffered saline (PBS) to block secondary antibody

3.8.3: Procedure

IHC procedure for trophoblasts, IDO, and macrophages using anti cytokeratin7 (Ck7), IDO, CD68 antibody kits, respectively, was done depending on the manual procedure of My BioSource Company, Inc, San Diego, CA. USA.

3.8.3.1: Tissue Processing

It was followed the standard protocol used as per routine histology methods.

1. Fixation: Tissues were fixed in 10% neutral buffered formalin for 24 hours and embedded in paraffin wax.

2. Sectioning: Tissues were cut by microtome to a thickness of 5 µm, then placed on positively charged slides.
3. Dewaxing: Slides were heated in oven for 30 min. at 60°C.
4. Clearing: Slides were washed two times for 15 min., each time, in xylene.
5. Rehydration: The tissues were transferred through a series of decreasingly concentrated alcohol solutions (100% ethanol 5 min., 90% ethanol 5 min., 80% ethanol 5 min., 70% ethanol 5 min.). Then, the tissues were rinsed in distilled water for 5 min.

3.8.3.2: Antigen Retrieval: Formaldehyde fixation results in methylene bridges which masks epitopes and can restrict antigen-antibody binding. Antigen retrieval method break these methylene bridges and expose antigenic sites, allowing antibodies to bind. It was done by placing the slides in TBS 1X solution and heated in the microwave at 100°C for 20 min., then, removed from heat and let stand at room temperature in buffer for 20 min. Slides were washed with Phosphate Buffer Saline (PBS) three times for 5 min., each time.

3.8.3.3: Immunostaining: Slides weren't allowed to dry at any time during the staining procedure by using humidity chamber.

1. Blocking with blocking reagent is essential to prevent non-specific binding of antibodies to tissue. It was done by adding 100 µl per slide of H2O2-methanol blocking buffer for 10 min.
2. The blocking buffer was drained from slides, Then, applied a 100 µl per slide of diluted primary antibody (1:100) with PBS and incubated for 60 min. at room temperature.

3. The slides were washed three times in PBS for 5 min. Then, applied a 100 µl per slide of biotinylated secondary antibody for 15 min.
4. After that, 100µl per slide was applied of streptavidin-horseradish peroxidase (SA-HRP) and incubated for 30 min. at room temperature.
5. The slides were washed 3 times in PBS for 5 min., Then, applied 100 µl per slide of DAB chromogen substrate for 30 min.
6. slides were washed 3 times in PBS for 5 min. Then washed in water for 1 min.
7. The slides were placed in hematoxylin dye for 10 sec., Then, it was washed again with water for 1 min.
8. Dehydration: The slides were washed in (90% ethanol for 1 min., 95% ethanol for 1 min., two times of 100% ethanol for 1 min.).
9. Clearing: The slides were washed three times with xylene 15 min., each time.
10. Mounting: Drop of DPX was put on the slide and applied the coverslip. IHC was evaluated under light microscope.

3.8.4. Image Analysis

Data analysis was done by Image J software (Rasband, W.S., 1997-2018). The immunohistochemical staining was assessed by two observers, and for each sample, 4 fields were evaluated at magnification 400x.

In case of CD68 marker, the stained cells were counted per mm^2. Regarding CK7 and IDO, the 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 follow: (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 (Ino *et. al.,* 2006; Kigasawa *et. al.,* 2017).

3.9: Apoptosis Assay: Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL)

3.9.1: Assay Principle

When cells undergo apoptosis, endonuclease enzymes are activated to cleave the genomic DNA between nucleosomes. When genomic DNA is cleaved, biotinylated dUTP (Biotin-dUTP) can be added to the exposed 3'-OH by terminal deoxynucleotidyl transferase (TdT), followed by the binding of streptavidin-labeled horseradish Peroxidase (Streptavidin-HRP). Thereafter, through the HRP catalysis, the apoptotic cells are marked by DAB chromogen substrate, which can be detected by ordinary microscopy.

3.9.2: Reagent Preparation

1. TdT Enzyme Working Solution: 450µl of TdT equilibration buffer was added to 10 µl of biotin-dUTP and 40 µl of TdT enzyme

2. Streptavidin-HRP Working Solution: 5µl of Streptavidin-HRP was added to 995µl of PBS

3.9.3: Procedure

Apoptosis assay using TUNEL kit, it was done depending on manufacturer procedure of My BioSource Company, Inc, San Diego, CA.

3.9.3.1: Tissue Processing

It was followed the standard protocol used as per routine histology methods.

1. Fixation: Tissues were fixed in 10% neutral buffered formalin for 24 hours and embedded in paraffin wax.
2. Sectioning: Tissues were cut by microtome to a thickness of 5 µm, then placed on positively charged slides.
3. Dewaxing: Slides were heated in oven for 30 min. at 60°C.
4. Clearing: Slides were washed two times for 15 min., each time, in xylene.
5. Rehydration: The tissues were transferred through a series of descending concentration of alcohol (100% ethanol 5 min., 90% ethanol 5 min., 80% ethanol 5 min., 70% ethanol 5 min.). Then, the tissues were rinsed in distilled water for 5 min.

3.9.3.2: Antigen Retrieval: Proteinase K (1X) solution was added on the slides, then, incubated at room temperature for 15-30 min. Samples were washed with PBS three times for 5 min., each time.

3.9.3.3: Blocking: One hundred µl per slide of blocking buffer was added and incubated at room temperature for 10 min. Then the blocking buffer was drained from slides. The slides were washed with PBS two times for 5 min., each time.

3.9.3.4: TUNEL Protocol

1. Fifty µl per slide of TdT enzyme working solution was added and incubated for 60 min. at room temperature.
2. The slides were washed with PBS three times for 5 min., each time.
3. Fifty µL Streptavidin-HRP working solution was added and incubated at 37°C for 30 min.
4. The slides were washed with PBS three times for 5 min., each time.
5. One hundred µL DAB substrate was added and incubated at room temperature 30 min.

6. The slides were washed with PBS three times for 5 min., each time.
7. The sections were counterstained by hematoxylin for 10 sec. and rinsed three times with PBS for 5 min., each time. Finally, the sections were rinsed again with distilled water for 1 min.
8. Dehydration: The slides were washed in (90% ethanol for 2 min., 95% ethanol for 2 min., two times of 100% ethanol for 2 min.).
9. Clearing: The slides were immersed in two changes of xylene 2 min., each time.
10. Mounting: Drop of DPX was put on the slide and applied the coverslip. The slides were examined using light microscope.

3.10: 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 $p \leq 0.05$. Statistical analysis was carried out using IBM® SPSS® Statistics version 24 predictive analytics software.

Chapter Four
Results

4: Results

4.1: The Effect of DEX on the Progesterone and Progesterone Receptor (PR)

The results of the present study have observed that the progesterone levels decreased significantly ($p \leq 0.05$) in all the treated groups as compared to control groups. The changes in progesterone levels in pregnant does following the administration (HD) and (LD) of (DEX) from 5 to 9 dG and sacrificed at 10 and 28 dG are shown in Table (4.1), and the administration (HD) and (LD) of (DEX) from 10 to 17 dG and sacrificed at 18 and 28 dG are presented in Table (4.2).

(Table 4.1): Serum progesterone level (ng\ml) in pregnant does following DEX administration from 5 to 9 dG.

Groups	No	dG	Mean ± SD
G1	7	10	$12.231^{a} \pm 3.426$
G2	7	10	$1.187^{b} \pm 0.161$
G3	7	10	$1.882^{b} \pm 0.690$
G2	3	28	$0.956^{b} \pm 0.105$
G3	3	28	$1.328^{b} \pm 0.234$

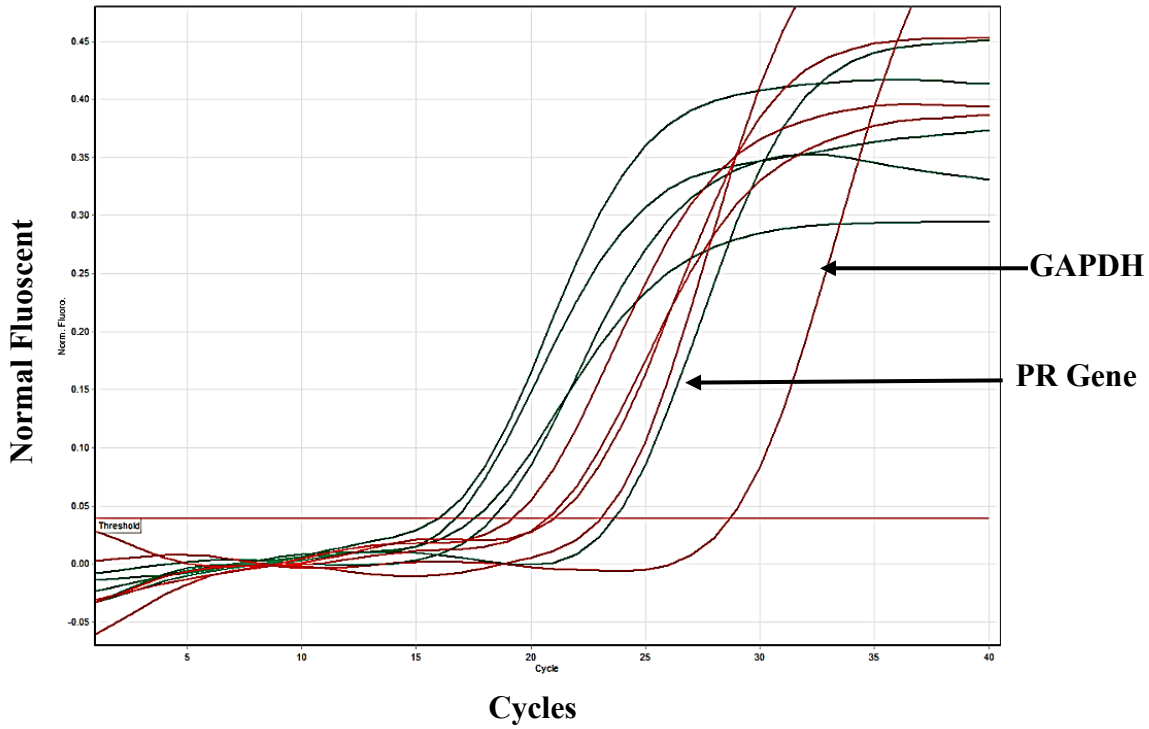
No= Number of animals, dG= day of Gestation, SD= Standard Deviation, a and b= Significant variation ($p \leq 0.05$).

(Table 4.2): Serum progesterone level (ng\ml) in pregnant does following DEX administration from 10 to 17 dG.

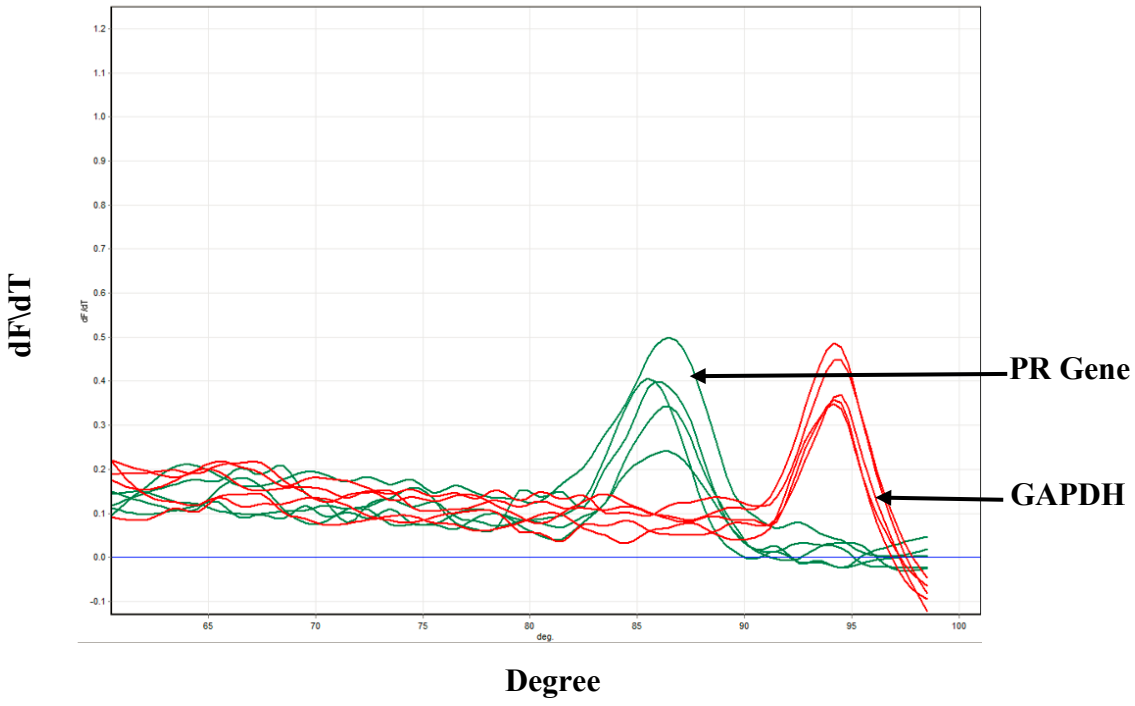
Groups	No	dG	Mean ± SD
G4	7	18	$10.441^{a} \pm 2.395$
G5	7	18	$1.492^{b} \pm 0.623$
G6	7	18	$2.080^{b} \pm 0.828$
G5	3	28	$2.252^{b} \pm 0.206$
G6	3	28	$1.668^{b} \pm 0.211$

No= Number of animals, dG= day of Gestation, SD= Standard Deviation, a and b= Significant variation ($p \leq 0.05$).

On another hand, the results have confirmed that the gene expression of PR in the uterus increased significantly ($p \leq 0.05$) in all treated groups compared to control group. Moreover, there have been significant variations in PR gene expression among treated groups in terms of doses and timing of gestation where; it increases significantly in the HD as compared to LD injected groups, and it significantly decreases in the 28th dG as compared to 10 and 18. Fig. (4.1) has shown Qrt, RT-PCR amplification curve of PR, and Fig. (4.2) has shown melting curve. Table (4.3) illustrates the procedure of calculation of fold differences of PR gene expression. The gene expression of PR of fetal-maternal placenta after pregnant does administrated with (HD) and (LD) of (DEX) from 5 to 9 dG and sacrificed at 10 and 28 dG are presented in Table (4.4), whereas administration (HD) and (LD) of (DEX) from 10 to 17 dG and sacrificed at 18 and 28 dG are presented in Table (4.5), PR gene expression has been normalization for that of GAPDH.



(Fig. 4.1): Qrt, RT-PCR amplification curve.



(Fig. 4.2): Qrt, RT-PCR melting curve.

(Table 4.3): Procedure of calculation of fold differences of PR gene expression.

Group	Cts Mean	Cth Mean	ΔCts	ΔΔCts Mean	EFC Mean
G1	23.1	18.35	4.744*	-0.007231569	0.995
G2	21.13	19.58	-1.55	3.188559176	9.117
G3	19.22	16.75	-2.47	2.273814245	4.836
G2	20.5	18.01	-2.49	2.244887059	4.740
G3	20.77	17.57	-3.2	1.54399072	2.916
G4	18.42	16.21	2.199*	-0.017417053	0.988
G5	18.91	20.11	1.20	3.394239735	10.514
G6	20.33	20.18	-0.144	2.049630768	4.140
G5	18.49	18.06	0.425	2.61800367	6.139
G6	24.07	22.76	-1.31	0.888304895	1.851

Cts: Concentration of PR gene, Cth: Concentration of housekeeping gene, EFC= Expression fold change, *: The value of ΔCts for control is obtained from the mean value of ΔCts of relevant groups.

(Table 4.4): Expression fold of PR\ GAPDH in pregnant does following DEX administration from 5 to 9 dG.

Groups	No	dG	EFC (Mean ± SD)
G1	5	10	$0.995^{a} \pm 0.123$
G2	5	10	$9.117^{b} \pm 2.251$
G3	5	10	$4.836^{c} \pm 1.056$
G2	3	28	$4.740^{c} \pm 1.227$
G3	3	28	$2.916^{d} \pm 0.760$

No= Number of animals, dG= day of Gestation, EFC= Expression fold change, SD= Standard Deviation, a, b, c and d= Significant variation ($p \leq 0.05$).

(Table 4.5): Expression fold of PR\ GAPDH in pregnant does following DEX administration from 10 to 17 dG.

Groups	No	dG	EFC (Mean ± SD)
G4	5	18	$0.988^{a} \pm 0.121$
G5	5	18	$10.514^{b} \pm 3.780$
G6	5	18	$4.140^{c} \pm 0.740$
G5	3	28	$6.139^{d} \pm 2.105$
G6	3	28	$1.851^{e} \pm 0.710$

No= Number of animals, dG= day of Gestation, , EFC= Expression fold change, SD= Standard Deviation, a, b, c, d and e= Significant variation ($p \leq 0.05$).

4.2: The Effect of DEX on the Levels of Calcitonin

The calcitonin levels have increased significantly ($p \leq 0.05$) in all the treated groups compared to control groups. The serum calcitonin levels in pregnant does following the administration (HD) and (LD) of (DEX) from 5 to 9 dG and sacrificed at 10 and 28 dG are presented in Table (4.6), while the administration (HD) and (LD) of (DEX) from 10 to 17 dG and sacrificed at 18 and 28 dG are presented in Table (4.7).

(Table 4.6): Serum calcitonin level (pg\ml) in pregnant does following DEX administration from 5 to 9 dG.

Groups	No	dG	Mean ± SD
G1	7	10	$152.329^{a} \pm 12.012$
G2	7	10	$329.216^{b} \pm 22.605$
G3	7	10	$322.725^{b} \pm 35.058$
G2	3	28	$315.726^{b} \pm 26.820$
G3	3	28	$324.083^{b} \pm 39.652$

No= Number of animals, dG= day of Gestation, SD= Standard Deviation, a and b= Significant variation ($p \leq 0.05$).

(Table 4.7): Serum calcitonin level (pg\ml) in pregnant does following DEX administration from 10 to 17 dG.

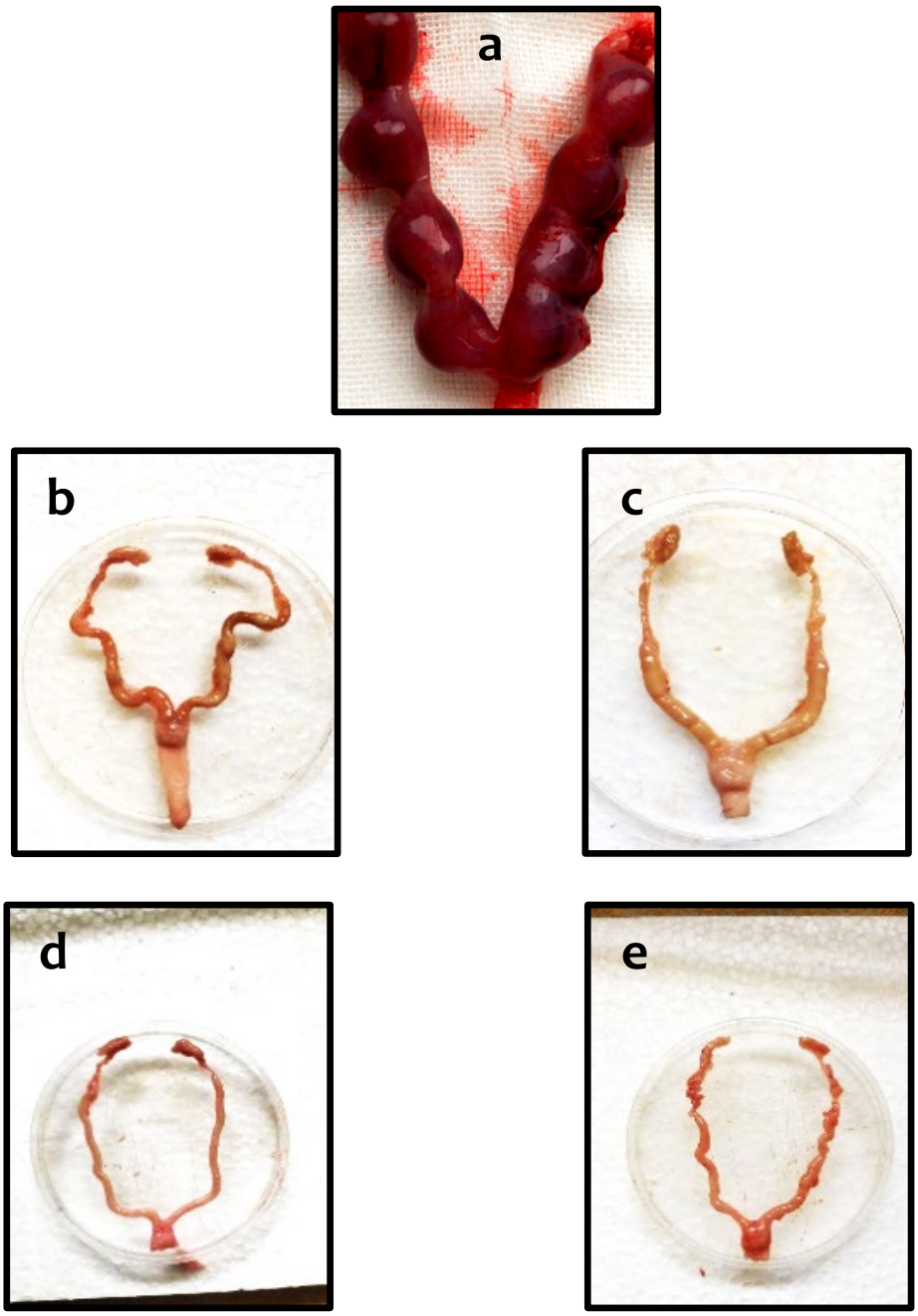
Groups	No	dG	Mean ± SD
G4	7	18	$262.067^{a} \pm 25.261$
G5	7	18	$342.654^{b} \pm 13.569$
G6	7	18	$320.830^{b} \pm 43.932$
G5	3	28	$340.577^{b} \pm 17.279$
G6	3	28	$329.145^{b} \pm 16.155$

No= Number of animals, dG= day of Gestation, SD= Standard Deviation, a and b= Significant variation ($p \leq 0.05$).

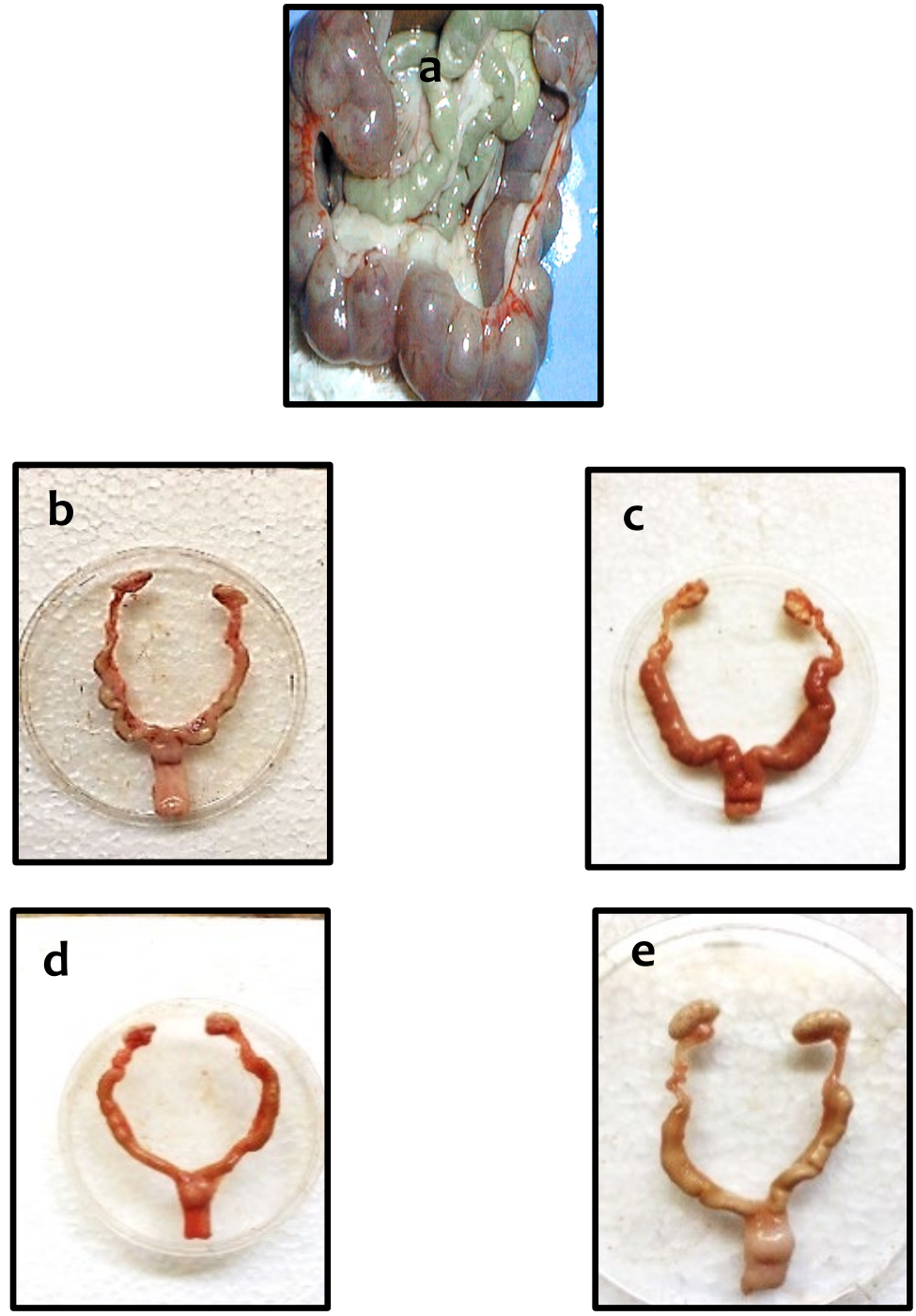
4.3: Effect of DEX on Gross Studies

The gross anatomy of the G1 (control) and G2,3 (treated with DX from day 5-9 dG) has shown a dramatic difference between the control and treated pregnant does. The results of control have demonstrated a beaded appearance of the uterus indicating the success of implantation manifested by presence of the embryos in G1 (Fig. 4.3 a). In contrast to that the uteri of G2,3 have shown a failure of implantation indicated by less marked beaded appearance of the uteri (Fig. 4.3 b,c,d, and e).

In the next groups, G4,5 and 6 in which the pregnancy period has been extended to day 17 dG. The most pronounced results of the control does (G4) is the increase in the size of the uterus and the presence of fetuses inside them (Fig. 4.4 a). On the other hand, the does of treated groups (G5 and 6) which had received the DX from day 10 to 17 have shown marked decrease in the size of the uteri suggesting resorption of the fetuses in these does (Fig. 4.4 b,c,d, and e).



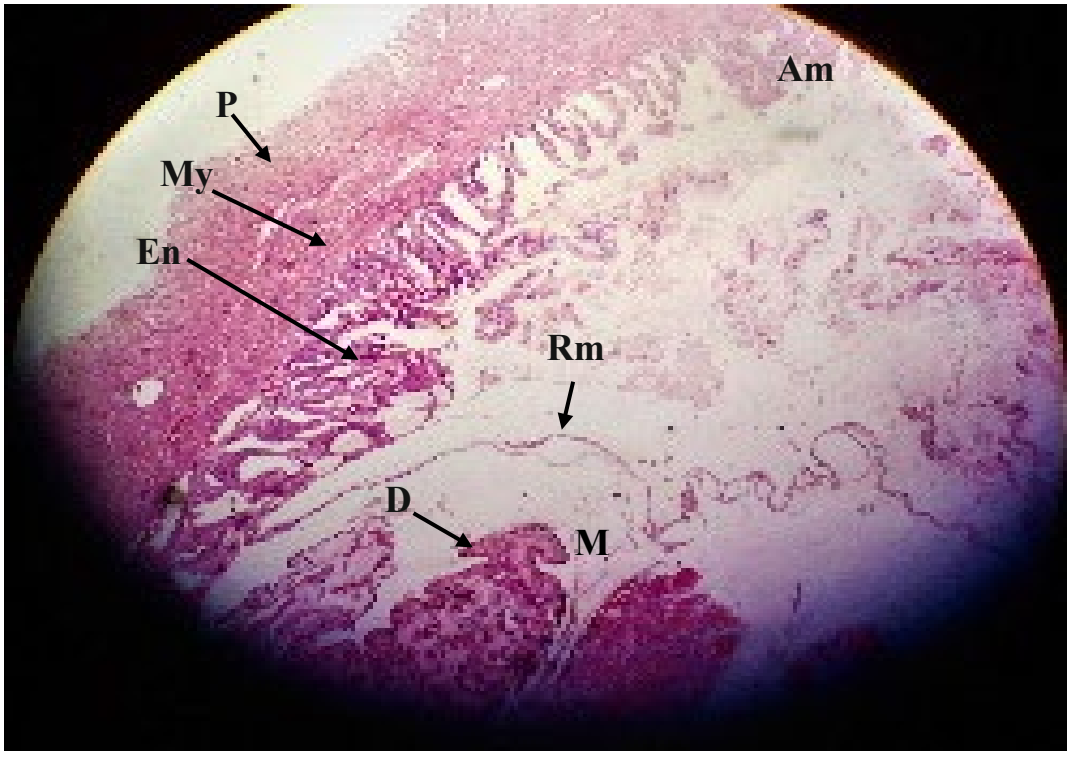
(Fig. 4.3): External appearance of uteri of pregnant treated groups with DEX from 5-9 dG and control group at different gestational periods. (a): Control at 10 dG showing beaded appearance of the uterus. Treated groups; (b): HD at 10 dG. (c): LD at 10 dG. (d): HD at 28 dG. (e): LD at 28 dG. are showing the less beaded appearance.



(Fig. 4.4): External appearance of uteri of pregnant treated groups with DEX from 10-17 dG and control group at different gestational periods. (a): Control at 18 dG showing increase in the size of the uterus. Treated groups; (b): HD at 18 dG. (c) LD at 18 dG. (d) HD at 28 dG. (e) LD at 28 dG. are showing decrease in the size of the uterus.

4.4: Effect of DEX on Histological Studies

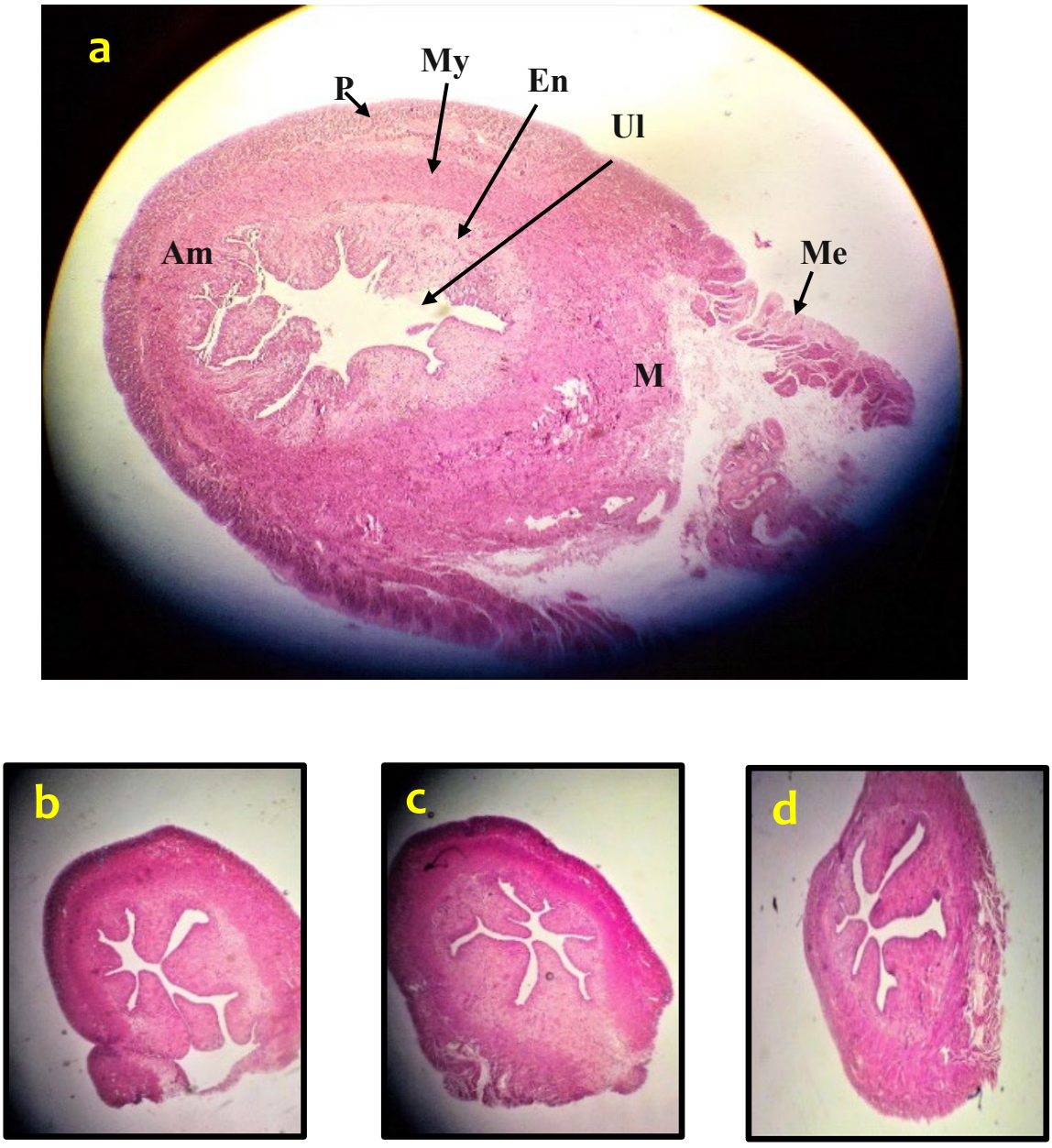
The histological results at 10 dG of G1 (control) have revealed the success of pregnancy. In the cross section of the uteri in the G1, the pregnancy has shifted from the anti-mesometrial side of the uterus to its mesometrial side. The Reichert's membrane and the decidual tissue have been well marked in the mesometrial side. The lumen of the uterus is wide to accommodate the fetus and the myometrium has thinned down (Fig. 4.5).



(Fig. 4.5): Cross section of uterus in the control pregnant does at 10 dG. The Reichert's membrane and the decidual tissue in the mesometrial side, the wide lumen of the uterus, and the thinned myometrium indicate the success of pregnancy. Am: Antimesometrial side, M: Mesometrial side, Rm: Reichert membrane, En: Endometrium, My: Myometrium, P: Perimetrium, D: Decidua, (Magnification: 100 x).

In the treated does of G2 and 3, the uterine histological observations gave the criteria of failure of pregnancy. There is an absence of fetuses and their Reichert's

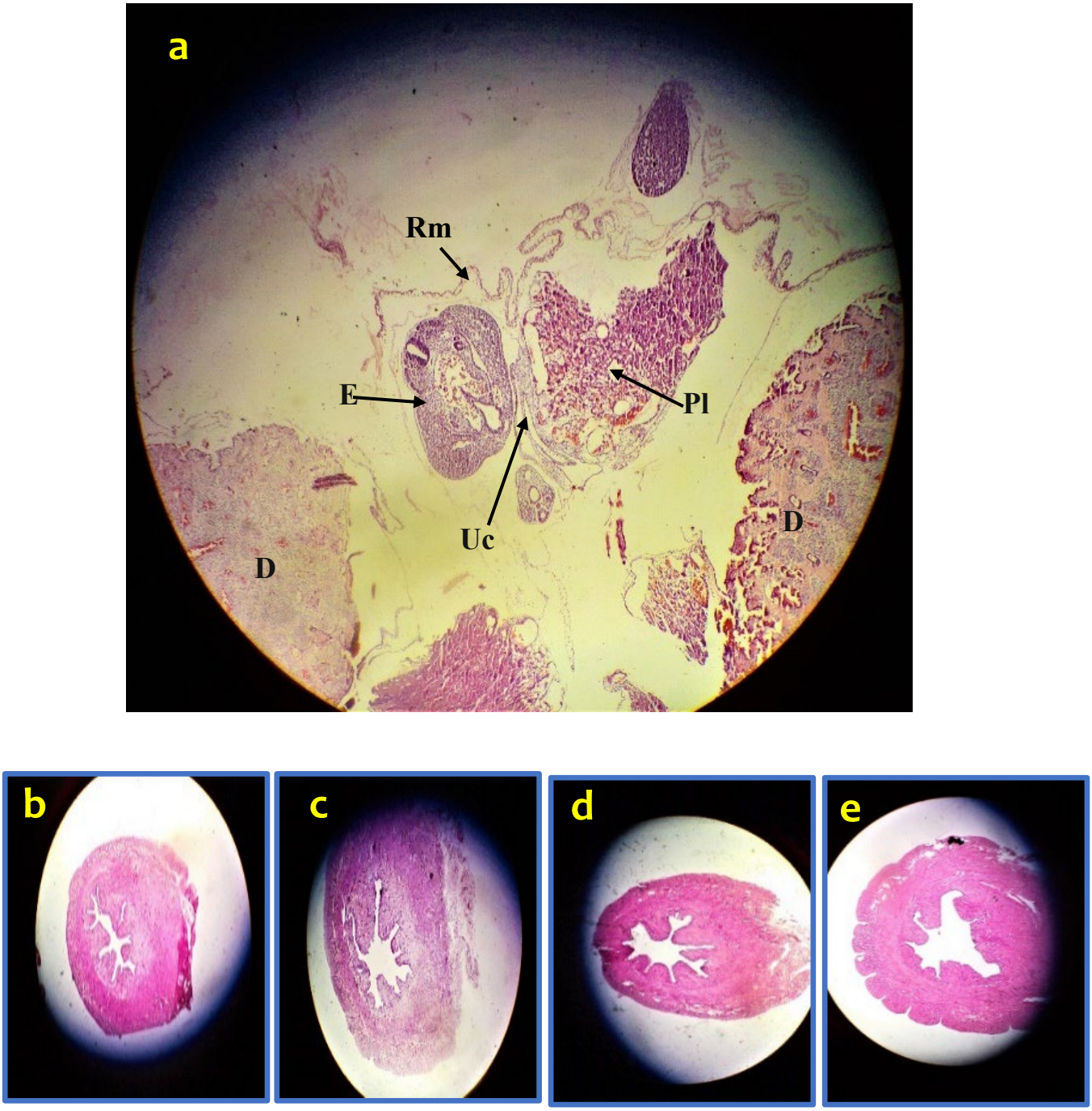
membranes and decidual reaction, the lumen of the uteri is narrow, the myometrium is considerably thicker compared with uterus of control group (Fig. 4.6 a,b,c, and d).



(Fig. 4.6): Cross section of uteri in the pregnant does treated with DEX from 5-9 dG. Absence of fetuses and their Reichert's membranes and decidual reaction, narrowing of the uterine lumens, thickness of the myometrium. (a): HD at 10 dG. (b) LD at 10 dG. (c) HD at 28 dG. (d) LD at 28 dG. Am: Antimesometrial side, M: Mesometrial side, En: Endometrium, My: Myometrium, P: Perimetrium, Me: Mesometrium, Ul: Uterine lumen, (Magnification: 100 x).

The cross sections of the pregnant uteri of the G4 (control) taken at 18 dG have shown well advanced pregnancy. The fetus has been connected with placenta via the umbilical cord. The stroma of the endometrium had differentiated into decidual tissue. The Reichert's membrane is enclosing both the placenta and fetus (Fig.4.7a).

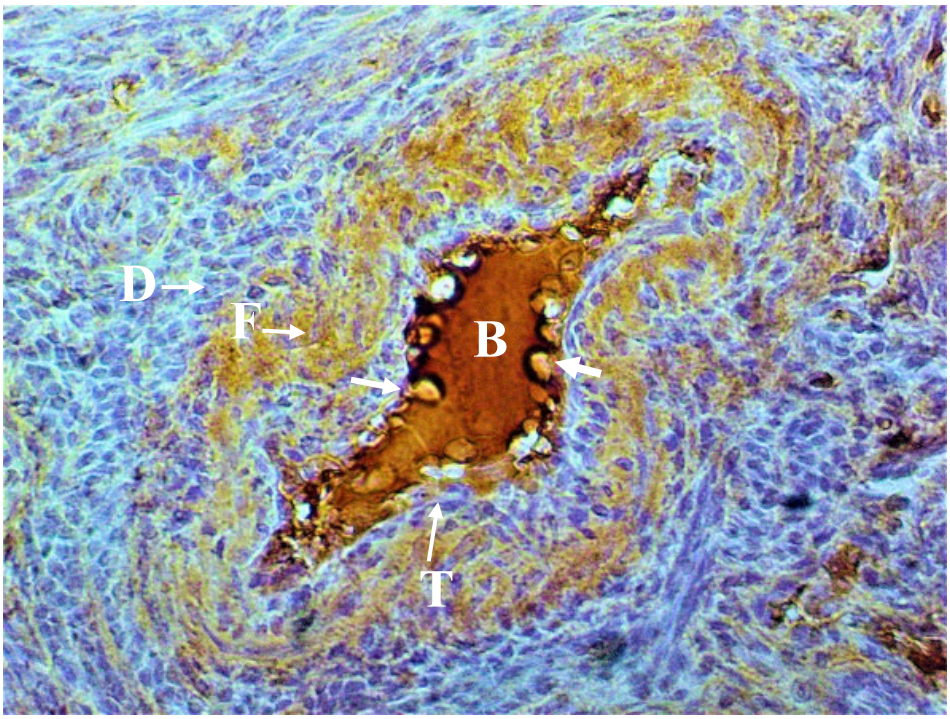
In contrast to that the effect of DX treatment in the G5 and 6 is manifested by absence of the fetuses, narrowing of the lumen, absence of the decidual reaction (Fig. 4.7 b,c,d, and e).



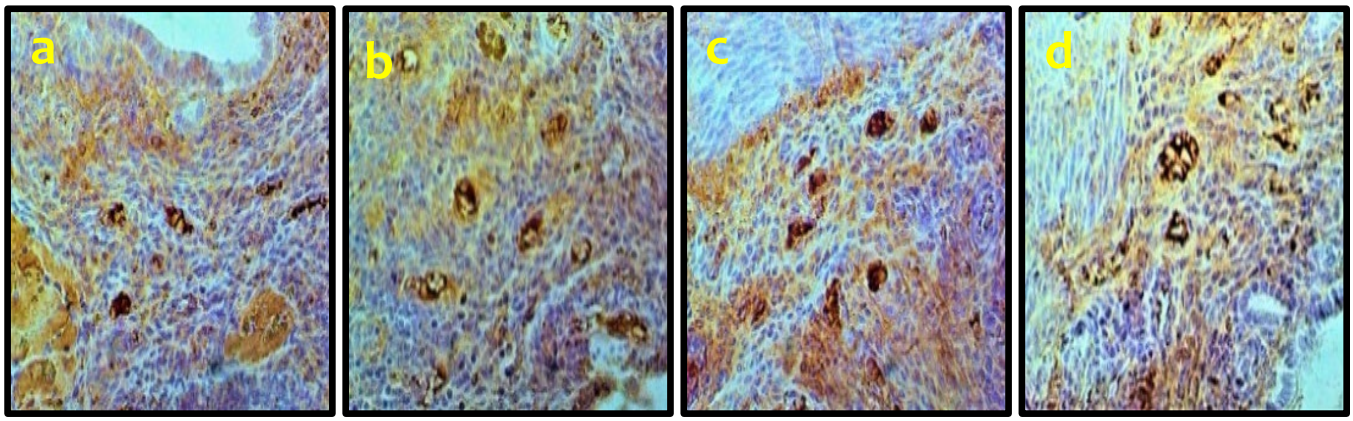
(Fig. 4.7): Cross section of uteri in the pregnant does treated with DEX from 10-17 dG and control group. (a): Control at 18 dG. The connected fetus with placenta via the umbilical cord, the differentiated stroma of the endometrium into decidual tissue, and the Reichert's membrane enclosing both the placenta and fetus indicate the advanced pregnancy. Treated groups; (b) HD at 18 dG. (c) LD at 18 dG. (d) HD at 28 dG. (e) LD at 28 dG. are showing absence of the fetuses, narrowing of the lumen, absence of the decidual reaction which indicate failure of pregnancy. E: Embryo, D: Decidua, Uc: Umbilical cord, Pl: Placenta, Rm: Reichert membrane, (Magnification: 100 x).

4.5: The Effect of DEX on Spiral Arteries Remodeling

The results at 10 dG of G1 (control) have shown well infiltration of the endovascular trophoblast into the wall of the spiral artery. The trophoblasts have lodged themselves in the tunica intima and tunica media. The trophoblasts have replaced the endothelial cells in the former tunica and smooth muscle cells in the latter tunica (Fig. 4.8). The cross section of the spiral artery has expressed a cuffing layer of light brown fibrinoid material at the outskirt of the wall of the artery. The lumen of the spiral artery is wide and filled by coagulated blood with brownish coloration filling the lumen, while the trophoblastic giant cells have taken the places of the endothelial cells. All these observations are suggesting early remodeling in the wall of the spiral artery, (Fig. 4.8). On the other hand, in the treated does of G2 and 3, the unremodeling spiral arteries in the treated groups are markedly narrowed compared with remodeled artery of control group. There is no evidence for the existence of the migrating trophoblasts in the wall of the artery, (Fig. 4.9 a,b,c, and d). As a matter of fact, the spiral arteries appearance is parallel to that of unremodled arteries.

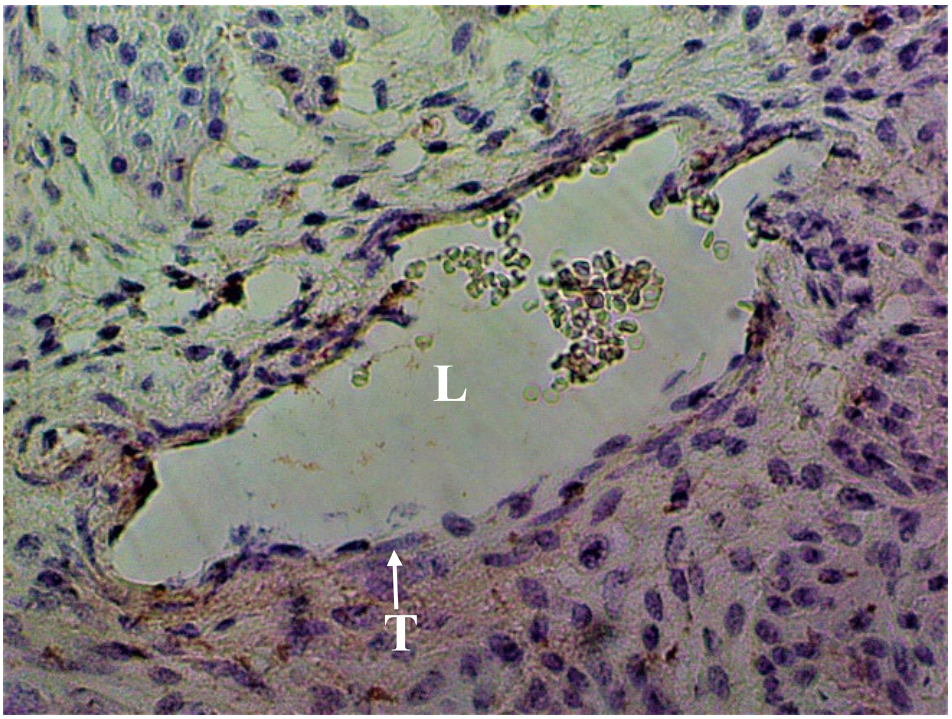


(Fig. 4.8): TUNEL assay shows absence of the apoptosis in spiral artery of the control pregnant does uterus at 10 dG. infiltration of the endovascular trophoblast into the wall of the spiral artery, embedding the in light brown fibrinoid material, the wide lumen of the spiral artery filled by coagulated blood with brownish coloration, and presence the trophoblastic giant cells (arrows) indicate the early stage of spiral artery remodeling. T: Trophoblast, F: Fibrinoid. (Magnification: 400 x).



(Fig. 4.9): TUNEL assay shows the apoptosis in spiral arteries (brown color) in the uteri of pregnant does treated with DEX from 5-9 dG. which indicates the unremodeling spiral arteries. (a): HD at 10 dG. (b): LD at 10 dG. (c): HD at 28 dG. (d) LD at 28 dG. (Magnification: 400 x).

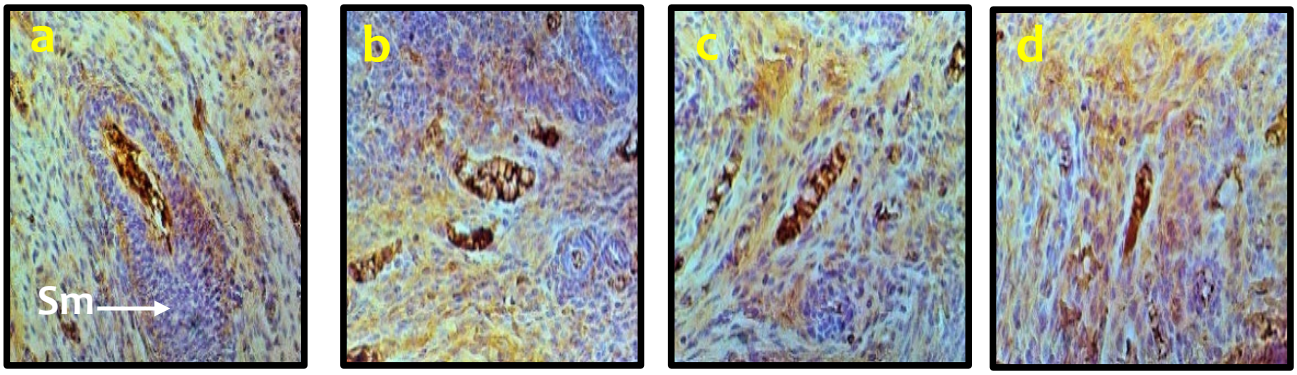
The results at 18 dG of G4 (control), during this period of pregnancy the obvious changes seen were the increase in the luminal diameter (Fig. 4.10) compared with treated group does. The endothelial cells and the internal elastic lamina are absent and have been replaced by the invading trophoblasts. Moreover, the smooth muscle cells of tunica media and the surrounding elastic lamina have been removed and have given their places to the invading trophoblasts (Fig. 4.10).



(Fig. 4.10): TUNEL assay shows absence the apoptosis in spiral artery of the control pregnant does uterus at 18 dG. the increase in the luminal diameter, absences the endothelial cells and the internal elastic lamina, removing the smooth muscle cells of tunica media and the surrounding elastic lamina indicate the advanced stage of spiral artery remodeling. L: Lumen, T: Trophoblast. (Magnification: 400 x).

In the treated does of G5 and G6, the remodeling of the spiral artery has been halted, the most characteristic features were dramatic narrowing of the lumen, and apoptotic changes have been inflicted on the migrating trophoblast rendering unable to induce their effect on the required remodeling of the spiral artery. The TUNEL

assay used is able to demonstrate the brown coloration in the lumen of the spiral artery, which is an indicative for insufficient and poor remodeling. The smooth muscle cells of the tunica media are nicely arranged around the narrow lumen (Fig. 4.11 a,b,c and d). Bearing in mind, the spiral arteries appearance is parallel to that of early remodeled arteries.



(Fig. 4.11): TUNEL assay shows the apoptosis in the lumen of spiral arteries (brown color) in the uteri of pregnant does treated with DEX from 10-17 dG. which indicates the insufficient early spiral arteries remodeling. Sm: Smooth muscle. (a): HD at 18 dG. (b): LD at 18 dG. (c): HD at 28 dG. (d) LD at 28 dG. (Magnification: 400 x).

4.6: The Effect of DEX on IHC Parameters (Trophoblasts, Macrophages, and IDO)

The results have shown that all parameters of immunohistochemical study (IHC) including trophoblasts, macrophages, and indoleamine-2,3 dioxygenase (IDO) in the placentae of pregnant does decreased significantly ($p \leq 0.05$) in all the treated groups compared to control groups. The changes in cytokeratin7 (Ck7) expression level on trophoblastic cells in pregnant does' placentae following the administration (HD) and (LD) of (DEX) from 5 to 9 dG and sacrificed at 10 and 28 dG are presented in Table (4.8), and the administration (HD) and (LD) of (DEX) from 10 to 17 dG and sacrificed at 18 and 28 dG are shown in Table (4.9). Table

(4.10) represents the scores of the percent positivity of stained cells and the staining intensity of Ck7 expression level. The immunohistochemical study of Ck7 in the placentae of pregnant does at different gestational periods and different DEX doses are shown in Figs. (4.12), (4.13), (4.14), and (4.15). CD68 expression level on macrophage in pregnant does' placentae after pregnant does administrated with (HD) and (LD) of (DEX) from 5 to 9 dG and sacrificed at 10 and 28 dG are presented in Table (4.11), and the administration (HD) and (LD) of (DEX) from 10 to 17 dG and sacrificed at 18 and 28 dG are shown in Table (4.12). Figs. (4.16), (4.17), (4.18), and (4.19) have shown immunohistochemical images of CD68 in the placentae of pregnant does at different gestational periods and different DEX doses. In addition, IDO expression level in pregnant does following the administration (HD) and (LD) of (DEX) from 5 to 9 dG and sacrificed at 10 and 28 dG are presented in Table (4.13), and the administration (HD) and (LD) of (DEX) from 10 to 17 dG and sacrificed at 18 and 28 dG are illustrated in Table (4.14). Table (4.15) represents the scores of the percent positivity of stained cells and the staining intensity of IDO expression level. Also, Figs. (4.20), (4.21), (4.22), and (4.23) have shown immunohistochemical images of IDO in the placentae of pregnant does at different gestational periods and different DEX doses.

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

(Table 4.8): Expression level (%/mm^2) of CK7 in pregnant does following DEX administration from 5 to 9 dG.

Groups	No	dG	Mean ± SD
G1	5	10	$40.18^a \pm 5.546$
G2	5	10	$15.45^b \pm 3.943$
G3	5	10	$17.49^b \pm 3.021$
G2	3	28	$19.26^b \pm 1.476$
G3	3	28	$20.18^b \pm 2.768$

No= Number of animals, dG= day of Gestation, SD= Standard Deviation, a and b= Significant variation ($p \leq 0.05$).

(Table 4.9): Expression level (%/mm^2) of CK7 in pregnant does following DEX administration from 10 to 17 dG.

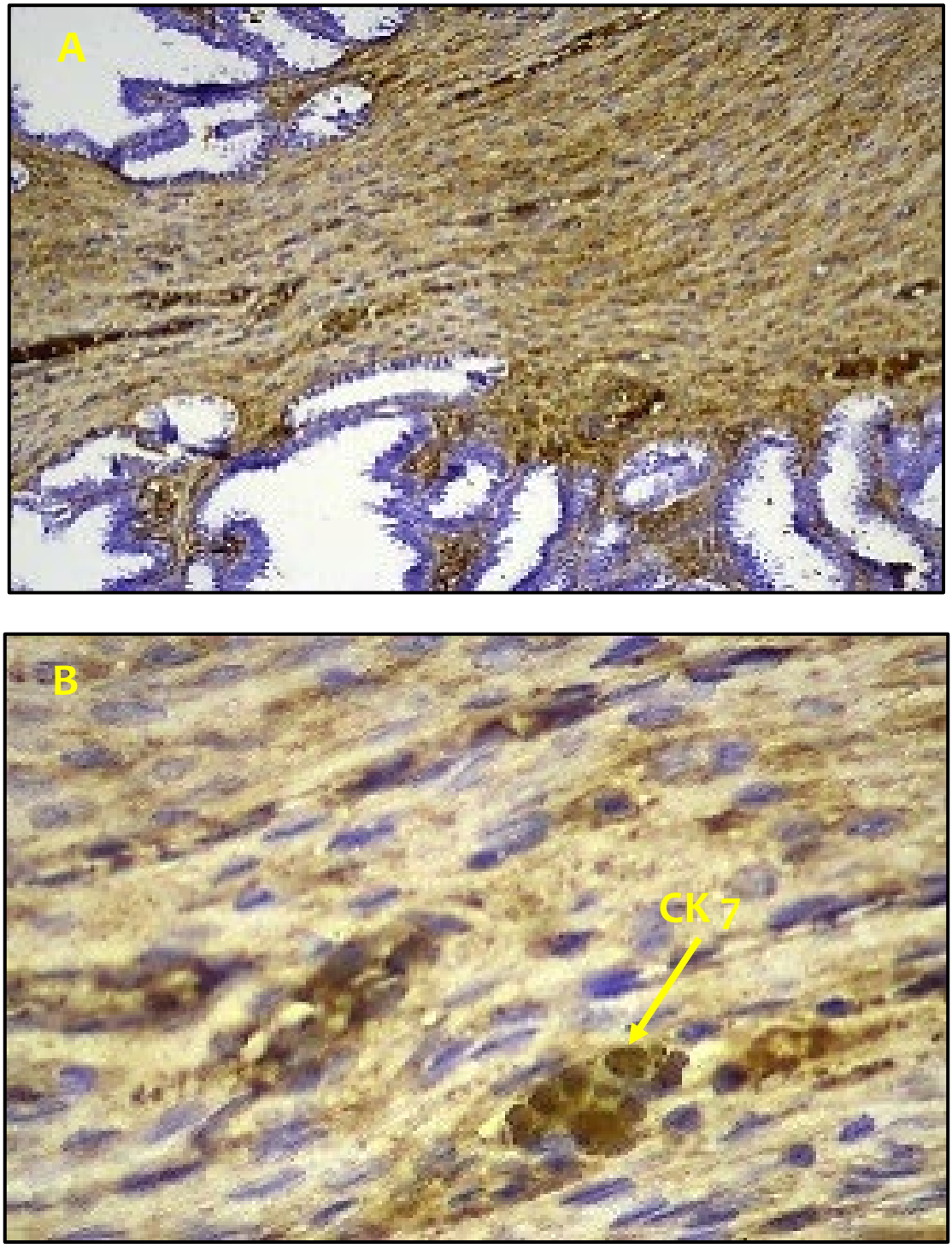
Groups	No	dG	Mean ± SD
G4	5	18	$38.27^a \pm 8.342$
G5	5	18	$18.62^b \pm 2.664$
G6	5	18	$21.24^b \pm 4.112$
G5	3	28	$15.27^b \pm 3.952$
G6	3	28	$18.94^b \pm 2.338$

No= Number of animals, dG= day of Gestation, SD= Standard Deviation, a and b= Significant variation ($p \leq 0.05$).

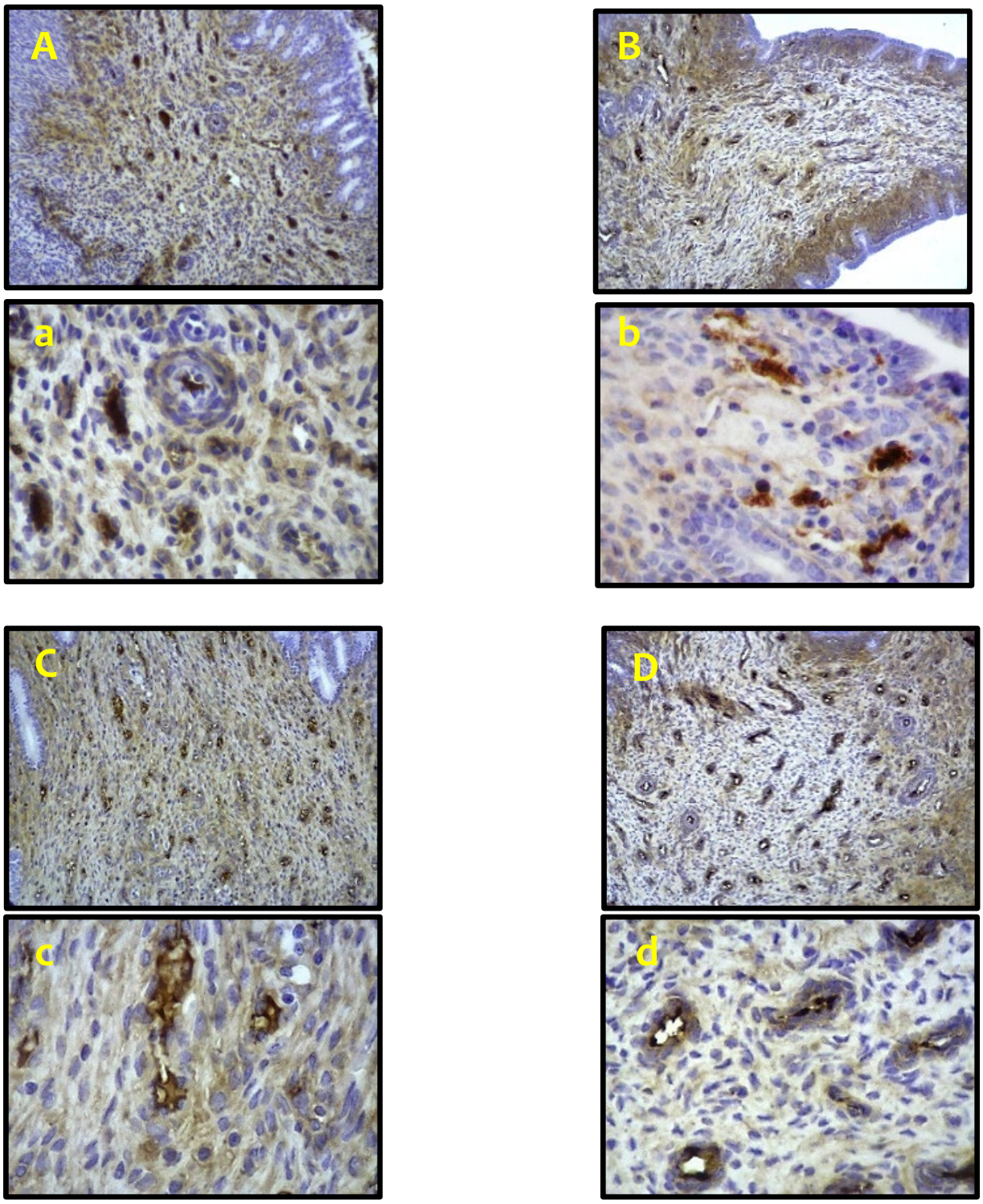
(Table 4.10): Ck7 expression level scores of the percent positivity of stained cells and the staining intensity.

Groups	No×4	dG	Score			
			0	1+	2+	3+
G1	5(20)	10	0	3	12	5
G2	5(20)	10	4	15	1	0
G3	5(20)	10	3	17	0	0
G2	3(12)	28	5	14	1	0
G3	3(12)	28	3	16	0	1
G4	5(20)	18	0	2	16	2
G5	5(20)	18	4	14	1	1
G6	5(20)	18	5	13	1	1
G5	3(12)	28	4	6	2	0
G6	3(12)	28	3	7	1	1

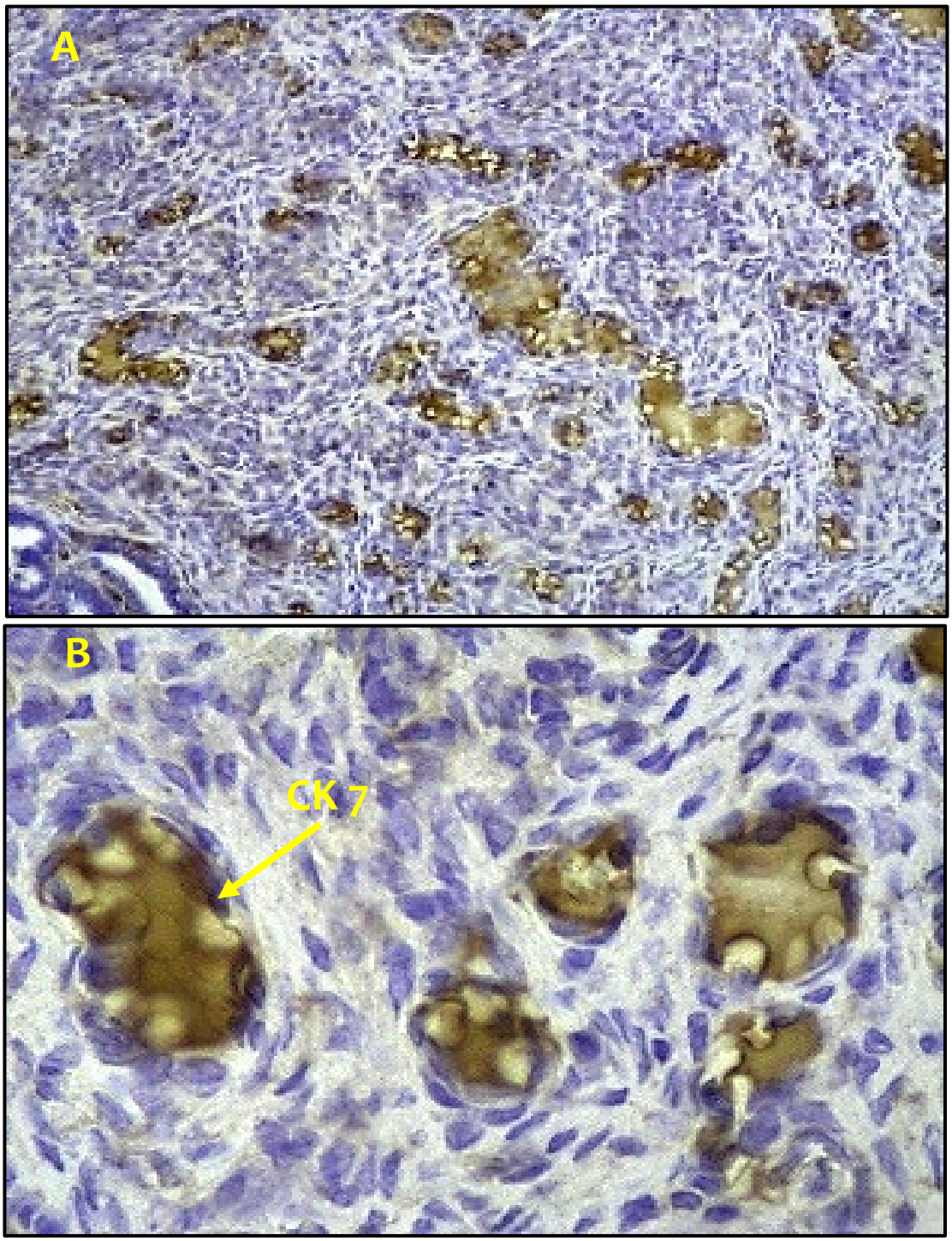
Note: 0= Negative, 1+= Weak, 2+= Moderate, 3+= Strong.



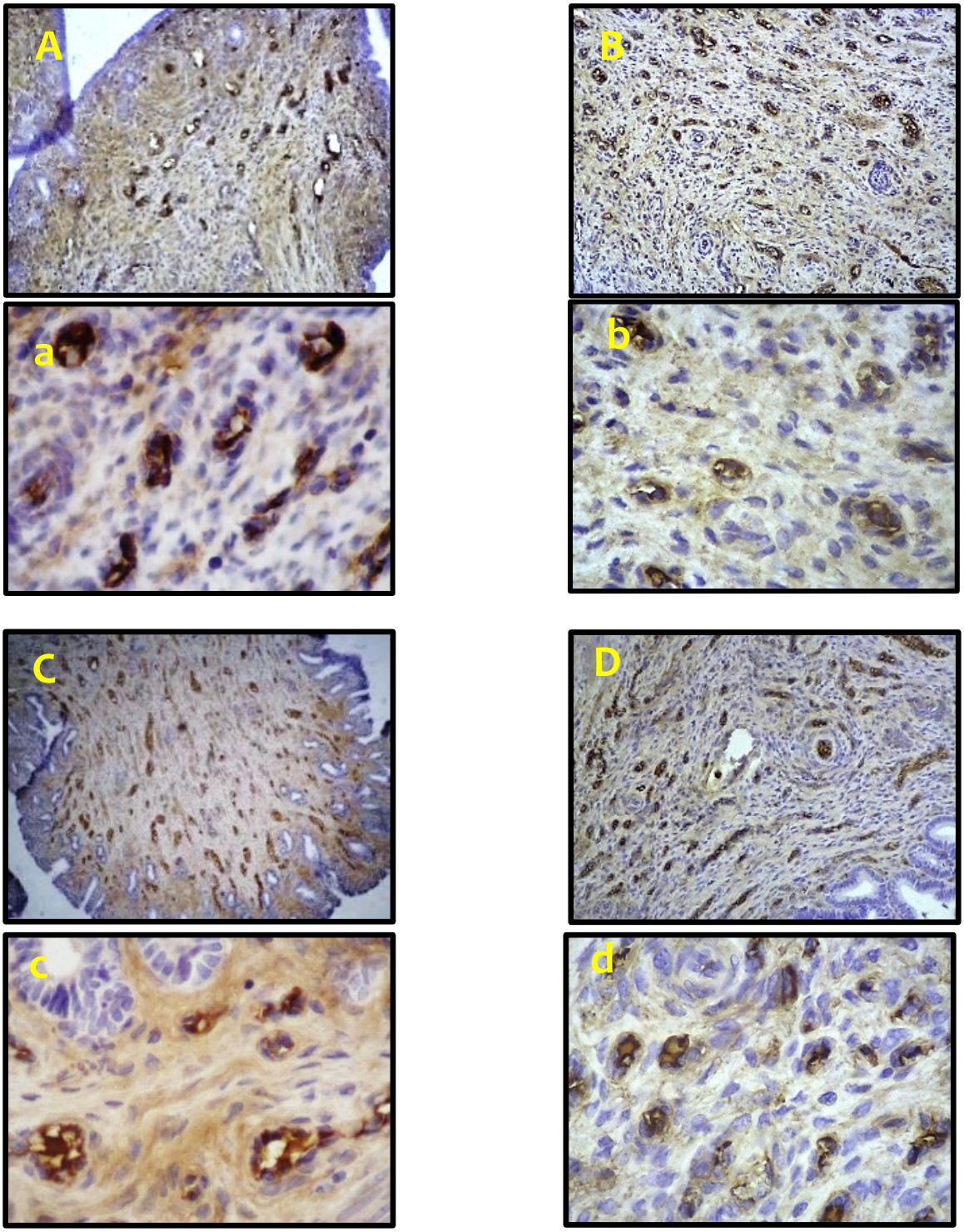
(Fig. 4.12): Immunohistochemical staining images of Ck7 (brown color) in the control pregnant does placenta at 10 dG. (A): Magnification: 100x. (B): Magnification: 400x).



(Fig. 4.13): Immunohistochemical staining of Ck7 (brown color) in the placentae of pregnant does treated with DEX from 5-9 dG. (A): HD at 10 dG. Magnification: 100x (a): HD at 10 dG. Magnification: 400x (B): LD at 10 dG. Magnification: 100x (b): LD at 10 dG. Magnification: 400x. (C): HD at 28 dG. Magnification: 100x. (c): HD at 28 dG. Magnification: 400x. (D) LD at 28 dG. Magnification: 100x. (d) LD at 28 dG. Magnification: 400x.



(Fig. 4.14): Immunohistochemical staining of Ck7 (brown color) in the control pregnant does placenta at 18 dG. (A): Magnification: 100x. (B): Magnification: 400x).



(Fig. 4.15): Immunohistochemical staining of Ck7 (brown color) in the placentae of pregnant does treated with DEX from 10-17 dG. (A): HD at 18 dG. Magnification: 100x (a): HD at 18 dG. Magnification: 400x (B): LD at 18 dG. Magnification: 100x (b): LD at 18 dG. Magnification: 400x. (C): HD at 28 dG. Magnification: 100x. (c): HD at 28 dG. Magnification: 400x. (D) LD at 28 dG. Magnification: 100x. (d) LD at 28 dG. Magnification: 400x.

(Table 4.11): CD68 macrophages (number/mm^2) in pregnant does following DEX administration from 5 to 9 dG.

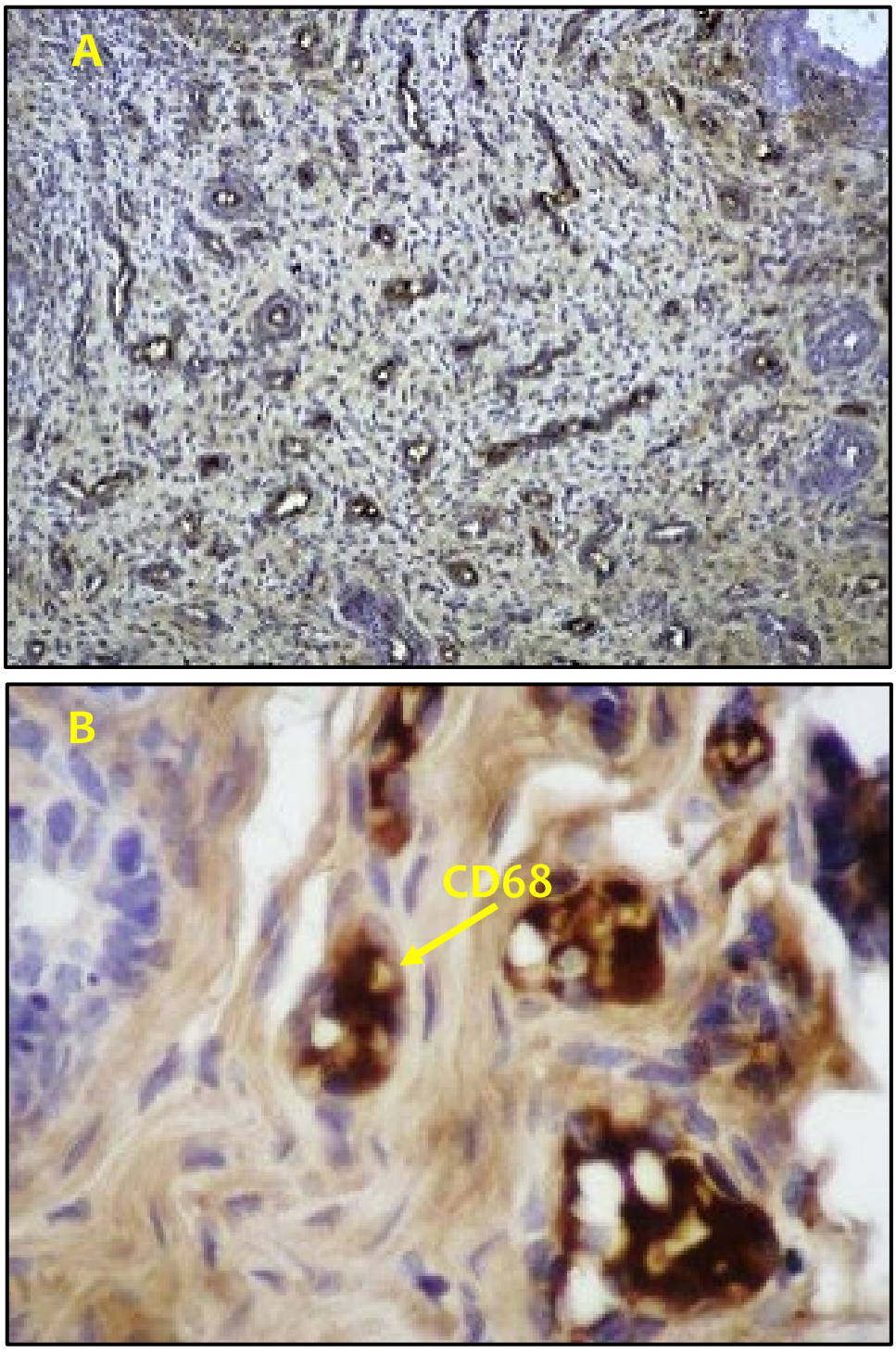
Groups	No	dG	Mean ± SD
G1	5	10	$6748^a \pm 139$
G2	5	10	$2681^b \pm 374$
G3	5	10	$3057^b \pm 125$
G2	3	28	$2928^b \pm 289$
G3	3	28	$2785^b \pm 311$

No= Number of animals, dG= day of Gestation, SD= Standard Deviation, a and b= Significant variation ($p \leq 0.05$).

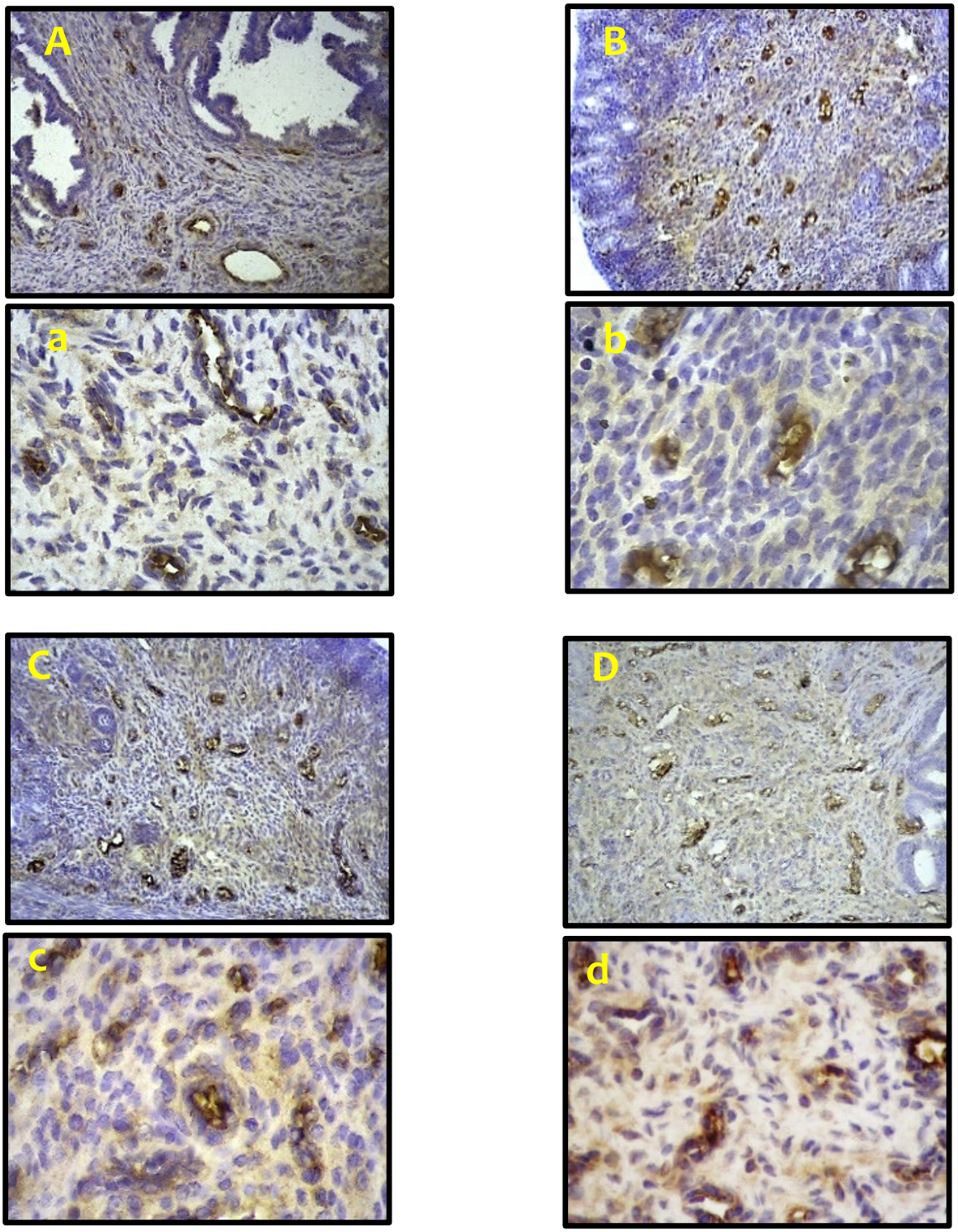
(Table 4.12): CD68 macrophages (number/mm^2) in pregnant does following DEX administration from 10 to 17 dG.

Groups	No	dG	Mean ± SD
G4	5	18	$5927^a \pm 553$
G5	5	18	$2148^b \pm 266$
G6	5	18	$1927^b \pm 218$
G5	3	28	$1989^b \pm 333$
G6	3	28	$2025^b \pm 229$

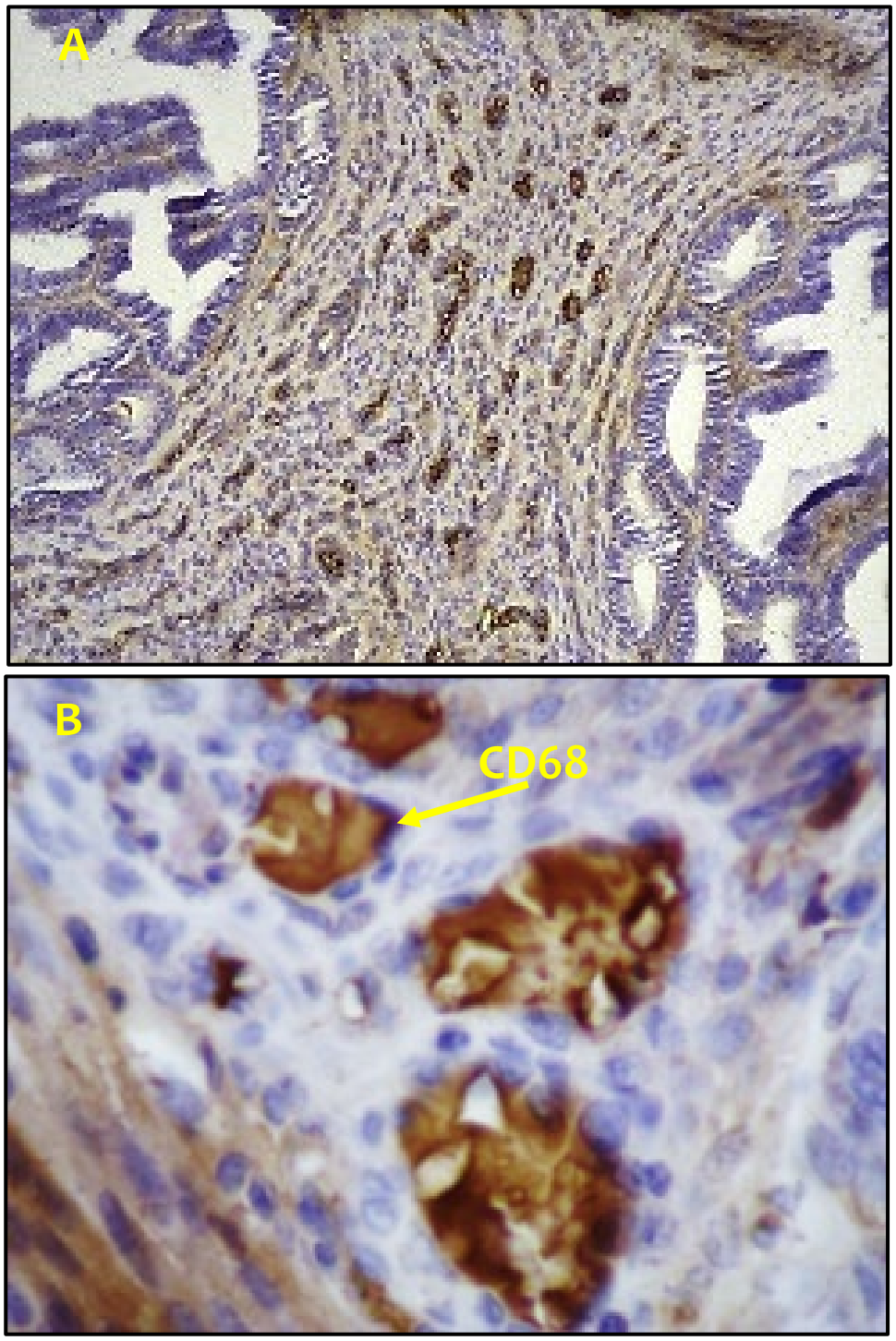
No= Number of animals, dG= day of Gestation, SD= Standard Deviation, a and b= Significant variation ($p \leq 0.05$).



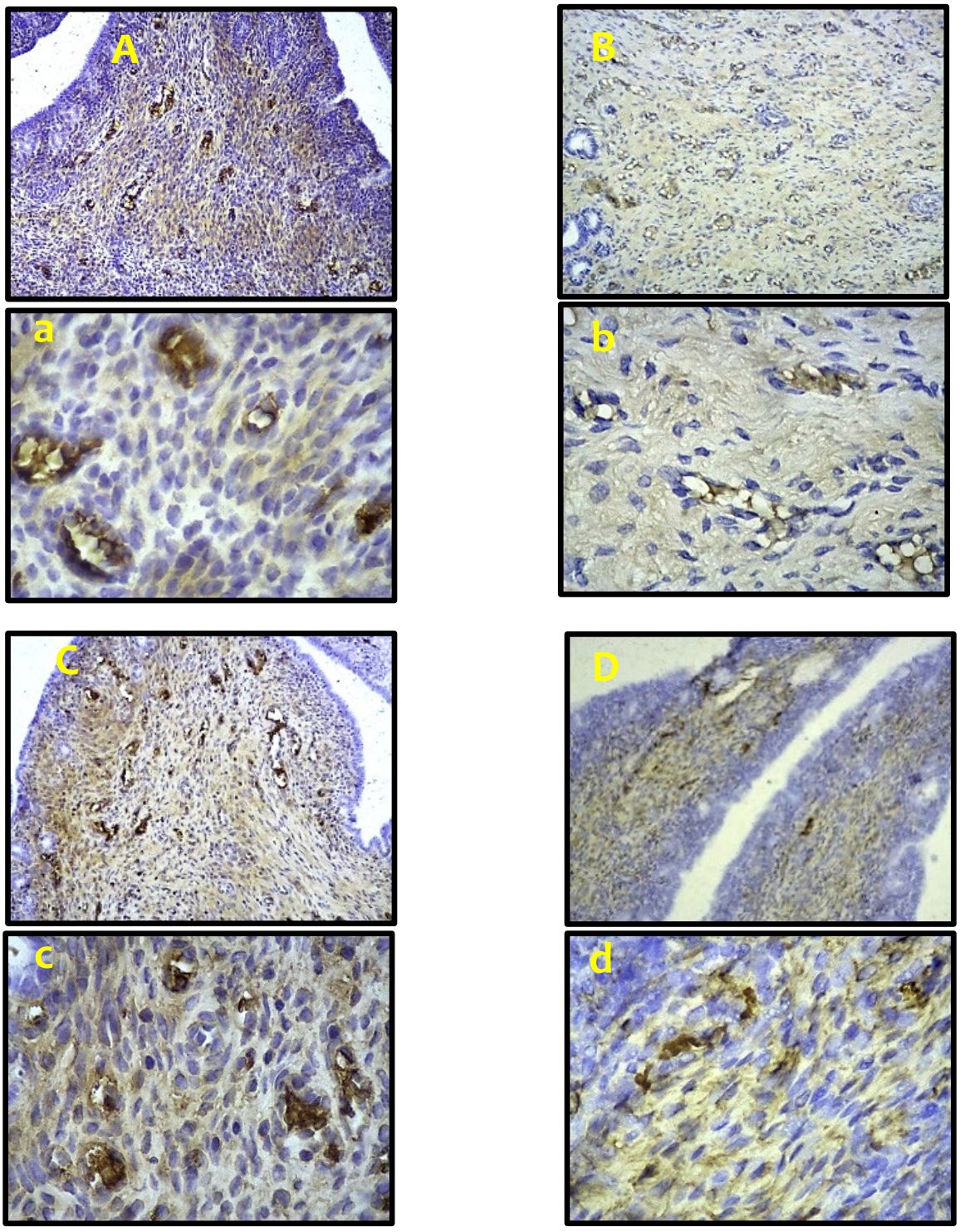
(Fig. 4.16): Immunohistochemical staining of CD68 (brown color) in the control pregnant does placenta at 10 dG. (A): Magnification: 100x. (B): Magnification: 400x).



(Fig. 4.17): Immunohistochemical staining of CD68 (brown color) in the placentae of pregnant does treated with DEX from 5-9 dG. (A): HD at 10 dG. Magnification: 100x (a): HD at 10 dG. Magnification: 400x (B): LD at 10 dG. Magnification: 100x (b): LD at 10 dG. Magnification: 400x. (C): HD at 28 dG. Magnification: 100x. (c): HD at 28 dG. Magnification: 400x. (D) LD at 28 dG. Magnification: 100x. (d) LD at 28 dG. Magnification: 400x.



(Fig. 4.18): Immunohistochemical staining of CD68 (brown color) in the control pregnant does placenta at 18 dG. (A): Magnification: 100x. (B): Magnification: 400x).



(Fig. 4.19): Immunohistochemical staining of CD68 (brown color) in the placentae of pregnant does treated with DEX from 10-17 dG. (A): HD at 18 dG. Magnification: 100x (a): HD at 18 dG. Magnification: 400x (B): LD at 18 dG. Magnification: 100x (b): LD at 18 dG. Magnification: 400x. (C): HD at 28 dG. Magnification: 100x. (c): HD at 28 dG. Magnification: 400x. (D) LD at 28 dG. Magnification: 100x. (d) LD at 28 dG. Magnification: 400x.

(Table 4.13): IDO expression level (%\mm^2) in pregnant does following DEX administration from 5 to 9 dG.

Groups	No	dG	Mean ± SD
G1	5	10	36.86^a ± 8.571
G2	5	10	10.88^b ± 2.382
G3	5	10	13.34^b ± 1.021
G2	3	28	14.05^b ± 1.934
G3	3	28	13.17^b ± 4.721

No= Number of animals, dG= day of Gestation, SD= Standard Deviation, a and b= Significant variation ($p \leq 0.05$).

(Table 4.14): IDO expression level (%/mm^2) in pregnant does following DEX administration from 10 to 17 dG.

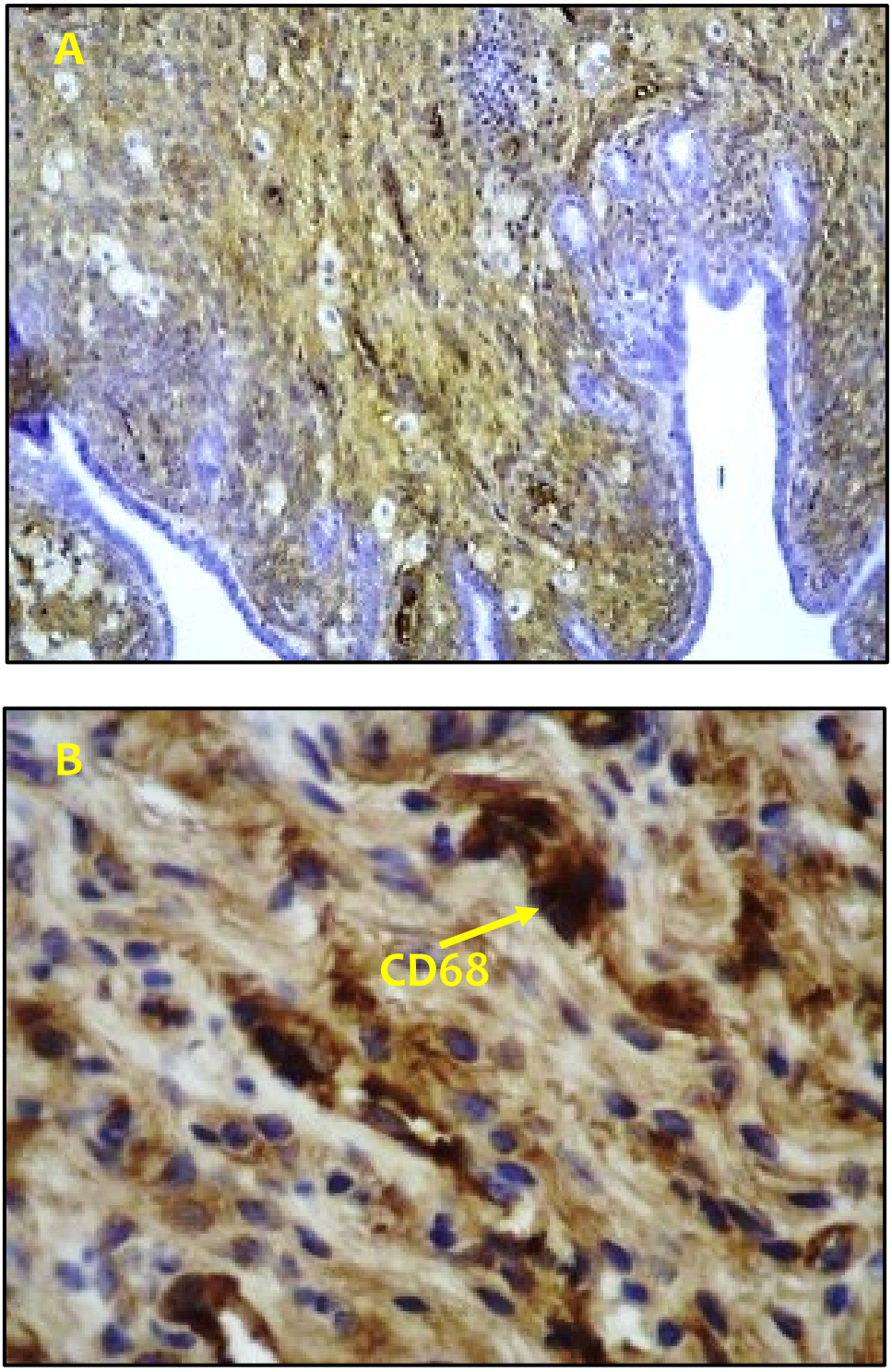
Groups	No	dG	Mean ± SD
G4	5	18	37.44^a ± 2.044
G5	5	18	12.47^b ± 3.621
G6	5	18	12.35^b ± 3.883
G5	3	28	15.32^b ± 3.366
G6	3	28	13.08^b ± 2.914

No= Number of animals, dG= day of Gestation, SD= Standard Deviation, a and b= Significant variation ($p \leq 0.05$).

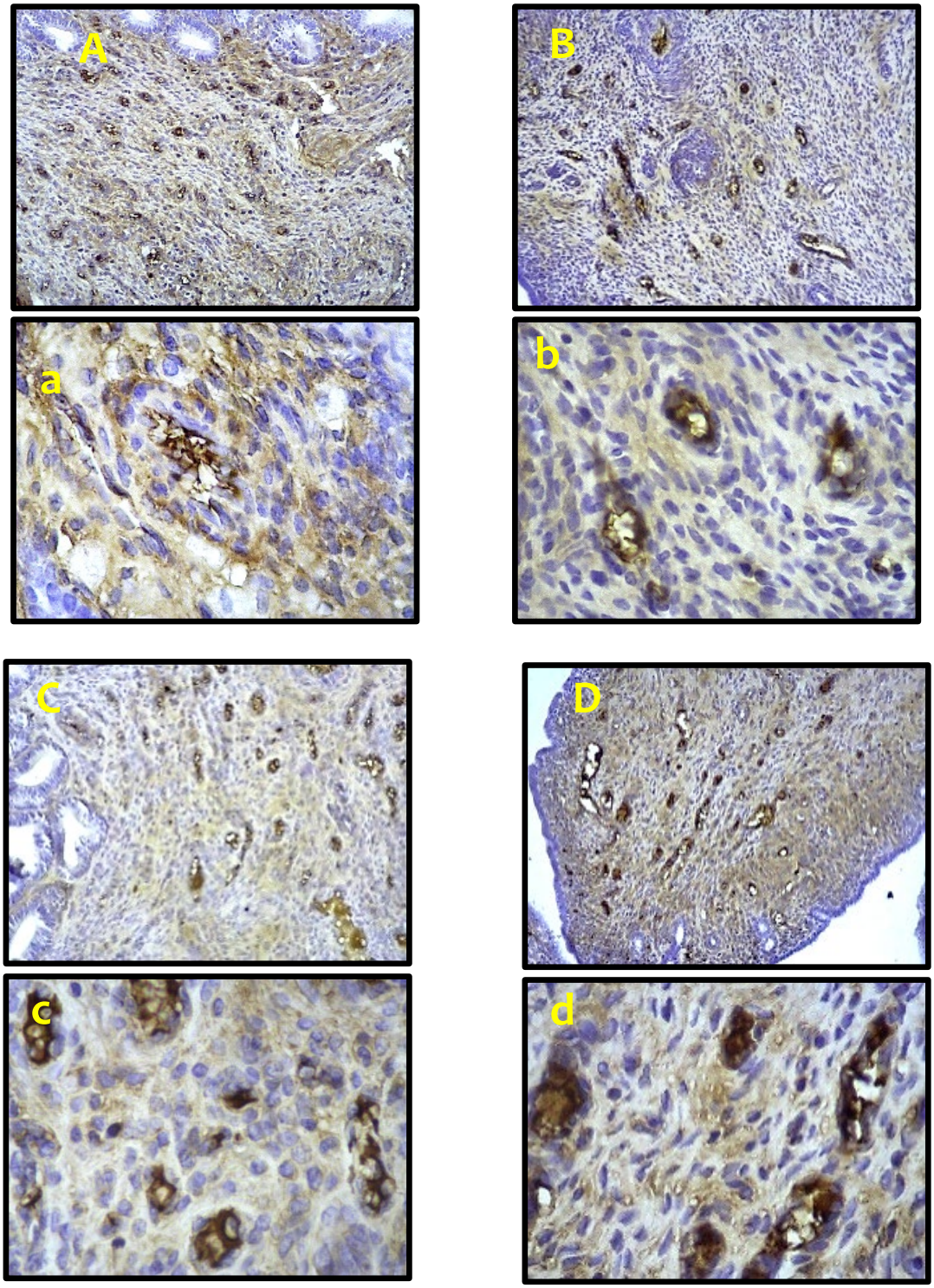
(Table 4.15): IDO expression level scores of the percent positivity of stained cells and the staining intensity.

Group name	No×4	dG	Score			
			0	1+	2+	3+
G1	5(20)	10	0	1	16	3
G2	5(20)	10	3	14	2	0
G3	5(20)	10	1	18	0	1
G2	3(12)	28	3	15	2	0
G3	3(12)	28	4	15	1	0
G4	5(20)	18	0	0	15	5
G5	5(20)	18	2	16	2	0
G6	5(20)	18	3	14	3	0
G5	3(12)	28	1	10	1	0
G6	3(12)	28	1	9	1	1

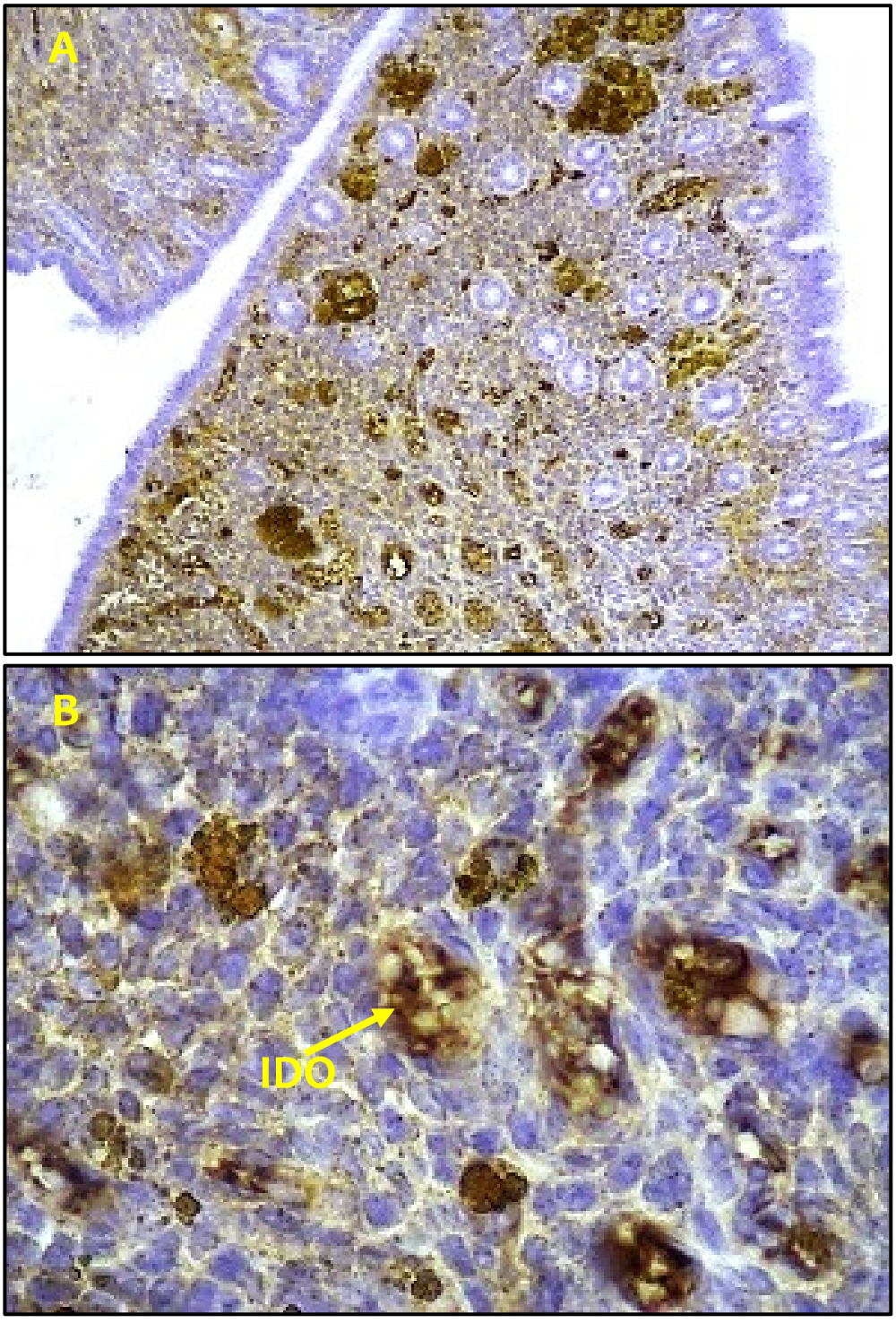
Note: 0= Negative, 1+= Weak, 2+= Moderate, 3+= Strong.



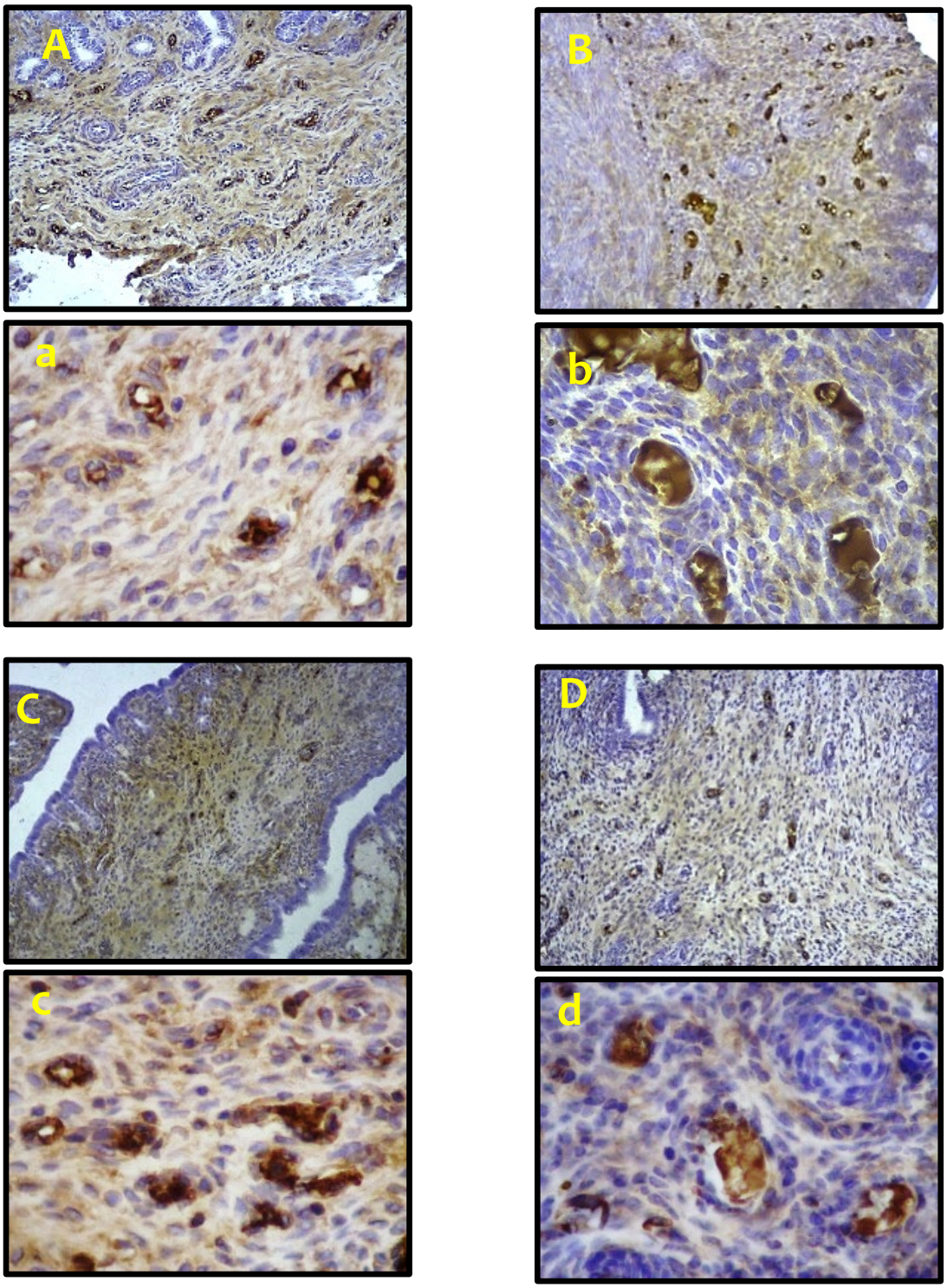
(Fig. 4.20): Immunohistochemical staining of IDO (brown color) in the control pregnant does placenta at 10 dG. (A): Magnification: 100x. (B): Magnification: 400x).



(Fig. 4.21): Immunohistochemical staining of IDO (brown color) in the placentae of pregnant does treated with DEX from 5-9 dG. (A): HD at 10 dG. Magnification: 100x (a): HD at 10 dG. Magnification: 400x (B): LD at 10 dG. Magnification: 100x (b): LD at 10 dG. Magnification: 400x. (C): HD at 28 dG. Magnification: 100x. (c): HD at 28 dG. Magnification: 400x. (D) LD at 28 dG. Magnification: 100x. (d) LD at 28 dG. Magnification: 400x.



(Fig. 4.22): Immunohistochemical staining of IDO (brown color) in the control pregnant does placenta at 18 dG. (A): Magnification: 100x. (B): Magnification: 400x).



(Fig. 4.23): Immunohistochemical staining of IDO (brown color) in the placentae of pregnant does treated with DEX from 10-17 dG. (A): HD at 18 dG. Magnification: 100x (a): HD at 18 dG. Magnification: 400x (B): LD at 18 dG. Magnification: 100x (b): LD at 18 dG. Magnification: 400x. (C): HD at 28 dG. Magnification: 100x. (c): HD at 28 dG. Magnification: 400x. (D) LD at 28 dG. Magnification: 100x. (d) LD at 28 dG. Magnification: 400x.

Chapter Five
Discussion

5: Discussion

The present project was demonstrating an intimate relationship between the migratory trophoblast and success of pregnancy.

After the embryo implantation; there were a series of physiological, morphological, histological and immunohistochemical changes in the uterus and spiral arteries supplying the placenta with maternal blood. These changes were coinciding with presence of the embryo inside the uterus and its need for adequate supply of nutrients and oxygen. In fact, modulation of these maternal tissues is considered as prerequisite for accepting the new comer i.e. the embryo and its growth inside the uterus till delivery. All these changes were coupled with new profile of the hormones of the pregnant does, certainly, in favor of the growing embryo. Moreover, regarding the results of current study obtained from the treated groups there were pronounced effects of DEX on the different parameters sought in the study. The effect of the drug was expressed by failure of implantation at the group which have injected two days prior to the implantation (day 5 dG). The groups which received the treatment in latter days of implantation (day 10 dG) were showing resorption of the fetuses. These results are contradicting the results of Nevagi and Kaliwal (2001) who pointed that a dexamethasone injection was having no interference with the implantation, its effect only seen in late stages of gestation.

5.1: Hormonal and Gene Expression Study

5.1.1: Progesterone Study

Normally, as seen in the present study, progesterone hormone level increases progressively as the pregnancy is advancing in order to maintain the uterus quiescent status during gestation which were compatible with the result of Yahi *et. al.,* (2017a). The adverse effects of dexamethasone were imposing changes in the normal

concentrations of progesterone and its receptors during pregnancy (Yahi *et. al.,* 2017b). Thus, the lower progesterone concentration of current study in treated groups compared to control groups could be attributed to DEX which has suppressed the secretion of LH and FSH through a direct effect on the anterior pituitary gland, as noticed in previous study on mice (Ahmadabad *et. al.,* 2016). In addition to that, 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 glucocorticoid receptors (GR). In mice, corticosterone administration reduced hypothalamic expression of KISS1 and suppressed the hypothalamic - pituitary-gonadal (HPG) axis. The second neuropeptide, GnIH inhibits the activity of GnRH neurons and KISS1 neurons (Luo *et. al.,* 2016 and Whirledge and Cidlowski, 2017). Many studies showed that dexamethasone caused significant decreases in progesterone levels in sheep (Yahi *et. al.,* 2017b), mice (Ahmadabad *et. al.,* 2016), and rats while progesterone receptors (PR) were upregulated (McDonald *et. al.,* 2003).

5.1.2: Progesterone Receptor (PR) Study

Glucocorticoids and progesterone are potent activators of glucocorticoids receptors (GR) and PR, respectively. Relative binding affinity of PR is progesterone 100% (Attardi *et. al.,* 2007), corticosterone 2.6% (Solano and Arck, 2020) and dexamethasone 0.2% (Issar *et. al.,* 2006). Its expression in immune cells is limited to specific cell but in uterus is too high (Whirledge *et. al.,* 2015). With respect to GR the relative binding affinity is progesterone 1–6% (Attardi *et. al.,* 2007), corticosterone 85% (Solano and Arck, 2020) and dexamethasone 100% (Issar *et. al.,* 2006). Its expression in immune cells is too high (Lissauer *et. al.,* 2015; Engler *et. al.,* 2017) but in uterus is lesser (Arenas-Hernandez *et. al.,* 2019). That means PR

and GR have exchangeable binding ability for each other but in limit manner. In this study, PR upregulation in treated groups might be a compensatory mechanism to enhance progesterone sensitivity. The mechanism is accomplished through increasing of PR mRNA levels and gene transcription (Yahi *et. al.,* 2017a). Moreover, the data of current study showed that there was significant increase of PR in HD treatment compared to LD treatment 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 size (Whirledge and Cidlowski, 2017). 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 dexamethasone effects may be reversible after DEX withdrawal (Cote and Gage, 1986).

5.1.3: Calcitonin Study

The current study results have shown that there was an increase in calcitonin hormone level of control groups at 18 dG compared to 10 dG. Similarly, Cote and Gage, (1986) found that the level of calcitonin hormone increases with pregnancy progressing. Silva *et. al.,* (1981) observed that parathyroid hormone (PTH) level was increased in women during pregnancy. In addition, they found that intestinal absorption of calcium increases in pregnancy, it could be attributed to increase in PTH. The hypercalcinemia serves to protect the maternal skeleton against demineralization (Cote and Gage, 1986) and the fetus calcium needs are met (Silva *et. al.,* 1981). However, the present results have observed that significant increase of calcitonin in all treated groups compared to control groups. Muszynski *et. al.,* (1983) found that the dexamethasone administration caused increase in calcitonin between

4 and 6 days of treatment, and demonstrated that dexamethasone 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 transcription (Cote and Gage, 1986).

5.2: Apoptosis and Histological Studies

5.2.1: Unremodeled Spiral Arteries and Implantation Failure Studies

The results of present study have revealed that there were early signs of spiral artery remodeling with subsequent success of pregnancy in control group at 10 dG. That entails conversion of the endometrial epithelium from non-receptive to receptive state which requires adhesion molecules, and receptor-ligand interactions (Nicholls *et. al.,* 2011). Many of these proteins essential in establishment of pregnancy and decidualization are synthesized as inactive precursors, then converted to active forms by proteolytic enzymes, proprotein convertases (PCs) (Khatib *et. al.,* 2002). In rabbit, PC5/6 mRNA was elevated prior to blastocyst attachment on day 6, and further increased on day 6.5 in the endometrial glands and epithelial lining cells. On the top of that it has increased in higher level on day 7-10 in the multinucleated luminal epithelial cells and decidual cells at the determined site of embryo implantation (Nicholls *et. al.,* 2011). Those data agree with Nie *et. al.,* (2003) in mouse and Heng *et. al.,* (2010) in woman. PC5/6 activates several essential proteins like propeptide hormones such as calcitonin, progrowth factors such as VEGF and EGF, cell surface receptors (Okada *et. al.,* 2005), and integrins (Stawowy *et. al.,* 2004; Mendes *et. al.,* 2019). The integrin is considered among the best components for implantation in the rabbit (Nicholls *et. al.,* 2011). It is a cell surface adhesion receptor, whose expression has been shown to be elevated in the

endometrium at the time of implantation in both humans and other mammalian species. In rabbits, the integrin is present on the trophoblast at 6.5 dG and appears to be involved in early materno-embryoic cross-talk (Illera *et. al.,* 2003). Similarly, calcitonin as an important mediator of implantation. It is synthesized in glandular epithelial endometrium cells during the window of implantation, where it is regulated by progesterone. Administration of antisense oligodeoxynucleotides against calcitonin mRNA resulted in a noticeable decrease in the number of implanted embryos in the rat (Kodaman and Taylor, 2004). The mode of action could involve the dissolution of gap junctions between cells because a calcitonin-induced increase in intracellular calcium, thus decreases endometrial cell expression of E-cadherin, a cell-surface glycoprotein that mediates cell–cell adhesion among epithelial cells. Such increased permeability is postulated to facilitate implantation of the blastocyst (Bagchi *et. al.,* 2001). Also, VEGF has crucial role in controlling of uterine artery remodeling during gestation (Babischkin *et. al.,* 2019). Inhibition of placental VEGF expression is linked to reduction in placental vascularization (Hewitt *et. al.,* 2006). Furthermore, PC5/6 mRNA levels were increased in mice treated with 17-estradiol plus medroxyprogesterone acetate but not with only17-estradiol during decidualization (Okada *et. al.,* 2005).

In contrast, the results from treated group administrated with DEX from 5-9 dG have revealed that there was failure of implantation compared to control group. Also, there were unremolding in spiral arteries in treated group administrated with DEX from 5-9 dG compared to control group, and that effect of unremodeling continued till 28 dG.

The failure of implantation and the unremolding in spiral arteries in treated group administrated with DEX from 5-9 dG may be attributed to that DEX decreased

progesterone, which in turn, downregulated PC5/6 mRNA, and subsequently there weren't activated components essential for implantation and decidualization. Moreover, the unremodeling in spiral arteries of the current study continued till 28 dG could be attributed to accompanying low progesterone level of pregnant does treated with DEX as our data demonstrated.

5.2.2: Early Spiral Arteries Remodeling and Resorption of Embryos Studies

The results of present study have revealed that there were structural modifications which ended up in remolding in the wall of spiral arteries, that was expressed by successful pregnancy in control group at 18 dG. Both of those events, remodeling and successful pregnancy, could be caused by high level of progesterone that leads to high ratio of trophoblasts and microphages as demonstrated by our results. Progesterone is essential hormone for successful pregnancy. It is acting in synchronization with estrogen which is paving the way for drastic changes in the uterus and its stromal and vascular tissues. Both of them have many functions viz: estrogen acts indirectly to attract macrophages (chemotactic) to the endometrium (Vishnyakova *et. al.,* 2019). It stimulates fibroblasts to produce cytokines which in turn draw macrophages inward, also it stimulates macrophage proliferation (Pepe *et. al.,* 2018). Progesterone promotes an immunotolerance in maternal-fetal interface by inducing differentiation of macrophages into M2 phenotype (Tsai *et. al.,* 2017). M2 macrophages is important for migration of trophoblast, angiogenesis, and early remodeling of spiral artery (Yao *et. al.,* 2019) by secreting vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) (Saikia *et. al.,* 2016; Vishnyakova *et. al.,* 2019; Chambers *et. al.,* 2021). Through the Spiral artery remodeling, IL-6 and CXCL8 secreted by extravillous trophoblasts, under progesterone control, induce uterine endothelial cells to express the chemokines

CCL14 and CXCL6. CCL14 is chemotactic of both uNK cells and decidual macrophages, whereas CXCL6 is chemotactic of uNK cells (Choudhury *et. al.,* 2017). Both macrophages and uNK cells express angiogenic factors; vascular endothelial growth factor, placental growth factor, and angiopoetin-2 (Hazan *et. al.,* 2010). As well as, they produce matrix metalloprotease7 and 9 which are important in degradation of the endometrial extracellular matrix (Pollheimer *et. al.,* 2018), and trophoblast invasion (Cohen *et. al.,* 2006; Pollheimer *et. al.,* 2018). Reduced progesterone may bring about fetal loss in mammals (Friebe *et. al.,* 2011; Prados *et. al.,* 2011 and Solano and Arck, 2020) by decreasing in placental macrophage and uNK related with defect in remodeling of uterine spiral artery and trophoblastic migration which lead to abortion (Guenther *et. al.,* 2012; Yao *et. al.,* 2019).

From above information, it could be deduced that there were coordinated events running concomitantly to reach to the target of spiral artery remodeling. Thus, the study is in consistence with Faas and De Vos (2017) who referred to the coordinating action of the invading trophoblast, macrophage and uNK cells in normal pregnancy to bring about optimal structural modifications in the materno-fetal tissues.

On the other hand, the results have observed that there was resorption of embryos in treated group administrated with DEX from 10-17 dG compared to control group. Also, there was early remolding in spiral arteries in treated group administrated with DEX from 10-17 dG compared to control group, and that effect, early remodeling, continued till 28 dG.

The present study confirmed that DEX caused decreases in progesterone levels in pregnant does, which was associated with decreased in macrophages and trophoblasts. Also, Previous studies observed that DEX caused decreases in

progesterone levels in sheep (Yahi *et. al.,* 2017a), progesterone, 17β-estradiol, placental macrophages, uNK cells, and placental growth, and increases in abortion rate (Ahmadabad *et. al.,* 2016). Moreover, dexamethasone reduced some populations of trophoblasts, placental cell- proliferating nuclear antigen, and fetal growth, and increased proapoptotic factors (Bax, p53) (Braun *et. al.,* 2015). As well as, maternal dexamethasone treatment results in disturbance in trophoblasts development 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 (Zong *et. al.,* 2016). Ozmena *et. al.,* (2015) found that DEX brings about a decrease in VEGF and PIGF expression (angiogenic factors) by affecting Akt/mTOR pathway which causes reducing in phosphorylation of Akt, p70S6K, and 4EBP1, in rat placenta. Phosphorylation of Akt is carried out by mTOR Complex 2 (Sarbassovet *et. al.,* 2005), Subsequently, mTORC1 phosphorylates p70S6K and 4EBP1 (Feldman *et. al.,* 2009). Reduced phosphorylation of p70S6K and 4EBP1 result in decreased expression of VEGF and PIGF (Ozmena *et. al.,* 2015). Besides, glucocorticoids, including DEX, may inhibit angiogenesis by affecting stem cells to secret VEGF (Greenberger *et. al.,* 2010). The above information explains the two consequences of present study: first, the early remolding in spiral arteries, second, resorption of embryos which is initiated by embryo endogenous apoptosis without maternal interference (Drews *et. al.,* 2020). Thus, the early remolding in spiral arteries continued till 28 dG could be attributed to accompanying low progesterone levels of pregnant does treated with DEX.

5.3: Immunohistochemical Study

The data of current study have shown that there were significantly decreases in all parameters, including trophoblasts, macrophages, and IDO of treated groups in comparison to controls groups.

First of all, progesterone promotes an immunotolerance in maternal-fetal interface by inducing differentiation of macrophages into M2 phenotype (Tsai *et. al.,* 2017), prevents dendritic cells maturation (Xiu *et. al.,* 2016), and consequently generating Treg cells and suppressing of CD8+ T cell cytotoxicity during pregnancy (Lissauer *et. al.,* 2015; Solano *et. al.,* 2015). Also, progesterone has effects on hemostasis and activity of trophoblasts such as stimulation of trophoblast through progesterone receptors to produce VEGF (Rätsep *et. al.,* 2015; Solano and Arck, 2020).

Previous study confirmed that DEX were associated with decreased progesterone and 17β-estradiol levels, and reduced uterine macrophages in pregnant BALB/c mice (Ahmadabad *et. al.,* 2016). 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 resulted in disturbance in trophoblasts development then dysregulation of PRL gene expression by inhibition of Akt/protein kinase B pathway. Diminished placental Akt activation, which was associated with decreased pro-apoptotic protein (BAD) phosphorylation and increased poly-ADP-ribose-polymerase (PARP) cleavage, an indicator of apoptosis (Zong *et. al.,* 2016). Besides, IDO is expressed by different cell types as antigen presenting cells such as macrophages (Schön, 2019; Krupa and Kowalska, 2021), and trophoblasts (Zong *et. al.,* 2016). 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 (YU *et. al.,* 2017). In addition, IDO promotes trophoblast proliferation and migration by decreasing STAT3 phosphorylation led to MMP9 expression (Suman *et. al.,* 2013; Halasz *et. al.,* 2013). From above information, the lowering in macrophages, trophoblasts, and IDO could be attributed to those reasons.

Conclusions and Recommendation

Conclusions

- The current research data have demonstrated that dexamethasone is very effective on progesterone, PR, and calcitonin levels. Also, it has highest degree immunosuppressive effects on placental parameters including trophoblasts, macrophages, and IDO. In sum total, those effects led to disturbance in spiral arteries remodeling which was associated with failure of implantation or resorption of embryos during early, mid, and late pregnancy. And its effects are lasted for long time of pregnancy.
- Negative impact of DEX has been manifested by failure of implantation if given during the period of implantation or resorption of fetus if given in later period of gestation.
- The results have highlights of the extreme caution which should be taken when necessity is there, especially in compromised certain medical cases which require DEX.
- The current study proved that the early exposure to DEX during prenatal life has its impact during postnatal life, therefore the effect may stay for long period of time after birth.

Recommendations

1. Following up of the long-lasting effect of DEX in the adult life, human or experimental animals, whom mothers were given drug during their pregnancy.
2. Studying the role of pericyte in the uterine angiogenesis during pregnancy.
3. Inventing a method to detect proprotein convertase 5\6 (P5\6) in any easily and accessible fluid in the body like the blood and saliva.
4. Studying the effective dose of DEX.
5. Localization and quantification of CD56 uNK affected DEX during pregnancy.
6. Estimation the level of transforming growth factor (TGF) affected DEX during pregnancy.
7. Studying the role of apoptotic gene expression.
8. Following up gradually increasing doses.
9. The advice of the author is to use the DEX with caution during pregnancy. It is a matter of compromise between the personal health and the outcome of pregnancy.

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النسخ العكسي الكمي لتقنية تفاعل البلمرة المتسلسل في الوقت الحقيقي (Qrt، RT-PCR) ، الدراسة النسيجية ، الكشف الكيميائي المناعي للأرومة الغاذية ، IDO ، والخلايا البلعمية باستخدام مضادات السيتوكراتين 7 (Ck7) ، مضاد IDO ، مضاد - مجموعات الأجسام المضادة CD68 ، على التوالي، وإعادة تشكيل الشرايين الحلزونية باستخدام تقنية Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL). تم إجراء التقييمات الإحصائية للبيانات باستخدام تحليل التباين أحادي الاتجاه (ANOVA) ، متبوعا باختبار Post Hoc-Duncan لتحديد الفروق المعنوية لجميع المقارنات. تم قبول فروق ذات دلالة إحصائية عند $P \leq 0.05$.

أظهرت النتائج أن مستوى هرمون البروجسترون في الدم ، وهجرة الأرومة الغاذية ، و IDO ، وخلايا البلاعم انخفضت بشكل ملحوظ ($p \geq 0.05$) في جميع المجموعات المعالجة مقارنة بمجموعات السيطرة. في المقابل ، ارتفع مستوى الكالسيتونين معنويا ($p \geq 0.05$) في جميع المجموعات المعالجة مقارنة بمجموعات السيطرة. زاد التعبير الجيني PR في المشيمة (الأم-الجنين) زيادة معنوية ($p \geq 0.05$) في جميع المجموعات المعالجة مقارنة بمجموعات السيطرة. علاوة على ذلك ، كانت هناك اختلافات كبيرة بين المجموعات المعالجة من حيث الجرعة وفترة الحمل حيث ؛ زاد بشكل ملحوظ في الجرع العالية مقارنة مع الجرع المنخفضة في المجموعات المعالجة ، لكنه انخفض بشكل ملحوظ في اليوم الثامن والعشرين من الحمل مقارنة باليوم العاشر واليوم الثامن والعشرين من الحمل. بالإضافة إلى ذلك ، لوحظت النتائج أنه كان هناك فشل في الانغراس الجنيني في المجموعة المعالجة المحقونة بالديكساميثازون من اليوم الخامس الى اليوم التاسع من الحمل ، وارتشاف الأجنة في المجموعة المعالجة المحقونة بالديكساميثازون من اليوم العاشر الى اليوم السابع عشرمن الحمل ، مقارنة بمجموعات السيطرة. أيضًا كان هناك انعدام إعادة تشكيل في الشرايين الحلزونية للمجموعة المعالجة التي تمت معالجتها بالديكساميثازون من اليوم الخامس الى اليوم التاسع من الحمل ، وإعادة تشكيل المبكر في الشرايين الحلزونية للمجموعة المعالجة التي تمت معالجتها بالديكساميثازون من اليوم العاشر الى اليوم السابع عشرمن الحمل ، مقارنة بمجموعات السيطرة. استمر كل من التأثيرات (انعدام إعادة تشكيل وإعادة تشكيل المبكر) حتى اليوم الثامن والعشرين من الحمل. وقد لاحظت النتائج أيضًا أن الحيوانات الثلاثة الحوامل من كل مجموعة سيطرة استمرت حتى الولادة في اليوم 30-33 من الحمل.

نستنتج من الدراسة الحالية أن الديكساميثازون له تأثيرات عميقة على جميع المتغيرات: مستوى البروجسترون ، مستوى الكالسيتونين ، هجرة الأرومة الغاذية ، IDO ، الخلايا البلعمية ، انغراس الجنين وارتشافه ، وعلى اعادة تشكيل الشرايين الحلزونية أثناء الفترة المبكرة والمتوسطة والمتأخرة من الحمل. كذلك استمرت آثاره طوال فترة الحمل.

الخلاصة

ديكساميثازون دواء يستخدم لعلاج العديد من الأمراض والحالات الطبية في كل من الحيوانات والبشر. لكن إعطاء القشرانيات السكرية الاصطناعية ، بما في ذلك الديكساميثازون ، كعلاج ، يؤدي إلى اختلال توازن الغدد الصماء. هذا الاختلال في التوازن هو أساس مضاعفات الحمل. تبحث الدراسة الحالية في تأثيرات الجرعات المختلفة من ديكساميثازون [جرعة عالية من ديكساميثازون (1.125 مجم) وجرعة منخفضة من ديكساميثازون (0.562 مجم)] على مستقبلات البروجسترون ، البروجستيرون ، الكالسيتونين ، ألأنسجة المشيمية (الام-الجنين) ، هجرة الأرومة الغاذية ، الخلايا البلعمية ، انزيم الإندولامين 2،3- ديوكسجيناز (IDO) ، وإعادة تشكيل الشرايين الحلزونية في اناث الارانب الحوامل المحلية (*Oryctolagus cuniculus*). أجريت الدراسة خلال المدة من كانون الثاني (2021) إلى تشرين الأول (2021) في قسم علوم الحياة / كلية العلوم / جامعة بابل. أستخدم في الدراسة ستين انثى حامل ، ووضعت في البيت الحيواني وقسمت إلى ست مجاميع (10 أرانب لكل مجموعة). يتم التعامل مع هذه المجاميع على النحو التالي: المجموعة الاولى (G1) (كمجموعة تجريبية) تم حقنها يوميًا تحت الجلد بواسطة (1 مل) من اليوم الخامس إلى اليوم التاسع من الحمل بجرعة عالية. ثم ضحي بسبع اناث في اليوم العاشر من الحمل ، وثلاث اناث في اليوم الثامن والعشرين من الحمل. المجموعة الثانية (G2) (كمجموعة تجريبية) تم حقنها يومياً تحت الجلد بمقدار (1 مل) من اليوم الخامس إلى اليوم التاسع من الحمل بجرعة منخفضة. ثم ضحي بسبع اناث في اليوم العاشر من الحمل ، وثلاث اناث في اليوم الثامن والعشرين من الحمل. المجموعة الثالثة (G3) (كمجموعة سيطرة) تم حقنها يومياً تحت الجلد بمقدار (1 مل) من الخامس إلى اليوم التاسع من الحمل بمحلول كلوريد الصوديوم (0.9٪). بعد ذلك ضحي بسبع اناث في اليوم العاشر من الحمل ، وثلاثة اناث استمروا في الحمل حتى الولادة. المجموعةالرابعة (G4) (كمجموعة تجريبية) تم حقنها يومياً تحت الجلد بمقدار (1 مل) من اليوم العاشرإلى اليوم السابع عشر من الحمل بجرعة عالية. ثم ضحي بسبع اناث في اليوم الثامن عشر من الحمل ، وثلاث اناث في اليوم الثامن والعشرين من الحمل. المجموعة الخامسة (G5) (كمجموعة تجريبية) تم حقنها يومياً تحت الجلد بمقدار (1 مل) من اليوم العاشر إلى اليوم السابع عشر من الحمل بجرعة منخفضة. ثم ضحي بسبع اناث في اليوم الثامن عشر من الحمل ، وثلاث اناث في اليوم الثامن والعشرين من الحمل. المجموعة السادسة (G6) (كمجموعة سيطرة) تم حقنها يومياً تحت الجلد بمقدار (1 مل) من اليوم العاشر إلى اليوم السابع عشر من الحمل بكلوريد الصوديوم (0.9٪). ثم ضحي بسبع اناث في اليوم الثامن عشر من الحمل ، وثلاث اناث استمروا في الحمل حتى الولادة. تم جمع مصل الدم للمقايسات الهرمونية للبروجسترون والكالسيتونين باستخدام تقنية الفحص المناعي المرتبط بالإنزيم (ELISA). تم أخذ خزعات المشيمة (الجنين-الأم) من أجل؛ التعبير الجيني باستخدام

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل / كلية العلوم / قسم علوم الحياة

هجرة الخلايا الغاذية في الاناث الحوامل للارانب المعاملة بالدكساميثازون

أطروحة

مقدمة الى مجلس كلية العلوم في جامعة بابل وهي جزء من متطلبات نيل درجة الدكتوراه فلسفة في العلوم / علوم الحياة / الحيوان

من

آمال فيصل لفتة عطية

بكالوريوس علوم حياة / جامعة بابل (2002)

ماجستير فرع الحيوان / علوم حياة / كلية العلوم / جامعة بابل (2006)

إشراف

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