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THE EFFECT OF HYPERSTIMULATION ON TRANSFORMING GROWTH FACTOR β_1 AND β_2 IN THE RAT UTERUS: POSSIBLE CONSEQUENCES FOR EMBRYO IMPLANTATION

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfillment of the requirements for the degree of Master of Science in Medicine

Johannesburg, 2007

DECLARATION

I, Aleksandra Jovanovic, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Aleksandra Jovanovic

_____ day of _____, 2007

To my son Andrej

I was once told that to bring up a child, one merely needs to set a good example… I had good examples when I was growing up, and now, I am trying to do the same for you…

Mojim roditeljima Mirjani i Dr. Petru Kosuticu (1932-2001)

Ocigledno da je moguce, u drugoj zemlji, na tudjem jeziku i cak 15 godina kasnije…….znam da ste od svih vas dvoje najvise ponosni

To my parents Mirjana and Dr. Petar Kosutic (1932-2001)

It is obviously possible in another country, in a foreign language and even 15 years later……I know you are the most proud

ABSTRACT

Ovarian hyperstimulation is achieved through ovarian gonadotropin stimulation, and thus associated with supraphysiological levels of oestrogen and progesterone. To investigate the effects of exogenous gonadotropins on the expression of TGF β_1 and TGF $\beta_{2,}$ which have been recognized as possible modulators of many endometrial functions, FSH and hCG were superimposed upon the normal hormonal milieu of the cycling rat, prior to mating. Endometrial tissue was collected at 4.5, 5.5 and 6.5 days after mating. ELISA was performed to estimate blood oestrogen and progesterone levels and immunohistochemistry was undertaken to localize TGF β_1 and TGF β_2 in the uterine endometrium. Apart from the known detrimental effects of hyperstimulation on gross morphology, hormone levels and endometrial histology, the hyperstimulation was also found to affect TGF β expression. An increase in the expression of TGF β_2 was distinct in the glandular epithelium of the hyperstimulated animals, while regionalized expression of both TGF β_1 and TGF β_2 was prominent in the stroma. In conclusion, hyperstimulation affects the expression of both TGF β_1 and TGF $\beta_{2,}$ which may contribute to the disruption of the endometrial environment required for successful embryo implantation.

ACKNOWLEDGEMENTS

With thanks to:

Professor Beverley Kramer for the heaps of work she had supervising an inexperienced, Serbian student. I am grateful for every minute of her professional and private life

Professor J.C. Allan and Mr. Jason Hemingway for help with statistics

Professor Paul Manger for donating the normal rabbit serum

Mrs. Hasiena Ali for exceptional technical and beyond technical assistance

Mrs. L. York, S. Rogers and A. Mortimer for technical support

Dr Mirjana Strkalj and Ms. Tanya Augustine for their friendship

Mr. Sinisa Jovanovic, for financial and computer skills support

A note on the collection and analysis of blood

The blood from the 36 animals used in this study was jointly collected by Dr. M. Strkalj, Embryonic Differentiation and Development Research Programme, School of Anatomical Sciences, and myself. Dr. Strkalj performed the Elisa test on half of the animals (n=18) and I performed the test on the other half of the animals (n=18). Analysis of the data obtained from the Elisa was carried out independently by Dr. M. Strkalj and myself, Mrs. A. Jovanovic.

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1. INTRODUCTION

Despite numerous innovations in assisted reproductive technologies (ART), implantation rates per human embryo transfer are still very low and rarely exceed 30% (Human Fertilization and Embryology Authority, 2006). The majority of these technologies utilize controlled ovarian superovulation (hyperstimulation) for oocyte collection before the replacement of the fertilized oocyte into the uterine tube or uterus.

Hyperstimulation, the artificial induction of superovulation by the administration of follicle stimulating hormone (FSH) and human Chorionic Gonadotropin (hCG) is routinely used in ART (http://www.advancedfertility.com/ovarstim.htm). It has been shown that FSH and hCG cause adverse morphological and molecular changes in the endometrium (Stein and Kramer, 1989; Kramer *et al*., 1990; Kramer and de Wet, 1994; Peverini and Kramer, 1995). In addition, changes have also been noted in the implanting embryo (Ertzeid and Storeng, 2001; Terry *et al.*, 2001), altering the conditions necessary for normal implantation.

Administration of exogenous hormones is found to increase the amount of circulating oestradiol prior to implantation (Kramer, 1990), affect the progesterone: oestradiol ratio (Kramer *et al.*, 1993, Kramer and de Wet, 1994), decrease the vascular permeability of the endometrium (Kramer, 1997), prevent decidualization of the subepithelial stromal cells (Stein and Kramer, 1989) and reduce carbohydrates in the glycocalyx of the endometrium (Kramer and de Wet, 1994; Peverini and Kramer, 1995).

Considering the effects of exogenous hormones on the endogenous hormonal milieu as well as the endometrial environment, and the low rate of implantation concomitant with these, it has been concluded that both gross and molecular factors play an essential role in priming the uterus for reception of the embryo.

1.1. Hormonal control of endometrial function

The human endometrium is the end organ for the signals derived from the hypothalamic-hypophysial-ovarian axis. It is thus subjected to changes that will occur if the normal functioning of this axis becomes disturbed. Endometrial cells in the rat and mouse are under tight control of the interaction between the ovarian steroid hormones, oestrogen and progesterone (Psychoyos, 1973; Tabibzadeh, 1998). In the adult non-pregnant mouse, oestrogen directs proliferation of uterine epithelial cells, while the same process in the stroma requires both oestrogen and progesterone. These hormones have similar effects in the pregnant uteri of mice. On days 1 and 2, pre-ovulatory ovarian oestrogen stimulates cell proliferation. On day 3, progesterone, from corpora lutea, induces stromal cell proliferation, which is further potentiated on day 4 by ovarian oestrogen secretion. On the same day, epithelial cells stop proliferating and become differentiated, which makes the uterine epithelium receptive to the blastocyst (Carson *et al*., 2000; Dey *et al*., 2004).

Furthermore, Yoshinaga *et al*. (1969) showed results of oestrogen secretion during the oestrous cycle and early pregnancy in the rat, by using an intravaginal bioassay. According to their observations, oestrogen expressed a 24-hr secretion periodicity during the pre-implantation period. They registered measurable oestrogen levels in the rat ovarian venous plasma only at noon of each day of the normal cycle. Oestrogen further showed a continuous rise on the day before pro-oestrus and including pro-oestrus, with its maximum at noon of pro-oestrus. However, in early pregnancy, a continuous rise of this hormone was absent, while daily rhythmicity persisted. At noon of day 4 of pregnancy, oestrogen levels reached their maximum.

In addition, results obtained using an ovariectomy procedure at different times during early pregnancy have shown that ovariectomy followed by progesterone treatment from day 4 of pregnancy in the rat can be performed without disturbing the events of normal implantation. Animals ovariectomized earlier, showed delayed implantation until oestrogen was added to the progesterone treatment (Psychoyos, 1973). Moreover, hypophysectomy performed on the afternoon of day 3 of pregnancy, blocked implantation in progesterone-treated rats. If this procedure was carried out on the afternoon of day 4, progesterone treatment alone was sufficient to allow normal and timely implantation (Psychoyos, 1973).

It may be concluded from the above, that events that take place during the pre-implantation and peri-implantation period are hormonally influenced. Any procedure that will temporarily or permanently interfere with normal hormonal functioning of the

hypothalamic-hypophysial-ovarian axis will interfere with normal implantation.

1.2. The molecular basis for embryo implantation

Endometrial receptivity is a transitory and unique stage during which the endometrial cells achieve their maximum growth and sensitivity to the blastocyst. This window of implantation or receptive phase has a limited time and lasts less than 24 hours in rodents, after which the uterus becomes refractory for embryo implantation (Psychoyos, 1986; Aplin, 1997; Adams *et al.*, 2004).

The events that take place during the receptive or peri-implantation period require precise synchronization between ovarian oestrogen and progesterone. In mice and rats, oestrogen is essential for preparation of the progesterone-primed uterus for the receptive state, when the uterine milieu becomes favorable to blastocyst acceptance and implantation (Psychoyos, 1973). This delicate coordination involves the synchronized production of corresponding molecules that bind the apical uterine epithelium and the implanting trophectoderm surface. This results in the attachment of two opposing membranes, which is the initial and necessary event that will consequently result in successful implantation (Carson *et al*., 2000).

Some cases of unexplained infertility may be due to disrupted endometrial function, which may originate from alterations in the molecular repertoire that are crucial for implantation. The molecular

members that make the endometrium receptive to implantation are gradually being recognized. Among these are cytokines, such as leukemia inhibitory factor (LIF). According to Bhatt *et al*. (1991), LIF is expressed on day four of pregnancy, the day of commencement of implantation in the mouse. Growth factors such as heparin binding epidermal growth factor (Lim *et al*., 2006), are proposed to be important during implantation, by accelerating the expression of αvβ3 integrin in the peri-implantation mouse uterus. Das *et al*. (1995) showed that amphiregulin, a progesterone-regulated uterine epithelial cell growth factor, is associated with epithelial cell differentiation during implantation. Das *et al*. (1997a) have also established that the expression of beta-cellulin and epiregulin are restricted to the mouse uterine luminal epithelium and underlying stroma adjacent to the implanting blastocyst. Moreover, glycoconjugates such us proteoglycans including heparan sulfate are also required for implantation-related processes and participate in the early stages of embryo attachment (Farach *et al*., 1987; 1988).

Tabibzadeh *et al*. (1996) emphasized that the function of heat shock proteins may be to protect cells against the cytotoxic damage of TNF-alpha, particularly during the critical period of the "window of implantation". In addition, Fukuda *et al*. (1995) highlighted the role of the tastin-trophinin adhesion molecule complex which has a function in embryo implantation. Suzuki *et al*. (1998) subsequently established that bystin, a cytoplasmic protein, interacts with trophinin, tastin and cytokertain to promote cell adhesion between trophoblast and endometrial cells.

Additional markers of endometrial receptivity are matrix metalloproteases (MMPs) and their inhibitors - tissue inhibitors of metalloproteinases (TIMPs). By inhibiting MMPs activities, TIMP's participate in alteration of the extracellular matrix and thus have a role in decidual tissue remodeling and regulation of trophoblast invasion (Fisher and Damsky, 1993; Alexander *et al*., 1996). Another molecular member of the receptive endometrium is COX-2 (cyclooxigenase-2), an enzyme that is responsible for formation of important biological mediators such as prostaglandins. Cox-2 deficient females have multiple reproductive failures that include defects in ovulation, fertilization and implantation (Lim *et al*., 1997). Integrins have also been found to be expressed during the "window of implantation" in both human and mice (Tabibzadeh, 1998). They represent the cell surface receptors that interact with the extracellular matrix and mediate various intracellular signals. αVβ3 integrin is expressed during the window of implantation, but is absent in certain types of infertility, including the luteal phase defect (Lessey *et al*., 1992).

1.3. Transforming growth factor β (TGF β)

The transforming growth factor βs (TGF βs) belong to a superfamily known as the transforming growth factor β superfamily. Apart from TGF βs, this family includes inhibins, activin, anti-mullerian hormone, bone morphogenic protein, decapentaplegic and Vg-1. TGF βs are structurally related dimeric, disulfide linked peptide hormones. Members of this family include five TGF β isoforms (TGF β1-5) of which three isoforms (TGF β 1, TGF β 2 and TGF β 3) are prevalent in mammals.

The peptide structures of the three proteins are similar. They are all synthesized as prepropeptides of 112-114 amino acids that dimerize to give rise to active growth factors (Das *et al*., 1992). TGF β dimer binds to specific receptors on the cell surface. Although four receptors have been cloned (type I, II, III and endoglin), only type I and II receptors have been proven to mediate TGF β signaling. They are both serine-threonine kinases that signal through the Smad family of proteins (Lawrence, 1996). Binding of TGF β to its cell surface receptor Type II leads to the phosphorylation of the Type I receptor. The Type I receptor is then able to phosphorylate and activate the Smad 2 protein, an intracellular transducer of the TGF β superfamily. The Smad 2 and Smad 4 complex enters the cell nucleus and becomes involved in recruiting other transcription factors. Through these actions TGF β control expression of target genes that mediate biological effects of these growth factors (Kawabata *et al*., 1999; Lin *et al*., 2006).

TGF βs are multifunctional growth factors that regulate many aspects of cellular activities including cell growth, proliferation and differentiation, tissue remodeling, extracellular matrix formation, control of cell surface molecules, immunoregulation, angiogenesis and apoptosis. Potential roles of TGF βs have been identified in gonad and secondary sex organ development, spermatogenesis and ovarian function, immunoregulation of pregnancy, embryo implantation and placental development (Ingman and Robertson, 2002).

TGF β isoforms express overlapping in their functions but they also have unique actions within the cell. For example, TGF β_1 was identified in human platelets as a protein with a potential role in wound healing (Assoian *et al*., 1983). TGF β_1 was found to be secreted by most

leukocytes (Letterio and Roberts, 1998). Some T cells release TGF β_1 to inhibit the actions of other T cells. Interleukin 1 and interleukin 2-dependant proliferation of activated T cells is prevented by the activity of TGF β_1 (Gilbert *et al*., 1997; Wahl *et al*., 2006). Similarly, TGF β_1 can inhibit the secretion and activity of many other cytokines such as interferon-γ, tumor necrosis factor-alpha (TNF-α) and various interleukins. It can also increase the expression levels of cytokine receptors, such as IL-2 receptor to down-regulate the activity of immune cells. However, TGF β_1 can also increase the expression of several cytokines in T cells and promote their proliferation, particularly in immature cells (Lettario and Roberts, 1998). In addition, TGF β_1 inhibits proliferation and apoptosis of B cells and plays a role in controlling the expression of antibodies on the immature and mature B cells (Lebman and Edmiston, 1999). The effects of TGF β_1 on macrophages and monocytes are predominantly deactivating, but according to Bogdan and Nathan (1993) they can have macrophage-activating effects as well.

TGF β_2 has a vital role during embryonic development (Pelton *et al*., 1991) but is also known to suppresses the effect of interleukin dependant T-cell tumors

Since embryo-uterine interactions during the process of implantation involve each of these processes, it seems that these growth factors may play an important role during the peri-implantation period (Das *et al*., 1992).

1.4. TGF βs and implantation

The endometrium represents a highly specialized uterine tissue which provides an optimal environment for implantation of the semi-allogenic embryo (Jones *et al*., 2006). Being an end organ for the signals derived from the hypothalamic-pituitary-ovarian axis, the uterus/endometrium is under the control of the ovarian steroids, oestrogen and progesterone. Following oestrogen-induced cell proliferation, progesterone promotes cell differentiation within the endometrium, establishing an environment receptive for embryo implantation (Salamonsen and Jones, 2003).

Studies have shown that apoptosis is increased in the rat endometrium during implantation and during regression of the decidua basalis (Abrahamsohn and Zorn, 1993; Pampfer and Donnay, 1999). mRNA for TGF β_1 has been shown to be present within the uterus during pregnancy in the rat and was localized to the luminal and glandular epithelial cells during early and late pregnancy (Chen *et al*., 1993). TGF β_1 and TGF β_2 mRNAs were also found in the mouse uterus in the luminal and glandular epithelia on days 1-4 of pregnancy, and in the extracellular matrix of the stroma and decidual cells (Tamada *et al.,* 1990; Lea *et al.*, 1992). TGF β_2 and TGF β_3 mRNAs were shown to be expressed in the uterus of the mouse in the peri-implantation period. TGF β_2 was found in the luminal and glandular epithelia, the myometrium and decidua, while TGF β_3 was mostly localized to the myometrium (Das *et al*., 1992).

Shooner *et al.* (2005) demonstrated an increase in TGF β_1 and TGF β_2 expression on days 5.5 and 6.5 in the rat, whereas TGF β_3 protein was not detected on these days of early pregnancy. Immunohistochemical analyses revealed that TGF β_1 and TGF β_2 were found surrounding the epithelium (luminal and glandular) in the stromal compartment at the implantation site, while TGF β_3 was present at the time of decidua basalis regression in late pregnancy of the rat (Shooner *et al.,* 2005).

1.5. AIM OF STUDY

The aim of this investigation is to determine if the administration of exogenous gonadotropins, FSH (follicle stimulating hormone) and hCG (human chorionic gonadotropin), which has a luteinizing effect (and which together cause hyperstimulation and therefore superovulation), adversely affect the expression of TGF β_1 and TGF β_2 in the rat uterus during the peri-implantation period. Differences in the normal expression of these growth factors in the endometrium/embryo following hyperstimulation, may indicate the requirement for transforming growth factors in successful embryonic implantation.

2. MATERIALS AND METHODS

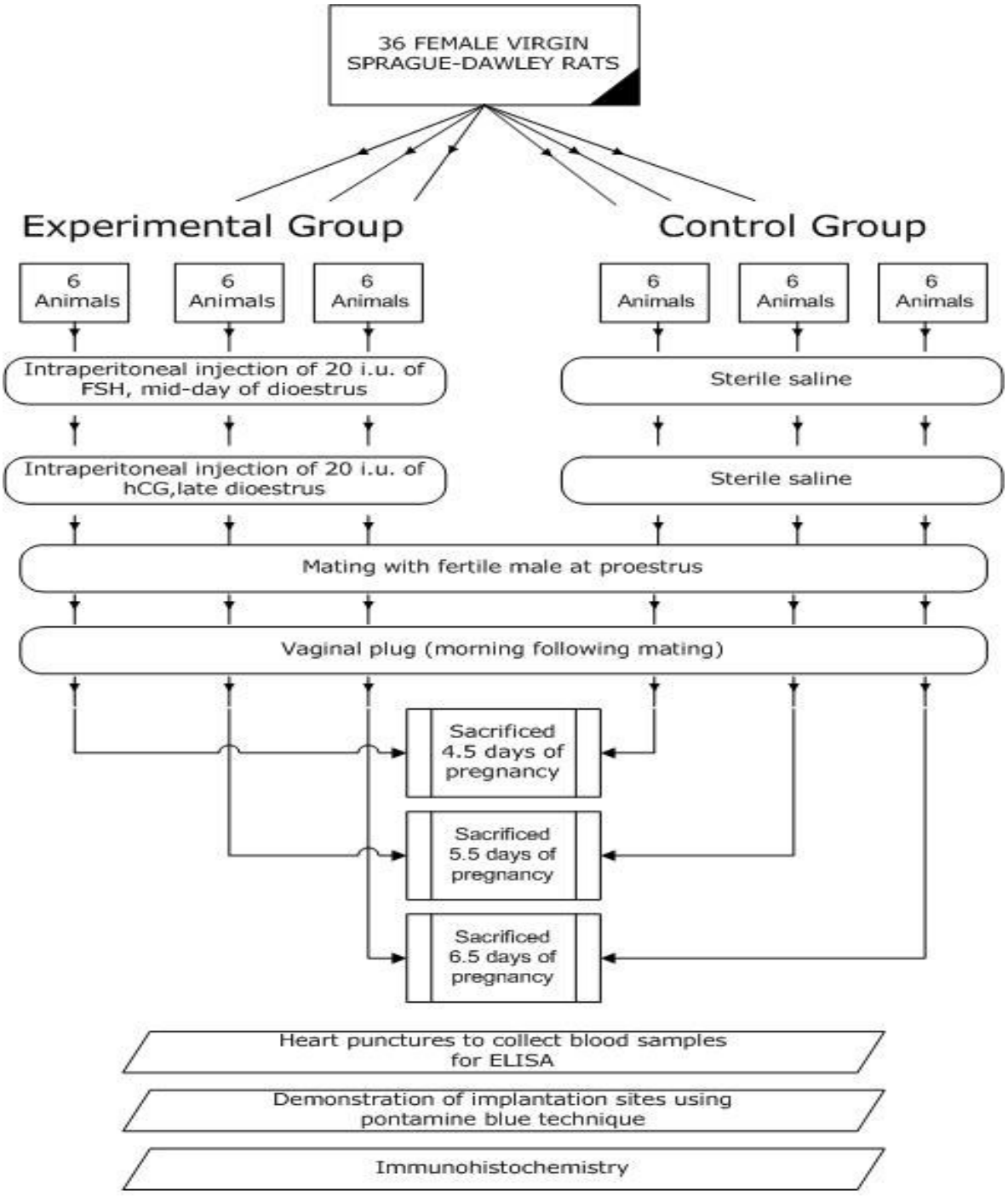
Clearance for this experiment was obtained from the Animal Ethics Committee, University of the Witwatersrand (clearance no 2004/100/4).

Thirty six adult, virgin, female Spraque-Dawley rats weighing between 200 – 250g and two male rats of proven fertility were housed in the animal unit of the University of the Witwatersrand. The animals had free access to food and water and were maintained at a constant temperature of 22° C. A regular 12h day and 12h night cycle was also maintained.

Vaginal smears of the female rats were taken daily until a regular 4-day oestrus cycle had been established. The daily smears were fixed in alcohol and stained using Shore's technique (Drury and Wallington, 1980) (Appendix 1). The stages of the oestrus cycle were assessed according to Kent and Smith (1945) (Appendix 2). The phases of the oestrus cycle are: oestrus, metoestrus, dieostrus (early, mid and late) and pro-oestrus. Only those animals showing at least three consecutive regular 4-day cycles were used in the study.

The 36 female rats were divided into six groups of six animals each. Three groups (n=18) represented the control animals and the remaining three, the experimental groups (n=18), which underwent hyperstimulation. After mating and establishing of the vaginal plugs or spermatozoa in the vaginal smears, animals from both experimental

(hyperstimulated) and control groups were further divided into groups of six animals each. Each group represented a different stage of pregnancy namely 4.5, 5.5 and 6.5 days (see flow diagram).



Flow diagram to show the plan of the experiment

2.1. Experimental Group (n=18)

Each animal received an intraperitoneal injection of 20 i.u. of FSH (Folligon, Intervet, JHB) at mid-day of mid-dioestrus followed by an intraperitoneal injection of 20 i.u. of hCG (Chorulon, Intervet, JHB) 24h later i.e. at mid-day of late dieostrus (*Kramer et al.,* 1993). Since it has been shown that the endometrial changes in the human female and in the rat are very similar at the time of implantation (Psychoyos and Martel, 1985), female rats in the present study have been hyperstimulated in phase with the oestrus cycle to simulate the conditions comparable to human IVF. Hyperstimulation results in the increased secretion of the ovarian hormones and the subsequent increase in production of oocytes (superovulation).

2.2. Control Group (n=18)

Each control animal received an intraperitoneal injection of 0.1ml of sterile saline at mid-day of mid-dioestrus followed by an intraperitoneal injection of 0.3ml of sterile saline 24h later i.e. at mid-day of late dieostrus. The volumes of the sterile saline given to the control animals at mid-dioestrus and late dioestrus were the same as the volumes of exogenous gonadotropins that were administered to the hyperstimulated animals at the relevant time of the oestrus cycle.

All animals from both the experimental and control groups were mated with proven fertile males on the evening of the day when they received the second injection i.e. when they were in pro-oestrus. The morning following mating was taken as day 0.5 of pregnancy. The presence of a mucous vaginal plug or spermatozoa in the vaginal

smears on the morning following mating indicated successful copulation. Vaginal smears were continued daily until the day of sacrifice, to confirm the maintenance of pregnancy. Pregnancy smears are predominantly leukocytic with an abundance of mucous secretion. Those animals still showing pregnancy smears on the day of sacrifice were used for further investigation.

Animals in both the experimental and control groups were sacrificed on days 4.5, 5.5 and 6.5 of pregnancy (the peri-implantation period). Rats were anaesthetized with a mixture of 0.6ml Chanazen (Centaur Labs, Johannesburg, South Africa) and 0.24ml Ketamine (Centaur Labs, Johannesburg, South Africa).

Heart punctures were performed and blood (5ml) from the ventricle of each animal was collected in sterile heparin tubes. The blood was immediately centrifuged. The plasma was stored at -70°C for an ELISA (enzyme-linked-immunosorbent assay), in order to estimate blood oestrogen and progesterone levels at the time of death.

The pontamine blue technique was used to demonstrate implantation sites (Finn and Porter, 1975). The inferior vena cava was surgically exposed and a 1 % pontamine blue solution (0.5ml) was injected into the vessel and allowed to circulate for 10-15 minutes to localize the implantation sites. The uterine horns were removed. Implantation sites (blue bands) were dissected out and fixed for 24 hours in 10% neutral buffered formalin solution (Appendix 3). Tissues were then dehydrated through a graded series of alcohols, cleared in chloroform (Automatic Tissue Processor, Shandon Citadel 1000) and embedded in paraffin wax. Tissues were maintained at room temperature until routine

histology and immunohistochemical localization of specific antigens were performed.

Following removal of the implantation sites, the animals were killed by exsanguination.

2.3. Progesterone and oestradiol ELISA

An ELISA (enzyme linked-immunosorbent assay) was carried out to determine the plasma concentrations of oestradiol and progesterone by using an oestradiol (E_2) enzyme immunoassay test kit and a progesterone enzyme immunoassay test kit (Linear Chemicals, Spain). The assay kits are based on the principle of competitive binding between unlabelled hormone in the test specimen (e.g. E_2) and a fixed quantity of E_2-HRP conjugate for a constant amount of rabbit anti- E_2. For the incubation, goat anti-rabbit IgG-coated wells are incubated with 25µl of the E_2 standards, controls (negative control in which an unknown antigen is omitted and a positive control which uses a known antigen), animal samples (test sample containing E_2), 100 µl oestradiol-HRP conjugated reagent and 50µl rabbit anti-oestradiol at room temperature for 90 minutes. During incubation, a fixed amount of HRP-labelled E_2 competes with endogenous E_2 in the standard, sample or quality control serum for a fixed number of binding sites of the specific E_2 antibody. Thus, the amount of E_2 peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of E_2 in the specimen increases.

Unbound E_2 peroxidase conjugate was then removed and the wells washed with diluted wash solution (phosphate buffer pH 7.4, NaCl and

0.05% Tween 20). Next, a solution of 3,3 ,5,5 -tetramethylbenzidine (TMB) reagent is added and incubated at room temperature for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of 1N HCL, and the absorbance is measured spectrophotometrically at 450nm. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled E_2 in the sample. A standard curve was obtained by plotting the concentration of the standard versus the absorbance. The E_2 concentration of the specimens and the controls were run concurrently with standards and were calculated from the standard curve.

2.4. Statistics

The unpaired Student "t"-test as well as the non-parametric Mann-Whitney test was used for the statistical analysis of the plasma concentrations for progesterone and oestradiol and comparisons between the control and hyperstimulated animal groups.

2.5. Histology

Paraffin wax embedded uteri were cut at 4 μm and the sections were placed on glass slides coated with silane (Appendix 4). Sections were deparaffinised in xylene, rehydrated in a series of graded alcohols and then stained with haematoxylin and eosin (Appendix 5) in order to establish the morphology of the uteri at different stages of pregnancy. The haematoxylin and eosin staining method demonstrates a variety of different tissue structures (Bancroft and Gamble, 2002). The haematoxyln component stains nuclei blue-black (basophilic), while

the eosin stains the cell cytoplasm and connective tissue fibres in different shades of pink and red (eosinophilic) (Bancroft and Gamble, 2002).

2.6. Immunohistochemical staining

Uterine tissue sections 4μm thick were mounted on silane-coated slides (Appendix 4). The sections were deparaffinized, rehydrated and then heated for 2x5 minutes in 10mM citrate buffer pH 6 (Appendix 6) containing Triton X-100 (Sigma Aldrich) 0.1% (v/v). Sections were washed in running water and Tris buffered saline pH 7.6 (Appendix 7) containing Triton x-100 and then incubated with 0.3% hydrogen peroxide in distilled water for 30 minutes to quench endogenous peroxidase activity. After washing in running tap water and Tris buffered saline (TBS) for 5 minutes each, the sections were incubated with normal blocking serum (Vectastain Elite ABC Kit, Vector Laboratories, CA, USA) at room temperature for 1 hour. Then, the primary antibody diluted in TBS (TGF beta-1 or TGF beta-2; 1:100 dilution, Santa Cruz Biotechnology, CA, USA) was added to the sections and incubated at 4°C overnight in a humidified chamber. After washing for 3x5 minutes in TBS containing Triton X-100, sections were incubated for 30 minutes with biotinylated secondary antibody (anti-rabbit, Vectastain Elite ABC Kit, Vector Laboratories, CA, USA). Subsequently, sections were washed with TBS for 3x5 minutes and incubated with an avidin-biotin complex reagent containing horseradish peroxidase for 30 minutes. Sections were then washed with TBS for 3x5 minutes and colour development was achieved by applying the diaminobenzidine (DAB) substrate (Appendix 8) for 5

minutes. Sections were than washed in running water for 5 minutes, counterstained with hematoxylin and mounted in entellan.

Preparation of mouse ovarian tissue (for control purposes) for sectioning and staining was carried out using the same method that was used for rat uterine tissue. Clearance for this part of the study was obtained from Animal Ethics Committee, University of the Witwatersrand (clearance no 06/17/01).

2.7. Immunocytochemistry controls

To establish the specificity of the immunolabelling, the following immunocytochemical controls were used.

Negative controls were performed using the same protocol as above, but substituting either the primary or secondary antibody with TBS or normal rabbit serum (NRS). Negative controls were always carried out on the section adjacent to a section of uterine tissue which showed immunolocalization.

For **absorption controls,** absorption of the primary antibody with the purified antigen was used to show specificity of the antibody. The highest antibody dilution at which a constantly positive result is achieved was determined first. The primary antibody (at the concentration determined by the aforementioned method) was pre-absorbed with its own antigen (blocking peptide sc-146 P for TGF β_1 and sc-90 P for TGF β_2, Santa Cruz Biotechnology), overnight at 4 °C. The amount of blocking peptide used was a twenty-five-fold excess (by weight) diluted in 500µl of TBS. We were advised by Santa Cruz

Biotechnology to increase the concentration of blocking peptide for the absorption control to 25x excess of peptide to antibody by weight, instead of 5x (which was the dose recommended in the Santa Cruz Biotechnology support protocol). Sections adjacent to the sections showing immunolocalization for TGF β_1 and TGF β_2 respectively , were then incubated with antibody-antigen complex overnight at 4 °C.

To show that the technique in each immunohistochemical run was successful, **positive controls** for both TGF β_1 and TGF β_2 were performed. The positive control consisted of the immunolocalization of the antibodies in a sample of mouse ovary known to contain TGF β_1 and TGFβ_2 (Santa Cruz Biotechnology datasheets for TGF β_1 (V): sc-146 and TGF β_2 (V): sc-90).

2.8. Photography and images

Uterine tissue sections were analyzed by light microscopy with the Zeiss Axioscope microscope (Axioscope 2, MOT, Carl Zeiss, Germany). Suitable images were taken with the digital camera (Sony 3 CCD) which was attached to the Axioscope.

3. RESULTS

3.1. General observation

Post-mortem examination of the ovaries and uterine horns showed distinct gross morphological differences between the control and hyperstimulated animals. The ovaries of the control animals at 4.5, 5.5 and 6.5 days of pregnancy appeared small and rounded (Fig. 1). The ovaries of the hyperstimulated animals obtained from the stages of pregnancy used in this study showed gross enlargement with numerous, large follicles (Fig. 2). When ovaries from the control groups were compared to the ovaries of the hyperstimulated groups, a marked increase in size of the ovaries and an increase in follicular numbers were observed in the hyperstimulated animals at all three stages of pregnancy.

The uterine horns of the control animals at 5.5 and 6.5 days of pregnancy showed numerous implantation sites (8 to 15 per animal), which were expressed as blue bands following the pontamine blue technique (Fig. 1). Only one control animal at 4.5 days of pregnancy showed three implantation sites indicated by the pontamine blue technique. The uterine horns in the hyperstimulated animals at 4.5, 5.5 and 6.5 days of pregnancy were noticeably dilated when compared to control animals at the same stages of pregnancy (Fig. 2). The majority of the experimental animals from all three stages of pregnancy did not show blue-stained implantation sites. However, two animals in the hyperstimulated group at 5.5 days of pregnancy showed one visible implantation site per animal.

3.2. ELISA

3.2.1. Progesterone ELISA

The summary of the statistical values for progesterone plasma concentrations obtained from both control and hyperstimulated animals at 4.5, 5.5 and 6.5 days of pregnancy is shown in Table 1 and Table 2 respectively. All values for progesterone plasma concentrations are given in ng/ml.

The mean progesterone level for **control animals** (Table 1) showed a statistically non-significant increase between 4.5 and 5.5 days of pregnancy ($p=0.054$). This was followed by a statistically non significant decrease in progesterone level on day 6.5 of pregnancy ($p=0.060$).

In the **hyperstimulated animals** (Table 2) the progesterone plasma concentrations showed a gradual increase from 4.5 days through 6.5 days of pregnancy. The increase from 4.5 to 5.5 days of pregnancy was statistically not significant ($p=0.104$) as well as the increase between 5.5 and 6.5 days of pregnancy ($p=0.978$).

When progesterone plasma concentrations in the control animals were compared to those in the hyperstimulated animal groups (Table 1 and 2), higher concentrations of plasma progesterone were evident within the latter group. However, a significantly higher ($p=0.006$) progesterone level was found only at 6.5 days of pregnancy, when the control and hyperstimulated animals were compared (Fig. 3).

There was no significant difference when progesterone plasma concentrations were compared between the 4.5 day control and hyperstimulated animal groups (p=0.362) or between the 5.5 day control and hyperstimulated groups (p=0.213).

Table 1: Summary of the statistical values for progesterone concentrations in the control animals

Factor	Hormone	Days of Pregnancy	Number of animals	Mean	SD	Min	Max
Control	Progesterone	4.5	6	22.52	6.03	11.92	28.90
Control	Progesterone	5.5	6	28.17	1.97	26.87	30.78
Control	Progesterone	6.5	6	23.49	5.03	16.10	31.35

Table 2: Summary of the statistical values for progesterone concentrations in the hyperstimulated animals

Factor	Hormone	Days of Pregnancy	Number of animals	Mean	SD	Min	Max
Hyper-stimulated	Progesterone	4.5	5	25.45	4.50	19.10	31.33
Hyper-stimulated	Progesterone	5.5	6	32.96	7.71	24.30	44.30
Hyper-stimulated	Progesterone	6.5	7	33.07	4.99	26.20	39.18

All values of progesterone concentrations are expressed in ng/ml

3.2.2. Oestradiol ELISA

The summary of the statistical values for oestrogen plasma concentrations obtained from both control and hyperstimulated animals at 4.5, 5.5 and 6.5 days of pregnancy is shown in Table 3 and Table 4 respectively.

In the hyperstimulated group at 5.5 days of pregnancy, statistical analysis was carried out on four samples only. The result obtained from one of those animals was discarded since it was extremely different (66.60 pg/ml) to the values obtained from the other animals at the same stage of pregnancy. The analyses were done with and without the high value and it made no difference to the outcome of the experiment (personal communication with statistician). Variability may have been due to variations in the time at which this animal was killed with respect to the other animals in this group. The second sample discarded was highly haemolysed and thus not suitable for the test.

In the **control group** (Table 3) the oestradiol levels did not show a significant increase from 4.5 to 5.5 days of pregnancy ($p=0.816$) while a non-significant increase in oestradiol plasma concentrations was observed between 5.5 days and 6.5 days of pregnancy ($p=0.164$).

In the **hyperstimulated group** (Table 4) oestradiol plasma concentrations were not significantly higher at 5.5 days of pregnancy when compared to those at 4.5 days of pregnancy ($p=0.632$). However, a significant decrease in oestradiol plasma concentrations

was observed in animals at 6.5 days of pregnancy when compared to those at 4.5 and 5.5 days of pregnancy (p=0.038).

When oestradiol plasma concentrations in the control animals were compared to those in the hyperstimulated animal groups (Table 3 and 4), higher concentrations of plasma oestradiol were obvious in the latter group at 4.5 and 5.5 days of pregnancy (Fig. 4). However, significantly lower oestradiol levels were found in the hyperstimulated group when compared to the control group at 6.5 days of pregnancy (p=0.039). Oestradiol plasma concentrations between the 4.5 day control and hyperstimulated animals were not significant (p=0.249). This, too, was the case at 5.5 days of pregnancy (p=0.117).

Table 3: Summary of the statistical oestradiol concentrations in the control animals

Factor	Hormone	Days of Pregnancy	Number of animals	Mean	SD	Min	Max
Control	Oestradiol	4.5	5	21.29	11.23	12.45	40.80
Control	Oestradiol	5.5	6	22.70	8.37	12.12	32.90
Control	Oestradiol	6.5	6	32.52	13.64	19.52	51.91

Table 4: Summary of the statistical oestradiol concentrations in the hyperstimulated animals

Factor	Hormone	Days of Pregnancy	Number of animals	Mean	SD	Min	Max
Hyper-stimulated	Oestradiol	4.5	6	28.46	8.15	19.50	41.10
Hyper-stimulated	Oestradiol	5.5	4	30.68	3.95	26.12	35.64
Hyper-stimulated	Oestradiol	6.5	6	17.88	6.44	12.95	29.87

All values of oestradiol concentrations are expressed in pg/ml

3.2.3. Progesterone - Oestradiol **($P:E_2$) ratio**

The summary of the progesterone:oestradiol ratio in the control and hyperstimulated animals is given in Table 5 and Table 6 respectively.

The $P:E_2$ ratio was calculated by dividing the P value in nm/ml by the E_2 value in nm/ml $\times 10^{-3}$. When comparing the $P:E_2$ ratio between the control and hyperstimulated groups, a slight increase was observed between 4.5 and 5.5 days of pregnancy. However, at 6.5 days of

pregnancy the P:E_2 ratio showed a marked decrease (i.e. oestrogen was dominant) in the control group. In the hyperstimulated group at 6.5 days of pregnancy the opposite happened, as the dominance of progesterone was noticeable and was demonstrated by a marked increase in the P:E_2 ratio (Figs. 5 and 6).

Table 5: Summary of the ratio between progesterone and oestradiol in control animals

Day and Factor	Progesterone (ng/ml)	Oestrogen (pg/ml)	P:E2 ratio
4.5 control	22.52	21.29	1.058
5.5 control	28.17	22.70	1.241
6.5 control	23.49	32.52	0.722

Table 6: Summary of the ratio between progesterone and oestradiol in hyperstimulated animals

Day and Factor	Progesterone (ng/ml)	Oestrogen (pg/ml)	P:E2 ratio
4.5 hyperstimulated	25.45	28.46	0.894
5.5 hyperstimulated	32.96	30.67	1.074
6.5 hyperstimulated	33.08	17.87	1.849

3.3. Histology

Histological examination of the sections of uterine tissue of both control and hyperstimulated animals was carried out prior to immunohistochemical analysis.

The outline of the uterine lumen in most **control animals at 4.5 days of pregnancy** was slightly wavy in appearance (Fig. 7). The luminal epithelial cells of the 4.5 days control animals were simple columnar with round to oval, basophilic, centrally placed nuclei (Fig. 8). The apical region of the luminal epithelial cells showed distinct and numerous microvilli (Fig. 8). Vacuoles were observed in the base of the luminal epithelial cells (Figs. 8 and 9). Several stromal cells at the antimesometrial pole were round with distinct round nuclei, indicating that decidualization had begun (Fig. 9). However, the majority of the stromal cells appeared flat and fibroblastic and the stromal tissue remained compact (Fig. 8). An abundance of glands was observed in the stroma. Glandular epithelial cells were simple cuboidal with round, centrally placed nuclei. Vacuoles surrounded the nuclei of the glandular epithelial cells (Fig. 9).

Distinct differences were observed between control and **hyperstimulated animals at 4.5 days of pregnancy**. The uterine lumen of the hyperstimulated animals was noticeably dilated and the epithelium was very folded (Fig. 10). Luminal epithelial cells were columnar with large, oval, basally placed nuclei and with an apically situated microvillus boarder (Fig. 11). Vacuoles were not evident in these epithelial cells. The majority of the subepithelial stromal cells

were flat and fibroblastic (Fig. 11). Numerous glands were present in the stroma. Glandular epithelial cells were simple cuboidal with large, basally placed nuclei. Vacuoles were not present in the glandular epithelial cells at 4.5 days of pregnancy in the hyperstimulated animals (Fig. 12).

The gross morphological features of the uterine tissue from both **5.5 and 6.5 day control animals** were similar to those at 4.5 days of pregnancy**.** The general outline of the luminal surface of 5.5 day control tissue was smooth (Fig. 13). In sections which were taken close to the implantation site, the opposite uterine walls were closer to each other (Fig. 14) and the blastocyst was engaged in the process of adhesion to the uterus. The luminal epithelial cells of the implantation chamber appeared reduced in size and layered, so that there were few cells between the trophoblast and epithelial basal lamina. At some places the epithelial cells were detached from the basal lamina (Fig. 15). The rest of the luminal epithelial cells were simple columnar with round to oval, centrally placed, basophilic nuclei. Microvilli were present at the apical surface. Some vacuoles were evident at the base of the luminal epithelial cells (Fig. 16). A decrease in the presence of vacuoles was observed when comparisons were carried out between the three stages of pregnancy. Vacuoles were abundant at 4.5 days, after which their number decreased towards 6.5 days of pregnancy (compare Figs. 8, 16 and 19). Additionally, a decline in the presence of vacuoles was observed at 5.5 day of pregnancy from the mesometrial side of the uterus to the antimesometrial side, where the cells were squamous and did not contain vacuoles (Figs. 18 and 19). In the subepithelial region, decidualization of the previously flat, fibroblastic stromal cells was visible (Fig. 16). The decidual cells contained round

nuclei and scanty cytoplasm. "Spaces" were evident between the decidual cells in this region. Blood vessels were also observed in the subepithelial compartment (Fig. 16). Clusters of uterine glands were apparent. The glandular epithelium remained simple cuboidal, with centrally placed, round, basophilic nuclei (Fig. 17). Vacuoles were present in the cytoplasm of the glandular epithelial cells.

Additional changes were seen when sections of control uterine tissue at 5.5 days of pregnancy were compared to that at 6.5 days of pregnancy. More extensive decidualization of the stromal cells was evident in the uterus of animals at 6.5 days of pregnancy. Several blood vessels were observed in the stroma (Fig. 19). A gradual change in the shape of the luminal epithelial cells from the mesometrial side to the antimesometrial side was noticeable (Figs 18 and 19).

Sections of the hyperstimulated uterine tissue of animals at 5.5 and 6.5 days of pregnancy showed a dilated uterine lumen with extensive mucosal folds (Fig 20). The luminal epithelial cells were simple columnar with oval nuclei, basally disposed. A microvillus border was visible at the apical pole of these cells. Vacuoles were not present in the cytoplasm (Fig. 21). Uterine glands were numerous with simple cuboidal epithelial cells and large basally placed nuclei. No vacuoles were present in the glandular epithelial cells (Fig. 22).

In some hyperstimulated animals at 5.5 days of pregnancy, unattached embryos were present, situated close to the luminal epithelium (Fig. 23).

Differences were observed in the morphology of the glands of hyperstimulated animals between 5.5 and 6.5 days of pregnancy. The

glands in the 5.5 and 6.5 day hyperstimulated animals had lumina which were more dilated (Fig. 22) than the lumina of the glands in the control groups at the same stages of pregnancy (Fig. 17).

3.4. IMMUNOHISTOCHEMISTRY

3.4.1. Controls for TGF β_1 and TGF β_2 immunohistochemistry

Mouse ovary was used as a **positive control** for both TGF β_1 and TGF β_2, since this tissue is known to express the above-mentioned growth factors (personal communication with Santa Cruz Biotechnology support group). Immunoperoxidase staining for the mouse ovarian tissue showed extracellular localization of both TGF β_1 (Fig. 24) and TGF β_2 (Fig. 25) as expected.

To confirm the specificity of immunolocalization of both TGF β_1 and TGF β_2, **negative controls** were included. Negative control sections were carried out on an adjacent section to a section of the uterine tissue which showed immunolocalization for that specific antibody. No immunolocalization was found in any of the tissue sections where primary or secondary antibody was omitted and replaced with either TBS (compare Figs. 27 and 28 with 26 and Figs. 30 and 31 with 29) or NRS (compare Fig. 33 with 32 and Fig. 35 with 34).

To show the specificity of the primary antibodies, **absorption controls** were performed. These were carried out on a section of uterus adjacent to a section that showed immunolocalization. The results obtained showed a significant reduction in the case of TGF β_1

antibody in the uterine tissue (Figs. 36a and 36b), while the absorption control for TGF β_2 antibody showed no immunolocalization (Figs. 36c and 36d).

According to the information provided by Santa Cruz Biology technical service (personal communications), both TGF β_1 and TGF β_2 antibodies are very "strong" antibodies. Thus "the complete disappearance of the immunolocalization should only be expected after the increase of the blocking peptide concentration to 10 or even 25 x (by weight)". Following the recommendation of Santa Cruz, the complete blocking/absorption was not achieved in the case of TGF β_1 antibody, despite the fact that the amount of blocking peptide was increased 25x (by weight) (compare Figs. 36 a and 36b). A slight residue remained.

3.4.2. Immunolocalization of TGFβ_1 in control and hyperstimulated animals at 4.5, 5.5 and 6.5 days of pregnancy

The distribution of TGF β_1 immunolocalization differed between the control and hyperstimulated groups of animals at different stages of pregnancy.

At **4.5 days of pregnancy in control animals,** TGF β_1 immunolocalization was weak in both the luminal and glandular epithelial cells (Fig. 37). However, it was evident in the stromal compartment (Fig. 37). Subluminal stromal cells at the antimesometrial side showed stronger expression of TGF β_1 than those at the mesometrial side of the lumen (figure not shown). The

myometrium of 4.5 days hyperstimulated animals had a weak expression of TGF β_1 (figure not shown).

The TGF β_1 immunolocalization in the **4.5 days hyperstimulated animals** was weak in the luminal and glandular epithelium (Fig. 37). Apical localization of TGF β_1 was observed in both the luminal and glandular epithelial cells (Figs. 38 and 39). There was a distinct separation of the immunolocalization of TGF β_1 into two regions in the stroma of 4.5 days pregnant hyperstimulated animals. The immediately subluminal zone had a weaker immunolocalization of TGF β_1 while the deeper stromal region had a more concentrated expression (Fig. 38). This kind of regional separation in immunolocalization was not observed in the control group at 4.5 days of pregnancy (Fig. 37). There was no difference in TGF β_1 immunolocalization between the antimesometrial and the mesometrial side of the lumen in the hyperstimulated group at 4.5 days of pregnancy (figure not shown). The myometrium of 4.5 days hyperstimulated animals had a weak expression of TGF β_1 (figure not shown).

There were no particular differences in TGF β_1 expression in the luminal and glandular epithelial cells **between the control and hyperstimulated groups at 4.5 days of pregnancy**. TGF β_1 immunolocalization was weak in this region in both groups but with well defined apical localization (compare Figs. 37 and 38). However, differences were observed in the stroma in regard to regionalization. No difference in the intensity of the immunolocalization of TGF β_1 was seen in the stroma of the control animals (compare with 4.5 day hyperstimulated animals, page 58).

At **5.5 days of pregnancy in control animals**, TGF β_1 immunolocalization was weak in the luminal epithelium (Fig. 40) but more intense throughout the stromal compartment without showing regionalization (Figs. 40 and 41). In addition, the expression of TGF β_1 was stronger at the antimesometrial side of the lumen when compared to the mesometrial side (figure not shown).

The TGF β_1 immunolocalization in the **5.5 day hyperstimulated animals** was weak in the luminal and glandular epithelium with well defined apical localization (Figs. 42 and 43). Regionalization i.e. the presence of two zones with different expression of TGF β_1 was noticeable in the stroma (Fig. 42) while the myometrium showed weak expression.

Thus, **differences in TGF β_1 expression between the control and hyperstimulated groups at 5.5 days of pregnancy** existed particularly in the stromal compartment. Two zones of variation in TGF β_1 expression i.e. subluminal zone with noticeably weaker TGF β_1 expression and deeper stromal zone where immunolocalization was more prominent, occurred in the stroma of the hyperstimulated animals (Fig. 42) but were not evident in the control group at the same stage of pregnancy (Fig. 40).

There was no **difference in the expression of TGF β_1 between 4.5 and 5.5 days of pregnancy in the control groups.** The luminal and glandular epithelium had a weak TGF β_1 immunolocalization in both groups, while the stroma showed a stronger expression but without regionalization (Figs. 37, 38 and 41).

Also, there were no **differences in the expression of TGF β_1 between 4.5 and 5.5 days of pregnancy in the hyperstimulated groups.** Weak TGF β_1 immunolocalization in the luminal and glandular epithelial cells existed in both stages of pregnancy (compare Figs. 38 and 42). However, a well defined apical concentration was present in the luminal epithelium at both stages, as well as in the glandular epithelial cells at 4.5 days of pregnancy. Apical TGF β_1 immunolocalization was absent from the glandular epithelia of the 5.5 days of pregnancy in the hyperstimulated group (compare Figs. 39 and 43). Regionalization in the stromal compartment was also present in both 4.5 and 5.5 days of pregnancy in the hyperstimulated groups (Figs. 38 and 42).

Luminal and glandular epithelial cells in **both control and hyperstimulated groups at 6.5 days of pregnancy** had weak TGF β_1 expression (Figs. 46, 47, 48 and 49). The major difference in TGF β_1 expression between the control and hyperstimulated groups at 6.5 days of pregnancy existed in the stromal compartment (Figs. 44 and 48). The entire stroma of the 6.5 control group had weak TGF β_1 immunolocalization (Fig. 44). Only at the antimesometrial side was TGF β_1 expression more apparent (Fig.45). However, two zones of TGF β_1 expression were evident in the stroma of the hyperstimulated animals; the subluminal zone had weaker TGF β_1 immunolocalization than the deeper, stromal zone (Fig. 48). This regionalization was not evident in the control group at the same stage of pregnancy (Fig.44). In the glands of control and hyperstimulated animals, the lumen contained some secretion which tended to take up the DAB chromagen (Figs. 47 and 49).

At 6.5 days of pregnancy in the vicinity of implantation sites, localization of TGF β_1 was evident in the stroma at the antimesometrial pole of the lumen and in the subluminal stromal cells surrounding the implanting conceptus (Figs. 44 and 45), while the stromal cells at the mesometrial pole did not show any TGF β_1 immunolocalization (Fig. 44).

The expression of TGF β_1 in the **conceptus** was localized to the trophoblast region, while the remainder of the embryo showed no immunolocalization of TGF β_1 (Fig. 45).

3.4.3. Immunolocalization of TGF β_2 in control and hyperstimulated animals at 4.5, 5.5 and 6.5 days of pregnancy

Immunolocalization of TGF β_2 was evident in the tissue of both control and hyperstimulated animals at different stages of pregnancy. However, differences in the distribution of TGF β_2 immunolocalization were evident between the two groups of animals.

At **4.5 days of pregnancy in the control group of animals,** TGF β_2 immunolocalization was strongly expressed in the cytoplasm of both the luminal and glandular epithelial cells (Figs. 50 and 51). Although immunolocalization of TGF β_2 was observed in the stroma, it was noticeably weaker than that of the epithelium (Figs. 50 and 51). No apparent differences in the immunolocalization of TGF β_2 were observed between the anti-mesometrial and mesometrial side of the lumen.

In the **hyperstimulated animals at 4.5 days of pregnancy,** the luminal epithelium showed a weak expression of TGF β_2 which was mainly located in the apical region of these cells (Fig. 52). The glandular epithelium showed a strong immunolocalization of TGF β_2 (Fig. 53). The stromal compartment was separated into two zones on the basis of TGF β_2 immunolocalization. The subluminal stroma had a weaker TGF β_2 expression, while the deeper zone had a more intense immunolocalization (Fig. 52).

The differences in TGF β_2 expression between the control and hyperstimulated group at 4.5 days of pregnancy was particularly evident in the luminal epithelial cells and the subluminal stromal compartment (Figs. 50 and 52). While TGF β_2 was intensely expressed in these areas in the control group, little immunolocalization occurred in these regions in the hyperstimulated group at the same stage of pregnancy (Figs. 50 and 52). The immunolocalization of TGF β_2 in the glandular epithelial cells was similar in the two groups (Figs. 51 and 53).

At **5.5 day**, a reduction in TGF β_2 expression was obvious in the luminal epithelial cells in the control group when compared to the 4.5 day control (Fig. 55). The immunolocalization of TGF β_2 in the stroma was not as intense, when compared to that in the epithelium (Figs. 54 and 55). Distinct expression of TGF β_2 occurred in the glandular epithelium (Fig. 56). A difference in TGF β_2 immunolocalization between the anti-mesometrial and the mesometrial side of the lumen was noticeable at 5.5 days of pregnancy in control animals (Fig. 54). In all the control animals at 5.5 days of pregnancy, strong TGF β_2 expression was evident in the myometrium of the uterus (Fig. 54).

In the **hyperstimulated animals at 5.5 days of pregnancy,** a strong apical localization of TGF β_2 in the luminal epithelial cells was apparent (Fig. 58). Regionalization in the expression of TGF β_2 was noticeable in the stroma. The zone immediately beneath the epithelium had scanty TGF β_2 expression, while in the deeper stromal region immunolocalization appeared to be much stronger (Fig. 57). The expression of this growth factor was prominent in the glandular epithelium (Fig. 59).

TGF β_2 immunolocalization was also observed in the myometrium in the uterine tissue sections of 5.5 days hyperstimulated animals (figure not shown).

Differences in TGF β_2 expression between the control and hyperstimulated groups at 5.5 days of pregnancy thus existed in the luminal epithelium and subluminal stroma (Figs. 54, 55, 57 and 58). The most obvious discrepancy was immunolocalization of TGF β_2 in the apical region of the luminal epithelial cells in the hyperstimulated animals (compare Figs. 55 and 58). Regionalization in the expression of TGF β_2 in the stroma of the hyperstimulated animals was another difference observed between the control and hyperstimulated groups at 5.5 days of pregnancy (compare Figs. 54 with 57 and 55 with 58).

At 6.5 days of pregnancy (control animals) a decrease in the localization of TGF β_2 was apparent in the luminal epithelium, stroma and glandular epithelium (Figs. 60, 61 and 63) when compared to 5.5 days control animals. However, TGF β_2 immunolocalization remained strong in the myometrium (Fig. 60). In sections through the

implantation sites, stromal cells at the mesometrial site did not show any TGF β_2 immunolocalization (Fig. 60), while those at the anti-mesometrial side had evident expression of TGF β_2 (Fig. 61). Glandular epithelium showed weak localization of TGF β_2 (Fig. 63).

At the implantation sites in 6.5 days pregnant animals, the implantation chamber had a heavy concentration of decidualizing stromal cells (Fig. 62). The blastocyst was elongated and adhered to both sides of the implantation chamber, almost entirely encompassed by the flattened luminal epithelial cells (Fig. 62). The location of the blastocyst was antimesometrial.

The flattened luminal epithelial cells as well as the entire blastocyst did not show any immunolocalization of TGF β_2 (Fig. 62). Intense TGF β_2 immunolocalization was expressed in the subluminal stroma surrounding the implanting conceptus (Fig. 62).

Hyperstimulated animals at 6.5 days of pregnancy expressed TGFβ_2 immunolocalization in the luminal epithelial cells concentrated at the apical pole (Fig. 65). Glandular epithelium in the hyperstimulated animals had strong TGF β_2 immunolocalization (Fig. 65), while the stroma was divided into two zones by the intensity of immunolocalization of TGF β_2. The subluminal zone was devoid of immunolocalization of TGF β_2 while the deeper zone had scanty TGF β_2 immunolocalization (Fig. 64).

Differences in TGF β_2 expression between the control and hyperstimulated groups at 6.5 days of pregnancy thus existed in the luminal epithelium in which the localization of this growth factor in

the hyperstimulated group was more distinct and more apically pronounced (Fig. 65) than in the control group at the same stage of pregnancy (Fig. 61). TGF β_2 immunolocalization was also more apparent in the glandular epithelium of the hyperstimulated animals at 6.5 days of pregnancy, when compared to the controls (compare Figs. 63 and 65). Control animals at 6.5 days of pregnancy did not show zonation in TGF β_2 immunolocalization in the stroma, when compared to the hyperstimulated animals.

Hyperstimulated animals at 6.5 days of pregnancy had a similar pattern of TGF β_2 expression to hyperstimulated animals at 5.5 days of pregnancy, but this was expressed in a much weaker form (Figs. 57 and 64). The luminal epithelial cells in the hyperstimulated animals at 5.5 days of pregnancy had strong TGF β_2 immunolocalization with prominent apical concentration (Fig. 58), while in animals at 6.5 days of pregnancy the immunolocalization in the same area was weaker with sparse apical localization (Fig. 65). Glandular epithelial cells had strong TGF β_2 expression in the hyperstimulated animals in both 5.5 days (Fig. 59) and 6.5 days of pregnancy (Fig.65). Immunolocalization in the stromal compartment was still divided into two zones at both 5.5 days and 6.5 days of pregnancy in the hyperstimulated animals (Figs. 57 and 64). However, this regionalization was considerably weaker in the hyperstimulated animals at 6.5 days of pregnancy, where, in the subluminal zone, it reached the point of complete absence (compare Figs. 57 and 64).

3.4.4. Summary of the comparisons of immunolocalization between TGF β_1 and TGF β_2 in control and hyperstimulated animals

The following differences were observed in the expression of TGF β_1 and TGF β_2 when the 4.5, 5.5 and 6.5 days pregnant control and hyperstimulated rat uterine sections were compared:

Immunolocalization of the TGF β_1 in the luminal and glandular epithelium of both the control and hyperstimulated animals was weak and apically localized in all three examined stages of pregnancy. TGF β_2 localization in the luminal and glandular epithelium decreased from 4.5 to 6.5 days in the control group. However, while the expression of TGF β_2 in the luminal epithelium was weak with distinct apical localization in all three examined stages of pregnancy in the hyperstimulated group, the glandular epithelium had strong expression of the TGF β_2.

Immunolocalization of TGF β_1 in the stroma of the control animals was strong throughout all the examined stages of pregnancy, with no obvious regionalization. However, a gradual decrease in the localization of the TGF β_2 was observed from 4.5 to 6.5 days of pregnancy in the stroma of the control animals. Immunolocalization of both TGF β_1 and TGF β_2 in the stroma of the hyperstimulated animals was regionalized into two zones, a subluminal zone with weak immunolocalization and deeper stromal region which expressed

stronger immunolocalization in all three examined stages of pregnancy.

Immunolocalization of the TGF β_1 in the myometrium was weak in the control as well as hyperstimulated groups in all the examined stages of pregnancy. However, immunolocalization of TGF β_2 in the myometrium was strong in the control and hyperstimulated animals throughout the peri-implantation period.



Figure 1. Representative photograph of dissected uterine horns of a control animal (5.5 day of pregnancy). Note the presence of numerous blue bands (Implantation sites, 13 in total). Ovaries appear small and rounded. Pontamine blue staining reaction.



Figure 2. Representative photograph of dissected uterine horns of a hyperstimulated animal (5.5 day of pregnancy). Note the dilated uterine horns and no evidence of blue stained implantation sites. Ovaries appear large with numerous follicles. Pontamine blue staining reaction.

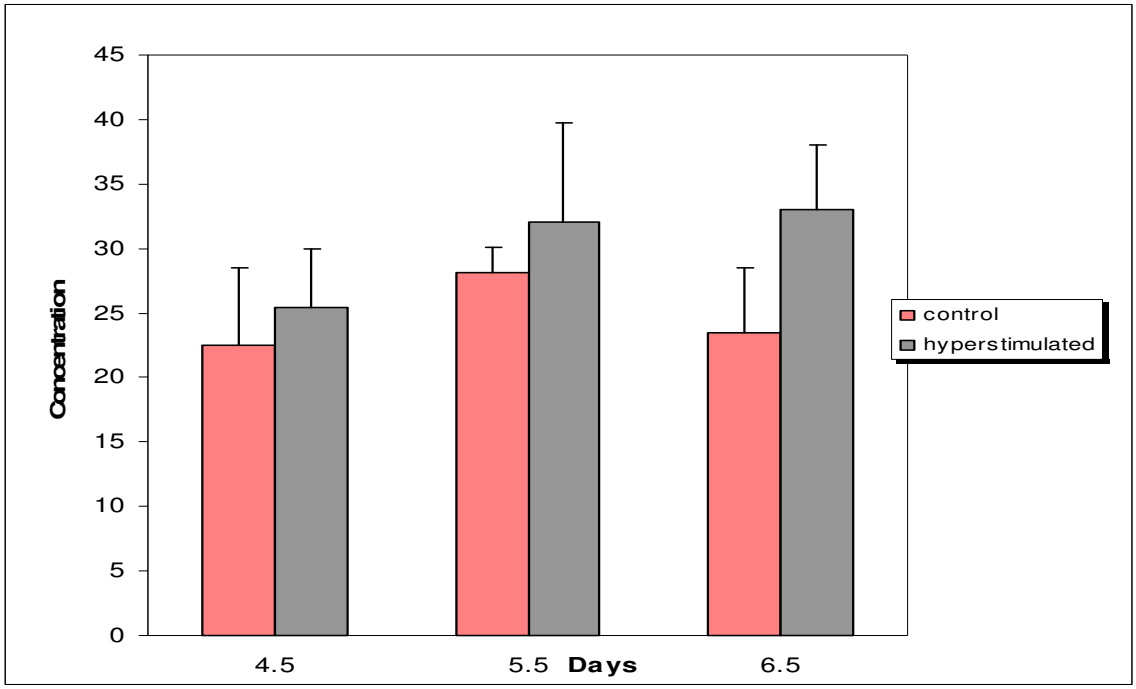


Figure 3. A graph representing progesterone concentrations (ng/ml) in control and hyperstimulated animals on different days of pregnancy

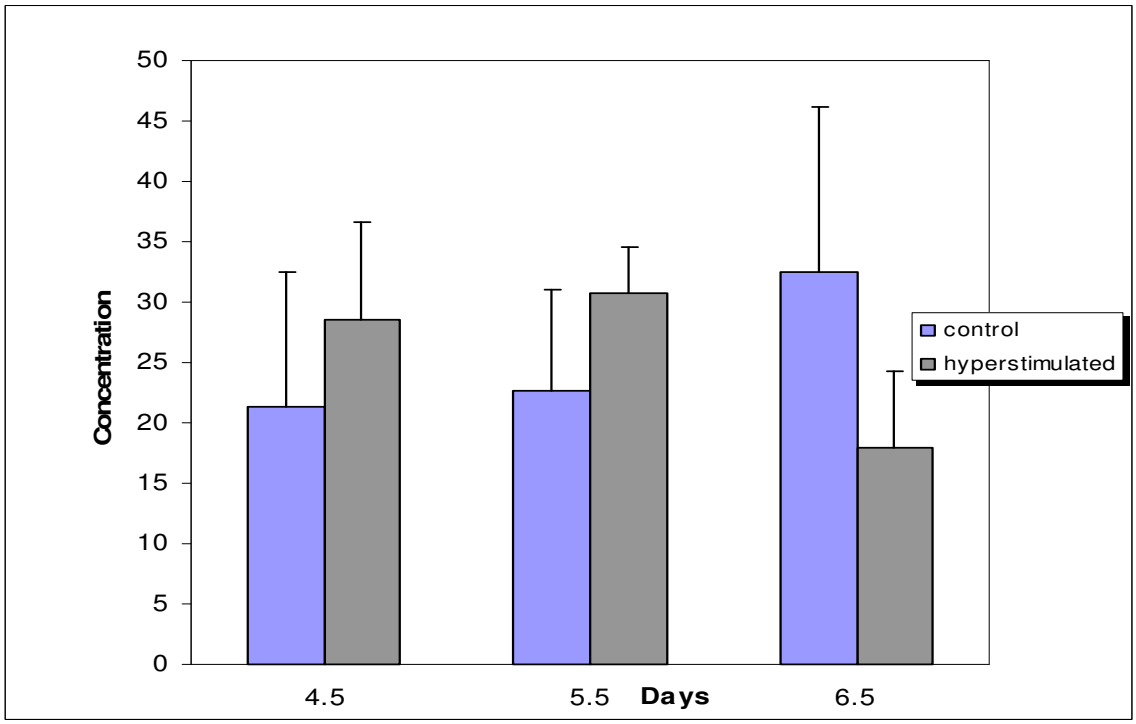


Figure 4. A graph representing oestrogen concentrations (pg/ml) in the control and hyperstimulated animals on different days of pregnancy

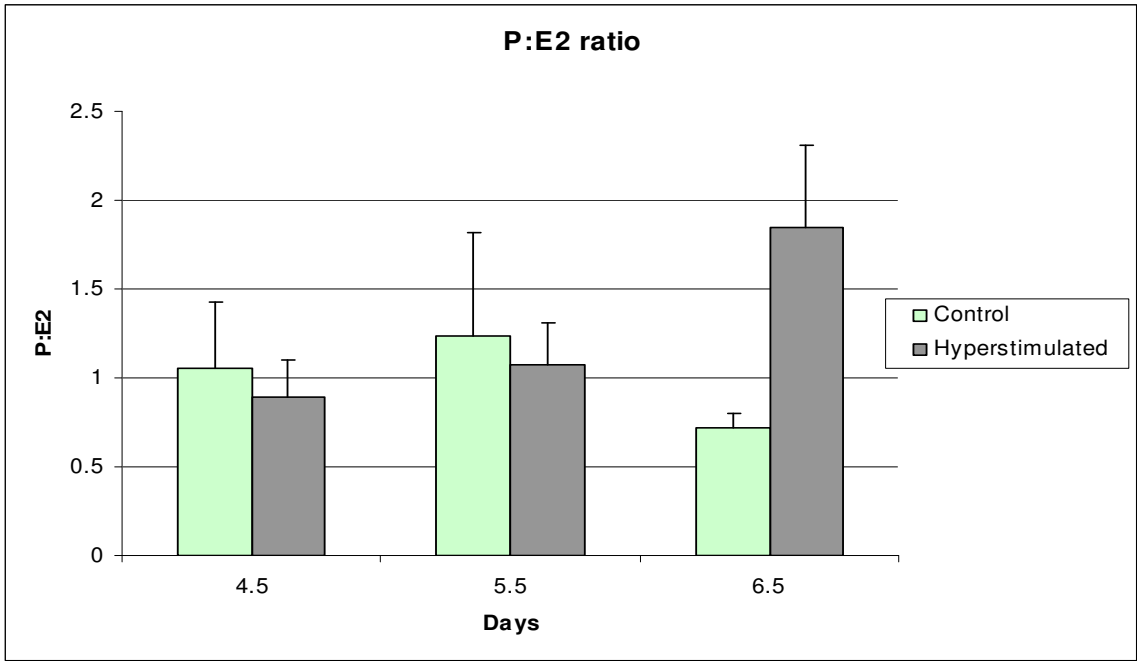


Figure 5. A graph representing the ratio between progesterone and oestradiol plasma concentrations ($P:E_2$ x 10^{-3}) in control and hyperstimulated animals on different days of pregnancy

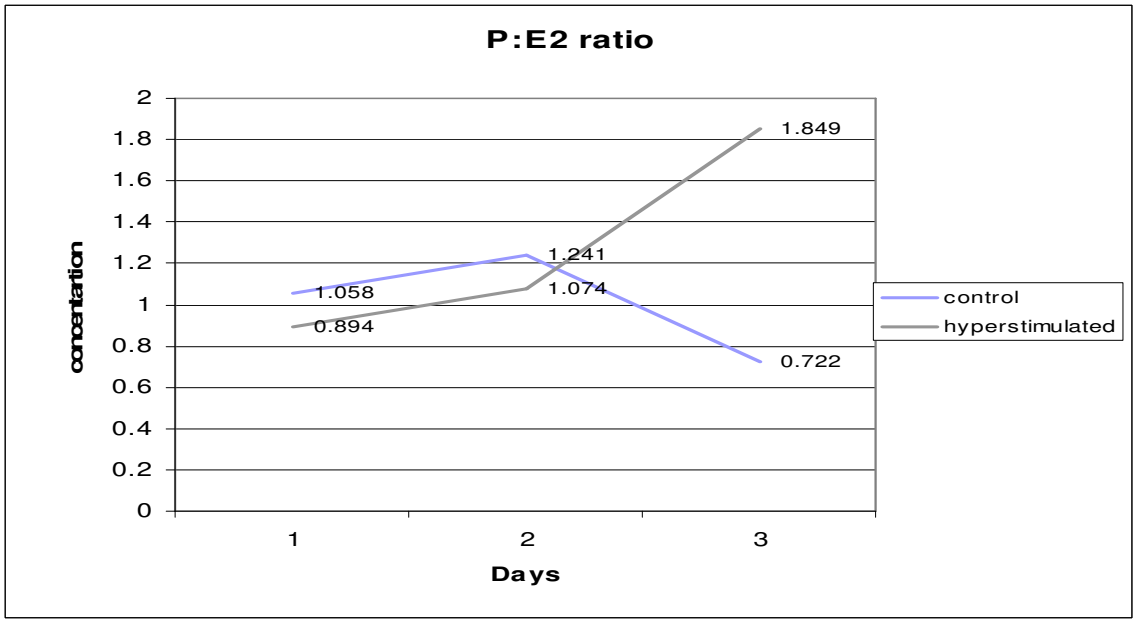


Figure 6. A graph representing the interaction between progesterone (ng/ml) and oestradiol (pg/ml) concentrations ratio in the control and hyperstimulated animals at different stages of pregnancy.



Figure 7. Representative histological section of the 4.5 day control uterine tissue. Note the wavy outline of the luminal surface (LE) and numerous uterine glands (GL) in the stroma (ST). L-uterine lumen. Haematoxylin and eosin stain. x100

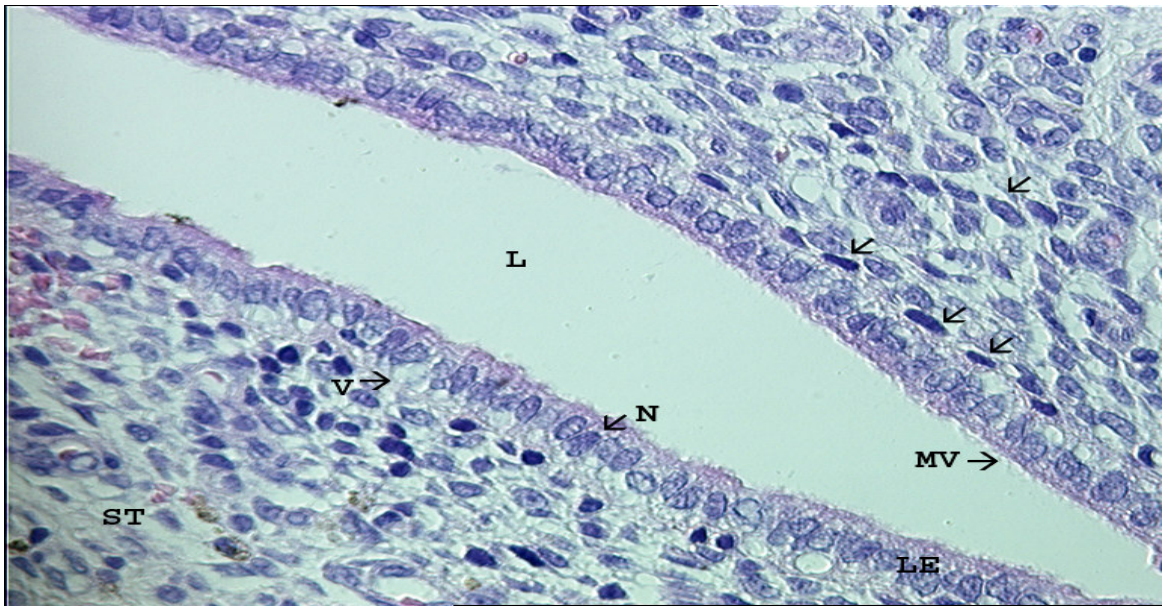


Figure 8. Representative histological section of 4.5 day control uterine tissue. Note the simple columnar luminal epithelial cells (LE) with round to oval, centrally placed nuclei (N), apical microvilli (MV) and vacuoles (V) in the base of the epithelial cells. Also note the flat and fibroblastic (arrow) cells in the stroma (ST). L-uterine lumen. Haematoxylin and eosin stain. x400

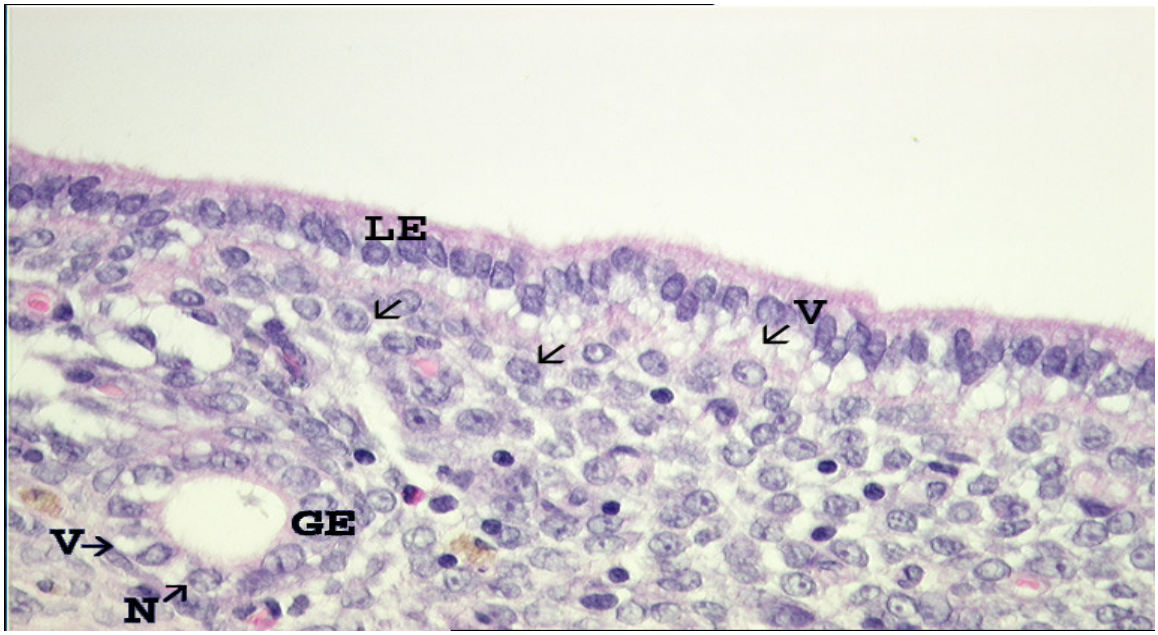


Figure 9. Representative histological section of 4.5 day control uterine tissue at the antimesometrial pole. Note the simple cuboidal glandular epithelial cells (GE) with round to oval centrally placed nuclei (N) and vacuoles basally disposed (V). Note several round cells with distinct round nuclei, indicating that decidualization had begun (arrow). Note the presence of vacuoles (V) in the base of the luminal epithelial cells (LE). Haematoxylin and eosin stain. x400

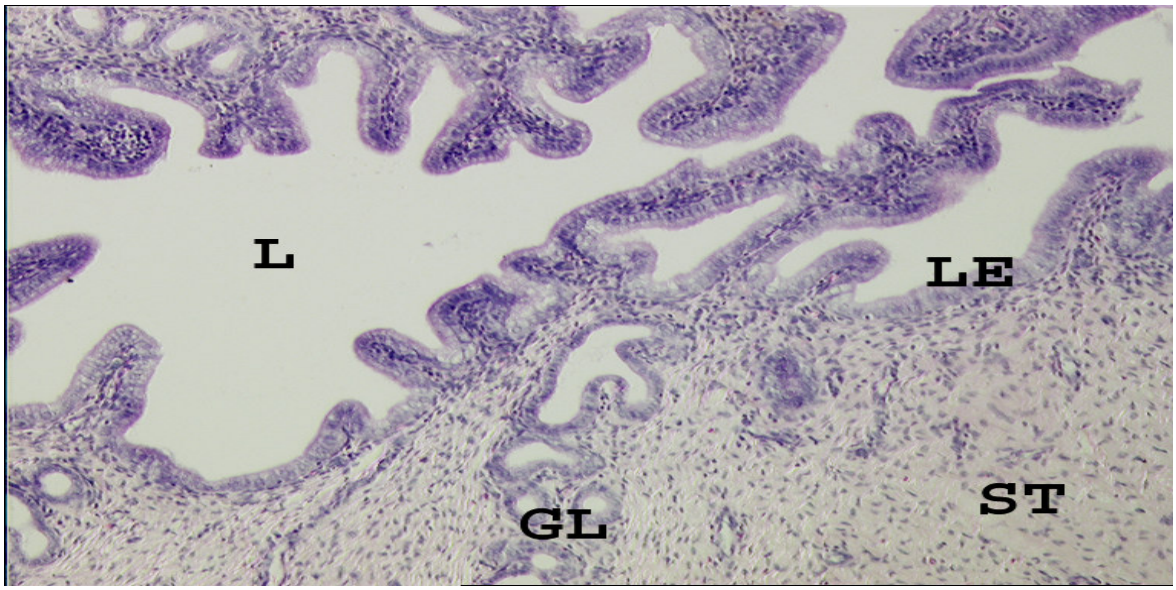


Figure 10. Representative histological section of 4.5 day hyperstimulated uterine tissue. Note that the luminal epithelium (LE) is thrown into folds. Several glands (GL) are present in the stroma (ST). L-lumen. Haematoxylin and eosin stain. x100

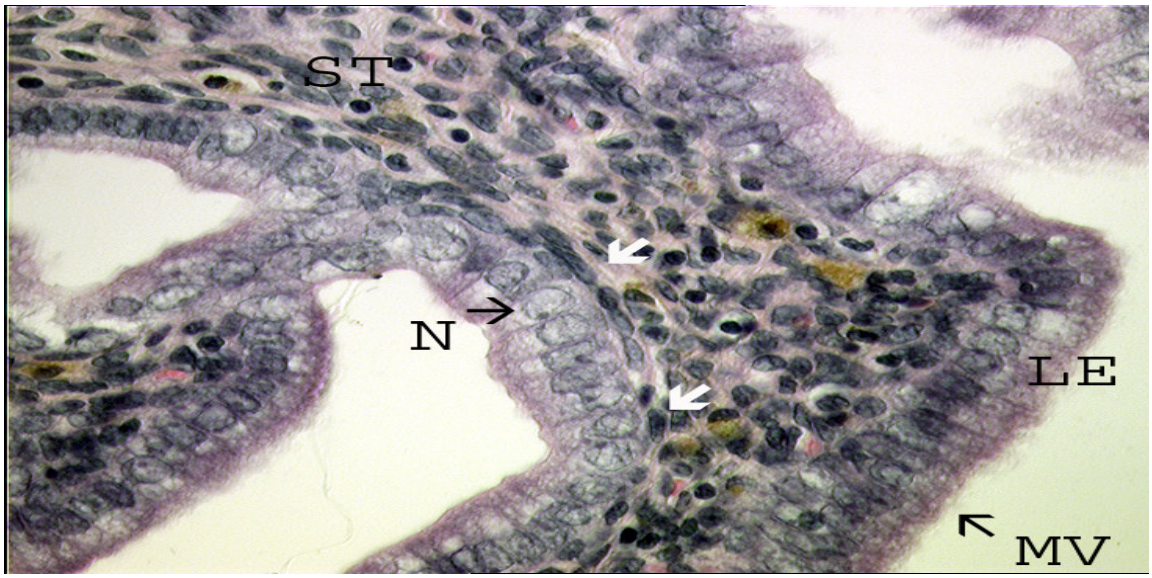


Figure 11. Representative histological section of 4.5 day hyperstimulated uterine tissue. Note the simple columnar luminal epithelium (LE) with apical microvillus border (MV) and large oval, basally placed nuclei (N). The majority of subepithelial stromal cells (ST) appear flat and fibroblastic (arrow). Haematoxylin and eosin stain. x400

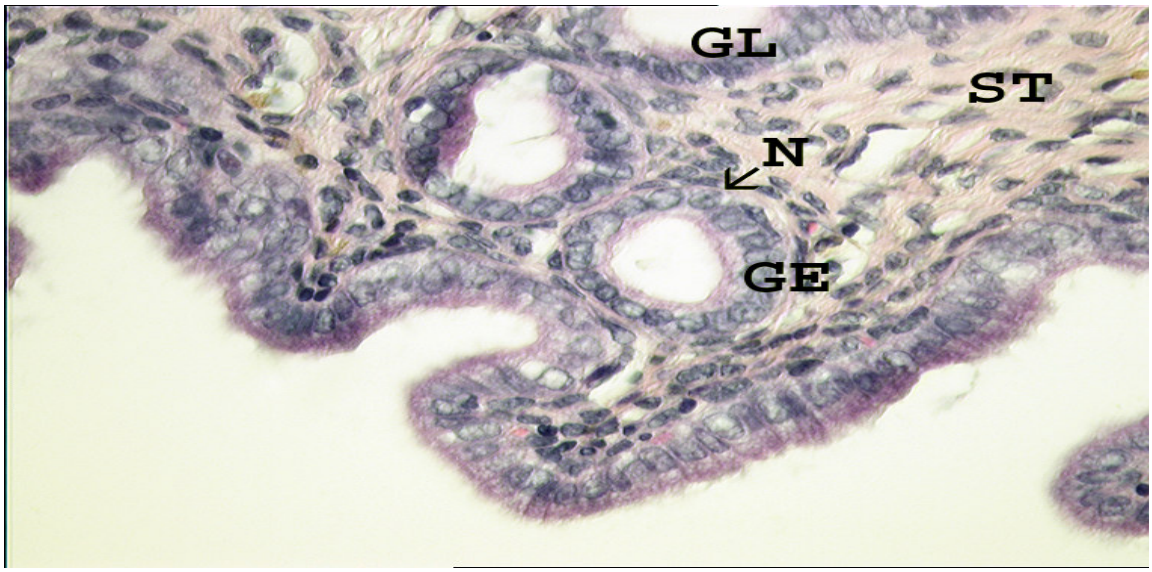


Figure 12. Representative histological section of 4.5 day hyperstimulated uterine tissue. Note the presence of glands (GL) in the subepithelial stroma (ST) which is populated by flat, fibroblastic cells. The simple cuboidal glandular epithelium (GE) has large, round to ovoid and basally placed nuclei (N) and an absence of vacuoles. Haematoxylin and eosin stain. x400



Figure 13. Representative histological section of 5.5 day control uterine tissue. Note the smooth luminal surface and decidualization (DEC) of the subepithelial stromal cells. Haematoxylin and eosin stain. x100



Figure 14. Representative histological section of 5.5 day control uterine tissue. Note the opposite uterine walls in the vicinity of the implanting embryo (EMB) are more closely positioned to each other than in the 5.5 control animal in Fig. 9. BV-blood vessels. Haematoxylin and eosin stain. x100

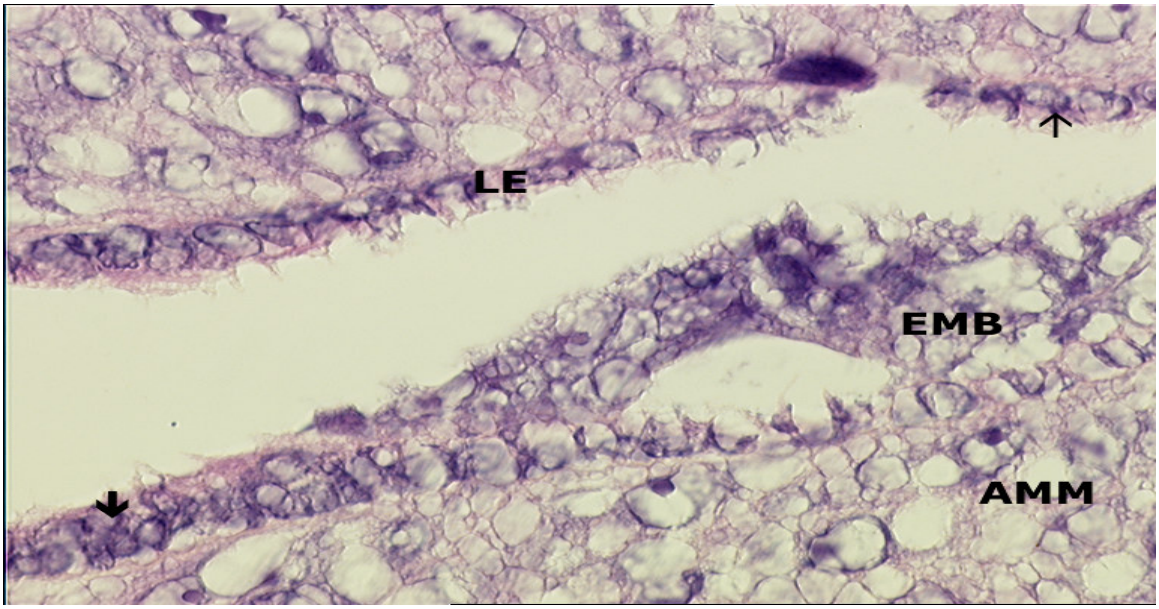


Figure 15. Representative histological section of 5.5 day control uterine tissue. Magnified view of the figure 14 to show an embryo (EMB) attached to one side of the luminal epithelium (LE) at the antimesometrial side (AMM). Also note the luminal epithelial cells appear reduced in size (arrow) and layered (thick arrow). Haematoxylin and eosin. x1000

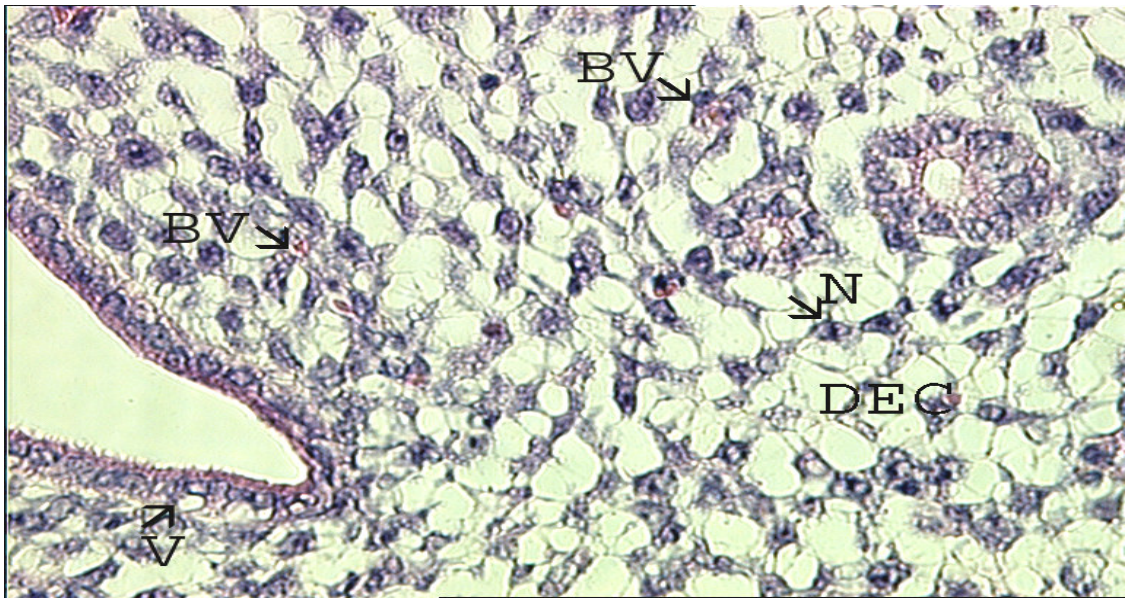


Figure 16. Representative histological section of 5.5 day control uterine tissue showing decidual cells (DEC) with round nuclei (N) and scanty cytoplasm. Occasional blood vessels (BV) are visible in the subepithelial compartment. V-vacuoles. Haematoxylin and eosin stain. x400

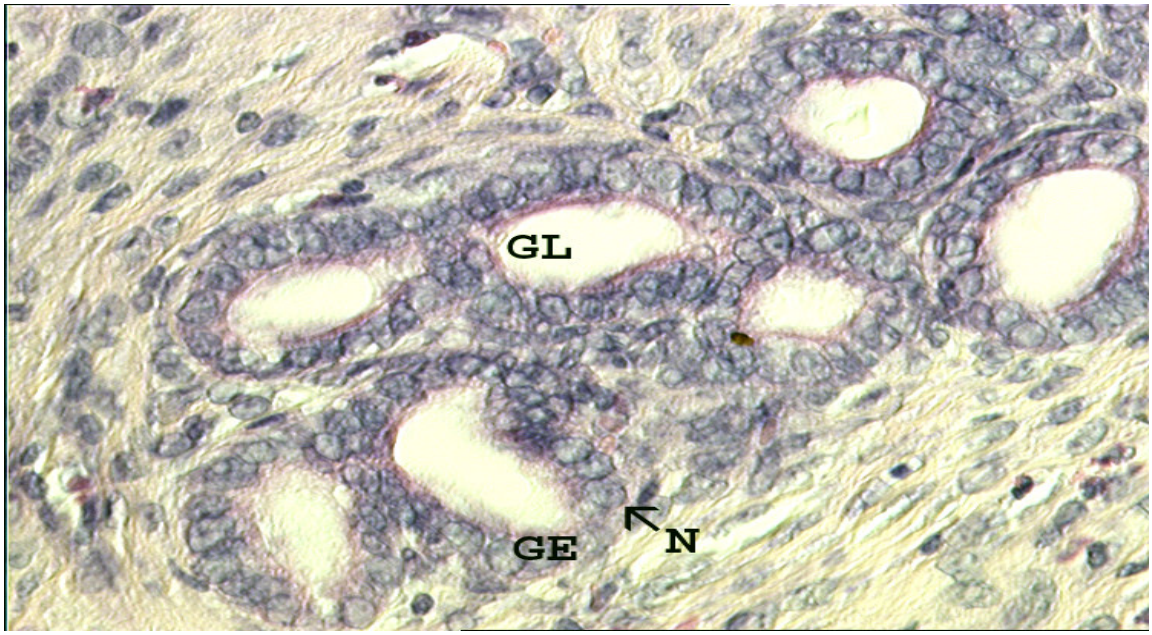


Figure 17. Representative histological section of 5.5 day control uterine tissue showing clusters of uterine glands (GL). Note simple cuboidal glandular epithelium (GE) with centrally placed, round and basophilic nuclei (N). Haematoxylin and eosin stain. x400

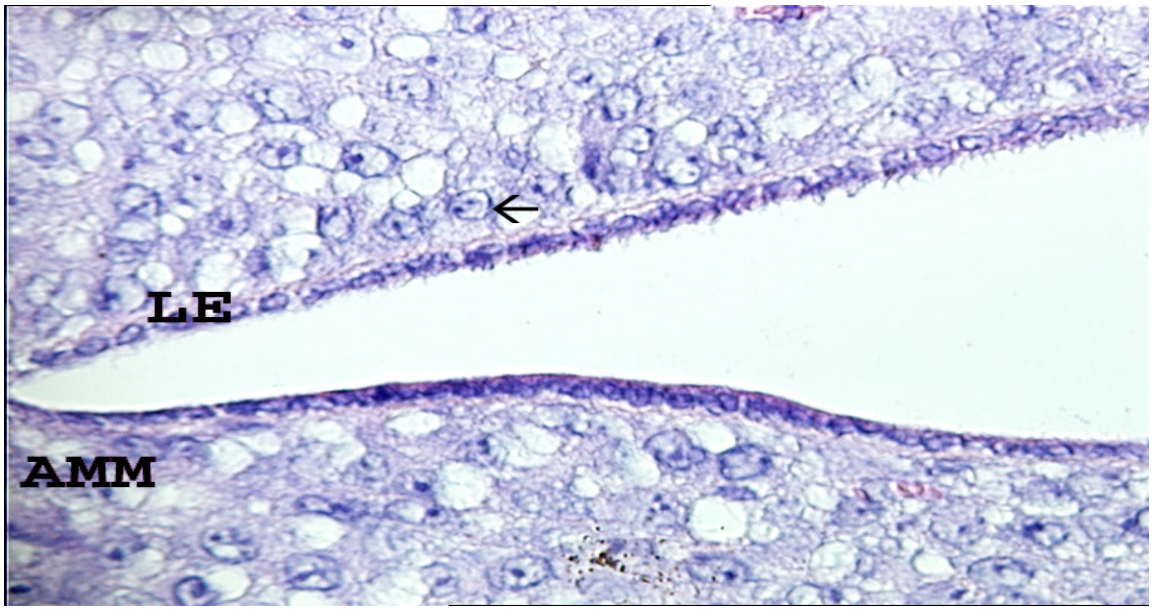


Figure 18. Representative histological section of 6.5 day control uterine tissue showing the simple cuboidal and simple squamous (antimesometrial side) shape of the luminal epithelial cells (LE). Note the well defined decidual cells with round nuclei (arrow) at the antimesometrial side (AMM). Haematoxylin and eosin stain. x400

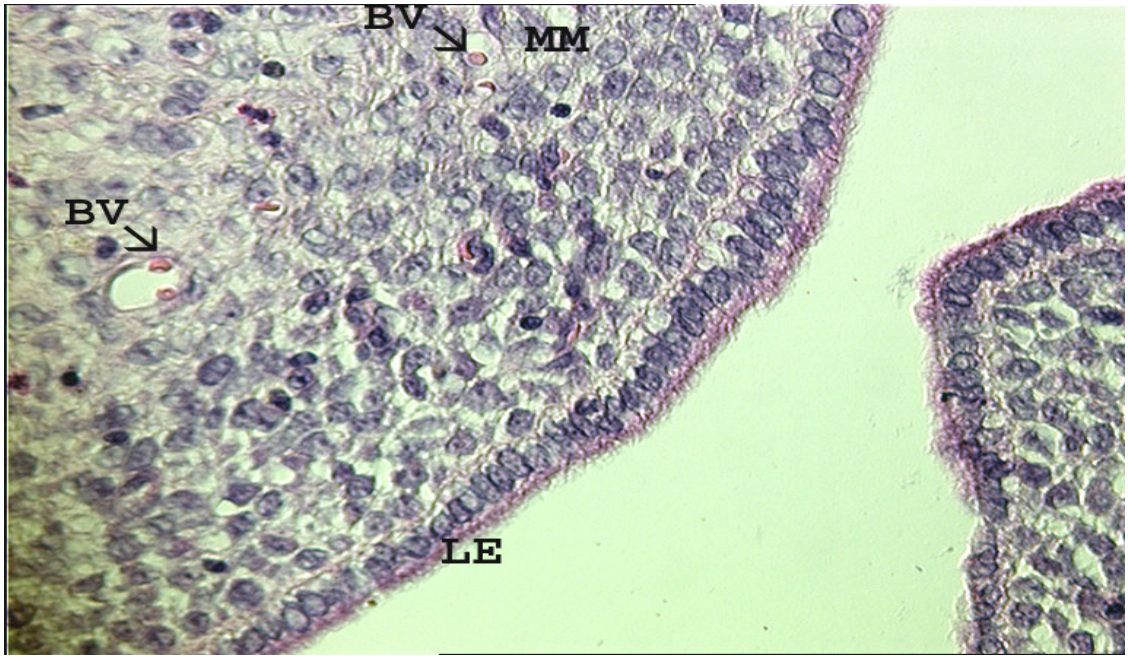


Figure 19. Representative histological section of 6.5 day control uterine tissue. Note the change in the shape of the luminal epithelial cells (LE) from simple cuboidal to columnar at the mesometrial side of the lumen (MM). BV-blood vessel. Haematoxylin and eosin stain. x400

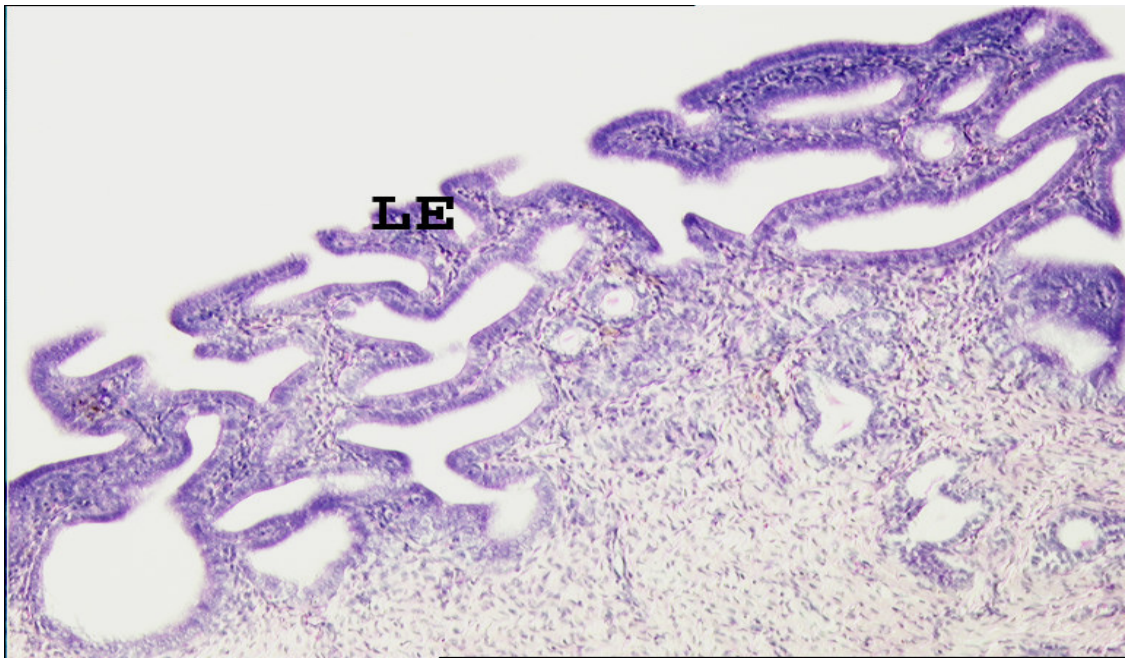


Figure 20. Representative histological section of 5.5 day hyperstimulated uterine tissue showing extensively folded luminal epithelium (LE). Haematoxylin and eosin stain. x100

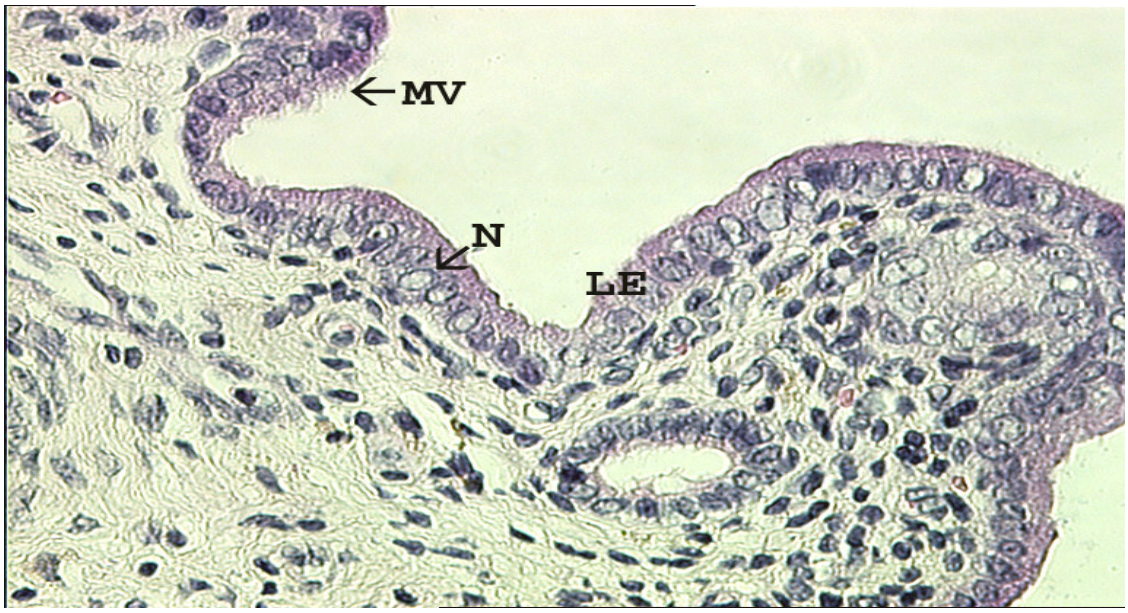


Figure 21. Representative histological section of 5.5 day hyperstimulated uterine tissue showing simple columnar luminal epithelial cells (LE) with oval nuclei (N), basally disposed. A microvillus border (MV) is visible at the apical pole of these cells. Vacuoles are not present in the cytoplasm. Haematoxylin and eosin stain. x400



Figure 22. Representative histological section of 5.5 day hyperstimulated uterine tissue. Uterine glands (GL) are observed in the stromal compartment (STR) filled with glandular secretion. Note the simple, cuboidal glandular epithelial cells (GE) with large basally placed nuclei (N). Haematoxylin and eosin stain. x400

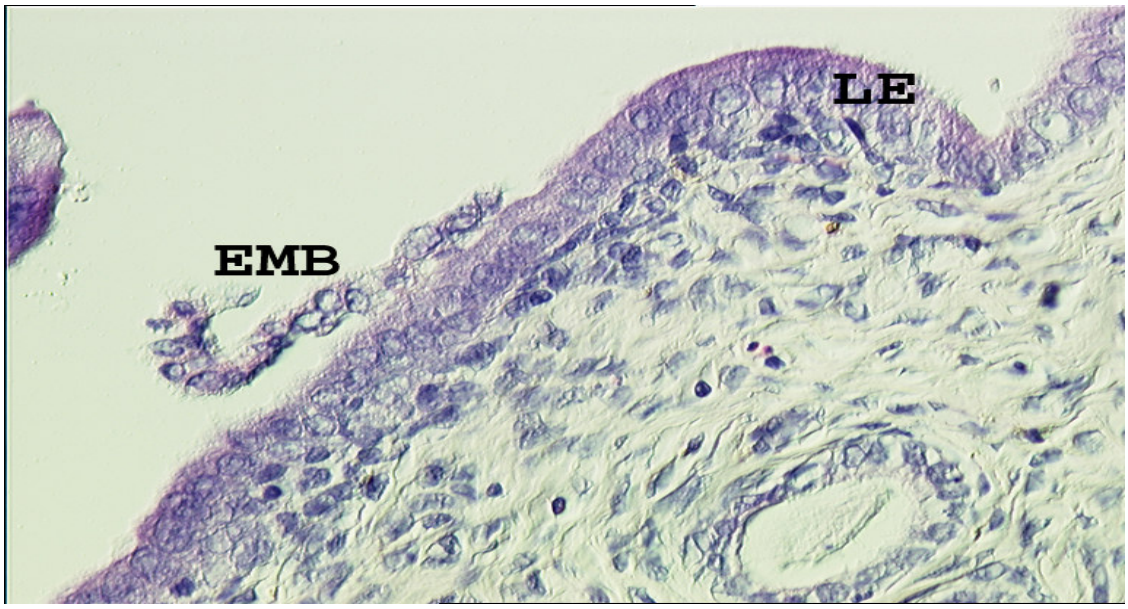


Figure 23. Representative histological section of 5.5 day hyperstimulated uterine tissue. Note the presence of incomplete (due to the sectioning) and unattached embryo (EMB) situated close to the luminal epithelium (LE). Haematoxylin and eosin stain. x400

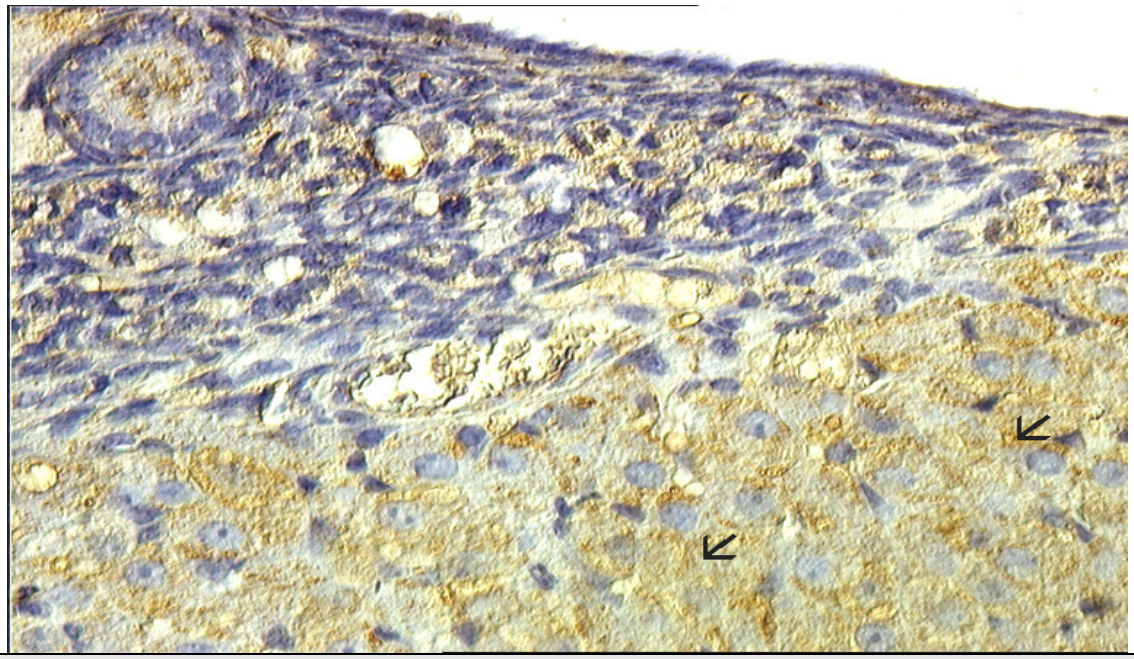


Figure 24. Mouse ovary, positive control. Photomicrograph of a histological section of mouse ovary incubated with TGF β_1. Note extracellular (arrow) immunolocalization of TGF β_1 (arrow). x400

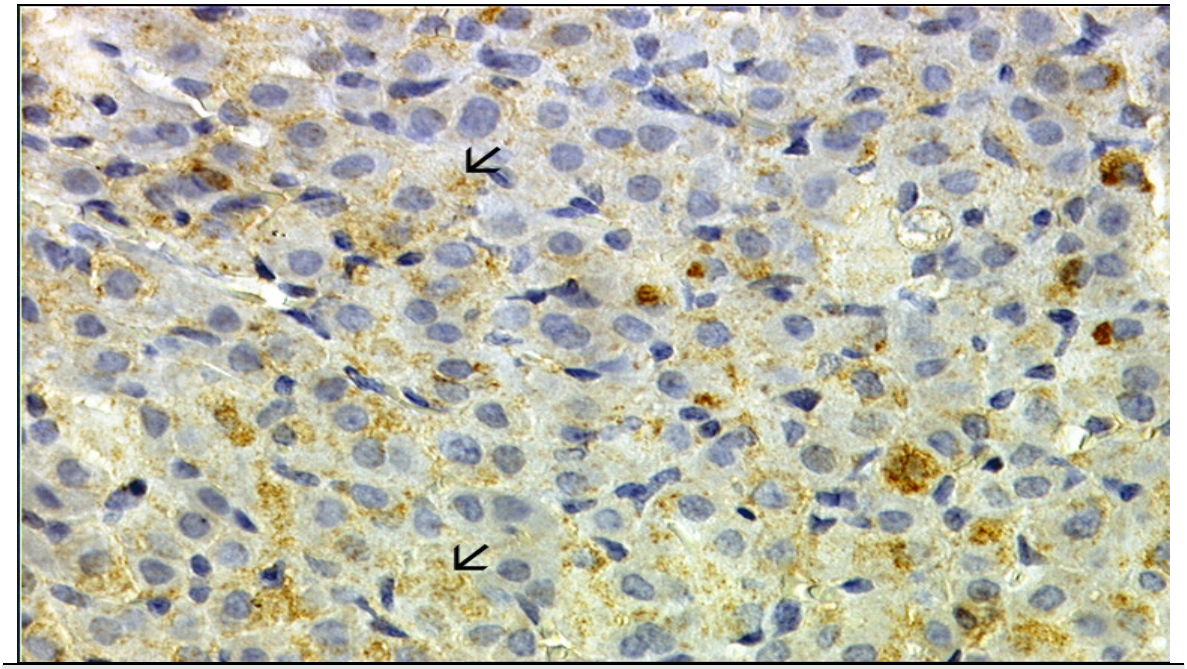


Figure 25. Mouse ovary, positive control. Photomicrograph of a histological section of mouse ovary incubated with TGF β_2. Note extracellular immunolocalization of TGF β_2 (arrow). x400

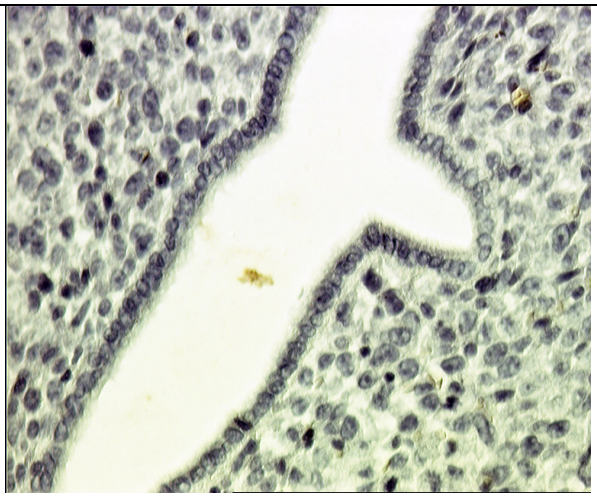
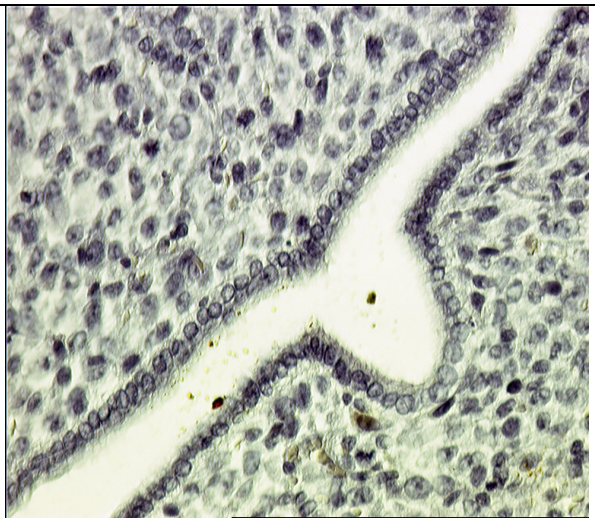
Figure 26. Photomicrograph of the control uterine tissue incubated with **TGF β_1 antibody**, showing cytoplasmic immunolocalization in the apex of the luminal epithelial cells and subluminal stroma (arrow). Counterstained with haematoxylin. x100	
Figure 27. Photomicrograph of the control uterine tissue where **primary antibody** was omitted and replaced with **TBS.** Note absence of the TGF β_1 immunolocalization. Counterstained with haematoxylin. x100	
Figure 28. Photomicrograph of the control uterine tissue where **secondary antibody** was omitted and replaced with **TBS**. Note absence of the TGF β_1 immunolocalization. Counterstained with haematoxylin. x100	

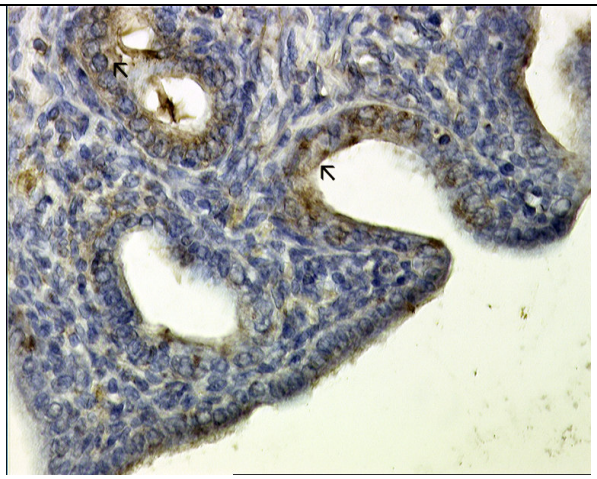
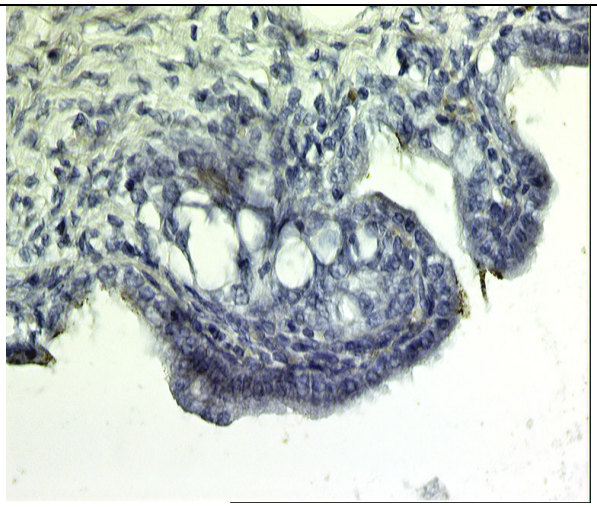
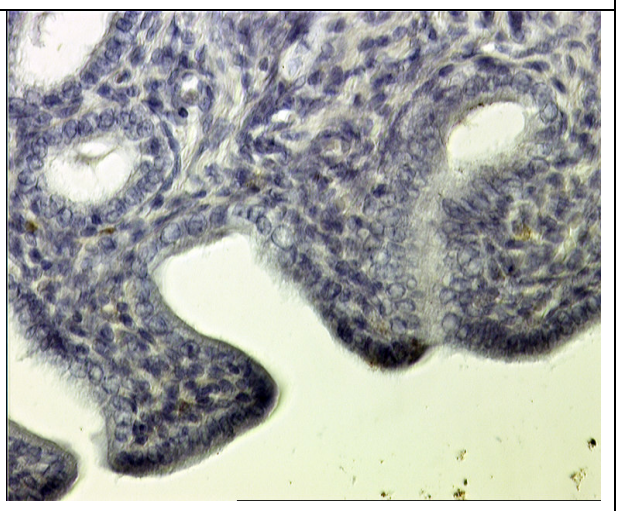
Figure 29. Photomicrograph of the hyperstimulated uterine tissue incubated with **TGF β_2 antibody**, showing TGF β_2 immunolocalization in the luminal (arrow) and glandular epithelial cells (arrow). Counterstained with haematoxylin. x400	
Figure 30. Photomicrograph of hyperstimulated uterine tissue where the **primary antibody** was omitted and replaced with **TBS.** Note absence of the TGF β_2 immunolocalization. Counterstained with haematoxylin. x400	
Figure 31. Photomicrograph of hyperstimulated uterine tissue where the **secondary antibody** was omitted and replaced with **TBS**. Note absence of the TGF β_2 immunolocalization. Counterstained with haematoxylin. x400	

Figure 32. Photomicrograph of the control uterine tissue incubated with **TGF β_1** antibody. Note **TGF β_1** immunolocalization in the subluminal stroma (arrow). Counterstained with haematoxylin. x400

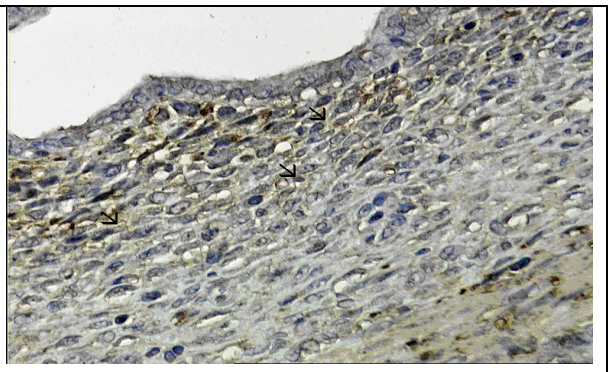


Figure 33. Photomicrograph of control uterine tissue where primary antibody was omitted and replaced with NRS. Note the absence of **TGF β_1** in the luminal epithelium and stroma. Counterstained with haematoxylin. x400

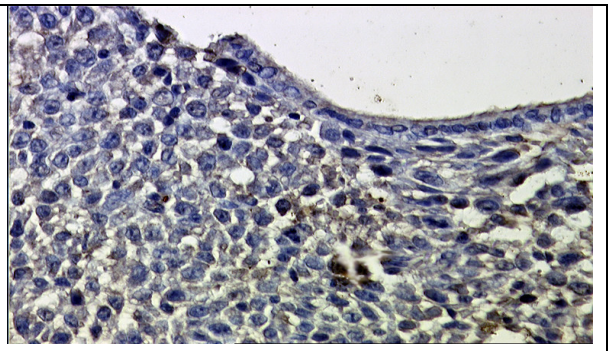


Figure 34. Photomicrograph of the hyperstimulated uterine tissue incubated with **TGF β_2** antibody. Note luminal and glandular (arrow) **TGF β_2** immunolocalization. Counterstained with haematoxylin. x400

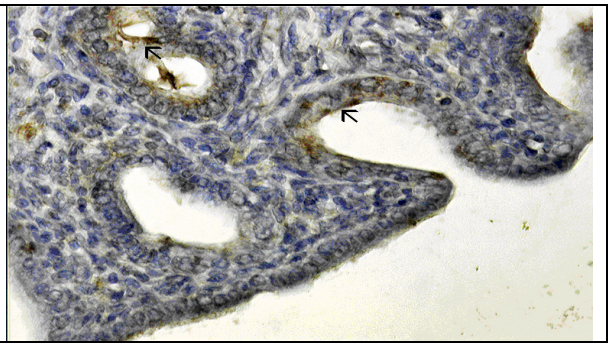


Figure 35. Photomicrograph of the hyperstimulated uterine tissue where **secondary antibody** was omitted and replaced with **NRS.** Note absence of TGF β_2 immunolocalization. Counterstained with haematoxylin. x400

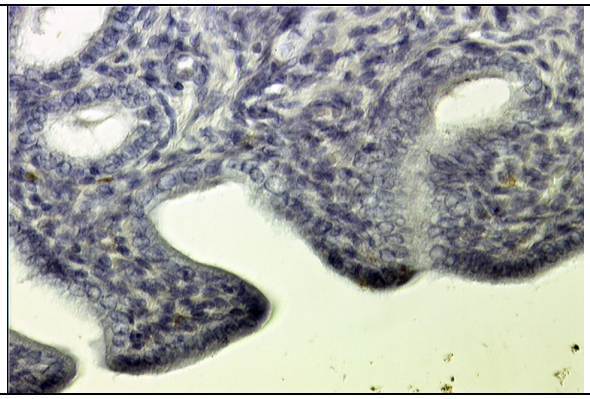
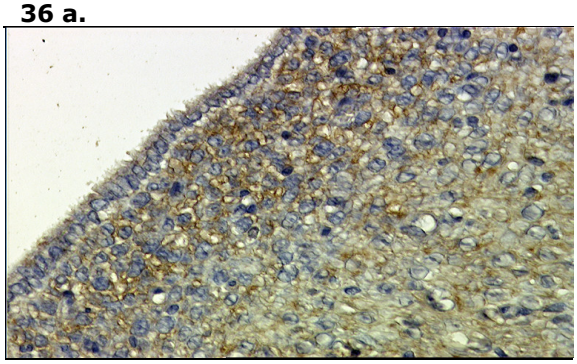


Figure 36 a and b. Photomicrograph of control uterine tissue where an **absorption control for TGF β_1** was performed. Note **TGF β_1** immunolocalization in the stroma (Fig. 31 a) and the marked reduction of TGF β_1 immunolocalization in the section in which the blocking of the primary antibody was performed (Fig. 31b). Counterstained with haematoxylin. x400

36 a.



36 b.

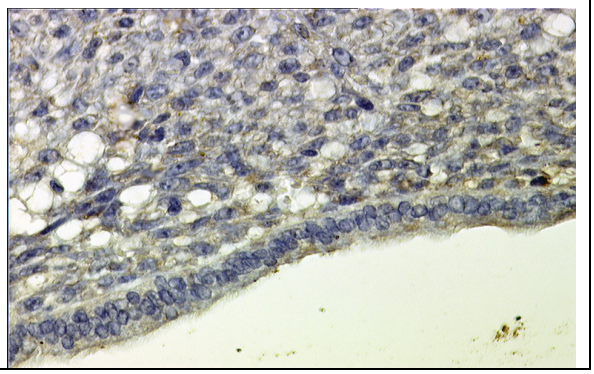
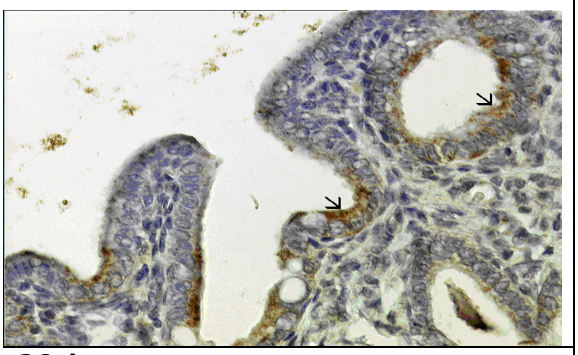
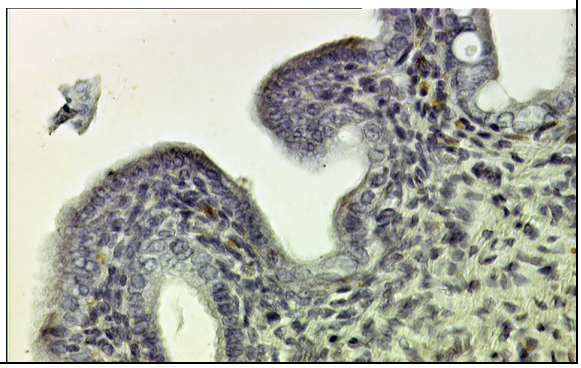


Figure 36 c and d. Photomicrograph of hyperstimulated uterine tissue where the **absorption control for TGF β_2** was performed. Note immunolocalization in the luminal (arrow) and glandular (arrow) epithelium (Fig. 31 c) and no immunolocalization in the section where complete absorption was achieved (Fig. 31 d). Counterstained with haematoxylin. x400

36 c.



36 d.



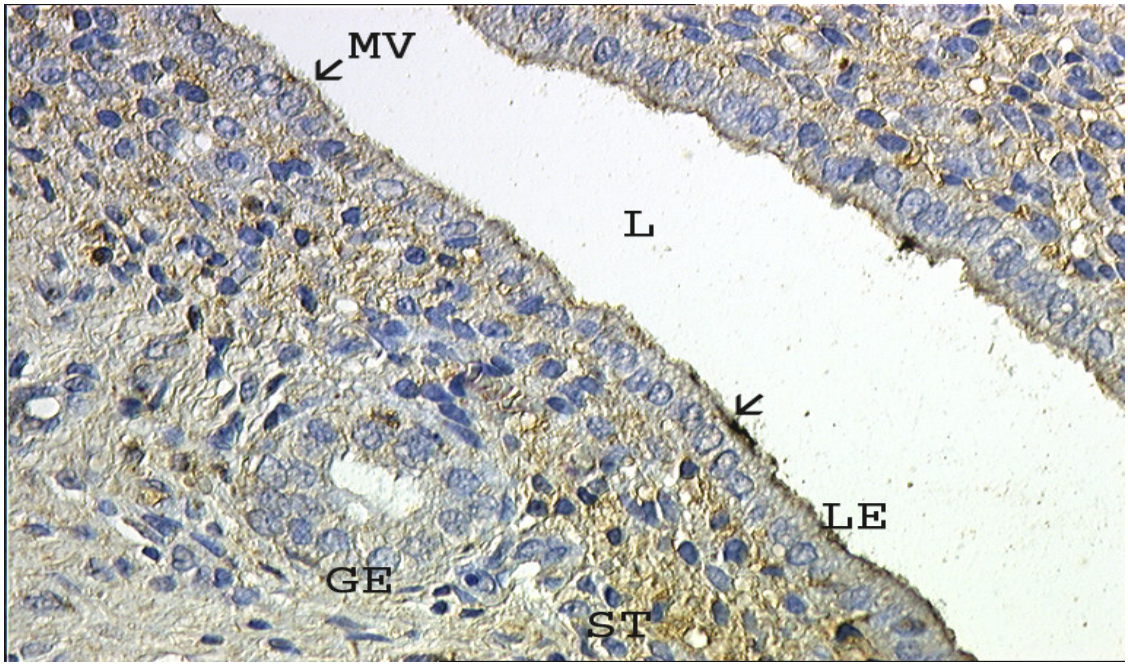


Figure 37. Representative section of 4.5 day control animal. Note the weak cytoplasmic immunolocalization of the TGF β_1 in the luminal (LE) and the glandular (GE) epithelial cells and the stronger immunolocalization in the stroma (ST). Also note apical immunolocalization of the TGF β_1 in the luminal epithelial cells (arrow). MV-microvillus border. Counterstained with haematoxylin. x400

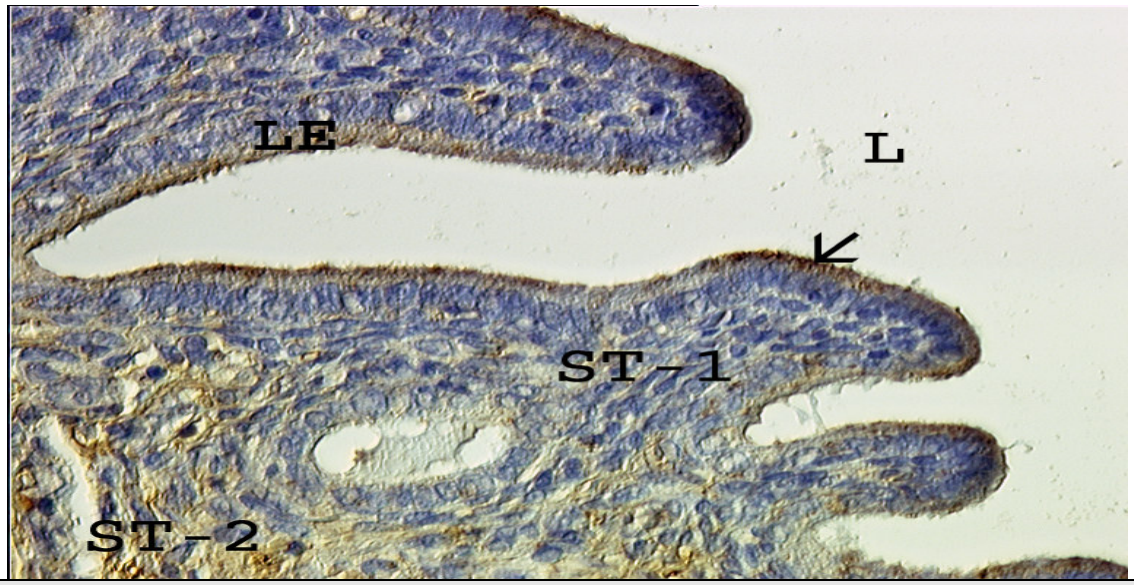


Figure 38. Representative section of 4.5 day hyperstimulated animal. Note the weak TGFβ_1 immunolocalization in the luminal epithelium (LE) with distinct apical concentration (arrow). Two regions (ST-1 and ST-2) of different TGF β_1 immunolocalization in the stroma were apparent. Counterstained with haematoxylin. x400

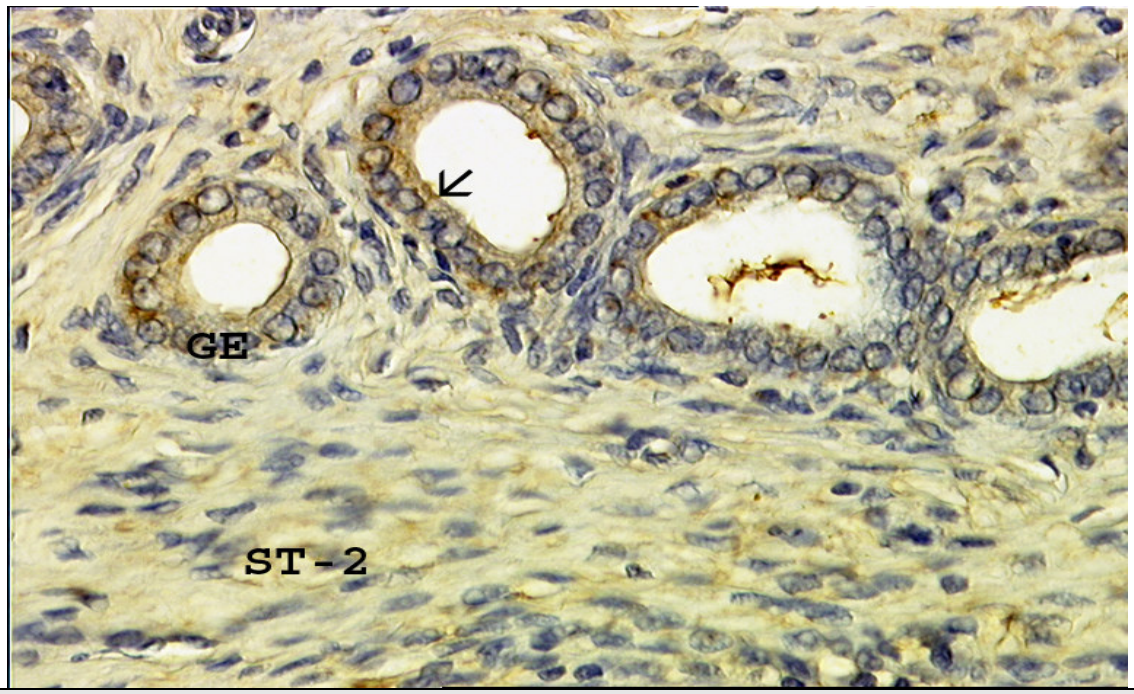


Figure 39. Representative section of 4.5 day hyperstimulated animal. Note the weak TGFβ_1 immunolocalization in the glandular epithelium (GE) with apical concentrations (arrow), surrounded with stromal, deeper region (ST-2), which has strong TGFβ_1 expression. Counterstained with haematoxylin. x400

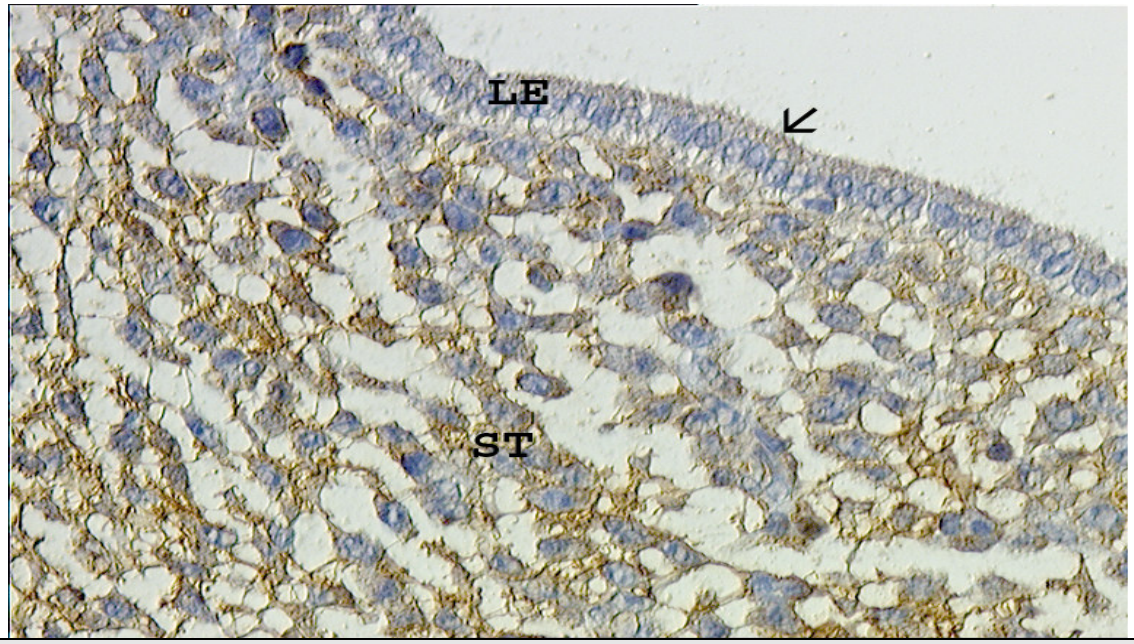


Figure 40. Representative section of 5.5 day control animal. Note apical (arrow) TGF β_1 immunolocalization in the luminal epithelial cells (LE) and strong subluminal stromal TGF β_1 localization (ST). Counterstained with haematoxylin. x400

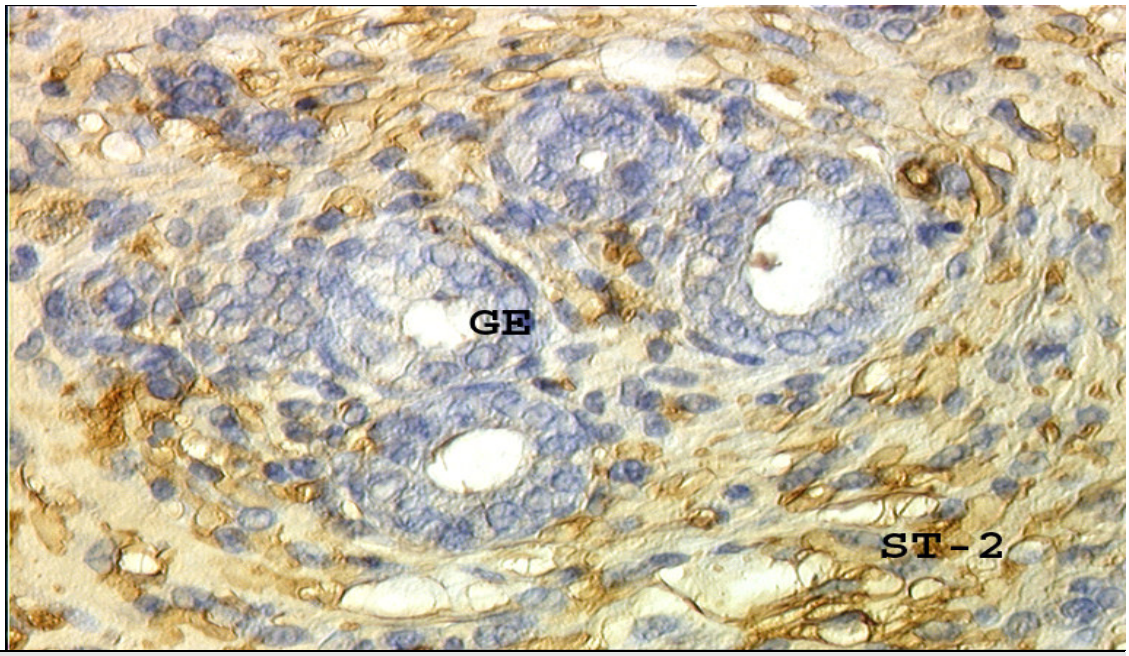


Figure 41. Representative section of 5.5 day control animal. Note the weak TGF β_1 immunolocalization in the glandular epithelium (GE) surrounded by stromal deeper region (ST-2) with strong TGF β_1 immunolocalization. Counterstained with haematoxylin. x400

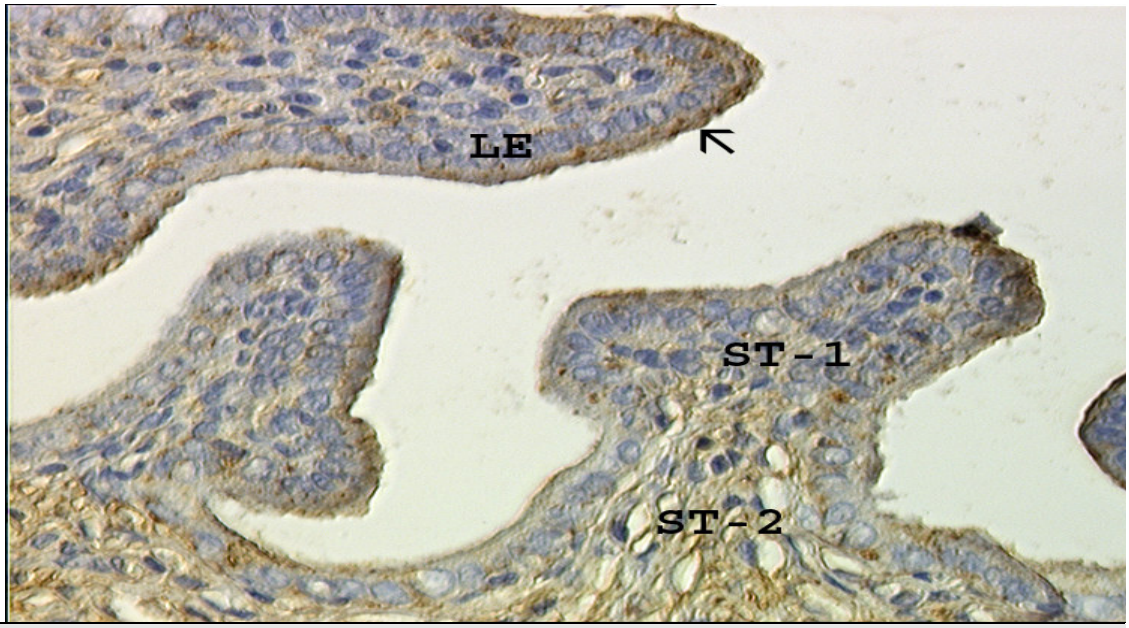


Figure 42. Representative section of 5.5 day hyperstimulated animal. Note the weak TGF β_1 immunolocalization in the luminal epithelium (LE) with well defined apical localization (arrow). Two zones of immunolocalization in the stroma can be observed (ST-1 and ST-2). Counterstained with haematoxylin. x400

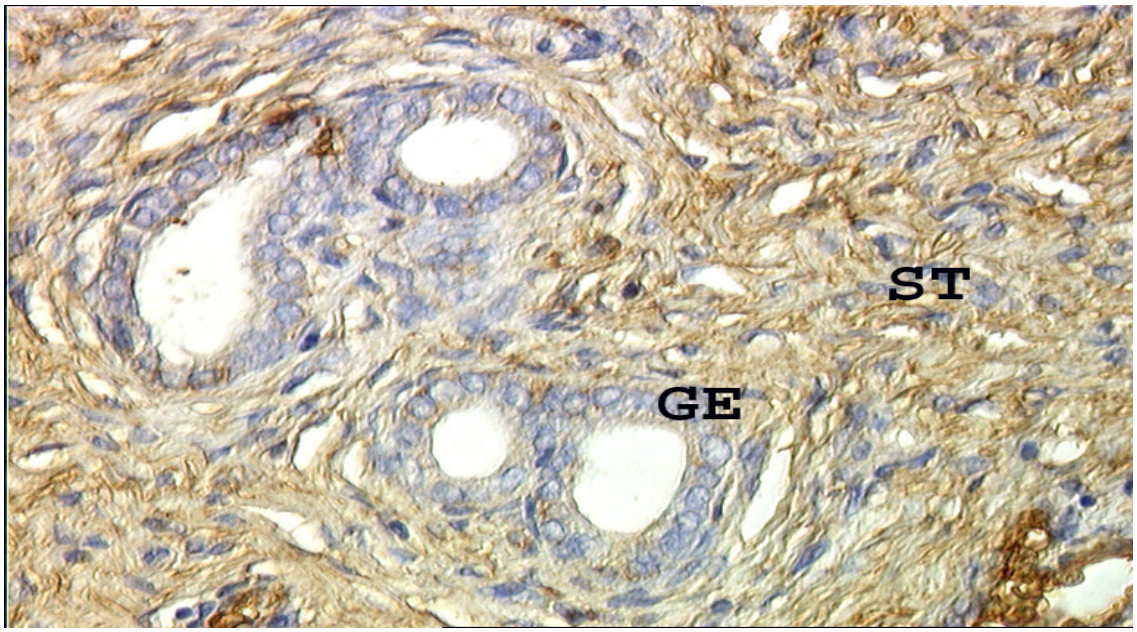


Figure 43. Representative section of 5.5 day hyperstimulated animal. Note the weak TGF β_1 immunolocalization in the glandular epithelium (GE) and strong in the stroma (ST). Counterstained with haematoxylin. x400

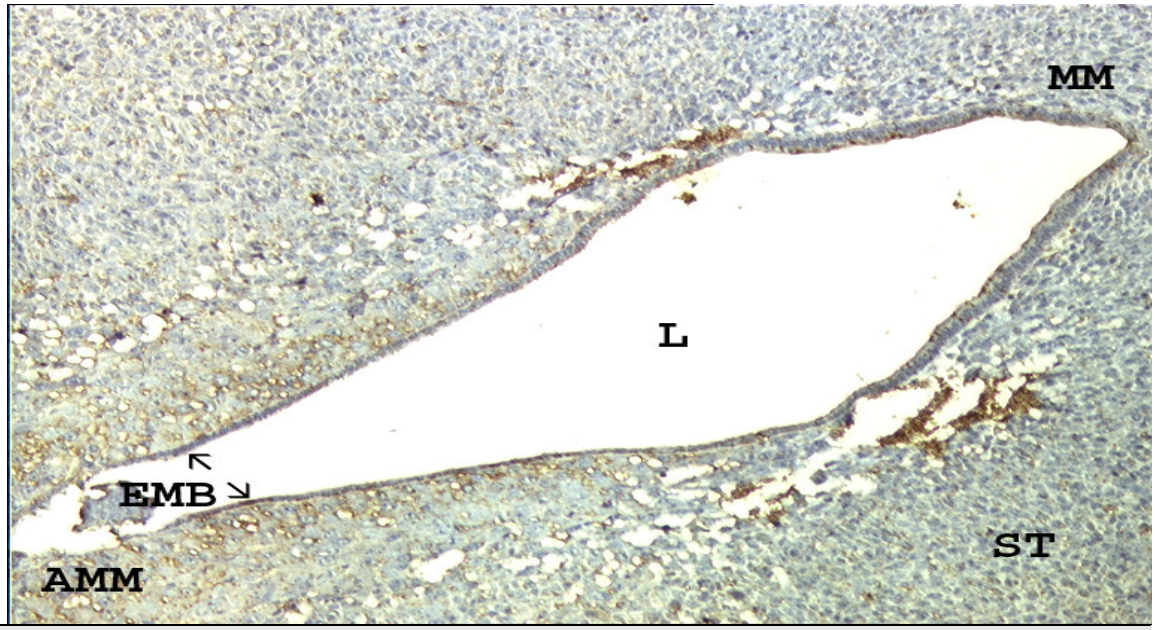


Figure 44. Representative section of 6.5 day control animal. Note the embryo (EMB) lying unattached in the uterine lumen (L) and the opposite sides of the luminal epithelium (arrow) positioned closer to each other. Also note the strong TGF β_1 immunolocalization at the antimesometrial pole (AMM) and weaker TGF β_1 expression in the surrounding stroma (ST). MM-mesometrial pole. Counterstained with haematoxylin. x100

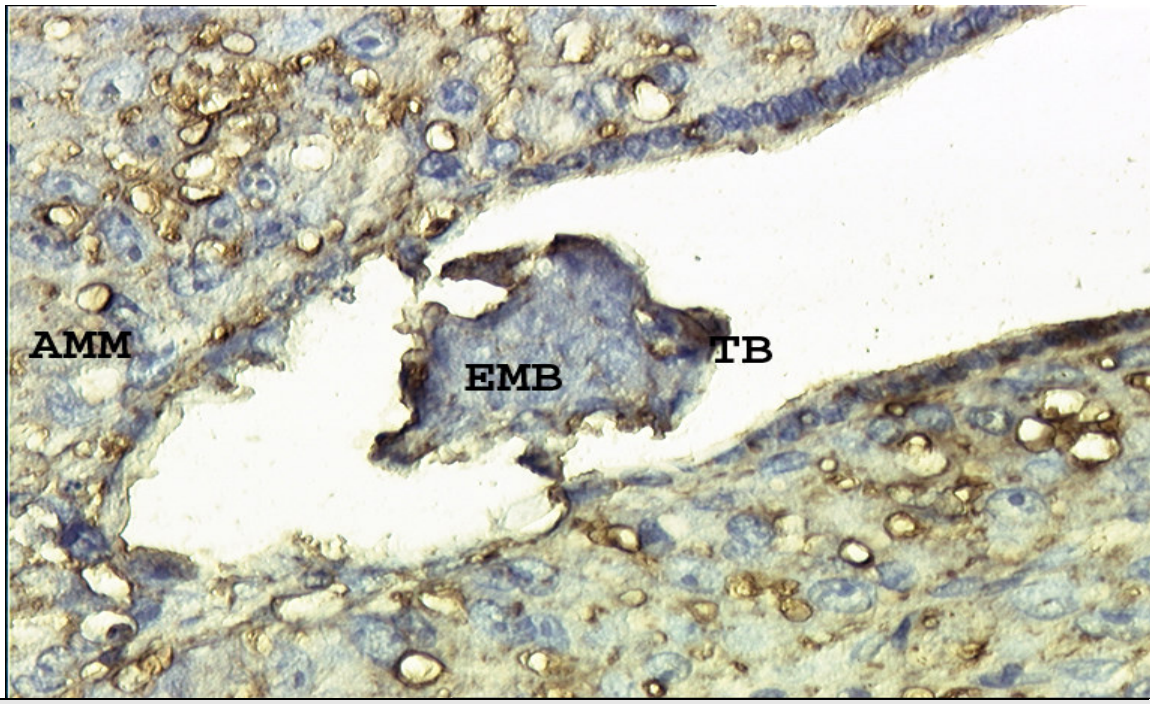


Figure 45. Representative section of 6.5 day control animal (antimesometrial side). Note TGF β_1 immunolocalization in the trophoblast (TB) of the implanting embryo (EMB). Also note strong TGF β_1 immunolocalization at the antimesometrial (AMM) pole surrounding the implanting conceptus. Counterstained with haematoxylin. x400

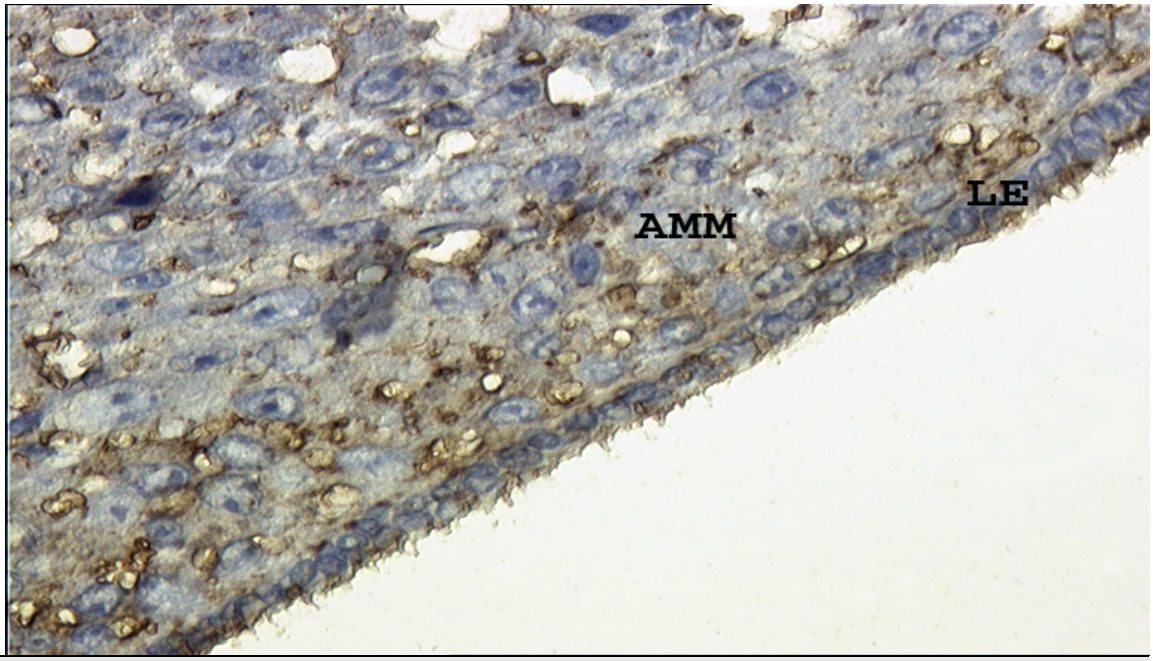


Figure 46. Representative section of 6.5 day control animal. Note the weak TGF β_1 expression in the luminal epithelium (LE). Note the subluminal TGF β_1 immunolocalization at the antimesometrial pole (AMM). Counterstained with haematoxylin. x400

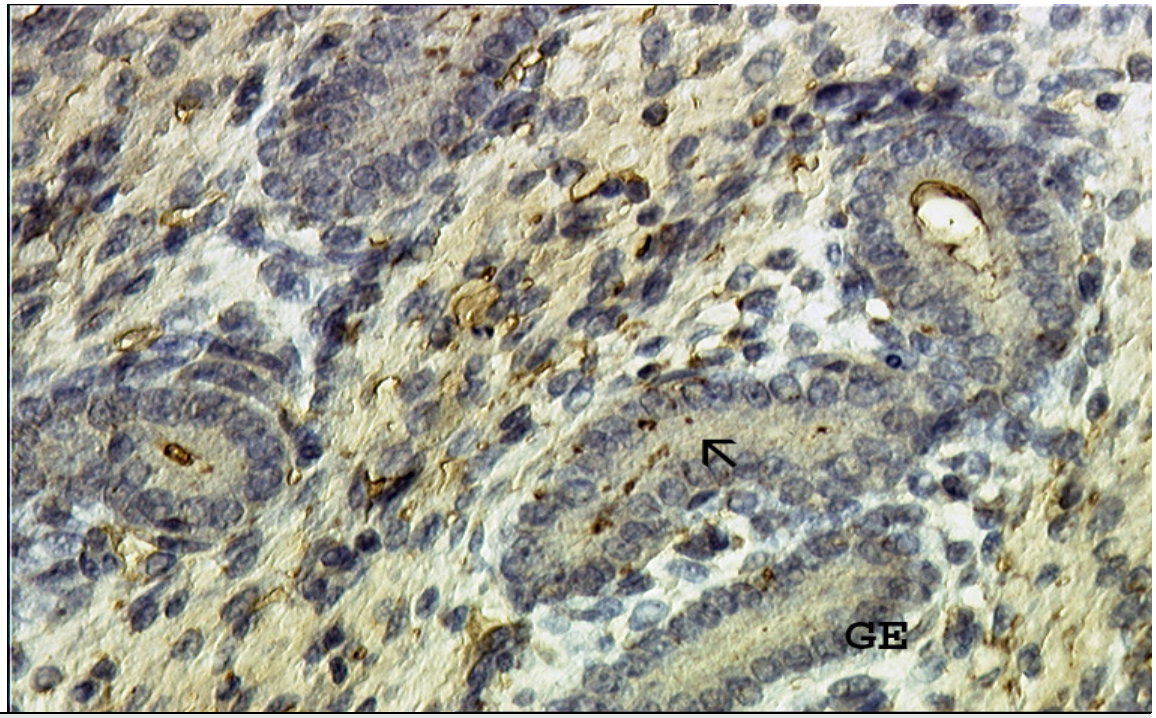


Figure 47. Representative section of 6.5 day control animal. Note weak TGF β_1 expression in the glandular epithelium (GE) and secretion in the lumina of the glands which took up the DAB chromagen (arrow). ST-stroma. Counterstained with haematoxylin. x400



Figure 48. Representative section of 6.5 day hyperstimulated animal. Note two distinct zones of TGF β_1 immunolocalization in the stroma, the weak ST-1 and strong, deeper zone ST-2. Counterstained with haematoxylin. x100

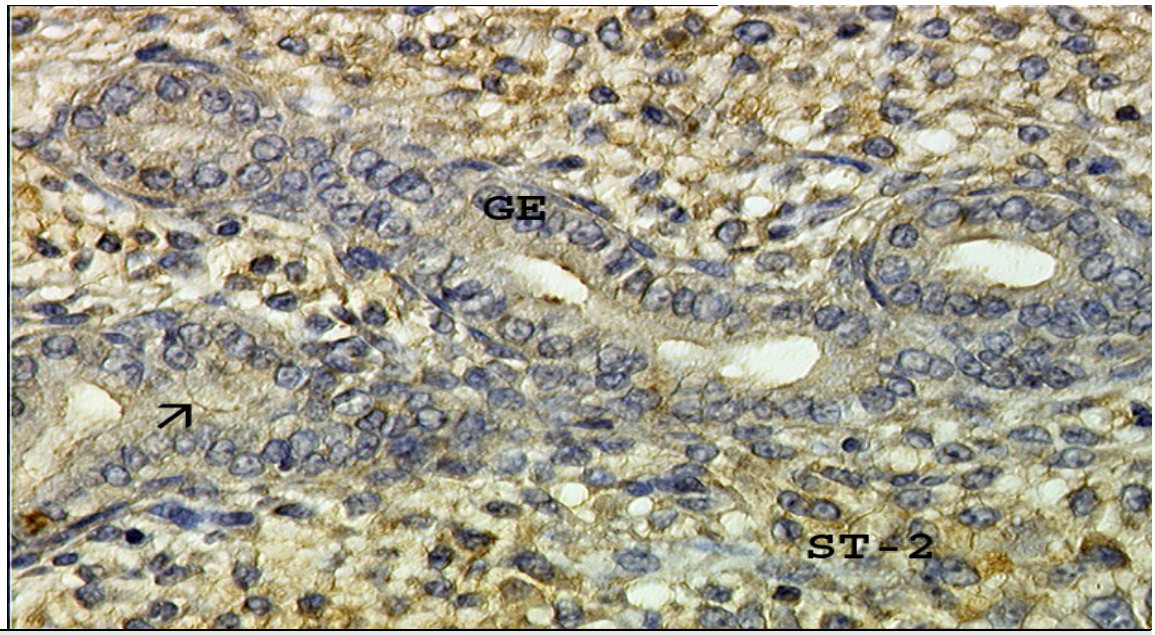


Figure 49. Representative section of 6.5 day hyperstimulated animal. Note the weak TGF β_1 immunolocalization in the glandular epithelium (GE) surrounded by the deeper region of the stroma, which has a stronger expression of TGF β_1. Also note the lumen with secretion (arrow) which took up the DAB chromagen. Counterstained with haematoxylin. x400

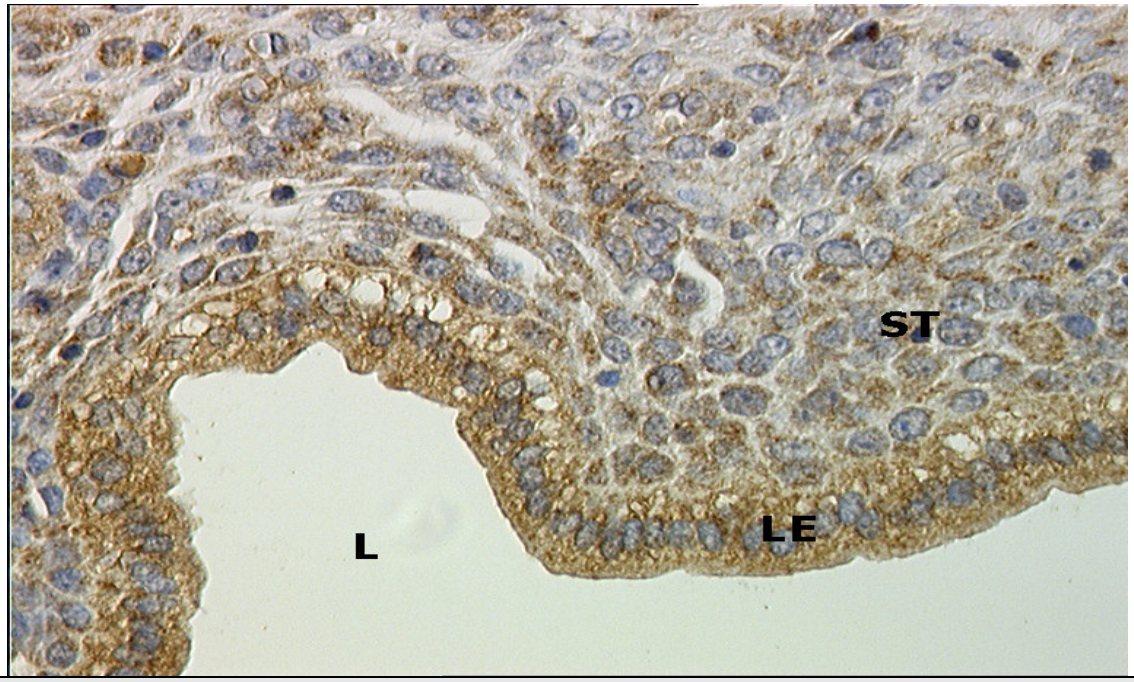


Figure 50. Representative section of 4.5 day control animal. Note the strong TGF β_2 immunolocalization in the luminal epithelial cells (LE). ST- stroma, L-lumen. Counterstained with haematoxylin. x400

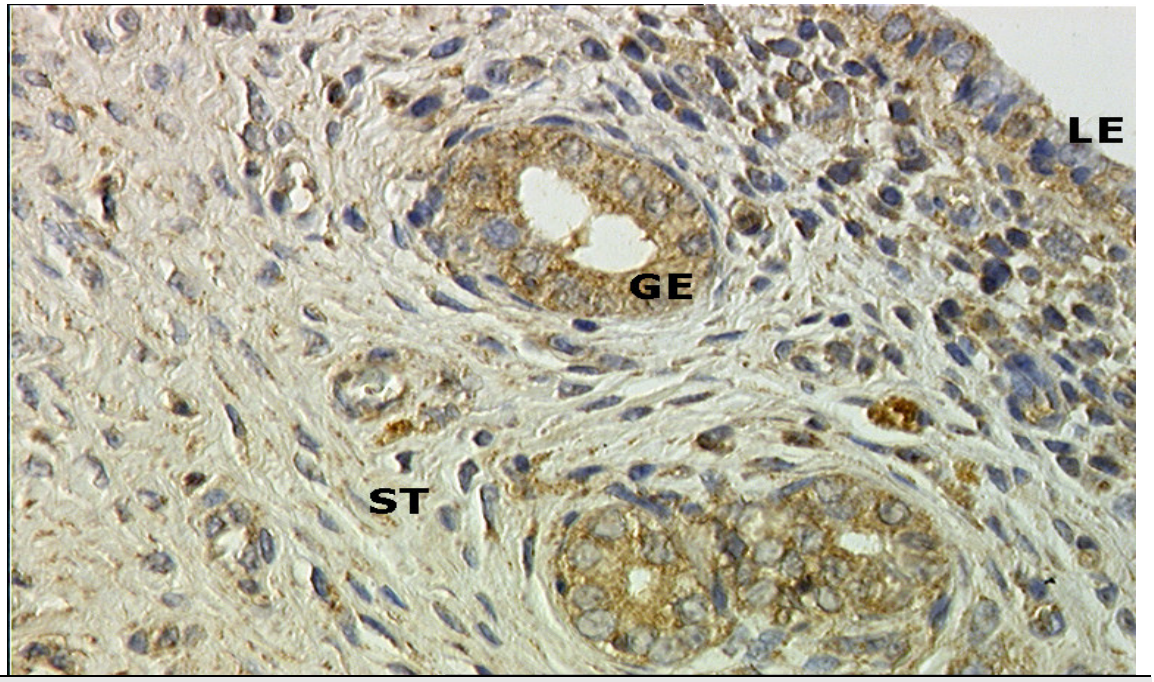


Figure 51. Representative section of 4.5 day control animal. Note the intense cytoplasmic TGF β_2 immunolocalization in the glandular epithelial cells (GE) and luminal epithelial cells (LE). ST-stroma. Counterstained with haematoxylin. x400

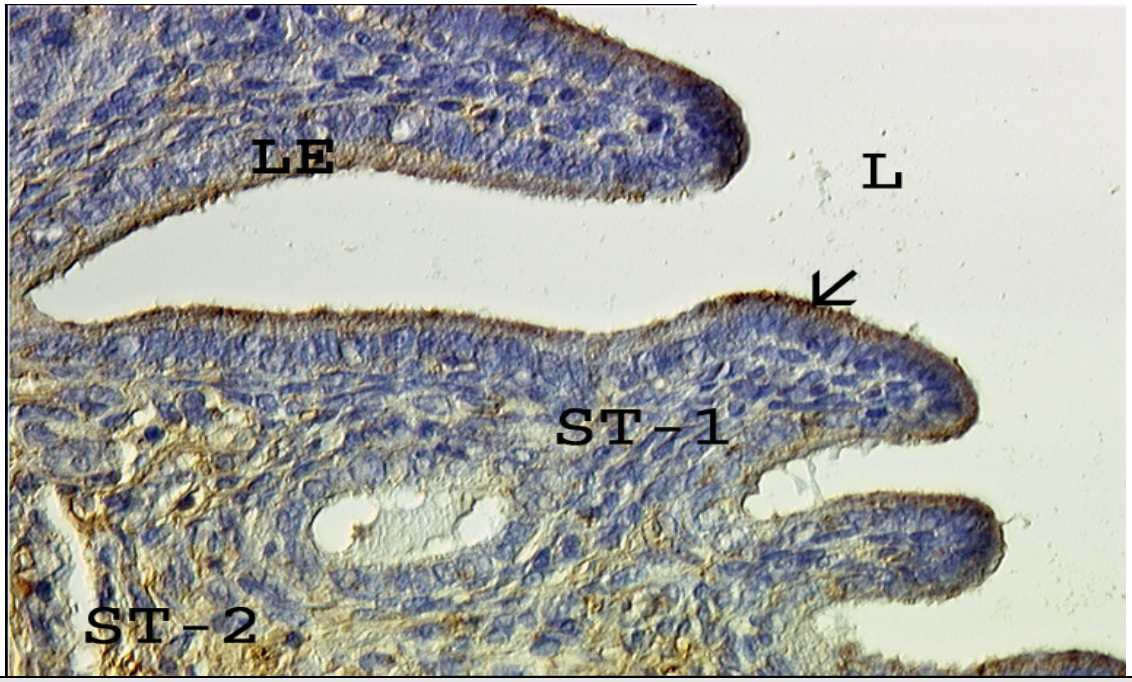


Figure 52. Representative section of 4.5 day hyperstimulated animal. Note the weak TGF β_2 immunolocalization in the luminal epithelial cells (LE), which was mostly concentrated at the apical pole of these cells (arrow). Also note the regionalization into two zones (ST-1 and ST-2) was present in the stroma. L-lumen. Counterstained with haematoxylin. x400

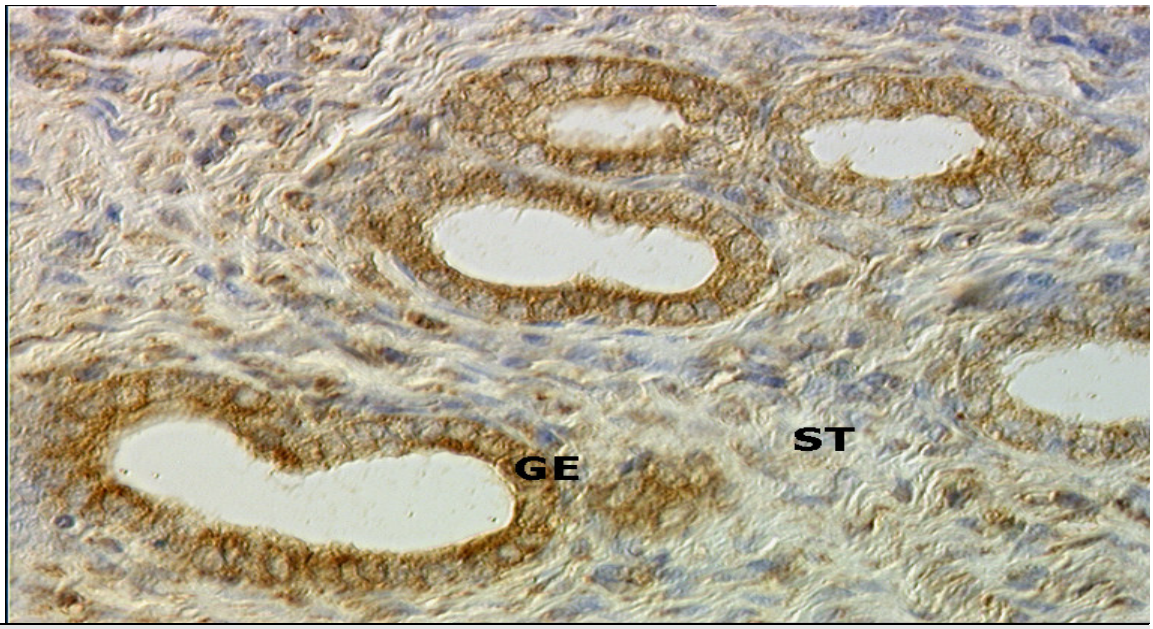


Figure 53. Representative section of 4.5 day hyperstimulated animal. Note the strong TGF β_2 immunolocalization in the glandular epithelial cells (GE) which appear more dilated than those in the control group at the same stage of pregnancy. ST-stroma. Counterstained with haematoxylin. x400

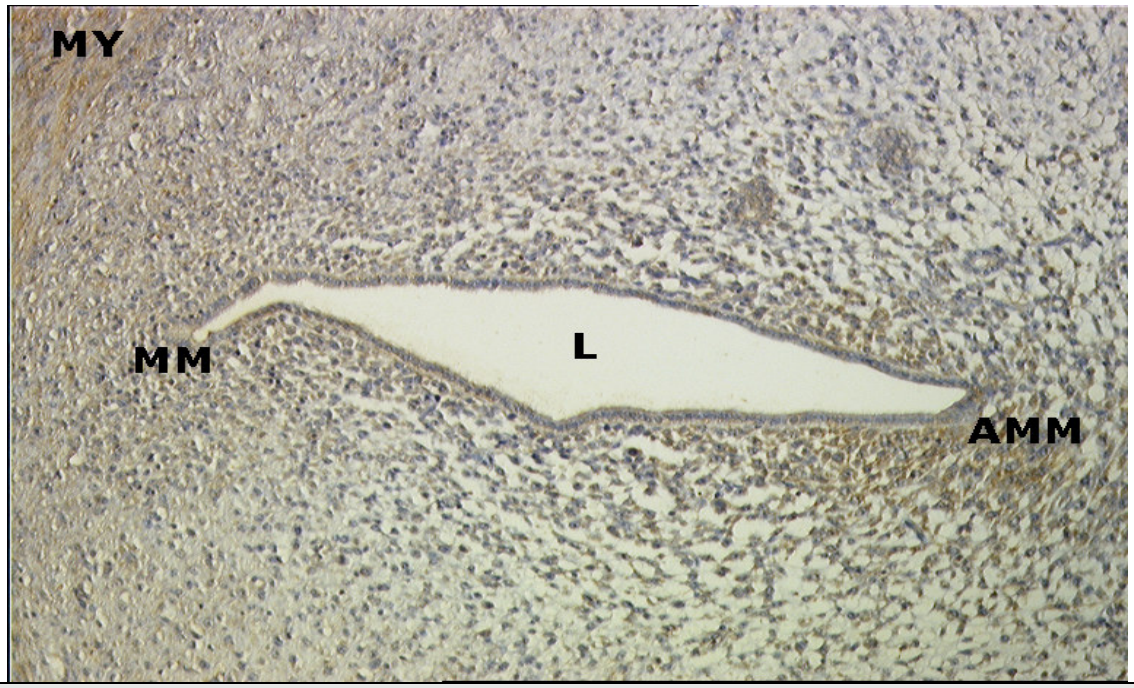


Figure 54. Representative section of 5.5 day control animal. Note the difference in TGF β_2 immunolocalization between the anti-mesometrial (AMM) and the mesometrial (MM) side of the lumen. L-lumen, MY-myometrium. Counterstained with haematoxylin. x100.

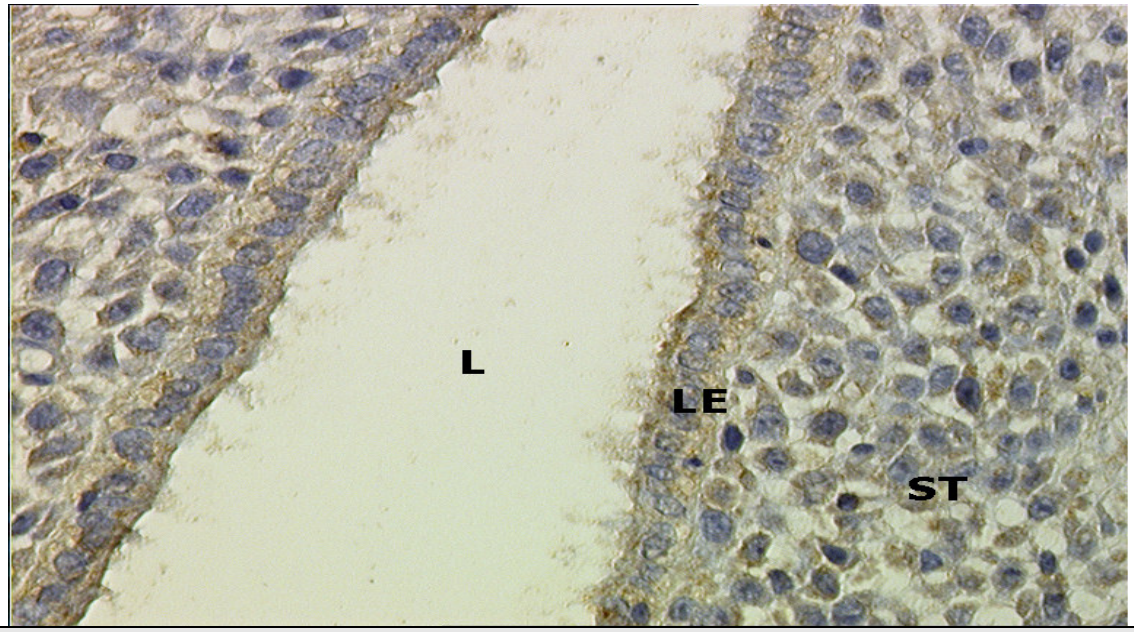


Figure 55. Representative section of 5.5 day control animal. Note cytoplasmic immunolocalization of TGF β_2 in the luminal epithelial cells (LE) and stroma (ST) which appeared weaker when compared to the same group of cells at 4.5 day of pregnancy (see Fig. 41). L-lumen. Counterstained with haematoxylin. x400

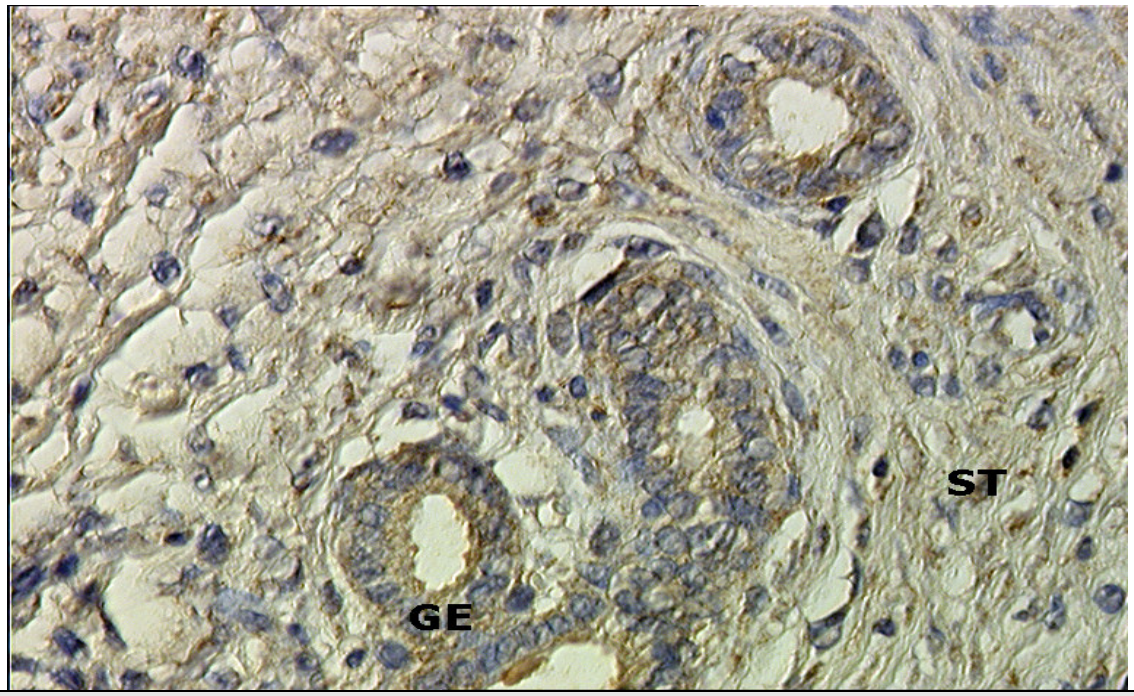


Figure 56. Representative section of 5.5 day control animal. Note distinct TGF β_2 immunolocalization in the glandular epithelial cells (GE). ST-stroma. Counterstained with haematoxylin. x400.

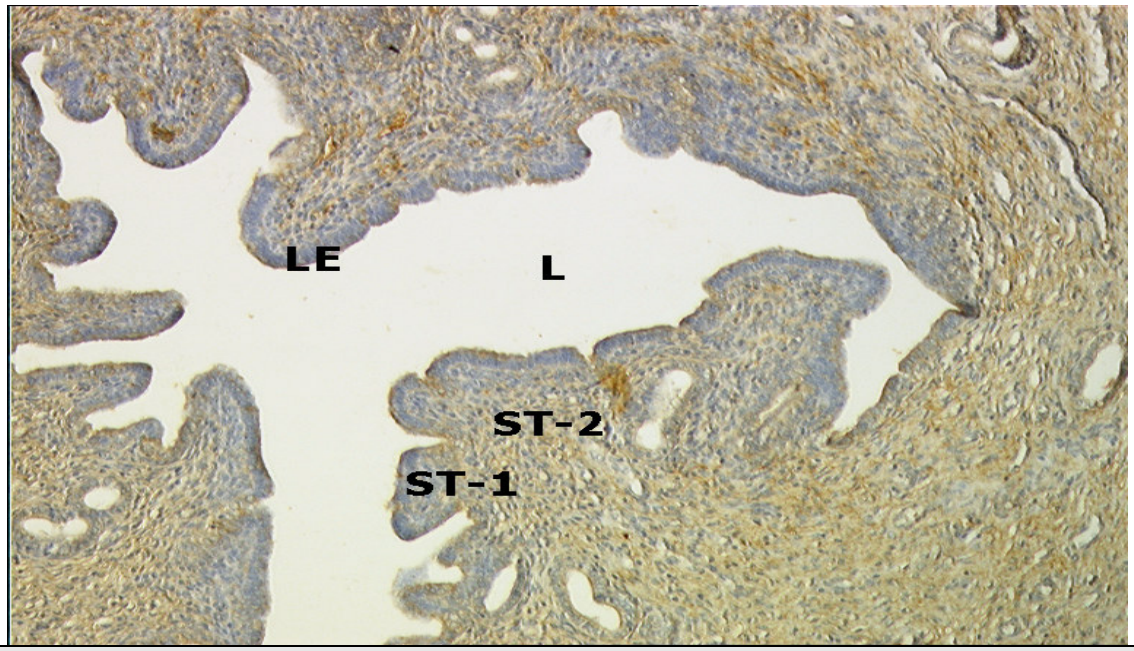


Figure 57. Representative section of a 5.5 day hyperstimulated animal. Note weak TGF β_2 immunolocalization in the luminal epithelium (LE) and first zone of subluminal stroma (ST-1)) and the much stronger localization in the deeper, second zone (ST-2). L-lumen. Counterstained with haematoxylin. x100.

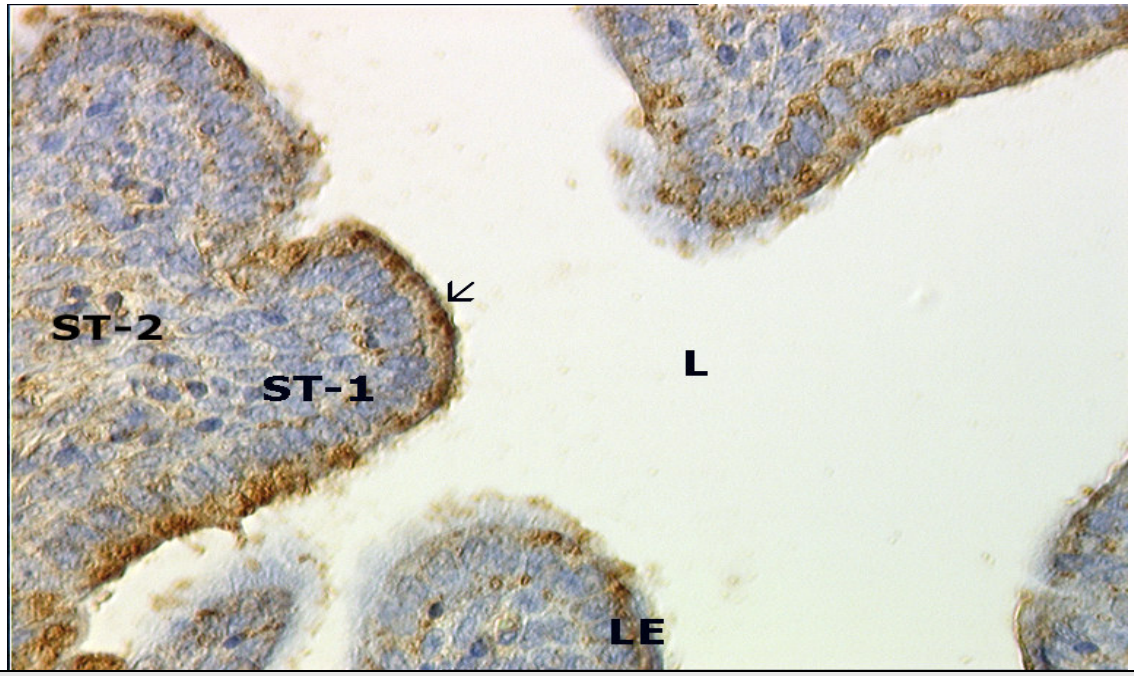


Figure 58. Representative section of 5.5 day hyperstimulated animal. Note the strong apical TGF β_2 immunolocalization (arrow) in the luminal epithelial cells (LE) and scanty TGF β_2 expression in the subluminal stromal zone (ST -1). ST-2- deeper, stromal zone. L-lumen. Counterstained with haematoxylin. x400.

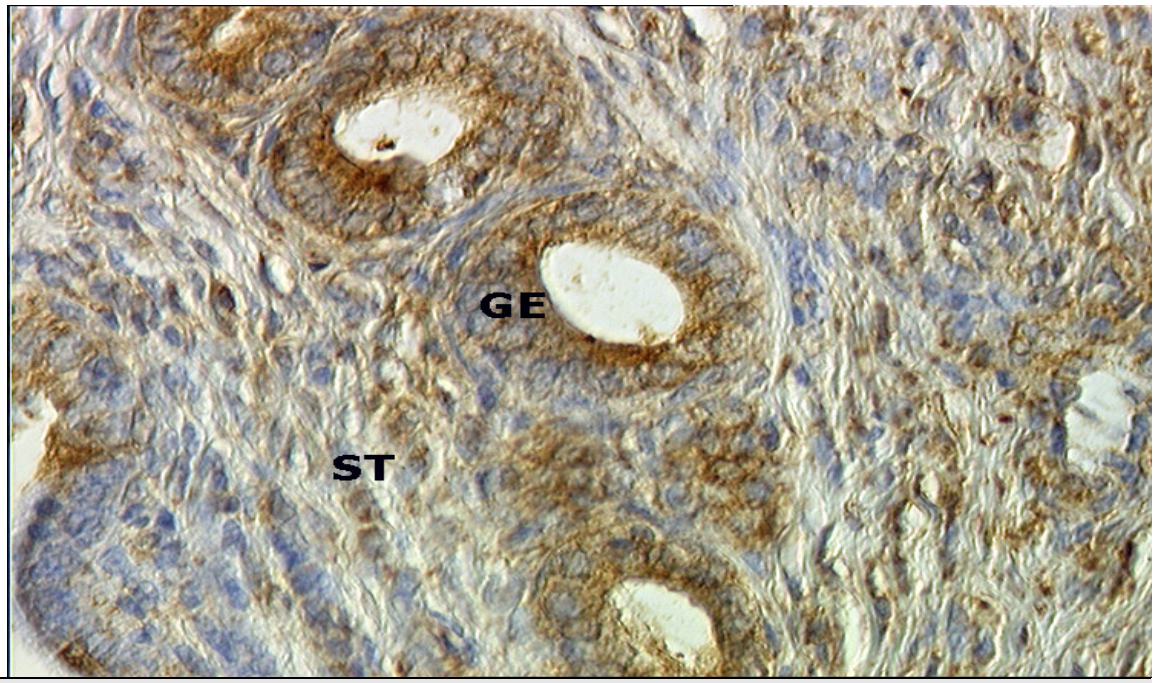


Figure 59. Representative section of a 5.5 day hyperstimulated animal. Note strong immunolocalization of TGF β_2 in the glandular epithelium (GE). ST-stroma. Counterstained with haematoxylin. x400.

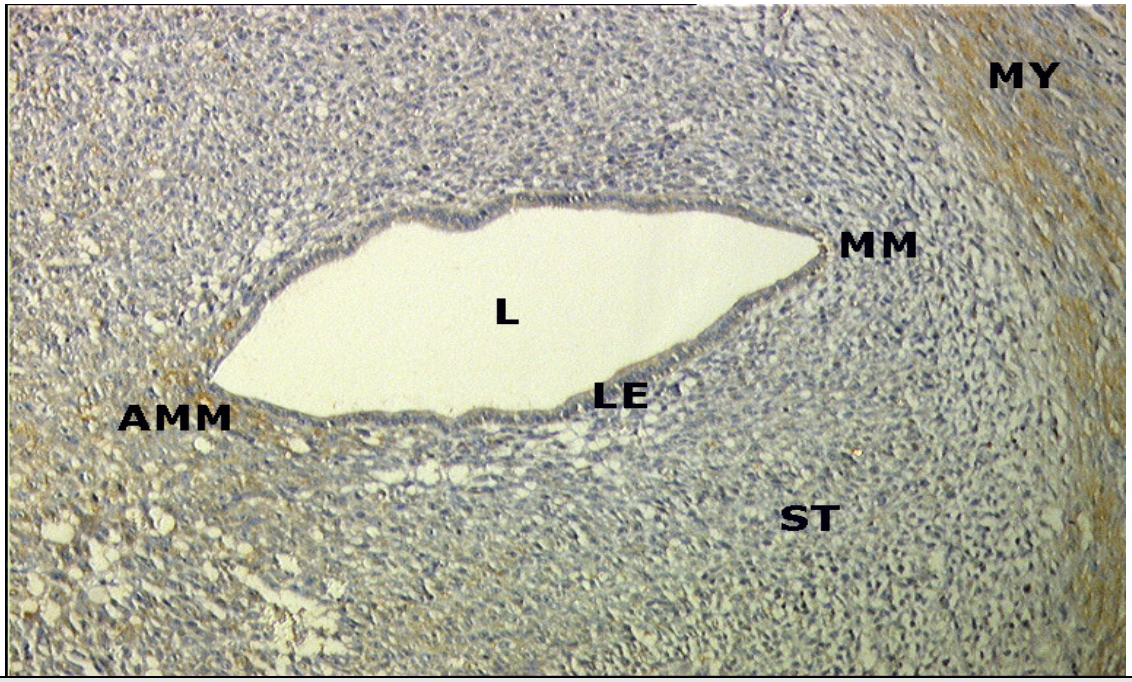


Figure 60. Representative section of 6.5 day control animal. Note weak TGF β_2 immunolocalization in the luminal epithelium (LE) and stroma (ST) and strong TGF β_2 immunolocalization in the myometrium (MY). AMM-anti-mesometrial side and MM-mesometrial side of the lumen. L-lumen. Counterstained with haematoxylin. x100

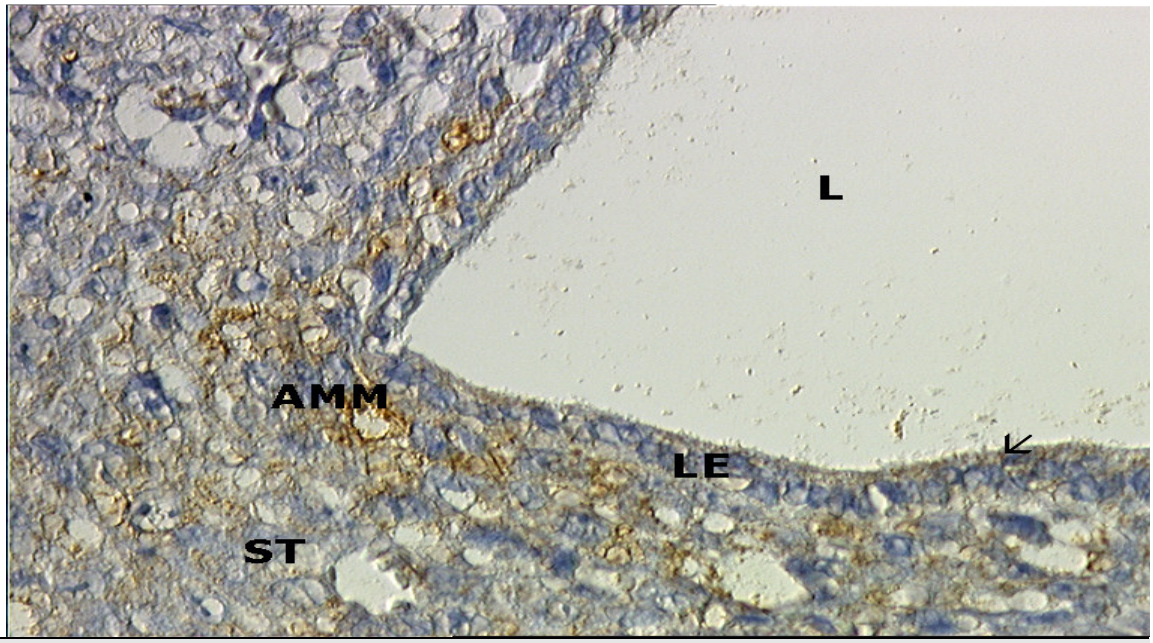


Figure 61. Representative section of 6.5 day control animal. Note TGF β_2 immunolocalization at the anti-mesometrial side (AMM) of the lumen and scanty apical expression (arrow) in the flattened luminal epithelial cells (LE). L-lumen, ST-stroma. Counterstained with haematoxylin. x400



Figure 62. Representative section of a 6.5 day control animal. Note an implantation chamber with an implanted embryo (EM) adhering to both sides of the implantation chamber and encompassed entirely by the flattened luminal epithelial cells (LE).Also note strong TGF β_2 immunolocalization of in the subluminal stroma (ST) surrounding the implanting conceptus. Counterstained with haematoxylin. x400



Figure 63. Representative section of a 6.5 day control animal. Note the weak the TGF β_2 immunolocalization in both the glandular epithelium (GE) and stroma (ST). Counterstained with haematoxylin. x400

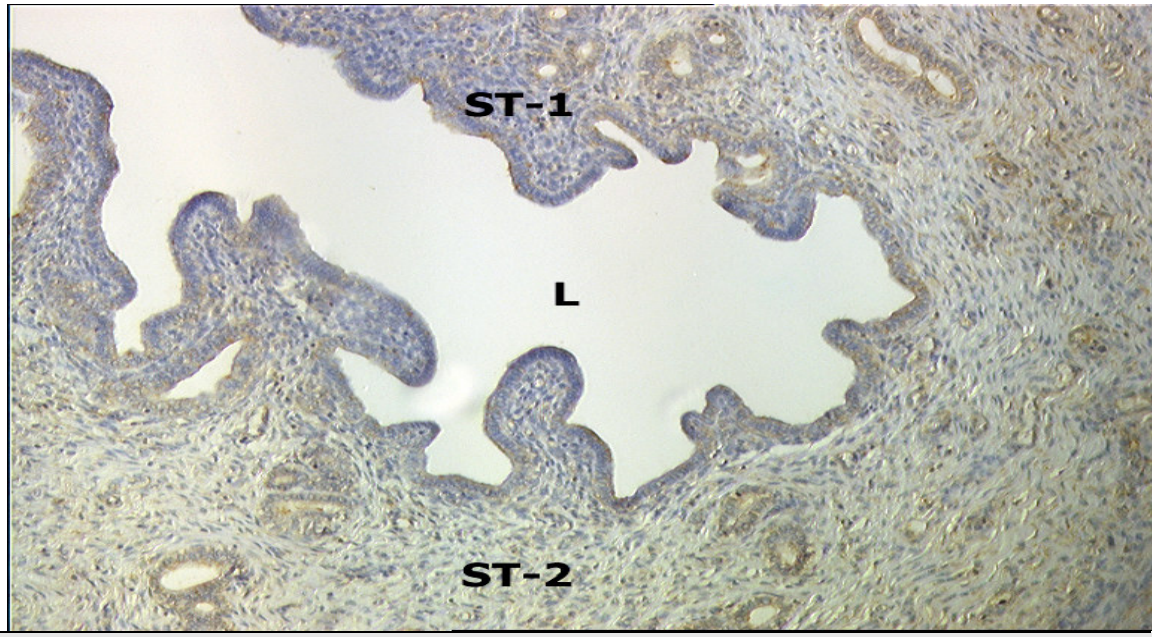


Figure 64. Representative section of 6.5 day hyperstimulated animal. Note TGF β_2 immunolocalization in the stroma is divided into two zones ST-1 –first zone with no TGF β_2 immunolocalization and ST-2 second, deeper zone with scanty TGF β_2 expression. L-lumen. Counterstained with haematoxylin. x100

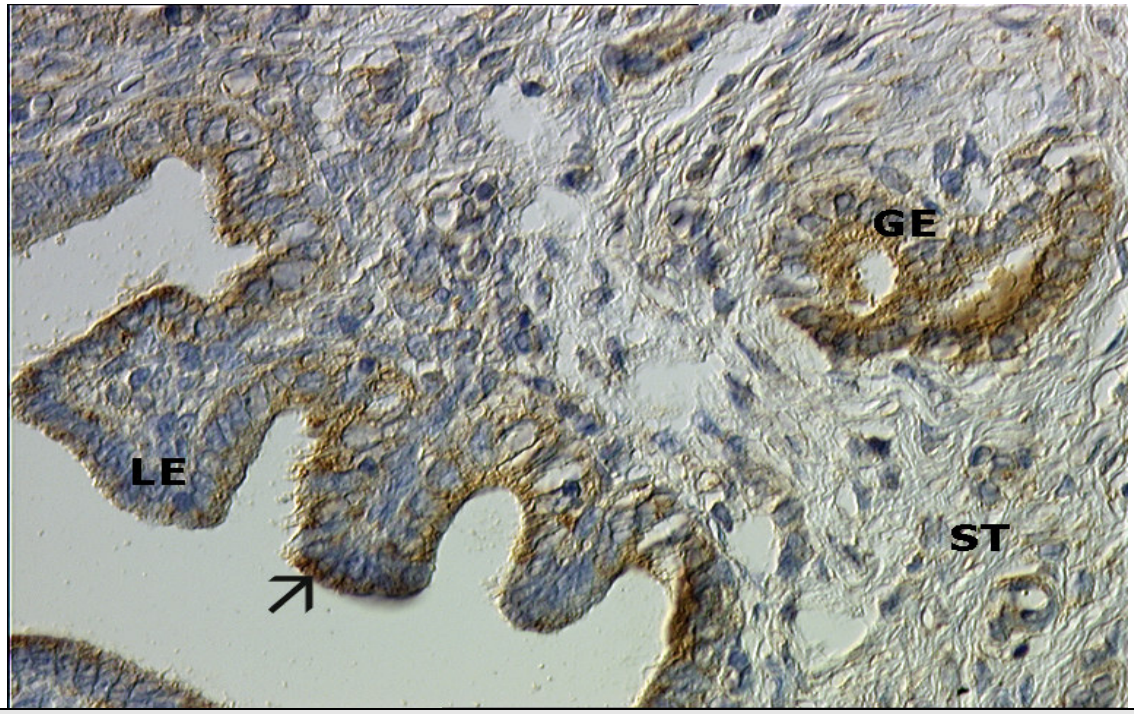


Figure 65. Representative section of 6.5 day hyperstimulated animal. Note apical TGF β_2 concentration (arrow) in the luminal epithelial cells (LE) and strong cytoplasmic TGF β_2 immunolocalization in the glandular epithelium (GE). ST-stroma. Counterstained with haematoxylin. ST-stroma. x400

4. DISCUSSION

Administration of exogenous gonadotropins in phase with the oestrus cycle of the rat was achieved by successful ovarian hyperstimulation. The latter was indicated by the enlarged ovaries with numerous large follicles and noticeably dilated uterine horns. These changes were not observed in the control groups at all of the examined stages of pregnancy.

Round ovaries and non-dilated uterine horns which contained implantation sites and which were evident in all the control animals, contrasted strongly with the majority of the hyperstimulated animals in which implantation sites were not observed. However, two animals in the hyperstimulated group at 5.5 days of pregnancy showed one visible implantation site per animal. There were no other blue bands (implantation sites) present in the remainder of the uterine horns in both of these hyperstimulated animals.

The incidence of the implantation sites and implanting embryos within the control uterine horns in the present study, support the hypothesis that the blastocysts within the uterine lumen are a stimulus which results in an increase in the permeability of endometrial capillaries (Psychoyos, 1973). If a macromolecular dye such as pontamine blue is injected intravenously around the peri-implantation period, it will leave the circulation only in areas where permeability of capillaries is greatly increased (Psychoyos, 1973). This method has been widely used to localize implantation sites, which appear as distinctive blue bands at

the sites of embryo implantation 15 minutes after the pontamine blue injection (Finn and Porter, 1975). Therefore, the pontamine blue reaction was not completely inhibited in two hyperstimulated animals at 5.5 days of pregnancy, in which one implantation site was present in each animal. In these two hyperstimulated animals, unattached embryos were found in the uterine lumina, but implanting embryos were never located. The possible reason for this exception could be the alteration in the hormonal levels known to occur in hyperstimulated animals (Kramer *et al*., 1990), their detrimental effect on the morphology of the endometrium as well as on the passage of the ova down the uterine tubes (Kramer *et al*., 1993) and an individual response of these animals to the abovementioned changes, that had altered the endocrine environment and uterine morphology due to the supraphysiological hormonal environment.

4.1. Dating of pregnancy

The presences of either a vaginal plug on the morning following mating or spermatazoa in a vaginal smear are used as markers of successful impregnation. Although the markers of pregnancy are indisputable, the dating of pregnancy varies within published studies. In the current study, the presence of the mentioned markers on the morning following mating is referred to as day 0.5 of pregnancy (Stein and Kramer, 1989; Kramer *et al*., 1993). As the animals mate around 8 p.m. and are killed at approximately 8 a.m. the following morning, the dating of pregnancy is held to be approximately 12 hours or 0.5 day of pregnancy. This contrasts with other studies which refer to this

occurrence as day 1 of pregnancy (Enders and Schlafke, 1967; Psychoyos, 1973; Lundkvist and Ljungkvist, 1977). This discrepancy should be taken into consideration during the ensuing chapters, in which comparisons are made between the results of this study and published material.

4.2. The effects of hyperstimulation on progesterone and oestrogen levels

The preparation of the endometrium for receipt of the embryo is dependent on the ovarian hormones, oestrogen and progesterone. In this study, the secretion of oestrogen and progesterone from the ovaries was affected by the administration of exogenous gonadotropins, which had been superimposed upon the normal hormonal levels of the intact animal. As a consequence, the release of ovarian steroids in the hyperstimulated animals was altered, causing a change in the endogenous hormonal environment, which severely affected the number of the implanting embryos and thus the pregnancy outcome.

The **progesterone** plasma concentrations showed an increase from 4.5 to 5.5 days of pregnancy in the control group, followed by a sudden drop at 6.5 days. The hyperstimulated group showed a gradual increase from 4.5 to 6.5 days of pregnancy. When comparisons were made between the control and the hyperstimulated groups at all three stages of pregnancy, the values of progesterone were higher in the

latter group, but statistically significantly higher only at 6.5 days of pregnancy.

Many controlled ovarian hyperstimulation cycles are associated with the early expression of endometrial histological features, the change in the expression of oestrogen and progesterone receptors and the shift in the timing of pinopode appearance (Develioglu *et al.*, 1999; Lessey, 2001a; Lessey, 2001b; Murphy, 2004). The above mentioned signs of hyperstimulation are said to be caused by the high levels of progesterone following hCG administration (Develioglu *et al.*, 1999). Additionally, the appearance of pinopodes observed within the limited time of 24-48h in mammals, is an indicator of the window of implantation (Nikkas *et al.*, 1995; Murphy, 2004) and depends on the ovarian hormones, especially progesterone (Singh *et al*., 1996). Moreover, premature elevations in plasma progesterone levels, after routine use of hCG in IVF and ET programs, induces an unexpected and greatly increased secretion of progesterone (Taieb *et al*., 1997). This sudden shift in progesterone level accelerates the secretory changes of the endometrium to the point of phasing out endometrial receptivity and embryo development (Taieb *et al*., 1997). As progesterone levels increased in the hyperstimulated animals during the peri-implantation period, it is postulated that this event could have shifted the window of implantation earlier in the treated animals, thus causing dyssynchrony between the maturity of the embryo and receptivity of the endometrium.

The **oestradiol** plasma concentrations showed an increase from 4.5 to 6.5 days of pregnancy in the control group. Following hyperstimulation, the oestradiol plasma levels increased from 4.5 to

5.5 days of pregnancy followed by a sudden drop from 5.5 to 6.5 days of pregnancy. When comparisons were made between the control and the hyperstimulated group, higher oestradiol concentrations were evident in the treated group but a statistically significant difference was only found at 6.5 days of pregnancy.

The findings of the present study on high oestrogen levels in the hyperstimulated animals are consistent with Ma *et al*. (2003), who showed that the uterus becomes refractive after raised oestrogen levels due to ovarian hyperstimulation procedures. On the other hand, in their work, Simón *et al.* (1998) compared the effects of a standard ovarian hyperstimulation protocol to the step-down regimen in which the patient received different gonadotropin doses prior to IVF treatment. The outcome was that the implantation and pregnancy rates were significantly improved in the patients who underwent the step-down procedure. Simón *et al*. (1998) indicated that the implantation rates could be improved when oestrogen levels are decreased during the pre-implantation period. In addition, Kramer *et al.* (1990) investigated the effects of exogenous gonadotropins on the rat endometrial morphology. They found that these hormones produced a number of significant changes, such as an increase in luminal and glandular epithelial height, an increase in the number and length of microvilli, a decrease in the glycocalyx, and a decrease in mitotic activity in the surface epithelial cells as well as the stromal cells. Similarly, Basir *et al.* (2001) morphometrically examined the peri-implantation endometrium in patients undergoing IVF treatment. They found that increased levels of oestradiol significantly alter endometrial morphological features and transform the endometrium, causing decreased implantation and pregnancy rates. The data of the

present study in which higher oestrogen levels have been shown in hyperstimulated animals are consistent with the work done by Simón *et al*. (1998) and Basir *et al*. (2001). It is thus postulated that the absence of implantation sites in the majority of the treated animals could be due to the change in the oestrogen levels following ovarian hyperstimulation and its effect on vascular permeability and decidualization of the subepithelial stromal cells.

Ma *et al.* (2003) offered another possible explanation for inhibition of implantation in the hyperstimulated animals. In their work using a progesterone-treated delayed-implantation model in mice, Ma *et al*. (2003) provided evidence that levels of oestrogen in a very narrow range, such as 3 ng, establish the duration of the window of uterine receptivity for implantation. In animals with high oestrogen levels e.g. 10-25 ng, the uterus was rapidly transformed from a receptive into a refractive state, suggesting an intense sensitivity of the uterus to oestrogen levels. Our findings in which higher oestradiol concentrations occurred in hyperstimulated animals at all stages of pregnancy when compared to those in the control groups support the work of Ma *et al*. (2003). The increased levels of oestrogen after ovarian hyperstimulation in the present study appear to have narrowed the window of implantation in the treated animals and led the uterus into a refractive state.

Following hyperstimulation, oestradiol levels were increased prior to implantation (day 5.5), followed by a rapid decrease at 6.5 days of pregnancy. The progesterone plasma concentrations were also affected by the treatment and showed a gradual increase from 4.5 day onwards. Consequently the **$P:E_2$ ratio** was lower in the

hyperstimulated group than in the control animals before implantation, and only statistically significantly higher at 6.5 days of pregnancy. According to Gidley-Baird *et al*. (1986) alterations in oestrogen and progesterone levels affect the reproductive outcome, but the $P:E_2$ ratio better predicts the implantation outcome than the absolute levels of either oestrogen or progesterone alone (Gidley-Baird *et al., 1986;* Ma *et al.,* 2003*).* The present study is consistent with the work previously done by Gidley-Baird *et al.* (1986) and Kramer *et al.* (1990) in which they showed that a change in the $P:E_2$ ratio had detrimental effects on the number of implanting embryos in the mouse and rat respectively. In the present investigation the comparatively lower $P:E_2$ ratio in the experimental groups at 4.5 and 5.5 days of pregnancy and the absence of a statistically significant difference in the $P:E_2$ ratio at 4.5 and 5.5 days of pregnancy in the hyperstimulated group support observations made by Gidley-Baird *et al.* (1986) and Kramer *et al.* (1990).

Another approach for explaining the influence of the $P:E_2$ ratio on the absence of implantation sites in hyperstimulated animals could arise from recent work by Ozcakir *et al.* (2004). They observed the outcome of premature luteinization which was defined as a progesterone-oestradiol ratio > 1 on the day of hCG administration. Group A consisted of patients whose $P:E_2$ ratio was 1 while Group B comprised patients with premature luteinization whose $P:E_2$ ratio was >1. The primary outcome measure in this study by Ozcakir *et al*. (2004) was oocyte quality, fertilization rate and clinical pregnancy rate, which were all adversely affected by premature luteinization defined as $P:E_2$ ratio >1. Indeed, the pregnancy outcome of the present study could have been affected by the dominance of progesterone ($P:E_2>1$) which

was observed at 5.5 and 6.5 days of pregnancy in the hyperstimulated animal.

Although the aim of this investigation was to observe the effects of hyperstimulation on the endometrium, it is noteworthy that hyperstimulation has an influence on the embryo as well as other parts of the female reproductive tract. According to Fossum *et al*. (1988) and also Ertzeid and Storeng (2001) ovarian hyperstimulation has effects on oocyte and/or embryo quality, oviductal and/or uterine environment and synchrony, which normally exists between the embryo and the endometrium at the time of implantation. Taking advantage of the fact that the uterus in mice has two horns, Ertzeid and Storeng (2001) established an embryo transfer model in which they transferred the embryos from either superovulated or non-stimulated females into separate uterine horns within the same superovulated or non-stimulated pseudopregnant recipients. The negative effect of ovarian hyperstimulation on oocyte/embryo development was observed, as a transfer of embryos from superovulated donors resulted in a significantly lower implantation rate in control recipients compared with that of embryos from control donors.

Ertzeid and Storeng (2001) also observed the negative effect of ovarian hyperstimulation on uterine receptivity. They suggested that the exogenous gonadotropin hormones, by altering the concentrations of circulating oestrogen and progesterone, also affected the expression of locally produced endometrial cytokines and hence they affect endometrial receptivity. Similarly, Klentzeris (1997) and Beier and Beier-Hellwig (1998) showed that progesterone and oestrogen mediate

their actions by locally produced cytokines which act in either an autocrine or paracrine manner. Beier and Beier-Hellwig (1998) explained that the endometrium requires cytokines to act locally or to mediate cell-to-cell communications thus regulating oestrogen and progesterone control of endometrial development and consequently embryo implantation.

Furthermore, Van der Auwera *et al.* (1999) demonstrated that superovulation and subsequent high concentrations of steroids have detrimental effects on pregnancy outcome by causing hormonal stimulation of the oviductal milieu and in that way producing harmful changes to pronucleate ova as well as on the developmental capacity of the pre-implantation embryo.

4.3. The effects of hyperstimulation on the histology of the rat uterine tissue

Factors influencing endometrial receptivity in ovarian stimulation for IVF are still poorly understood (Bourgain, 2004). The general trend that emerges from available studies shows that abnormalities in the luteal phase have been detected in almost all stimulation protocols used in *in vitro* fertilization programs, on both the hormonal and endometrial levels (Tavaniotou *et al*., 2001; Bourgain and Devroey, 2003). Furthermore, it is a well known fact that IVF treatments are usually achieved through ovarian stimulation and are thus associated with supraphysiological serum concentrations of oestradiol and progesterone. These conditions which have been forced upon the

intact animal in order to produce superovulation have their influence on the histology of the uterine tissue and as a consequence, on endometrial receptivity and embryo implantation.

In the present study, the non-dilated uterine lumina and relatively smooth luminal epithelium in most of the control animals contrasted strongly with the grossly dilated lumina and exceptionally folded epithelium of the hyperstimulated animals. These changes are in agreement with findings made by Stein and Kramer (1989), Kramer *et al*. (1993) and Valbuena *et al*. (1999) whose results indicate both stimulation (dilatation of the uterine horns, accumulation of uterine fluid, changes in the luminal epithelial height and microvillus border) and damage (presence of necrotic and damaged cells) of the endometrium by the gonadotropic hormones. Additional differences were observed in the cytology of the epithelial cells in the current study. In the luminal and glandular epithelium, the position of the nuclei and vacuoles varied between the different groups. Also, the glands of the hyperstimulated animals had dilated lumina which were often filled with secretion when compared to the glands of the control groups, in which these glandular aspects were absent.

Furthermore, in control animals, a gradual modification of luminal epithelial cell height starting from the mesometrial side and proceeding to the antimesometrial side was observed in control animals in the sections that were taken close to the implantation site. The implantation chamber containing the conceptus consisted of flattened luminal epithelial cells which were in a close association with the trophoblast cells (Murphy, 2004), and decidual cells, whose developing stages were more pronounced at the anti-mesometrial side of the

uterine lumen (Psychoyos, 1967; Welsh and Enders, 1983). This preparation of both the luminal epithelium and decidualizing stroma will consequently result in a displacement of the epithelium, which will further facilitate contact between the trophoblast and the maternal blood (Schlafke *et al*., 1985). None of the above mentioned findings have been observed in the hyperstimulated animals in the present study, even though embryos were found in the lumen of the uteri of two hyperstimulated animals at 5.5 days of pregnancy. It is postulated that hyperstimulation had a negative effect in the preparation of the luminal epithelium as well as the trophoblast cells, thus preventing the events necessary for successful implantation.

Hyperstimulation with gonadotropins caused increased uterine epithelial height (Nilsson, 1967) and increased mitotic activity of the luminal, glandular epithelial cells and stromal cells in the hyperstimulated animals (Finn and Martin, 1970). In gonadotropin agonist cycles, mid-luteal biopsies showed increased glandulo-stromal dyssynchrony and postponement in endometrial development, strong positivity of endometrial glands for progesterone receptors and advanced appearance of pinopodes of the surface epithelium (Tavaniotou *et al*. 2001). Additionally, an advanced maturation of the endometrium in the peri- and post-ovulatory period, followed by "normal" features of the endometrium in the early luteal phase, resulted in frequent glandulo-stromal dyssynchrony in the mid- and late luteal phase (Bourgain and Devroey, 2003). More specifically, advanced endometrial maturation was said to be present on the day of oocyte retrieval in IVF cycles, using either GnRH agonist or antagonist (Papanikolaou *et al.,* 2005). In contrast, in natural cycles such an advancement of the endometrium is not present (Bourgain *et al.,*

2002). Macklon *et al.* (2006) explained that in biopsies taken 7 days after ovulation from patients undergoing hyperstimulation for the IVF procedure, there is endometrial delay and glandular-stromal dissociation. It seems that stimulation with a GnRH agonist and gonadotropins induces early endometrial development with consequent glandular maturation arrest in the mid-luteal phase (Basir *et al.*, 2001). In the same study, Basir *et al*. (2001) further explained that the glands with varying luminal filling represent glands at different stages of secretory development and that each patient responds differently to the ovarian stimulation regimen. In high responders whose glandular secretion was disrupted, a prolonged retention and retarded emptying of the secretory material was present (Basir *et al*., 2001).

Further differences between control and hyperstimulated animals were found in the stroma in the present study. During the observed peri-implantation period, the stromal cells of the control animals changed their shape from fibroblastic at 4.5 days of pregnancy, into round cells with scanty cytoplasm towards day 6.5 of pregnancy. By contrast, the majority of the stromal cells in the hyperstimulated animals remained flat and fibroblastic and the stromal tissue remained compact without any signs of decidualization throughout the peri-implantation period.

Preparation of the endometrium as is reflected in its morphology is one of the crucial prerequisites for successful implantation (Schlafke and Enders, 1975; Psychoyos and Martel, 1985). In their work, Stein and Kramer (1989) and Kramer *et al*. (1990), showed that exogenous gonadotropins administered in phase with the oestrus cycle of the rat, cause numerous endometrial changes which affect the attachment and

implantation of the embryo. Hyperstimulation i.e. high levels of oestrogen, have harmful effects on the endometrial morphology such as increased stromal vascularity and luminal dilatation (Spaziani, 1963), a decrease in the vascular permeability of the endometrium (Kramer, 1997) and an absence of decidualization of the subepithelial stromal cells (Stein and Kramer, 1989). Although detailed observations of stromal vascularity and vascular permeability were not included in the present study, similarities are present between results found in the current study and the findings of Kramer (1997). Firstly, the presence of the pontamine blue reaction in 4.5 (only one control animal), 5.5 and 6.5 days control animals demonstrated an increase in the permeability of uterine vessels, which is according to Psychoyos (1973) an indispensable condition for deciduoma formation. Psychoyos (1984) also adds that any procedure that inhibits this increase in permeability also inhibits decidualization. Comparable observations were not seen in the hyperstimulated animals which indicated that the vascular permeability was suppressed with increased oestradiol and progesterone levels. Secondly, while the decidual reaction was observed in all of the control animals, a non-decidualized stroma was present in all the hyperstimulated animals. Thus, the absence of the pontamine blue reaction as well as the decidual reaction in the majority of the hyperstimulated animals provided additional evidence for yet another detrimental effect of hyperstimulation in the current study.

In addition, another characteristic was observed in the control animals in this study, which demonstrated further development of the endometrium in the anticipation of embryo. “Spaces” which were evident between decidual cells of the stroma in control groups at 5.5

and 6.5 days of pregnancy are said to represent oedema (Psychoyos, 1973). Oedema was not observed in the stroma of the hyperstimulated animals in this study. According to Psychoyos (1973) oedema is the most pronounced transitional stromal change during the period of uterine receptivity. It occurs in the mid-luteal period independent of the presence of an implanting ovum (Psychoyos, 1973; Psychoyos, 1984). Psychoyos showed that oedema occurs in rats on day 5 (day 4.5 this study) of pregnancy and pseudopregnancy, and at about noon of the same day, develops into a widespread oedema dispersing the decidual stromal cells which results in the gradual obliteration of the uterine cavity. Shelesnyak (1957) followed the further events involved in the decidual cell reaction and found that histamine was involved in deciduogenesis through the activation of the relevant enzyme located in the endothelium of uterine capillaries. Moreover, Dey *et al*. (1979) showed that endometrial cells contain histamine receptors. When histamine binds to the endothelial receptors it affects vasodilatation and influences vascular permeability. Histamine-induced vascular leakage is potentiated by prostaglandins which are involved in the initiation and differentiation of decidual cells (Kennedy, 1985). As a result of the decidual reaction as well as increased vascular permeability, the uterine lumen gradually becomes occluded, facilitating the primary contact between the blastocyst and the luminal epithelium (Psychoyos, 1973).

In the two 5.5 day hyperstimulated animals in which the pontamine blue reaction was not completely inhibited, an unattached embryo was found in the lumen of each of the animals. However, other characteristics important for implantation, such as the decidual reaction, oedema and obliteration of the lumen, as well as close

contact of the opposite sides of the luminal epithelium were not observed in these animals. The above mentioned findings support the work by Kramer (1997), who showed that absence of decidualization in the rats that underwent ovarian hyperstimulation is due to a decrease in vascular permeability, which is an indispensable condition for deciduomata formation.

In their study Ku *et al*. (2004) obtained endometrial stromal cells from patients undergoing hysterectomy procedures and cultured specimens in serum-containing media. They monitored the effects of different doses of human menopausal gonadotropin (hMG), FSH or hCG on cumulative radioactive thymidine incorporation into these endometrial stromal cells. The study demonstrated that hMG and FSH induced inhibition of radioactive thymidine incorporation into the endometrial stromal cells at all the concentrations used, while inhibition with hCG was dose dependant. Ku *et al.* (2004) postulated that gonadotropins inhibit proliferation of the human endometrial stromal cells *in vitro*, which has implications for endometrial receptivity and embryo implantation.

4.4. The effect of hyperstimulation on the expression of TGF β_1 and TGF β_2

The findings of the present study showed marked differences in the expression of both TGF β_1 and TGF β_2 between the control and hyperstimulated animals.

Immunohistochemical comparisons of expression of TGF β_1 between control and hyperstimulated animals revealed discrepancies in the stromal compartment. This segment of the uterine endometrium expressed strong and even TGF β_1 immunolocalization in all three stages of pregnancy in the control groups, while hyperstimulated animals at all stages of pregnancy showed regionalization in the expression of TGF β_1. Hyperstimulation appears to have caused two zones in the expression of TGF β_1. The subluminal stromal zone had a weaker expression of TGF β_1 in all the examined stages of pregnancy in the hyperstimulated animals, when compared to the deeper stromal zone, which strongly expressed this growth factor. The expression of TGF β_1 in the luminal and glandular epithelium as well as myometrium did not differ between the control and hyperstimulated animals in all the examined stages of pregnancy.

Compared to the TGF β_1 findings, immunohistochemical comparisons of expression of TGF β_2 between the 4.5, 5.5 and 6.5 days pregnant control and hyperstimulated animals did not show as marked differences in immunolocalization. The control animals had a gradual decrease in the expression of TGF β_2 in the luminal epithelium, glandular epithelium and the stroma, while myometrium showed strong immunolocalization of this growth factor. The most prominent effect of hyperstimulation was the presence of an increased expression of TGF β_2 in the glandular epithelial cells in all examined stages of pregnancy. The immunolocalization of TGF β_2 was not affected by hyperstimulation in the remainder of endometrium, apart from the stroma which also had regionalization in the expression of TGF β_2.

The diversity in the expression of both TGF β_1 and TGF β_2 in the present study is not surprising, knowing that the transforming growth factors present multifunctional cytokines which are dynamically expressed in the endometrium. Through their actions TGF β's are associated with cell proliferation, differentiation, apoptosis, angiogenesis, tissue remodeling and immune responses (Jones *et al*., 2006). Because of the involvement that TGF β's have in the cellular and molecular processes such as those mentioned above, these polypeptides have been said to play an important role in modulating cellular events involved in menstruation, decidualization, establishment of pregnancy, trophoblast attachment, immunotolerance and embryogenesis. They thus have been recognized as possible modulators of many endometrial functions (Godkin and Dore, 1998; Jones *et al.,* 2006). Although TGF β isoforms overlap in their biological actions, their potencies may vary (Das *et al*. 1992). They also have unique roles in embryo-uterine interactions during implantation (Das *et al*. 1992). Oestrogen and progesterone have previously been shown to regulate changes in the endometrium and stroma of pregnant and non-pregnant uteri. The actions of these steroid hormones are usually not direct, but are mediated through their stimulatory or inhibitory effects upon different endometrial cells. This results in the control of autocrine/paracrine actions of TGF β's. The hormones may also modulate the production of specific isoforms of TGF β's (Tang *et al*., 1994; Bruner *et al.*, 1995; Robinson *et al.*, 1996; Ashcroft *et al.,* 1997; Wira and Rossoll, 2003; Luo *et al.*, 2004).

In the present study, TGF β_1 immunolocalization in the control animals had weak expression in the luminal and glandular epithelium with a distinct apical concentration, while stronger immunolocalization was

observed in the stroma throughout the peri-implantation period. TGF β_2 showed a gradual decline in the luminal epithelium, stroma and glandular epithelium from 4.5, 5.5 and 6.5 days of pregnancy in the control animals. This is in accordance with Godkin and Dore (1998) who found TGF β_1 present in both the epithelium and the stroma of the human endometrium during early pregnancy, while TGF β_2 was predominantly localized in the stroma. Moreover, from their *in vitro* study which used monolayer cultures of endometrial stromal or epithelial cells to determine the molecular expression associated with decidualization, Kim *et al*. (2005) proposed that TGF β_1 was the principal mediator for steroid action which leads to stromal decidualization. Polli *et al.* (1996) and Slater and Murphy (2000) indicated that TGF β_1 is secreted apically from endometrial glands in the rat and is present in the uterine fluid at the time of implantation. This corresponds with the findings of the present study in which TGF β_1 was found to be apically localized in the luminal epithelial cells throughout the peri-implantation period. Although some secretion has been found in the lumina of the glands in the control animals at 6.5 days of pregnancy, it was not possible to confirm the nature of the secretion. However, these findings further support the investigation done by Slater and Murphy (2000). Lin *et al.* (2004) observed that during pre-implantation, both Smad 2 and Smad 4 were accumulated in the luminal and glandular epithelium, while in the peri-implantation period they were present in the luminal epithelial cells, subepithelial stromal compartment and the primary decidual zone. These findings by Lin *et al*. (2004) suggested that both Smads, being the intracellular transducers of the TGF β superfamily, are involved in tissue remodeling of the pregnant rat uterus. Together with previously mentioned observations regarding the presence of TGF β_1 in the

epithelium and stroma (Godkin and Dore, 1998) and TGF β_2 in the glandular epithelium, including the apical boarder and uterine lumen (Polli *et al.*, 1996; Slater and Murphy, 2000), the current data showed expression of both TGF β_1 and TGF β_2 in the luminal epithelium and stroma.

The results of the present study showed no differences in the expression of TGF β_1 in the luminal and glandular epithelium between the control and the hyperstimulated animals. Differences between the control and hyperstimulated animals were however observed in the stroma of the hyperstimulated animals, which had regionalization in the expression of TGF β_1 in all examined stages of pregnancy. This feature was absent in all relevant stages of pregnancy in the control groups. Thus it can be concluded that hyperstimulation had an influence on TGF β_1 in the stromal compartment, which may contribute to a hostile environment for embryo implantation.

Ovarian steroid hormones have a primary role in the regulation of temporal and cell-type specific proliferation and differentiation in the uterus. Also, the actions of oestrogen and progesterone are not direct, but are mediated through stimulatory or inhibitory effects on other molecules, such as TGF β's (Murphy *et al*. 1987; Huet-Hudson *et al*. 1990; Tamada *et al*. 1990; Tamada *et al*. 1991). In their study on the expression of different TGF β isoforms in the mouse uterus and the effects that ovarian steroids have on these growth factors during the peri-implantation period, Das *et al*. (1992) explained that different mechanisms are involved in the production of TGF β's in this organ. In addition, Wakefield *et al*. (1990) proposed that regulation of production of TGF β isoforms by oestrogen and progesterone is target

tissue specific. Similarly, in their study on the mammary gland concerning the activation of TGF-β by ovarian hormones, Ewan *et al.* (2002) showed that during the periods of proliferation i.e. puberty, oestrus and pregnancy, which are under control of ovarian hormones, TGF β_1 activation decreased in some cells, consistent with preparation for proliferation. In contradiction, other cells simultaneously increased TGF β_1 immunoreactivity. Ewan *et al*. (2002) explained that this switch in the synthesis indicates that ovarian hormones regulate TGF-β_1 activation, which in turn restricts the proliferative response to hormone signaling. The present study showed that the rat uterus is under the control of ovarian steroid hormones. Differences in the expression of the TGF βs between the control and hyperstimulated animals have been shown. In the light of the results obtained by Ewan et al. (2002), it is postulated that the regionalization in the expression of TGF β_1 and TGF β_2 i.e. the presence of two zones in the stroma of the hyperstimulated animals, occurred as a result of raised oestrogen and progesterone levels, which in turn influenced the response of the stromal cells. Consequently, the altered stromal environment contributed to the unfavorable state of the uterus for embryo implantation.

Control animals showed TGF β_1 expression at the antimesometrial pole, which was not observed in the hyperstimulated animals. Moulton (1994) showed that the TGF β_1 and TGF β_2 treatments induced apoptosis on cultured rat endometrial stromal cells as well as controlled apoptosis in the rat uterus during early pregnancy. Thus, the findings from the current study which showed localization of TGF β_1 and TGF β_2 at the antimesometrial side of the uterine lumen and around the implanting conceptus, suggests that both TGF β_1 and TGF

β_2 may play a role in the initiation of embryo implantation as well as in apoptosis.

Apoptosis, programmed cell death, is said to be a crucial event in the physiology of endometrial stroma of normal cycling as well as pregnant uteri which enables the trophoblast cells to gain access to the maternal blood vessels (Moulton, 1994; Chatzaki, *et al*., 2003). Abrahamson and Zorn (1993) and Pampfer and Donnay (1999) observed some morphological characteristics of apoptosis in endometrial epithelial cells at embryo implantation sites in rodents. TGF β_1 both inhibits cell proliferation and increases apoptosis of uterine epithelial cells in culture (Nawaz *et al*., 1987). TGF β_2 secretion by endometrial cells controls stromal apoptosis during early pregnancy *in vitro* (Moulton, 1994). Schooner *et al*. (2005) indicated that both TGF β_1 and TGF β_2 were found in epithelial and stromal cells during early pregnancy in the rat. During late pregnancy, TGF β_1 was immunolocalized mainly in the stroma, whereas TGF β_2 was found mostly in the epithelial cells, and according to Shooner *et al.* (2005), at this particular time of the rat pregnancy, was involved in the regression of the decidua basalis.

Comparisons between the control and hyperstimulated animals revealed a prominent difference in the expression of both the TGF β_1 and TGF β_2 restricted to the antimesometrial side of the lumen as well as in the area surrounding the implanting conceptus. It is postulated that the hyperstimulation had a deleterious effect on the expression of both growth factors in this study, since the presence of neither TGF β_1 nor TGF β_2 was observed at the antimesometrial side of the lumen in the hyperstimulated animals. Thus, it is believed that hyperstimulation provided an unfavorable environment for embryo implantation by

totally inhibiting the presence of TGF β_1 and TGF β_2 at the implantation sites.

Furthermore, Chatzaki *et al*. (2003) suggested that endometrial stromal TGF β_1 induced apoptosis of endometrial epithelial cells by having a major autocrine and/or paracrine pro-apoptotic effect on human endometrial cells in culture. The use of neutralizing antibodies for blockage of TGF β_1, decreased apoptosis only by 22%, which suggested that endogenous TGF β_1 does exert a pro-apoptotic effect on stromal cells, but only when its latent form is activated (Chatzaki *et al*., 2003). In addition, Moulton (1994) exposed stromal cells to steroid treatment for the experimental induction of decidualization, which caused, according to Lea *et al*., (1992), a switch from TGF β_1 expression to TGF β_2. This suggests that TGF β_2 may be more involved in inducing apoptosis of the rat decidua than the TGF β_1 isoform. Indeed, results obtained from the present study, support findings made by Lea *et al*. (1992), Chatzaki *et al*. (2000) and Chatzaki *et al*. (2003) since, although TGF β_1 was present around implantation chambers, the immunolocalization potency of the TGF β_2 isoform, as well as its spatial and temporal expression was more pronounced than the TGF β_1 isoform.

Compared to the expression of TGF β_2 in the control group, a gradual decline in the expression of TGF β_2 in the luminal epithelium, stroma and glandular epithelium throughout the peri-implantation period was not observed in the hyperstimulated animals of the present study. It appears that hyperstimulation had an effect on the expression of TGF β_2 in the stroma which resulted in regionalization of this growth factor. Moreover, hyperstimulation had a stimulatory effect on the expression

of TGF β_2 in the glandular epithelium and myometrium. This resulted in the up-regulation of TGF β_2 expression in these parts of the endometrium in the treated animals.

According to Tamada *et al*. (1990), the luminal and glandular epithelial cells are primary sites of TGF β_1 in mice uteri, while the immunolocalization of this peptide in the stroma, presents an accumulation of TGF β_1 that has been synthesized and secreted from the epithelium. Additionally, Tamada *et al*. (1990) showed that mRNA for both TGF β_1 and TGF β_2 is limited to the uterine luminal and glandular epithelium during the pre-implantation period in mice and to the decidua during the post-implantation period. Also, Chen *et al*. (1993) found mRNA for TGF-β_1 present within the rat uterus during early and late pregnancy and restricted to the luminal and glandular epithelial cells. TGF β_2 gene expression is similar to that of TGF β_1, except that the former is also found in the uterine myometrium in mice (Das *et al*., 1992). Indeed, in the present study, the myometrium showed weak expression of TGF β_1 and strong immunolocalization of TGF β_2, throughout the peri-implantation period in both the control and hyperstimulated animal groups.

The existence of yet another function of TGF βs is worthwhile mentioning in the context of our findings, by introducing an appealing immunological model which may explain the mechanisms that prevent the mother's immune system from rejecting her concepti. Kauma *et al*. (1990) found that TGF β and TGF β_1 mRNA was localized at the human maternal-fetal interface, including the first trimester decidua, placenta and placental membranes. On the basis of their findings Kauma *et al*. (1990) presumed that TGF β s control the local maternal

immune response and prevent rejection of the fetus. Also, McLennan and Koishi (2004) compared the roles of both TGF β_1 and TGF β_2 as potential regulators of the immune system as well as factors that are involved in the continuation of pregnancy. In their findings McLennan and Koishi (2004) showed that maternal and fetal TGF β_1 co-operate with each other to maintain the pregnancy within an immuno-competent mother. Although TGF β_2 was produced by the placenta (Das *et al*., 1992) and has a potential role in immune tolerance of the placenta (Clark *et al*., 1999), McLennan and Koishi (2004) believed that TGF β_1 is a more suitable candidate for this role since it is ubiquitously present in fetal tissues (Heine *et al*., 1987).

Additionally, Wira and Rossoll (2003) pointed out that increased levels of oestradiol during the reproductive cycle and after the treatment with exogenous hormones restrain the capability of stromal antigen-presenting cells (APC) to accomplish their role. Moreover, since the epithelium and stroma work together by producing factors that regulate their different functions, oestrogen inhibition of stromal cell antigen-presentation is mediated through its stimulatory effect on TGF β production by epithelial cells (Wira and Rossoll, 2003). In the present study oestrogen levels in the control animals showed a steady rise throughout the peri-implantation period. The weak presence of TGF β_1 immunolocalization in the luminal epithelial cells with apical concentration and strong within the stroma was also observed. However, TGF β_2 expression showed a gradual decrease in all relevant areas of endometrium, from 4.5. 5.5 to 6.5 days of pregnancy, and absence of apical concentration in the luminal epithelium. Additionally, TGF β_1 was immunolocalized at the antimesometrial stromal side of the lumen, as well as in the trophoblast region, while the expression of

TGF β_2 was not observed in the trophoblast region or any other part of the implanting blastocyst. Taken together findings of the present study support the work made by Wira and Rossoll (2003) and imply that the TGF β_1 presents a more suitable candidate for the function in the uterine immunological response. It is believed that oestrogen could have had a stimulatory effect on TGF β_1 production in the luminal epithelial cells, which then got released into the stroma to inhibit antigen presentation by uterine APC. The presence of TGF β_1 at these sites of the maternal-fetal interface, support the above-mentioned presumptions made by Wira and Rossoll (2003), and offers an essential role of TGF β_1 in the immune protection of the female reproductive tract and preservation of pregnancy.

5. CONCLUSION

Despite the fact that hyperstimulation with exogenous gonadotropins is effective in multiple follicular development, there is a growing evidence of the deleterious effects of this procedure on the uterine endometrium. The results of the present investigation suggest that exogenous administration of FSH and hCG, which has been routinely used in IVF and ET programmes, interferes with the regular expression of TGF β_1 and TGF $\beta_{2,}$ whose actions are necessary for normal endometrial development in preparation for embryo implantation and successful pregnancy. This is yet another important consideration that needs to be addressed when assessing the low pregnancy rate in assisted reproductive programmes.

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APPENDIX

1. Shorr's staining technique (Drury and Wallington, 1980)

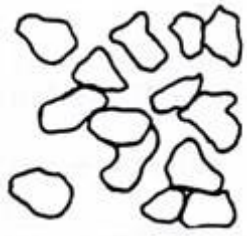
Ethyl alcohol (50 per cent)	100 cm^3
Biebrich scarler (water soluble)	0.5 g
Orange G	0.25 g
Fast green FCF	0.075 g
Phosphotungstic acid	0.5 g
Phosphomolybdic acid	0.5 g
Glacial acetic acid	1 cm^3

Method

1. Mix all ingredients by shaking
2. Filter
3. Stain smears for 1 minute
4. Rinse in 70% alcohol for 2x1 minute
5. Dehydrate through graded alcohols (95% and 100%)

2. Assessment of vaginal smears (Kent and Smith 1945)

Oestrus



Abundance of cornified polygonal cells

Metoestrus



Abundance of elongated cornified cells. Some nucleated cuboidal cells. Occasional leucocytes

Early-dioestrus



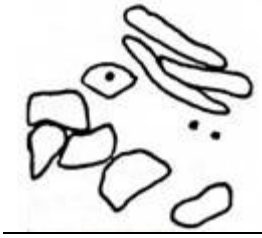
Numerous nucleated cuboidal cells. Some cornified polygonal cells. Abundance of leucocytes

Mid-dioestrus



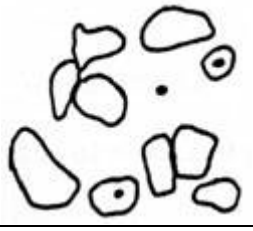
Numerous nucleated cuboidal cells. Occasional elongated polygonal cornified cells. Some leucocytes

Late-dioestrus



Elongated to polygonal cornified cells fairly numerous. Some nucleated cuboidal cells. Occasional leucocytes

Proestrus



Polygonal cornified cells numerous. Some nucleated cuboidal cells. Some nucleated cuboidal cells. Very few leucocytes

3. 10% Neutral buffered formalin

Formalin	100 ml
Sodium dihydrogen phosphate	3.5 g
Di-sodium hydrogen phosphate	6.5 g
Distilled water	900 ml

4. Silane coated slides (Mutter, 1988)

1. Soak slides in 10% Contrad or Super 10 overnight
2. Rinse in hot running water – minimum – 2 hours
3. Dry in oven at 60°C
4. Dip in Acetone (optional)
5. Dip in 2% Silane in Acetone for 30 minutes (6ml Silane + 294ml Acetone)
6. Wash in two changes of acetone 1-2 dips
7. Wash briefly in distilled water
8. Dry in 42°C incubator overnight

5. Haematoxylin and eosin staining technique (Bancroft and Gamble, 2002)

Acid haematoxylin (modified Mayer's)

Haematoxylin	1 g
Sodium Iodate	0.2 g
Potassium Alum	50 g
Citric Acid	1 g
Chloral Hydrate	50 g
Distilled water	1 l

Allow haematohylin, alum and sodium iodate to dissolve overnight. Add chloral hydrate and citric acid and bring to the boil. Continue boiling for 5 minutes after which the solution is cooled and ready for use.

Stock Eosin

Eosin	8 g
Erythrosin	2 g
Distilled water	1 l

Eosin working solution

Stock Eosin	250 ml
Distilled water	750 ml
Calcium Chloride	20 g

Method

1. Dewax and hydrate sections
2. Stain in haematoxylin for 6-20 minutes depending on the strength of the stain and the fixative used
3. Wash well in running tap water. The sections may be examined microscopucally at this stage to confirm a sufficient degree of staining. If insufficient, return to the stain
4. Remove the excess stain by differentiating in 1% hydrochloric acid in 70% alcohol (usually a few quick dips). The blue staining of the haematoxylin is changed to red by the action of the acid.
5. Regain the blue colour by washing in alkaline running tap water. The stain should be again checked microscopically until correct degree of staining is obtained.

6. Citrate Buffer pH 6

Solution A	0.1 M Citric acid 10.5 g Citric acid in 500ml distilled water
Solution B	0.1 M Sodium Citrate 29.4g Sodium Citrate in 1000ml distilled water

Mix 9ml of solution A and 41 ml of solution B and make up to 500 ml at distilled water
Boil for 2x 5 minutes at high power.
Allow slides to cool for 20 minutes

7. Tris buffered saline 7.6 pH

Tris base	12.12 g
NaCl	17.54 g
Destilled water	2l pH to 7.6

8. D.A.B.

Weight 1mg (0.001g) D.A.B. into new bijou bottle
At start of second day of run put measuring cylinder containing 29ml distilled water into fridge at 4C
Just before use add 2ml Tris HCl pH 7.6 to D.A.B. in bijou bottle
Then add 1ml 30% Perhydrol to the now cold distilled water to make a 1% solution and mix well.
Add 20ul of the 1% perhydrol to the D.A.B. solution, mix on whirlimix
Filter before use
Pippete onto section timing each individually for 5 minutes