EFFECT OF ROSIGLITAZONE ON THE PATHOLOGY OF EXPERIMENTALLY INDUCED ENDOMETRIOSIS IN BABOONS

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DECLARATION.

'This is my original work and it has not been presented for a degree in any other University'

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DEDICATION

I would like to dedicate this work first to Our Lord Jesus Christ whose blessings I have seen. Secondly to my family; my wife Gladys Tatu, daughters Pendo, Rehema and son Chivatsi for standing by me through the study.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS.

Δ_1	Spectrophotometer absorbance reading 1
µl	microliter
ANOVA	Univariate analysis of variance
ART	assisted reproductive techniques
CAM	Cell adhesion molecules
CA19	Cancer antigen 19
CA-125	Cancer antigen 125
COCs	Combination estrogen-progestin oral contraceptives
DIE	Deep infiltrating endometriosis
EMB	Endometrial biopsy
ESCs	endometriotic stromal cells
FDA	Food and Drug Administration of United States of America.
FSH	Follicle stimulating hormone
GA	GnRH group after treatment.
g/dl	grams/deciliter
GOT	Glutamate Oxaloacetic acid, also called Aspartate transaminase (AST) /serum glutamic oxaloacetic transaminase (SGOT) / aspartate aminotransferase (AAT)
GnRH	Gonadotrophin releasing hormone
H & E	Haematoxylin and Eosin stain

IL-8	Interleukin –8
IPR	Institute of Primate Research
IVF	In-vitro fertilization
LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
LH	Leutenising hormone
LSD	Least Significant Difference
MDH	Malate dehydrogenase
mg/dl	milligrams per deciliter.
nm	nanometer
NADH	Nicotinamide adenine dinucleotide, as reducing agent form
NK- κB	transcription factor nuclear factor- κB
PA	Placebo group after treatment.
PAI-I	plasminogen activator inhibitor-1
PB	Placebo group before treatment.
PF	Peritoneal fluid
RA	Rosiglitazone group after treatment.
RB	Rosiglitazone group before treatment.
ROS	reactive oxygen species
Rosiglit	Rosiglitazone.
SIV	Simian Immunodeficiency virus
STLV	Simian T-cell lymphotrophic virus

TNF- α	Tumour Necrosis Factor – alpha
VEGF	Vascular endothelial growth factor

ABSTRACT

The objective of this study was to determine the effect of rosiglitazone, on the pathology of endometriosis in a baboon model. It was a prospective, randomised, placebo-controlled study, conducted at the Institute of Primate Research in Nairobi, Kenya. Endometriosis was induced using intrapelvic seeding of eutopic menstrual endometrium in 12 female baboons that had at least one menstrual cycle while in captivity.

Laparoscopy was performed on each of the 12 baboons. Menstrual endometrial tissue was extracted from each baboon by curettage, and one gram of endometrium was then seeded onto several peritoneal sites to induce endometriosis. About 34 – 68 days after the induction of endometriosis, a pre-treatment laparoscopy was performed in the baboons to assess the extent of endometriosis . Thereafter, the 12 baboons were randomised into three groups that were treated daily for 30 days as follows; placebo group (n = 4) received phosphate – buffered saline (PBS) tablets once a day orally, for 30 days. The positive control group (n = 4) received a gonadotrophin releasing hormone antagonist, (Ganirelix acetate®), at a daily dose of 125 μg/day subcutaneous for 30 days. Finally, the experimental group (n= 4) received 2 mg rosiglitazone, (Avandia®) by mouth daily for 30 days. A third and final laparoscopy was performed 30 days after the start of treatment to record the extent of endometriosis.

Endometriosis lesions were examined at laparoscopy and classified morphologically as typical blue-black, white plaques with pinpunctal spots, red vesicular, red hemorrhagic, red

adhesion. During the procedure, a video recording was performed to document the number and surface area of the endometriosis lesions. Blood for haematology and serum for clinical pathology was collected during the time of pre-treatment laparoscopy and at the final laparoscopy. The baboons were then euthanised by administrating 60mg/kg body weight of Sodium Pentobarbitone, (Euthatal®), intravenously and immediately thereafter a complete post mortem was performed.

Lesion samples were obtained and fixed in 10% neutral buffered formalin to confirm the histological features of endometriosis. The endometriosis lesions were scored on a scale of 0 to +4 in order to monitor the effects of the drug. The scoring scale was designed on the basis of a trial run that identified all the possible histological features that enabled an allocation of scores of 0 - 4. Intensity, severity and quantitative parameters within the lesions formed a subjective basis of scoring. This was not a blinded scoring.

Biochemical tests were carried out in serum to assess the adverse effects of the drug on the liver function. The liver function assays included analyses for total protein content, alkaline phosphatase (ALK), aspartate aminotransferase (AST/SGOT/AAT) and bilirubin. Kidney function tests included creatinine and urea tests. The endometriosis lesions were assessed and scored for histopathologic changes and features.

It was noted that the surface area of endometriotic lesions were significantly lower in [illegible] treated baboons when compared with the placebo group. Baboons treated

(mm^2) of peritoneal endometriotic lesions than controls. The extent of epithelial degenerative changes, including nuclear pyknosis and focal epithelial erosions were increased in treated baboons.

Rosiglitazone effectively reduced the burden of endometriosis disease in a baboon model. In one rosiglitazone treated animal, the level of degeneration in the endometriosis lesion was severe enough to cause epithelial necrosis. There were also significant degenerative changes of endometriotic stromal cells (ESCs) in the treated group. The experimental induction also produced some Stromal endometriosis (i.e. endometriosis without glands).

The systemic effects of rosiglitazone on baboons were analysed by comparing the frequency of appearance of histological features among the groups. Briefly, the following significant observations were recorded in rosiglitazone treated group compared with the placebo and GnRH treated group; pulmonary edema & hemorrhages; hepatic bile cananaculi surrounded by inflammatory cells and bile duct hyperplasia. Haematology also showed significant changes when a pre and post-treatment data was compared; there was an increase in haemoglobin values in the placebo and Rosiglitazone treated group ; a significant increase of total plasma protein in GnRH group; an increase in eosinophil count in the rosiglitazone treated group and an increase in plasma fibrinogen in the GnRH group. Serum chemistry showed no significant changes.

observation that gross and histopathology changes showed a significant reduction in surface area of lesions and an increase in degenerative and epithelial desquamation of endometriosis in rosiglitazone and GnRH treated baboons. The baboon model holds promise that a thiazolidinedione drug may be helpful in women with endometriosis.

Key Words: Baboon, endometriosis, rosiglitazone, thiazolidinedione, GnRH-antagonist, systemic pathology.

CHAPTER ONE

1.1. INTRODUCTION

Endometriosis is a benign gynaecologic disease of man that is defined and histologically diagnosed as presence of both endometrial glands and stromal cells outside the uterine cavity. Endometriosis is not a malignancy but it is associated with pelvic pain, subfertility, abnormal menstrual bleeding and disrupted gastrointestinal tract due to partial obstruction. It may also contribute to periodic cyclic abdominal pain from irritation of pain fibres on peritoneal surface. In the uterus, endometrial cells have exquisite sensitivity to sex hormones, depending on the cell type and location (McClellan *et al.*, 1990; Slayden and Brenner 2004; Brenner and Slayden 2005). Endometriosis is an oestrogen dependent disease, and endometriotic lesions can produce their own oestrogen after 6 months of experimental induction (Atar and Bulun 2006). In women endometriosis is the second leading reason, after uterine fibroids, for hysterectomies (http://womwnhealth.gov 1 July 2006). The prevalence of endometriosis in women is reported to be as high as 22% in asymptomatic cases, 40 – 60 % in dysmenorrhoeal cases and 20 – 30 % in subfertile women (Farquhar 2000).

Grossly, endometriosis lesions may appear as small reddish peritoneal petechiae, white cystic peritoneal vesicles, brownish diffuse peritoneal lesions, or classic blue-black folded lesions on the peritoneum (Martin *et al.*, 1989). Endometriosis may also affect the ovary, resulting in large cyst-like lesions that contain chocolate-fluid called *endometriomas* (Olive and

Because it is not possible to study mechanisms of endometriosis in affected women *in vivo*, a baboon model has been developed in which intrapelvic injection of menstrual endometrium results in the induction of endometriotic lesions (D'Hooghe *et al.*, 1994a; Fazleabas *et al.*, 2002). The disease has also been reported to occur naturally in other non-human primates (Schenken *et al.*, 1984; D'Hooghe *et al.*, 1991; Cornillie *et al.*, 1992; Dick *et al.*, 2003; Story and Kennedy 2004).

The clinical data of this study, represented here in Section 4.1.1; Section 4.1.2 and Section 4.1.3 have been published before (Lebovic *et al.*, 2007). The author of this thesis was a co-author of this publication (Lebovic *et al.*, 2007). He performed all the surgeries, documentation, photography, video recording used in this publication.

This thesis represents a report (sections 4.1.4 to 4.4) on the clinical pathology and pathology based on blood samples collected at Laparoscopy II, Laparoscopy III and tissues at necropsy, and can be considered as a spin-off of the published study (Lebovic *et al.*, 2007) initiated by Dr Dan Lebovic.

1.2. OBJECTIVES OF THE STUDY

The major objective of the study was to determine the effects of rosiglitazone on pathology of experimental endometriosis in a baboon model.

in endometriosis lesions in rosiglitazone treated baboons

1.2.1.2. To determine the adverse effects of rosiglitazone using serum chemistry parameters in baboons.

1.2.1.3. To establish the target organs of rosiglitazone toxicity in baboons based on histopathology.

These objectives will be studied comparing the data in the study group (rosiglitazone) with a negative control group (placebo) and a positive control group (GnRH antagonist)

1.2.2. Rationale and study justification

Preclinical studies in the rodent model of endometriosis (Lebovic *et al.*, 2004; Demirturk *et al.*, 2006; Aytan *et al.*, 2007) have shown that using a peroxisome proliferator-activated receptors gamma (PPAR-γ) agonist may have merit to treat endometriosis lesions since it reduces the size of experimentally induced endometriosis. These promising results observed in the rat formed a basis of this investigation using nonhuman primates (Lebovic *et al.*, 2007).

Two common medical treatment options available for endometriosis are gonadotrophin releasing hormone (GnRH) antagonist, and a combination of oestrogen and progestin oral contraceptives (COC). This combination, referred to as the "pseudopregnancy" regimen, has been used for several decades, and many physicians use it as first-line therapy for endometriosis (Moore *et al.*, 2000). These hormonal drugs were first used for the treatment of endometriosis more than 40 years ago but interfere with fertility (Moore *et al.*, 2000).

eliminate endometriotic lesions, prevent recurrence and not impede ovulation or cause infertility. Rosiglitazone (a PPAR-γ) is a possible new candidate for treatment of endometriosis. However, controlled studies of endometriosis in women are restricted due to ethical reasons of performing repeated invasive surgical procedures. Experimentally induced endometriosis in the baboon provides a non-human primate model for endometriosis, allowing the evaluation of new drugs in the prevention or treatment of endometriosis (D'Hooghe *et al.*, 1995). Baboon endometriotic implants show histological transformations similar to those seen in human endometriotic lesions. In the current study, the baboon model of endometriosis will be utilized to assess if rosiglitazone can impede the growth of induced ectopic endometriosis tissue.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. PATHOGENESIS OF ENDOMETRIOSIS

Endometriosis is the presence of functional endometrial glands and/or stroma in ectopic locations outside the uterine cavity.

Pathogenesis is poorly understood and remains controversial. Three concepts can be discerned. The oldest concept that of *in-situ* development is that endometriosis develops on the spot where it is found (Ridley 1968). The mechanism of *in situ* development of lesions could be as a result of local induction, development from Mullerian remnants or alternatively from metaplasia of the peritoneal or ovarian tissue into endometrium (Ridley 1968; Lauchlan 1972). This can explain a reported case of endometriosis in a man (Pinkert *et al.*, 1978). While the disease is associated with significant morbidity, it is uncommon for endometriosis to cause death in humans.

A second concept, the induction or metaplasia theory, is that endometriosis results from differentiation of mesenchymal cells, activated (induced) by substances released by degenerating endometrium that arrives in the abdominal cavity (Levander and Norman 1955; Merrill 1966; Matsuura *et al.*, 1999).

theory in pathogenesis of endometriosis, one study observed that in patients with obstructed menstrual outflow, endometriosis occurred in 77% of patients with a functional endometrium and patent tubes and in up to 89% of those with hematocolpos (a condition in which the vagina fills with menstrual blood) or hematometra (a collection or retention of blood in the uterine cavity) (Olive and Henderson 1987). Extrapelvic endometriosis can be explained by the transplantation of menstrual cellular components or stem cells with potential to undergo changes into endometriosis to these sites via haematogenous or lymphatic system.

Retrograde menstruation is a proposed and widely accepted mechanism that explains mostly the presence of endometrial cells in ectopic sites (Sampson 1927). Although widely accepted, this theory does not wholly explain the disease progression and the reason why it occurs only in some women yet; all women have some menstrual reflux. Several other theories of the aetiology and pathophysiology of endometriosis have been proposed. These include inability of immune-system to clear retrograde menstruation and vascular dissemination of endometrial cells during menses (Akoum *et al.*, 2002; Giudice and Kao 2004).

The disease may also involve the interaction and combined effects of several pathways: hormonal (Hastings and Fazleabas 2006), cell adhesion, angiogenic, hypersensitivity and immunological mechanisms (Lebovic *et al.*, 2001; Kyama *et al.*, 2003). Aberrant immune response (Mori *et al.*, 1992; Lebovic *et al.*, 2001; Akoum *et al.*, 2002), inflammatory reaction (Ota *et al.*, 1996; Giudice and Kao 2004), inherited genetic polymorphism in oestrogen

Wieser *et al.*, 2002) and estrogens dependency (Noble *et al.*, 1997) are responsible for separate pathophysiologies that support progression of endometriotic lesions. This combination of processes is accompanied by synthesis of prostaglandins endoperoxidase synthetase – 2 leading to greater levels of prostaglandins E_2, which, in turn, is a potent stimulator of the aromatase II promoter in endometriotic stromal cells (Noble *et al.*, 1997).

It has been postulated that endometriosis has an autoimmune aetiology, but the nature of this disorder has not been properly characterized. Patients with endometriosis have a higher incidence of autoantibodies of both IgM and IgG isotypes directed against phospholipids, histones, or DNA (Gleicher *et al.*, 1987). Endometriosis lesions are characterized by the presence of abundant plasma cells, many of which produce IgM, and macrophages that produce a TNF-α implicated in other autoimmune diseases (Hever *et al.*, 2007). To support this theory, epidemiological studies have documented a higher incidence of other autoimmune diseases among endometriosis patients. Studies have shown that hypothyroidism, fibromyalgia, chronic fatigue syndrome, autoimmune diseases, allergies and asthma are all significantly more common in women with endometriosis than in women without endometriosis (Sinaii *et al.*, 2002). Other characteristics of endometriosis, like its recurring nature, are suggestive of autoimmunity.

Inflammation and endometriotic tissue growth are the two pathological processes which are responsible for chronic pelvic pain and infertility in endometriosis. Estrogens, growth factors and metalloproteinases enhance the endometriotic tissue growth and invasion of

endometriotic tissues, where as prostaglandins and cytokines mediate pain, inflammation and infertility (Bulun 2009).

2.2. PREVALENCE OF ENDOMETRIOSIS

Endometriosis affects 6 – 10% of women in the reproductive –age group (Eskenazi and Warner 1997). Endometriosis is the most common cause of pelvic pain in women and occurs in 10 – 70% of women with pelvic pain (Lapp 2000), 30-40% of women with infertility (Lapp 2000; Giudice and Kao 2004) and representing the third leading cause of gynaecological hospitalisation in the USA. Some clinical investigations show that the prevalence may be lower in black Africans than the Caucasian population. Some authors have reported no difference (Kyama *et al.*, 2007b) possibly due to under reporting of prevalence of endometriosis in Indigenous African women and African -Americans, thus the erroneous perceived low prevalence in this group.

In general endometriosis is commonly increased in infertile Caucasian or African -American women more than Indigenous - African women. The proposed possible low risk factors of endometriosis in Indigenous – African women are those that limit the cumulative number of menstrual cycles with retrograde menstruation a woman undergoes. These factors include giving birth at low age, increased number of children, tubal infertility (that causes 57% of female infertility) (Kyama *et al.*, 2007b). Also the low prevalence of endometriosis in Indigenous - African women could be due to low awareness, poor diagnostic and therapeutic facilities, limited training in diagnosis and treatment and underdeveloped endometriosis

African women often show cervical and umbilical endometriosis and manifest more cases of endometriosis- related ascites and pulmonary/thoracic endometriosis than infertile Caucasian or African-American women (Kyama *et al.*, 2004).

2.3. ENDOMETRIOSIS IN ANIMAL MODELS

Current understanding of endometriosis is limited due to the difficulty in studying the disease in humans. Non-human primates develop spontaneous endometriosis that is morphologically identical to its human counterpart (Schenken *et al.*, 1984) and lesions are found at similar sites (D'Hooghe *et al.*, 1991). Most published studies describe the disease in rhesus macaques (*Macaca mulatta*) and baboons (*Papio anubis anubis*) (Dick *et al.*, 2003), but it has also been reported in cynomolgus monkeys (*Macaca fascicularis*) (Fanton and Hubbard 1983).

Baboons are widely found in Africa and are considered as pests in many places. They are also not endangered. They have a menstrual cycle of 33 days, close to that of a women. Baboons are also an established model for cardiovascular, endoscopic, endocrinology, teratology and toxicological studies. Various methods of experimental inductions of endometriosis in monkey have been tried but only the intraperitoneal seeding of menstrual flow contents results in more profound lesions (D'Hooghe *et al.*, 1994a).

Endometriosis has also been experimentally induced in rats and rabbits and used to study the pathophysiology of the disease. This is achieved by suturing a piece of endometrium on the

an advantage due to their low cost relative to the monkey, but they are disadvantaged by lack of a menstrual cycle and absence of spontaneous endometriosis in rodents. The lesions in rodents are not physiologic, do not damage the uterus, or cause adhesions that interfere with fertility as in human disease (Lebovic *et al.*, 2004). Thus the ectopic autologous transplantation of uterine tissue in these models may not sufficiently replicate human endometriosis. The baboon endometriosis model was therefore established as a better model of the disease by intrapelvic seeding of menstrual eutopic endometrium on top of the pelvic organs (D'Hooghe *et al.*, 1995).

2.4. GROSS AND MICROSCOPIC PATHOLOGY OF ENDOMETRIOSIS

Grossly, typical endometriosis lesions occur as white plaques with pigmented spots or blue-black cysts in the peritoneum. Subtle lesions are red and occur either as red-orange vesicles, red polyps, hemorrhagic zones, or as petechiae or as white plaques with or without vesicles. Subtle lesions are considered as indicative lesions for endometriosis. Generally, active lesions are red and inactive ones are blue-black or white and have been reported to have increased estrogen receptors E_2R (ER – α) expression compared with inactive (bluish- black) (Matsuzaki *et al.*, 2001) thus making them more responsive to estrogen. Endometrial and stromal glands that occur in endometriosis show an overt endometrioid appearance that can be inactive or proliferative, secretory or hyperplastic nature (Clement 2007).

Endometriosis is a progressive disease and remodelling of endometriotic lesions from one type to another occurs. Subtle lesions were shown to be more frequent in younger women

lesions was found (Thomas and Cooke 1987). This was confirmed in baboons by that the incidence of typical lesions, which are considered to be older and/or burnt out endometriosis, decrease with age (D'Hooghe *et al.*, 1996a).

Endometriosis lesions are graded according to the classification system of the American Society for Reproductive Medicine (ASRM) (American Society for Reproductive Medicine 1997). In this system, scores are determined from lesion size, depth of penetration as determined during the excision, status of the posterior cul-de-sac, presence and size of ovarian endometriomas, and presence and nature of tubo-ovarian adhesions. Adhesions involving ovary, Fallopian tube and cul-de sac of the uterus are the greatest risk factors for infertility to occur in endometriosis and a given higher scores. The ovarian endometriomas that occur as large, cyst-like structures containing chocolate-fluid, represent a more severe form of the disease and are given a higher ASRM score. The ASRM classification system has been adapted for use in the baboon (D'Hooghe *et al.*, 2006; Falconer *et al.*, 2006; Lebovic *et al.*, 2007).

Deeply infiltrating endometriosis (DIE) is another form of endometriosis that penetrates more than 5 mm under the peritoneal surface (Koninckx and Martin 1994; Chapron *et al.*, 2003). These lesions are often not visible during laparoscopy. These lesions are very active and they are strongly associated with pelvic pain (Koninckx *et al.*, 1991).

Endometriosis may show cyclic changes and signs of cyclic haemorrhage, but not all

complicated by alterations or by the absence of glandular or stromal components in the lesion. The appearance of endometriosis may also be altered by hormonal and metaplastic changes, as well as cytologic atypia and hyperplasia (Clement 2007).

Although endometriosis is usually confined to the pelvis, extrapelvic sites of the lesions have been reported in nearly all organs of the abdominal cavity. The most common locations of intrapelvic endometriosis include abdominal sites including the ovaries, fallopian tubes, ligaments that support the uterus, the area between the vagina and the rectum (rectovaginal septum), the outer surface of the uterus, and the lining of the pelvic cavity. Extrapelvic endometriosis can be observed sometimes in abdominal surgery scars, on the intestines or in the rectum, appendix, on the urinary bladder, vagina, diaphragm, cervix, and vulva. Deep endometriotic lesions in the recto-vaginal septum are usually extensions from the cul-de-sac of the uterus. Extrapelvic endometriosis occurs less commonly outside the abdomen, in the lung, skin, muscles, peripheral nerves, brain, arm, thigh, spinal column, genital tract, urinary tract, and lymph nodes (Robboy *et al.*,1994). In extremely rare cases, endometriosis can occur in men. Scattered case reports in men exist of lesions that are histologically indistinguishable from endometriosis. These have occurred all in men with cancer of the prostate who were undergoing high-dose oestrogen therapy. In these men, endometriosis was found in the prostate (Pinkert *et al.*, 1978).

Infertility is associated with endometriosis. Several studies aimed at explaining the mechanisms of infertility in endometriotic women; have reported ultrastructural differences

normal and diseased baboons. Ultrastructurally, diseased endometrial glands showed abnormalities in secretory vacuoles and an intracellular accumulation of glycogen; in later stages of the disease, glands resembled those of the late secretory phase endometrium. The abnormalities within the endometrial of the endometriotic women were seen as changes in glycan expression. In early disease, there was an increased binding of lectin from *Dolichos biflorus* agglutinin (DBA) to fucosylated N-acetylglucosamine residues, whereas in later stages, this binding generally decreased in association with the appearance of a late secretory phenotype. They concluded that endometriosis is accompanied by progressive changes in the gland architecture and biochemistry resulting in dyssynchrony within the window of uterine receptivity, which may result in the reduced fertility associated with this disease (Jones *et al.*, 2006). This glandular variation was also reported in two ultrastructural studies of human endometriosis (Schweppe and Wynn 1984: and Schweppe *et al.*, 1984). Other studies in baboons have shown that gene expression changes occur in eutopic endometrium of experimentally induced endometriosis (Hastings and Fazleabas 2006). The significance of these changes is not yet known.

2.5. DIAGNOSIS OF ENDOMETRIOSIS

Surgical laparoscopy, with histology, is the only accepted mode of diagnosis of endometriosis (Dmowski 1984; Pittaway 1992). Diagnosis of endometriosis requires surgery because imaging techniques, such as ultrasound and Magnetic Resonance Imaging, are not reliable in the diagnosis or grading of the disease.

A true diagnosis of endometriosis is achieved only when endometrial glands and/or typical stroma are found on histological examination of the lesion. There is no correlation between histological and visual findings at laparoscopy (Jansen and Russell 1986; Walter *et al.*, 2001).

Wide variations in histological features that support the presumptive diagnosis of endometriosis have been described. Sometimes glands and stroma are absent in lesions such as chocolate cysts of the ovary or in the brownish-pigmented implants on serosal surfaces. Hemosiderin-laden macrophages and fibrous connective tissue containing inflammatory cells are often present in these lesions.

In his article focusing on diagnostic problems and unusual morphologic features of pelvic endometriosis, Clement (2007) documented some causes that can explain the under-diagnosis or misdiagnosis of endometriosis. He classified these into problems that include alterations of the stromal component, glandular component, tumour-like findings, unusual inflammatory and reactive changes, site related and other rare associated lesions. In some cases it may require special histological stains for diagnosis of endometriosis. CD10 positivity is present in normal and neoplastic stromal cells. It is helpful when dealing with limited materials and when glandular tissue is absent in the lesion. It is also helpful in distinguishing endometriotic stromal cells from ovarian cells (which are CD10 negative). Although McCluggage *et al.*, (2003), found that normal endocervical stromal cells are immunoreactive for CD10, those

endocervical stromal cells were predominantly $CD34^{+}/CD10^{-}$, whereas endometrial stromal cells were predominantly $CD34^{-}/CD10^{+}$. Additionally, Orlandi *et al.*, (2004) found that cellular Retinol-Binding-Protein-1, is more specific for endometriotic stromal cells than CD10 and that it stains endocervical stromal cells.

No clinical laboratory methods have been proven to be specific in confirmatory diagnosis of endometriosis. Current evidence suggests that endometriosis induces local and systemic inflammatory processes. Consequently, numerous studies have focused on markers of inflammation in diagnosis of endometriosis (Barberi *et al.*, 1986; Pittaway and Fayez 1986; Mol *et al.*, 1998; Bedaiwy *et al.*, 2002; Somigliana *et al.*, 2004; Cho *et al.*, 2007; Seeber *et al.*, 2008). Several cytokines such as interleukin-6 (IL-6), IL-8, and tumour necrosis factor-alpha (TNF-α) have been shown to differ between women with endometriosis compared with those without (Bedaiwy *et al.*, 2002). Tumour markers that are useful in the diagnosis of cancer [Cancer antigen 19 (CA19), Cancer antigen 125 (CA-125) and Cancer antigen 15-3 (CA15-3)] have also been tested in women with endometriosis (Mol *et al.*, 1998; Somigliana *et al.*, 2004). The most extensively studied marker is CA-125. Elevated CA-125 levels have been observed in serum, menstrual effluent, and the peritoneal fluid (PF) of women with endometriosis (Barberi *et al.*, 1986; Pittaway and Fayez 1986; Koninckx *et al.*, 1992). Although CA-125 is often elevated in advanced endometriosis, the low sensitivity of this assay limits its usefulness for detecting minimal and mild disease. A meta-analysis based on 23 articles showed limited diagnostic performance of serum CA-125 for detecting endometriosis (Mol et al., 1998). Cancer antigen 125 (CA-125) levels may be elevated in

advanced cases of endometriosis but are rarely elevated in mild-to-moderate disease. The test lacks adequate sensitivity or specificity to be of clinical value.

Recent studies have made use of white blood cell (WBC) subtypes and the neutrophil-to-lymphocyte ratio (NLR) as simple indices of systemic inflammatory response in critically ill patients and as prognostic indicators for various diseases. Evaluation of differential WBC counts and NLR in patients with endometriosis and in conjunction with CA-125, has been proposed as diagnostic markers for endometriosis (Cho *et al.*, 2008).

The same can be said of proangiogenetic factors, such as vascular endothelial growth factor (VEGF), and C - reactive protein found in elevated quantity in inflammatory processes (Xavier *et al.*, 2006). An additional problem is that the serum concentration of molecules such as C-reactive protein and CA 125 varies with menstrual cycle (Xavier *et al.*, 2006). Some studies have proposed the use of a panel of markers which include IL-6, TNF-α, MIF, MCP-1, IFN-γ, leptin, and CA-125 (Seeber *et al.*, 2008).

2.6. EFFECTS OF ENDOMETRIOSIS

The main complication of endometriosis is that it causes pain and impaired fertility. It is second to pelvic inflammatory disease as the leading cause of infertility in females that ovulate normally. Endometriosis can produce adhesions that can trap the egg near the ovary. It may inhibit the mobility of the fallopian tube and impair its ability to pick up the egg. In

peritoneal fluid and disorders of fertilization and immunoregulatory function explain the infertility that occurs in endometriosis (D'Hooghe *et al.*, 2003; Hastings and Fazleabas 2006). Other suggested causes of infertility range from impaired folliculogenesis, ovulatory dysfunction, hyperprolactinaemia, luteal phase defect, accelerated ovum transport, sperm phagocytosis, impaired fertilization, embryotoxicity, defective implantation (Garrido *et al.*, 2000), diminished ovarian reserve and dysregulation of activation of complement system (Kabut *et al.*, 2007).

Other complications include: internal scarring, adhesions, pelvic cysts, chocolate cysts, ruptured cyst, bowel and urethral obstruction resulting from pelvic adhesions. Rarely, endometriosis can be extraperitoneal in the lungs and central nervous system.

2.7. ADVANTAGES OF THE BABOON MODEL FOR REPRODUCTIVE RESEARCH

The baboon is a unique preclinical model for research in human reproduction for various reasons (D'Hooghe 1997; Kyama *et al.*, 2007b; D'Hooghe *et al.*, 2008).

2.7.1. Advantage number 1: Reproductive anatomy, endocrinology and physiology

The baboon is comparable to women with respect to the cycle length (33 +/- 2 days); and a duration of menstruation (3 +/- 1 days); the time interval between Lutenising hormone peak and menstruation (17 +/- 1 days); maximum serum estradiol level attained per cycle (245 +/- 30 pg/mL) and maximum serum progesterone level attained per cycle (11.5 +/- 2 ng/ml)

baboon is in some ways comparable to, and in other ways different from placentation in women and in rhesus monkeys (Pijnenborg *et al.*, 1996).

2.7.2. Advantage number 2. Non-invasive cycle monitoring based on perineal changes

In baboons, but not in rhesus monkeys or in cynomolgus monkeys, it is possible to perform noninvasive perineal skin monitoring to determine the phase of the menstrual cycle in baboons. Perineal inflation and deflation correspond with follicular and luteal phase, respectively, whereas ovulation occurs about 2 days before perineal deflation (Stevens 1997). A code has been developed for this perineal swelling and monitoring is performed by trained animal attendants at the IPR on a daily basis, and this allows detailed follow-up of individual baboons over a long period of time.

2.7.3. Advantage number 3. Continuous breeding

Baboons have continuous breeding in captivity (Birrell *et al.*, 1996), in contrast with seasonal breeding observed in rhesus monkeys (Zondervan *et al.*, 2002). This advantage allows investigators to carry out fertility follow up studies throughout the year and saves costs (D'Hooghe *et al.*, 2008).

2.7.4. Advantage number 4. Baboon's size and strength

Adult female baboons are stronger and larger (8-15 kg) than adult female rhesus monkeys (4-

large cervix, which allows taking of endometrial biopsies and other intrauterine procedures with ease.

2.7.5. Advantage number 5. Spontaneous peritoneal fluid (PF)

Baboons, but not rhesus monkeys or cynomolgus monkeys have spontaneous presence of PF in sufficient amounts (about 2 ml after ovulation) that can be harvested and used for research. This is important since the peritoneal cavity and PF are key players in the pathogenesis of endometriosis (D'Hooghe *et al.*, 1991)

2.7.6. Advantage number 6. Cross-reactivity of biological markers between baboons and humans

Due to a closely shared genetic composition and a close phylogenetic relation (evolutionary relatedness among various species) between baboons and humans, cross-reactive human steroid assays, antibodies or PCR primers can be used in baboons in the context of research in endometriosis or other reproductive disorders (D'Hooghe *et al.*, 1996b; D'Hooghe *et al.*, 2001a-b; Overbergh *et al.*, 2005; Kyama *et al.*, 2007a; D'Hooghe *et al.*, 2008). They have 42 chromosomes compared to human genome, which is composed of 23 pairs of chromosomes (46 in total).

2.7.7. Advantage number 7. Vaginal transcervical uterine access

In baboons, but not in rhesus monkeys or in cynomolgus monkeys, it is possible to have [illegible] vaginal transcervical access to the uterine cavity allowing endometrial biopsy

1996c; D'Hooghe *et al.*, 2004; Nyachieo *et al.*, 2007; Chai *et al.*, 2007). Alternative techniques have been developed where routine uterine access via a vaginal speculum proves difficult. Combined abdominal-cervical manipulation "the Chai technique" (Chai *et al.*, 2007; D'Hooghe *et al.*, 2008) allows transvaginal uterine access in nearly all difficult cases. Alternatively, endometrial biopsy is also possible by transabdominal insertion of a Novak curette through the uterine fundus under direct laparoscopic control (Nyachieo *et al.*, 2007; D'Hooghe *et al.*, 2008).

2.8 DEVELOPMENT OF BABOON MODEL FOR RESEARCH IN ENDOMETRIOSIS AT IPR

Baboons have spontaneous retrograde menstruation (D'Hooghe *et al.*, 1996d), display human-like minimal to severe spontaneous endometriosis (D'Hooghe *et al.*, 1991; Cornillie *et al.*, 1992; Dick *et al.*, 2003), offer an *in vivo* culture model for endometrial-peritoneal interaction and develop induced endometriosis within 25 days after intrapelvic injection of menstrual endometrium (D'Hooghe *et al.*, 1995). The baboon model also allows the study of evolution of both spontaneous and induced endometriosis by serial laparoscopies with detailed and repeated quantitative pelvic assessment of this condition.

The baboon model has been used to test new drugs for treatment or prevention of endometriosis at the IPR (D'Hooghe *et al.*, 2006; Falconer *et al.*, 2006; Lebovic *et al.*, 2007) and the baboon is important for testing general and reproductive safety of new anti-

The baboon model has also been developed for standardized and controlled fertility studies of endometriosis through laparoscopy assessment; ovulation by monitoring of the perineal cycle or for sexual activity through observation of timed intercourse with male baboon and postcoital test (D'Hooghe *et al.*, 1994b; D'Hooghe *et al.*, 1996e) and sperm analysis (Amboka and Mwethera 2003). Endometriosis-associated subfertility has been observed in baboons with mild, moderate or severe endometriosis that is either spontaneous or induced. In baboons, it is possibly related to an increased incidence and recurrence of the Luteinized Ruptured Follicle Syndrome, in the absence of ovarian endometriotic cysts (D'Hooghe 1997; D'Hooghe *et al.*, 1996c).

2.9. TREATMENT OF ENDOMETRIOSIS

Professional guidelines for the clinical management of endometriosis, like the ESHRE Guidelines (Kennedy *et al.*, 2005) and the Practice of Guidelines for the American Society for Reproductive Medicine (Practice Committee of the American Society of Reproductive Medicine 2006), provide a reference information.

Treatment of endometriosis, the impact of the disease and the effect on quality of life must be individualized. Pain symptoms of endometriosis may persist despite the extent of medical and/or surgical treatment. In humans, such cases require a multidisciplinary approach involving a pain clinic and counseling in the treatment plan.

Currently, there is no known cure for endometriosis in humans, though in some patients menopause abates the process. A hysterectomy and/or removal of the ovaries does not guarantee that the endometriosis lesions/or the symptoms of endometriosis will not recur. Adhesions can affect other organs besides those of reproductive system and even on the abdominal walls. It is suggested but unproven that pregnancy and childbirth can cease the progression of endometriosis. There is no guarantee that the endometriosis will not reccur after pregnancy (Stormert 2005).

Several strategies employed in the treatment of endometriosis and these can be classified into four major categories: 1) expectant management; 2) medical hormonal therapies; and 3) surgery therapy and 4) Assisted reproductive technologies (ART).

Medical therapy and conservative surgical therapy (uterus and ovaries retained) are only suppressive, not curative. Much of the concern pertains to appropriate treatments of minimal and mild disease. Severe endometriosis is usually treated surgically and is often associated with or followed by IVF (*in-vitro* fertilization) treatment for the infertility associated with endometriosis. In the past, numerous trials that address the question of optimal therapy for endometriosis have been completed (Stormert 2005). It is only now that well-designed prospective, randomised and controlled studies are been introduced. The presence of a control group that includes patients who do not receive any therapy for endometriosis is essential to demonstrate conclusively that treatment of endometriosis is better than expectant

2.9.1. Expectant management

This method consists of a period of observation with symptomatic treatment using antiprostaglandin medications to relieve pain. This method can be used in women with mild or minimal endometriosis who wish to become pregnant.

The document "ESHRE Guidelines for the diagnosis and treatment of endometriosis" several recommendations are given (Kennedy *et al.*, 2005). The guide categorises the forms of disease management as shown below. Among other things, the guide also recommends patient self-help groups as a source of invaluable counselling, support and advice (Kennedy *et al.*, 2005).

2.9.2. Empirical treatment of pain symptoms without a definitive diagnosis.

Empirical treatment of pain symptoms of presumed endometriosis includes counselling, adequate analgesia, nutritional therapy, progestagens or the combined oestrogen-progestin contraceptives (COCs). Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) are typically used to relieve mild-to-moderate pain associated with dysmenorrhea (Allen *et al.*, 2005). NSAIDs not only reduce pain but also reduce menstrual flow. They are commonly used in conjunction with other therapy. For more severe cases narcotic prescription drugs may be used.

2.9.3 Treatment of endometriosis-associated pain in confirmed disease.

Treatment may involve Non-steroidal anti-inflammatory drugs, Hormonal treatment and

2.9.3.1 Hormonal treatments

A fundamental strategy for treating endometriosis employs agents that decrease estrogen levels or increase androgen or progestin action. COCs, gonadotropin-releasing hormone (GnRH) agonist analogues, (Danazol®) and progestins are all effective in relieving pelvic pain caused by endometriosis. GnRH agonist analogues are the most effective; COCs are the least expensive (Barberi 2000). Hormonal therapy is not a cure for endometriosis since the disease recurs once treatment is stopped.

Progestins: Are synthetic progestagens (also known as, progestogens, progesterone or gestagens) that produce similar effects to those of progesterone. They cause (1) suppression of LH and FSH secretion, which suppresses estradiol production; (2) direct antiestrogenic effects that cause atrophy of endometriosis lesions and (3) induction of pseudodecidualization and endometrial atrophy.

Medroxyprogesterone acetate (Depo-Provera®), a long-acting depot injection is a convenient and a low-cost treatment for those patients unwilling or unable to tolerate (Danazol $^{(R)}$) or GnRH agonist therapy.

Androgenic agents: Danazol is a weak synthetic oral androgenic agent that induces amenorrhoea through suppression of the hypothalamic-pituitary-ovarian axis, accompanied by increased serum androgen concentrations and low serum estrogen levels (Valle and Sciarra 2003). The improvement (atrophy) of endometriosis may also be mediated by the effects of

COCs cause inhibition of ovulation, decreased gonadotropin levels, reduced menstrual flow and decidualization of endometriotic implants (Rice 2002); they reduce the swelling, bleeding, and inflammation of endometriotic lesions. COCs are progestin-dominant and they have been shown to down-regulate cell proliferation and increase apoptosis in the eutopic endometrium of women with endometriosis (Meresman *et al.*, 2005). COCs can be taken indefinitely, are cost-effective and cause relatively mild adverse effects.

GnRH analogues / analogs are synthetic peptide drugs modeled after the human hypothalamic gonadotrophin releasing hormone (GnRH). They interact with the GnRH receptor and modify the release of pituitary gonadotropins, FSH and LH, for therapeutic purposes (Wieser *et al.*, 2007). Two types of analogues exist: GnRH *agonist* and GnRH *antagonist.*

GnRH *agonist* act by causing constant stimulation of the pituitary GnRH receptors and initially causes stimulation (flare), but thereafter decreases pituitary secretion (downregulation) of LH and FSH. Examples of GnRH *agonist* are leuprolide acetate, goserelin acetate, and nafarelin. Some experiments have shown that GnRH agonists may have direct action on ovarian steroidogenesis independent of their action on the pituitary and direct effect on endometriosis growth. Laboratory data have confirmed direct action of GnRH agonists on ectopic endometrial cells (Meresman *et al.*, 2003; Meresman *et al.*, 2005; Wang *et al.*, 2006). Endometriosis cells exposed to GnRH agonist (leuprolide acetate) showed increased apoptosis with decreasing release of promitogenic cytokines such as

the growth of endometriosis. These effects were reversed by the addition of antide, a GnRH antagonist. Vascular endothelial growth factor may be involved in maintenance of endometriosis (Donnez *et al.*, 1998; Mc Laren 2000). Furthermore, GnRH receptors have been identified in ectopic endometrium (Lebovic *et al.*, 2000) suggesting that GnRH may be a direct regulator of endometriosis growth. These actions of GnRH would explain in part the regression of endometrial lesions seen following GnRH agonist therapy (Meresman *et al.*, 2003) as related to more than just the induced hypoestrogenic state. GnRH agonist therapy also influences eutopic endometrium function in patients with endometriosis either as a consequence of the induced hypoestrogenic state (Ishihara *et al.*, 2003) or by direct action such as demonstrated by Wang *et al.*, (2006). The studies imply an autocrine – paracrine action on local GnRH receptors within endometrium or ectopic endometrial tissue.

GnRH *antagonists* act by competitively blocking the GnRH receptor resulting in an immediate drop in gonadotropins (FSH, LH) secretion (Mahutte and Arici 2003; Shalev 2003). An example is (Ganirelix®) (used in this study as the positive control). Their action is immediate, time related and reversible. There is no initial flare of gonadotropins either before or after the onset of action. But unlike GnRH agonists, gonadotrophins are not depleted though the similar end effect of a hypoestrogen state is achieved (Rice 2002).

Suppression of the ovarian function for 6 months reduces endometriosis-associated pain. The hormonal drugs so far investigated are COCs, (Danazol®), gestrinone, medroxyprogesterone [illegible]® and GnRH agonists have been found to be effective but their side

effective at reducing endometriosis- associated pain, but there is insufficient evidence to make recommendations (Kennedy *et al.*, 2005). Duration of GnRH agonists treatment is for 3 months and may be effective for 6 months in terms of pain relief. Treatment for up to 2 years with combined estrogen progestagen "add back" appears to be effective and safe in terms of pain relief and bone density protection.

Compared with medical therapy, expectant management is less costly, and avoids treatment-induced anovulation and medication-related side effects. The disadvantages of expectant management are that it does not specifically treat the endometriotic implants, and in the majority of patients who fail to conceive with expectant management, progression of the disease may occur (Stomert 2005).

Though they are most expensive, GnRH antagonists, have added advantage of acting on the endometriotic lesions (Batzer 2006).

2.9.3.2. Surgery

Depending upon the severity of the disease, the ideal practice is to diagnose and remove endometriosis surgically, provided that pre-operative adequate consent has been obtained. Conservative surgery during laparoscopy is attempted to remove all areas of visible endometriosis through excision, fulguration (tissue destruction using an electrical current), or laser vaporization, without damaging normal tissue. Surgical treatment of severe disease has been shown to increase pregnancy rates (Olive and Martin 1987). Success of surgery is

endometriosis has been documented to result in 60% and 35% pregnancy rates, respectively (Olive and Lee 1986). Pregnancy rates are improved when minimal and mild disease is treated, but not as much as with more advanced disease (Marcoux *et al.*, 1997). Guzick and Rock (1983) demonstrated a rapid rise in the cumulative pregnancy rate with endoscopic laser therapy.

Removing the entire lesions in severe and deeply infiltrating disease can reduce endometriosis-associated pain. If a hysterectomy is performed, bilateral salpingo-oophorectomy should be considered, provided that all visible endometriotic tissue is removed at the same time (Kennedy *et al.*, 2005). In summary laparoscopic removal of minimal to mild endometriosis appears to have some therapeutic benefit. Treatment for severe endometriosis with either laparoscopic surgery or assisted reproductive techniques (ART) significantly improves pregnancy rates (Kodama *et al.*, 1996). In women with severe endometriosis, the only treatment of the disease may be a total hysterectomy with bilateral oophorectomy (Barberi 2000).

Ablation of endometriotic lesions plus laparoscopic uterine nerve ablation (LUNA) in minimal – moderate disease reduces endometriosis-associated pain at 6 months compared to diagnostic laparoscopy; the smallest effect is seen in patients with minimal disease (Jacobson *et al.*, 2004). However, there is no evidence that LUNA by itself is a necessary component, as it has no effect on dysmenorrhoea associated with endometriosis (Kennedy *et al.*, 2005). Surgical uterine suspension has been used as a form of treatment (Kennedy *et al.*, 2005).

However, there is no data supporting the use of uterine suspension but, in certain cases, there may be a role for pre-sacral neurectomy (Kennedy *et al.*, 2005).

2.9.3.3. Post-operative treatment

Combined Medical-Surgical Therapy may offer several advantages in advanced endometriosis. It is effective for pain relief; although it is not effective in improving fertility. Surgery alone or in combination with medical therapy, remains a common treatment method for all stages of endometriosis. Treatment with danazol or a GnRH agonist for 6 months after surgery reduces endometriosis-associated pain and delays recurrence at 12 and 24 months compared with placebo and expectant management. However, postoperative treatment with a COC is not effective (Kennedy *et al.*, 2005). At the same time there is no data to justify hormonal treatment prior to surgery to improve the success of surgery (Kennedy *et al.*, 2005).

2.9.3.4. Hormone replacement therapy (HRT)

HRT is recommended after bilateral oophorectomy in young women but the ideal regimen is unclear (Kennedy *et al.*, 2005). Adding progestrerone after hysterectomy is unnecessary but should protect against the unopposed action of estrogens on any residual disease. This theoretical benefit must be balanced against the small risk of recurrent disease and the increase in breast cancer risk reported to be associated with both tibolone and combined estrogen and progestagen HRT (Kennedy *et al.*, 2005)

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2.9.4. Treatment of endometriosis associated infertility

For patients with endometriosis-associated infertility, treatment of infertility may be necessary. Although the lack of prospective, controlled trials and inconsistency in staging contribute to the uncertainty about endometriosis as a cause of reduced fertility. The cumulative pregnancy rate after 5 years without treatment is 90% in women with minimal disease and is only slightly more in women without endometriosis (Stormert 2005). Based on these findings, it is recommended that a period of expectant management prior to any other therapy be allowed. This means additional infertility factors such as ovulatory disorders should be diagnosed and treated. The use of hormonal treatment to improve fertility is not effective and should not be offered for this indication alone (Kennedy *et al.*, 2005).

2.9.4.1. Surgical methods to improve fertility

Ablation of endometriotic lesions plus adhesiolysis to improve fertility in minimal –mild endometriosis is effective (Kennedy *et al.*, 2005). It is not known whether surgical excision of moderate to severe endometriosis enhances pregnancy rates. There seems to be a negative correlation between the stage of endometriosis and the spontaneous cumulative pregnancy rate after surgical removal of endometriosis. Laparoscopic cystectomy for ovarian endometriomas more than 4 centimeters diameter improves fertility compared to drainage and coagulation. Coagulation or laser vaporization of endometriomas without excision of the pseudocapsule is associated with a significantly increased risk of cyst recurrence (Kennedy *et al.* 2005).

Post operative treatment with danazol or a GnRH agonist after surgery does not improve fertility compared with expectant management.

2.9.4.2. Assisted reproduction in endometriosis

2.9.4.2.1. Intra uterine insemination (IUI)

Treatment of mild endometriosis with IUI combined with ovarian stimulation improves fertility, but the role of IUI alone is uncertain (Kennedy *et al.*, 2005).

2.9.4.2.2. Use of Clomide

In patients with mild endometriosis, treatment with fertility medication clomiphene may lead to success. This drug stimulates ovulation. Flushing with Lipiodol, an iodinated radio-opaque contrast poppyseed oil, may increase fecundity (Johnson 2005). There have been a few studies that suggest that flushing the lipiodol media through the tubes gives a short-term rise in fecundity in patients with unexplained infertility. A systematic review has suggested a significant increase in fertility, especially in those women who have endometriosis after using lipiodol flushing (Johnson 2005).

2.9.4.2.3. In vitro fertilization (IVF)

IVF procedures are effective in improving fertility in many women with endometriosis. IVF makes it possible to combine sperm and eggs in a laboratory and then place the resulting embryos into the woman's uterus. IVF is one type of assisted reproductive technology that [illegible] for women and families affected by infertility related to endometriosis

compromised, if there is also male factor infertility, and/or when other treatments have failed. IVF pregnancy rates are lower in patients with endometriosis than in those with tubal infertility. Also prolonged treatment with GnRH agonist before IVF in moderate-severe endometriosis should be considered and discussed with patients because improved pregnancy rates have been reported (Kennedy *et al.*, 2005).

2.9.5. Side effects of treatment

Analgesics: The use of analgesics has adverse effects of gastrointestinal nature and include nausea, abdominal pain, anorexia, constipation, and gastrointestinal bleeding. Some aspirin based NSAIDs have an anti-ovulatory effect when taken at mid-cycle (Kennedy *et al.*, 2005). When NSAIDs are ineffective, a narcotic pain reliever may be necessary.

Progestogens may cause androgenic effects, antiandrogenic, estrogenic, glucocorticoid, or antimineralocorticoid. Many women using progestogens gain weight or experience symptoms typical of the premenstrual period such as mood changes and bloating. Other common adverse effects of progestogens include nausea, breast tenderness, irregular bleeding, amenorrhea, and reduced libido. Progestogens are not an appropriate choice for women who wish to conceive quickly.

Androgenic agents: With Danazol, menstruation and ovulation usually cease and then resume after treatment is discontinued. Although Danazol is effective, its extended use may cause androgenic and metabolic side effects. These include acne, weight gain, mild

usually cease when danazol is discontinued, androgenic effects, such as deepening voice or increased hair growth may be irreversible. Danazol is also a known teratogen and causes side effects including migraine headaches, impaired liver function, seizure disorders and it may increase thromboembolic disease. Danazol is also important as a cancer risk factor for women with endometriosis (Modugno *et al.*, 2004).

COCs: Common adverse effects associated with COCs include nausea, breakthrough bleeding (or 'spotting' is the occasional, irregular bleeding/spotting experienced by women while using a hormonal method of birth control), breast tenderness, headache, and weight gain.

GnRH agonists and GnRH antagonists: The main effect of GnRH agonists and GnRH antagonists is hypoestrogenism and it is associated with amenorrhea, hot flushes, dry vagina and loss of libido (Story and Kennedy 2004). Other side effects of GnRH agonists are breakthrough bleeding, mild breast swelling or tenderness, mild depression, and headaches. These drugs may also cause disordered eating, stress fractures, osteoporosis, and a potential increase in the risk of premature cardiovascular disease. All GnRH analogues are contraindicated in pregnancy.

Disadvantages of Surgery

The disadvantage with laparoscopy is that not all endometriosis can be treated this way. There are risks involved in laparoscopic surgery and it may not cure the pain. Also

Hysterectomy and removal of endometriosis also have their own risks which are greater than those of laparoscopy. Hysterectomy removes fertility and some women grieve for uterus loss. If a total hysterectomy with bilateral oophorectomy is performed, it results in hypoestrogenism and associated symptoms may need Hormonal Replacement Therapy (HRT). Hysterectomy may at times not cure pain.

Furthermore, the emphasis on targeting the endometriotic lesions, by surgical removal or hypo-estrogen inactivation, does not necessarily correct the aberrant underlying molecular mechanism(s) of pathogenesis of endometriosis.

2.10. THIAZOLIDENEDIONES

Thiazolidenediones (TZDs) are synthetic compounds that are currently used for treatment of Diabetes type II. The drug is currently not used to treat endometriosis. TZDs act by binding to peroxisome proliferator-activated receptors (PPARs), a group of receptor molecules inside the cell nucleus, specifically *PPAR-γ* (gamma). The normal endogenous ligands for these receptors are free fatty acids (FFAs) and eicosanoids. TZDs have important and sustained effects on glycemic control.

PPAR-γ are expressed in the following organs; adipose tissue, liver, spleen, colon, adrenal gland, muscle tissue, macrophages and endometrial epithelial and stromal cells. Previous work has shown that the endometriotic lesions do express *PPAR-γ*. Since endometrial epithelial (including endometriosis) and stromal cells contain *PPAR-γ* (Pritts *et al*, 2002;

rosiglitazone, on a proof-of- principle study using the baboon model of endometriosis (Lebovic *et al.*, 2007).

2.10.1. Mode of action of Thiazolidenediones

PPAR-γ is a pleiotropic nuclear hormone receptor that binds to specific DNA response elements and may regulate the expression indirectly, negatively, or positively, through competition with other transcription factors. When activated, the receptor (PPAR) migrates to the DNA, activating transcription of a number of specific genes. TZDs agonists regulate adipocyte differentiation and glucose homeostasis among other actions through the *PPAR* (Hornung *et al.*, 2003). TZDs thus act as nuclear immune modulators.

Considerable evidence suggests that *PPAR-γ* ligands, such as TZDs, (1) are potent cell growth inhibitors (Yee *et al.*, 1999; Houston *et al.*, 2003); (2) are inducers of apoptosis (Yee *et al.*, 1999; Heaney *et al.*, 2003); (3) have anti-angiogenic effects mediated by diminished vascular endothelial growth factor production and (4) act through PPAR-gamma. TZDs inhibit proinflammatory cytokines (Pritts *et al.*, 2002; Wanichkul *et al.*, 2003) as well as NK-κß (Ricote *et al.*, 1998), an important nuclear transcription factor for the production of many cytokines (Lebovic *et al.*, 2001).

NK- κB appears to be activated in endometriotic cells and thus may play a central role in the pathophysiology of endometriosis (Guo 2006). Nuclear Factor-kappa beta (NK-κß) levels

immunomodulatory drug such as rosiglitazone to ameliorate the immunological dysfunction seen with endometriosis represents an alternate novel treatment. Several in vitro studies with thiazolidiones (TZDs) and endometriotic cells (Pritts *et al.*, 2002; Peeters *et al.*, 2005) led to their use in endometriosis animal models. There is evidence in the rat model of endometriosis that TZDs results in decreased induction of endometriosis lesions (Demirturk *et al.*, 2006) and in regression of established induced disease (Lebovic *et al.*, 2004; Aytan *et al.*, 2007).

2.10.2 Toxicology of rosiglitazone

Troglitazone (Rezulin®), the first TZD to be approved by Food, Drug Administration (FDA) in the US, proved to be hepatotoxic and was withdrawn from the market (Scheen 2001). Rosiglitazone (Avandia®) and pioglitazone (Actos®) are the other two TZDs still in the market. Although there are reports of clinical data that seem to exonerate rosiglitazone from the increased hepatotoxicity risk (Lebovitz *et al.*, 2002), cases of acute hepatotoxicity have been reported at pathology (Al-Salman *et al.*, 2000; Forman *et al.*, 2002).

The main side effect of all TZDs is fluid retention leading to edema, weight gain, and these potentially aggravate heart failure. (Avandia®) has been ordered in USA to have a strong warning on its label, advising users of an increased risk of heart failure (www.wikipedia. 12th November 2007).

2.10.3 Use of TZDs in experimental treatment of endometriosis

al., 2004; Demirturk *et al.*, 2006; Aytan *et al.*, 2007) and baboons (Lebovic *et al.*, 2007). In first experiment by Lebovic *et al.*, (2004), ciglitazone (a TZD) was first used in the rat model of endometriosis to evaluate a new approach to immunomodulation of endometriosis. Histological assessment showed maintenance of folliculogenesis and normal eutopic endometrial architecture in all animals. There was a significant decrease in the volume of ectopic uterine tissues and the mean explant wet weight, at a 52% reduction of volume and 70% lower weight, respectively. The ciglitazone -treated group showed marked epithelial regression and diminution of persisting epithelium score compared with the control group.

In the second experiment, by Demirturk *et al.*, (2006) it was shown that rosiglitazone interfered with the development of an endometriosis model in rats. In this experiment surgical induction of endometriosis was done and 0.2 mg/kg/d rosiglitazone was administered to the study group orally. Scoring systems were used to evaluate preservation of epithelia. The results showed that there was a significant difference in post-treatment spherical volumes and explant weights between control and rosiglitazone-treated groups. The epithelia were found to be preserved significantly better in the control group when compared with the rosiglitazone-treated group. Rosiglitazone was found to affect the induction of endometriosis negatively in the experimental rat model and it seemed to interfere with the growth and maintenance of the uterine explants.

In the third experiment by Aytan *et al.*, (2007), endometriosis was surgically induced in rats by transplanting an autologous fragment of endometrial tissue onto the inner surface of the

vehicle treatment was given to the control group. There was a significant difference in post-treatment length, width, height, and spherical volumes between control and rosiglitazone-treated groups. The epithelia were found to be significantly better preserved in the control group when compared with the rosiglitazone-treated group (Aytan *et al.*, 2007).

And finally this study was designed to further investigate and replicate these effects in a nonhuman primate model, which is phylogenetically closer to the humans and studies in this model are a requirement by law in most countries before potential human trials can be done.

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Animals

A total of 12 adult female baboons (*Papio anubis anubis*) weighing 9-15 kgs were used in this study. The animals were part of a large troop trapped in the wild and were kept in captivity at Institute of Primate Research, Nairobi, Kenya. A detailed capture technique has been described elsewhere (Muchemi and Yole 2008).

The animals were kept in quarantine for 3 months and were confined in single cages. During this period the animals were screened for TB (five times), de-wormed and any medical condition taken care of. They were bled and the blood stored in the serum bank, an aliquot was used to screen for SIV and STLV. From the quarantine, the animals were kept in group cages with males to enhance their cyclicity. They were kept for 9 months to 1 year, long enough for them to adapt to captivity and start cycling and menstruating normally. Capture stress tends to make the animals to stop cycling for some time. The animals were moved from the group cages to single cages for the duration of the study.

The choice of only 12 adult females baboons was determined on the basis of the following three methods for determining sample size; i) Previous experience or custom ii) Power analysis and iii) The "Resource equation" method. It takes huge samples to detect tiny

is a huge difference as seen in the previous rat experiments (Lebovic *et al.*, 2004). A replication of 4 times (animals) in a group should be able to gives us a scientifically significant effect that is also statistically significant.

3.1.2. Laboratory Equipment

The equipments used in this project were partly available at the Institute of Primate Research (IPR) and partly at the Department of Veterinary Pathology, Microbiology and Parasitology, University of Nairobi. Equipments include that for video laparoscopic surgery, histopathology processing, serum chemistry and hematology.

The Video laparoscopy equipment used was manufactured by Karl-Storz, Germany and is available at IPR.

For the laparoscopy viewing system, a Xenon light source, (STORZ KARL STORZ – ENDOSKOPE Model 201315 20, Tuttlingen, Germany) attached to an endoscope with a video camera (KARL STORZ –ENDOSKOPE Telecam SL Pal Camera 202120 20, Tuttlingen, Germany) was attached to a television monitor (SONY Trinitron Monitor, Japan). Each examination was video documented in a Video Cassette recorder, (SONY, Multisystem VCR, Model SLV- GA45AV, Japan). Once identified, the individual lesions were given an identification number, measured (width, length and depth) and photographed and the photos printed using a color printer (SONY Color Video Printer Model UP-2300P, Japan).

3.1.3. Drugs

The PPAR - γ agonist, Avandia® rosiglitazone, used in this study is manufactured by GlaxoSmithKline (Avandia, Research Triangle Park, North Carolina). The GnRH-antagonist, (Ganirelix®) Organon, Roseland, New Jersey), was used as an active comparator / positive control. The placebo tablets made of PBS (Phosphate Buffered Saline), were provided by Dr. Dan Lebovic, the University of Michigan Investigational Drug Services.

3.2. METHODS

3.2.1. CLINICAL PHASE PROCEDURES

3.2.1.1. Monitoring of Menstrual cycle

For each baboon, changes in the pattern of the menstrual and perineum skin cycle were carefully monitored during the study. Daily perineal inspection of each baboon allowed determination of the onset of perineal inflation (start of the perineal cycle, corresponding to the initiation of the follicular phase) and perineal deflation during the total duration of the study period. Perineal inflation is achieved when oestrogen levels in the body cause the soft tissue around the perineum to swell. As the levels of estrogens diminish, the tissue flattens in about 2 days. The flat perineum stays for about 7 to 10 days before the animal menses; after a further two days, the perineum tissue slowly starts to swell. The swelling increases slowly until it peaks at around day 19 to 21 of the cycle.

3.2.1.2 Induction Laparoscopy

Laparoscopy was conducted under a closed gaseous anaesthesia with the baboon in a dorsal

and to create a working space. For this reason, a Verres needle was used to make a puncture hole on abdominal skin near the umbilicus through which CO_2 gas under pressure was forced inside the abdomen. The abdominal pressure was maintained at an equivalent of 12 cm H_2O. The small puncture hole created by the Verres needle was enlarged and a 10mm trocar and cannula was pushed into the abdominal cavity. The purpose of the trocar and cannula was to create a 10 mm diameter opening for inserting the video camera which was attached on the endoscope. To achieve this, the cannula was removed and the endoscope with light source and camera was inserted through the trocar. Two other 5 mm secondary cannula and trocar were introduced laterally to the linea alba, a few centimetres caudal to the first opening, to create ports used for manipulations. The internal abdominal organs were then inspected and examined for any spontaneous endometriosis.

On the first or second day after onset of menses, endometrial tissue was extracted from each baboon by uterine curettage and suction using Novaks Curettes attached to a 10 ml syringe. The curettes were inserted from the perineum end, through the vagina, the external cervical opening into the uterus. The collected menstrual flow was fragmented through an 18-gauge needle. Menstrual flow used for induction of endometriosis was collected and any amount over 1 gram was seeded into the pelvic cavity using a standardized method (D'Hooghe *et al.*, 1994a). The menstrual paste was autologously seeded onto various peritoneal sites namely; uterosacral ligaments, uterovesical fold, pouch of Douglas, ovaries, and ovarian fossae (D'Hooghe *et al.*, 1995). The seeding sites were carefully recorded on individual pelvic map and video documented. It is known that there is a positive correlation between the amounts

1 gram may lead to induction of no lesions due to clearance by the animal's immune system (D'Hooghe *et al.*, 1994a).

3.2.1.3 Staging pre-treatment laparoscopy

A second video laparoscopy was performed 52.3 ± 12.3 days (mean ± SD; range, 34 – 68 days) after the endometriosis induction laparoscopy. Staging laparoscopy was done during luteal phase, 5 – 6 days post perineal skin deflation. The number and surface area of the endometriotic lesions were documented and the presence, localization, and extent of adhesion were determined during the staging laparoscopy.

In the process each lesion was photographed, identified with a number (e.g. 2998N1; 2998N2; 2998N3 etc), their sizes noted, video recorded and indicated on the pelvic map (Appendix 2). The surface area (mm^2) of endometriotic lesion and or presence of an endometriotic-related adhesion was determined. The type of endometriotic lesion was also recorded (See nomenclature in Appendix 2).

3.2.1.4. Drug treatments

The 12 baboons were randomly divided into three groups, the placebo, positive control and rosiglitazone treatment group (Table 1). The treatment schedule was as follows: group I (n = 4) were given a placebo tablet orally (per os) daily using a banana as a vehicle; group II (n=4) were injected a GnRH antagonist, (Ganirelix®) 125 μg subcutaneous (sc) daily, and

The mean baboon weight at the time of treatment was 13.3 kg. An equivalent dose of rosiglitazone in a 65 kg woman would be 9.92 mg daily of rosiglitazone. The current Food and Drug Administration (FDA) approved dose of rosiglitazone in women is a maximum of 8 mg daily. Because of uncertain absorption after oral administration of rosiglitazone hidden in a banana, in this study, the dose of 2mg daily is within the FDA – approved range of 4- 8 mg daily in women (since the baboons weighed 13.3 kgs).

In Group 3, one half the recommended human dose of Ganirelix® was used despite the baboon weighing roughly one-fifth of the weight of an average woman (the absorption of drugs given subcutaneously in baboons can be variable). All subcutaneous injections were given using an animal squeeze cage-mechanism.

Table 1: The treatment groups, drugs and routes of administration of rosiglitazone and Ganirelix(R) in endometriotic baboons.

Treatment Group	Animal Number	Treatment	Dose-Frequency
Placebo I	PAN 2991 PAN 2985 PAN 2631 PAN 2912	Phosphate Buffered Saline (PBS) tablets (Negative control)	Daily per os in banana
GnRH II	PAN 3014 PAN 3036 PAN 2871 PAN 2993	Ganirelix® GnRH-antagonist (Positive control)	125 mcg sc daily
Rosiglitazone III	PAN 3030 PAN 2998 PAN 3032 PAN 2788	Avandia (R) Rosiglitazone	2 mg per os in banana

3.2.1.5. Post-treatment staging laparoscopy.

The third (and last) laparoscopy was done on or about day 25 after the start of treatment (following the second laparoscopy). Pelvic inspection and documentation was done as described for the second laparoscopy. The main task was to identify and document the lesion as in the second laparoscopy. The type of lesion was recorded and categorised as described by (D'Hooghe *et al.*, 1995). The number, size and surface area (mm^2) of endometriotic lesions was recorded on the pelvic map. Video recording was done and photographs were taken as described in Section 3.2.1.3. The changes between the second pre-treatment laparoscopy and the third post-treatment laparoscopy were then analysed and compared.

3.2.1.6 Post mortem procedure

After the third laparoscopy, all animals were euthanised and subjected to a full post-mortem examination. Euthanasia was achieved by administration of Sodium pentobarbitone (Euthanaze®, CENTAUR LABS, Johannesburg, South Africa) intravenously at a dose of 60 mg/kg body weight.

The carcass was placed in a supine (dorsal) position and a midline skin-deep incision was made from the ventral region of the mandible down the midline of the neck, thorax and abdominal through the *linea alba* to the pelvic symphysis. Another skin-deep incision was made from the midline of the carcass, at the level of the clavicle bone down the fore limbs to the wrists for both arms. The same lines were done for the hind limbs from the pelvic symphysis at the femoral area of the hind limbs to the pyrdial tarsal joints. The skin was

carcass. The neck arteries and femoral arteries were cut so as to bleed the animal as much as possible to minimise the interference of blood with the visualization of endometriosis lesions in the abdominal cavity once the animal was opened.

At post-mortem, the pelvic maps and photographs that were taken during the final laparoscopy were placed in an orderly manner on a table next to the post mortem table. Before removing the endometriosis lesions, the maps and photographs were carefully studied to get their proper anatomic positions before the abdominal cavity was opened. The lesions were then collected according to their identification numbers in an orderly manner starting with lesion number 1 to the last one. Once a lesion was identified it was placed in a (Nunc™) vial containing 10% neutral buffered formalin that was then labelled with animal number, lesion number and date. New lesions that were identified in other parts of the abdominal cavity especially on other sites like descending colon, omentum were given new numbers and documented as new lesions. After all lesions were harvested into formalin, a complete necropsy was done as briefly described below:

The omentum was carefully removed by incising its attachment to the mesenteric side of the large and small intestines. The small intestine were double ligated at the pyloric end and cut at between the ligatures. The rectum also was double ligated and cut between the ligatures. Then the small and large intestines were removed by cutting through their dorsal attachments. The adrenals were identified and removed. The liver was then removed by cutting through the hepatic ligament and then carefully separated from the diaphragm, noting any gross

examined for adhesion of capsule to the kidneys. The stomach was ligated twice at the oesophageal end and cut at the middle of the ligatures.

The tongue was removed by cutting the inside of the medial mandible bones and pulled out. Two parallel incisions were done on both sides of the tongue and oesophagus up to the thoracic inlet. Then the thorax was opened by cutting through the intercostals muscles first, to expose the ribs, which were then cut with a (Stryker®) electric saw. The tongue, trachea, oesophagus and lungs were pulled out and carefully separated using scissors and placed on a dissecting board for examination. All organs were then examined carefully for any lesions.

Representative organ and lesion samples were fixed in neutral buffered 10 % formalin for histopathology.

3.2.2 Serum chemistry procedures

A toxicological investigation of the side effects of the drug was done through analysis of blood collected for haematology and serum for biochemical tests (mainly for liver functions tests). The liver function tests included assays of total proteins, alkaline phosphatase (ALK) , Aspartate aminotransferase (AST/SGOT) and Bilirubin. Kidney function was tested using Creatinine and Urea assays. All the tests were done using the wet serum chemistry method. A Spectrophotometer, CECIL Elegant Technology- CE 6606 Superscan Graphic Plotter 6000 Series Multimode Computing UV was used at IPR.

3.2.2.1 Total protein determination

Method (Human Laboratories Ltd, Wiesbaden, Germany). The test was based on the principle that cupric ions form a purple complex after reacting with protein in an alkaline solution. The absorbance of this complex was then measured using a spectrophotometer. In this test, the absorbance of the purple complex is proportional to the amount of protein.

A blank was prepared by using 1000 microliter of a mixture of Sodium hydroxide 200mmol/L, Potassium sodium tartrate 32 mmol/L, Copper sulfate 12 mmol/L and Potassium iodide 30 mmol/L. This was used to get the blank reading on the spectrophotometer set at a wavelength of 540 nm.

Thereafter the pipetting scheme involved taking 20 microlitre of sample or standard into 1000 microlitre of the blank. Each test tube was labelled appropriately. These were mixed, incubated for 10 minutes at room temperature. Then the absorbance of the samples and Standard (which contained protein 8 g/dl and Sodium azide 0.095%) were measured within 30 minutes.

The calculation of the protein concentration was done using the following formula:

Concentration of Total Protein = 8 x (Absorbance of sample / Absorbance of Standard).

Where 8 is a multiplication factor given by the manufacturer and correspond to the concentration of the protein in the standard.

3.2.2.2. Bilirubin determination

Germany. The trade name for Direct (D) and Total (T) Bilirubin- Modified Jendrassik/Grof Method (Mori 1978).

The principle behind the test is that bilirubin reacts with diazotised sulphanilic acid (DSA) to form a red azo dye. The absorbance of this dye at 546 nm is directly proportional to the bilirubin concentration in the sample. The water-soluble bilirubin glucuronide reacts directly with DSA whereas the albumin - conjugated indirect bilirubin will only react with DSA in the presence of an accelerator. Therefore the total bilirubin was the summation of the direct bilirubin and indirect bilirubin.

In summary the reaction is as follows;

1) Sulphalinic acid + sodium nitrite forms DSA.

2) Bilirubin + DSA gives absorbance readings for DIRECT Azobilirubin.

3) Bilirubin + DSA + accelerator gives absorbance readings for TOTAL Azobilirubin.

The reagents were mixed according to the instructions from the manufacturer of the kit. There were two sets of tests each with a different procedure 1) Total Bilirubin and 2) Direct Bilirubin. The Total Bilirubin test was run first, followed by Direct Bilirubin where Total Bilirubin results showed abnormality.

1) Total Bilirubin

A blank test tube was used as a standard for zeroing the spectrophotometer . The blank was made of a mixture of serum and contained Sodium nitrite 390 mmol/L . The wavelength was set at 546 on the spectrophotometer.

One ml of the mixture which contained Sulphanilic acid 14 mmol/L, Hydrochloric acid 300mmol/L, Caffeine (accelerator) 200 mmol/L and Sodium benzoate 420 mmol/L) was put in each of the differently labelled test tube. A drop (40 µl) of Sodium nitrite 390 mmol/L was then added to the above test tubes and mixed thoroughly with a mixer (Super-Mixer, LAB-LINE Instrument, Inc, Melrose Park, Illinois). The test tubes were incubated for 5 minutes at room temperature, and then 100 µl of each of the samples were added into the respective test tubes. These were mixed and incubated at room temperature for 10 to 30 minutes. The absorbency for each test tube was then measured against the blank.

2) Direct Bilirubin

The determination of Direct Bilirubin was done to the samples which were found to have Total bilirubin. The procedure was as follows: One ml of a mixture of Sulphanilic acid 14 mmol/L and Hydrochloric acid 300 mmol/L was put into each labeled test tube. One-drop (40 µl) of Sodium nitrite 25 mmol/L) was added to the above test tubes and mixed thoroughly and 100 µl of the sample added within 2 minutes. This was again mixed and incubated at room temperature for exactly 5 minutes. The absorbance was then measured against the sample blank.

The calculation of the concentration of total and direct bilirubin was obtained using the formula:

Bilirubin concentration [mg/dl] = [Absorbance reading] x 13.0

3.2.2.3. Alkaline phosphatase determination.

The determination of alkaline phosphatase in serum was based on a commercial diagnostics kit called "ALKALINE PHOSPHATASE liquicolor" DBA Buffer, DGKC. The kit was manufactured by Human Laboratories Ltd, Wiesbaden, Germany. It is both a colorimetric kit, (i.e. based on determination of colour change) and kinetic kit.

The Reaction Principle for the Test is that, p-Nitrophenylphosphate $+H_20 \Leftrightarrow$ phosphate + p-nitrophenol (yellow coloured). The enzyme involved in this reaction is Alkaline Phosphatase.

The working reagent was made by pipetting 2 ml of substrate (containing p-Nitrophenyl phosphate 55 mmol/l) into one bottle containing Diethanolamine buffer (pH 10.35 ± 0.2) 1.25 mol/l and Magnesium chloride 0.625 mmol/l) and mixed thoroughly. These were dispensed at 1 ml into test tubes, and then 100 µl of the sample was added. This was mixed, and the absorbance read at wavelength of 410 nm after 1 minute and at the same time the stopwatch was started. Four readings were taken after 1, 2, 3 and 4 minutes. The mixture turned from clear to yellow with time since it was a kinetic kit.

From the readings the mean absorbance change per minute (ΔA/min.) was calculated. The calculation of the alkaline phosphatase activity in the sample was done using the following factor:

3.2.2.4 Creatinine determination

The determination of creatinine was done using a commercial diagnostics kit called "CREATININE liquicolor, Jaffe Reaction". The kit used was acquired from Human Laboratories Ltd, Wiesbaden, Germany. It is a photometric colorimetric test for kinetic measurements of the reaction principle as Creatinine + Picric acid → Creatinine-picrate complex. According to the manufacturer's instruction, the method is done without deproteinisation.

Creatinine forms an orange-red coloured complex with picric acid in alkaline solution. The absorbance of this complex is proportional to the creatinine concentration in the sample.

The Sodium Hydrochloride (1.6 mol/l) in the kit was diluted with distilled water in the ratio 1+4 (i.e. 4 mls plus 16 mls of distilled water) and stored in a plastic bottle. The diluted Sodium Hydrochloride was mixed with the Picric Acid (26 mmol/l) to produce a working reagent in the ratio 1 to 1. The standard was made of Creatinine 2 mg/dl or 176.8 umol. The wavelength on the spectrophotometer was set at 490 nm.

A volume of 1000 ul of the Standard working reagent was pipetted into cuvettes and used to get the blank reading. Thereafter, 100 ul of samples were mixed with similar volumes of Standard working reagent. After 30 sec. the absorbance Δ_1 was read. Then the second absorbance Δ_2 was read exactly after 2 min. The difference between the two readings, $\Delta_2 - \Delta_1$ = change of absorbance of sample .

The calculation of serum creatinine was done using the following formula:

(Difference between 1st and 2nd absorbance of Sample ÷ Difference between 1st and 2nd absorbance of Standard) X 2.0 = Creatinine [mg/dl]

Where 2.0 is a multiplication factor given by manufacturer.

3.2.2.5. Aspartate Aminotransferase (AST/SGOT) determination

Aspartate transaminase (AST) also called serum glutamic oxaloacetic transaminase (SGOT) or aspartate aminotransferase (ASAT/AAT). The test was done based on a commercial diagnostics kit called **"GOT (AST) IFCC modified liquiUV Test"** Aspartate Aminotransferase (EC 2.6.1.1). The kit used was manufactured by Human Laboratories Ltd, Wiesbaden, Germany.

The reaction is as follows:

2-Oxoglutarate+L-aspartate ⇔ (Aspartate transaminase) ⇔ L-glutamate+ oxaloacetate

Oxaloacetate + NADH + H^+ ⇔ (Malate dehydrogenase) ⇔ L-malate + NAD^+

The test is a kinetic method for determination of ASAT activity.

The entire contents of one of the bottles of the substrate, which contained 2-oxoglutarate 60 mmol/l and NADH 0.9 mmol/l, were poured into one of the other bottle provided containing the enzyme for the reaction, which was made of TRIS buffer (pH 7.8) 100 mmol/l, L-aspartate 300 mmol/l, LDH >0.9 kU/l and Malate dehydrogenase > 0.6 kU/l, and mixed thoroughly.

The wavelength was set at 340 nm. 200 µl of the sample and 1000 µl of working reagent were pipetted into cuvettes. This was mixed and the absorbance read after 1 minute, further reading of the absorbance was done again exactly after 1, 2 and 3 minutes.

Concentration of AST was determined by multiplying the change of absorbance per minute by 1151 = U/l.

3.2.2.6. Albumin determination

The test was based on a commercial diagnostics kit called "**Albumin liquicolor Photometric Colorimetric Test for Albumin BCG- Method**". The kit used was acquired from Human Laboratories Ltd, Wiesbaden, Germany. The test is based on the fact that Bromocresol green forms a coloured complex with albumin in citrate buffer. The absorbance of this complex is proportional to the albumin concentration in the sample.

A standard first reading was acquired and recorded. The reagent blank consisted of 1000 µl of a mixture of Citrate buffer (ph 4.2) 30 mmol/L and Bromocresol green 200 µmol/l). 10 µl of sample or working standard made of Albumin 4 gm/dl or 40 g/l and Sodium azide 0.095 %) was added to 1000 µl of the mixture of Citrate buffer (ph 4.2) 30 mmol/L and Bromocresol green 200 µmol/l. This was mixed; incubate for 5 minutes at room temperature 20 to 25^{O}C. The absorbance of the sample and the standard was measured against the reagent blank within 30 minutes. The spectrophotometer was set at a 578.0 wavelength.

Calculation of the albumin concentration was determined by the following formula:

3.2.2.7. Urea determination

The test was based on a commercial diagnostics kit called **"UREA liquicolor, Enzymatic Colorimetric Test for Urea"**. The kit used was acquired from Human Laboratories Ltd, Wiesbaden, Germany.

Urea hydrolysed in the presence of water and urease to produce ammonia and carbon dioxide. In a modified Berthelot reaction the ammonium ions react with hypochlorite and salicylate to form a green dye. The absorbance increase at 578 nm is proportional to the urea concentration in the sample.

The "Enzyme reagent 1a " was prepared by mixing the contents of the bottle labelled [ENZ] (which was made of Urease > 500 KU/l) with bottle labelled [RGT1](which was made of Phosphate buffer (pH 7.0) 120 mmol/l, Sodium salicylate 60 mmol/l, Sodium nitroprusside 5 mmol/l and EDTA 1 mmol/l). This was mixed at a ratio of e.g. 1 ml of [ENZ] with 100 ml [RTG1]. This mixture was named "Enzyme reagent 1a".

The other reagents, [RGT2] (which was made of Phosphate buffer (pH < 13) 120 mmol/l, Hypochlorite 0.6 g/l) and [STD] (which was made of Urea 80 mg/dl or 13.3 mmol/l, equivalent to BUN 37.28 mg/dl or 6.2 mmol/l, and Sodium azide 0.095%) were ready for use and did not need preparation.

The reagent used as the blank was made of 1000 µl of "Enzyme reagent 1a" which was prepared as described above. The wavelength was set at 580 nm. A 10 µl of sample or

to 25°C. This turned to a greenish colour. The absorbance was read for the sample and the standard against the reagent blank within 60 minutes.

Concentration of blood Urea = $\frac{\text{Absorbance of sample}}{\text{Absorbance of Standard}}$ x 80 mg/dl

3.2.3. Haematology

Complete haemograms were done on the EDTA blood samples. The parameters taken were white blood cells (WBC), Red blood cells (RBC), Haemoglobin (HB), Packed cell volume (PCV), Mean Corpuscular volume (MCV), Mean cell haemoglobin concentration (MCHC), Mean cell haemoglobin (MCH), Total plasma, plasma fibrinogen, Band neutrophils, segmented neutrophils, lymphocytes, monocytes and eosinophils. The hematology data collected was summarized in Appendix 8.

3.2.4. Histopathology procedures

Many tissues were collected for histopathology. These included adrenal glands, colon, heart, ileum, jejunum, kidneys, liver, ovaries, pancreas, spleen, uterus (Fundus, horn and cervix) and all gross lesions. Only small sections (1 cm^3) was randomly cut from the larger organs like the liver, lungs and spleen; for the small organs like the heart, adrenal glands, kidneys, ovaries, and uterus, they were collected whole. The tissues were immersed and stored in 10% neutral buffered formalin in separate specimen jars for each animal and labelled. Tissues from endometriosis lesions (Table 7) were fixed in a smaller (Nunc®) 2 ml tubes.

sections other the organs were held in neutral buffered formalin for 48 – 36 hours after fixation. They were then transferred into 4 % neutral buffered formalin until they were processed for histopathology. The tissues were then processed through routine histopathology procedure (Humason 1962; Luna 1968). Sectioning was done at 5 µm thickness. Serial sections across the endometriosis lesions were done and about five sections mounted per slide. The approach was to completely section the lesion until there was no tissue left on the paraffin block. Depending on the size of the block, a tissue section was selected for staining after about 10 to 20 cuts to produce about 5 slides per endometriotic lesion. Representative tissues were sectioned from the other organs with or without lesions.

The cut tissue sections were spread on slides which were earlier pre-coated with egg albumin. Following section mounting, the slides were placed on a vertical rack and allowed to drain for at least one minute. The slides were then dried in a 60^0C dust free oven for 30 -60 minutes or overnight at 37°C. For staining purposes, the slides were then placed for 5 minutes in xylene to dewax/deparaffinize. To prepare the slides for staining, they were first re-hydrated by rinsing for 5 minutes respectively in 100% isopropanol, 96% industrial spirit and water. After rehydration, the slides were then transferred into haematoxylin for 5-8-10 minutes. For purposes of bluing, they were put in water for 1 minute then dipped twice in 5% Acid alcohol while closely monitoring the diminishing of the blue colour. A water wash for 5 minutes followed. Eosin counterstaining was done for 4 minutes. The slides were then dipped into water while checking the contrast between haemaoxylin and eosin. The slides were then prepared for mounting by first dehydrating then in 96% industrial alcohol (by dipping 3

100% isopropanol. The slides were ready for mounting, and DPX mounting media was used to secure the cover-slip to slide.

3.2.5. Scoring of endometriosis lesions

The lesions were scored microscopically in order to measure and monitor the effects of the drug on endometriosis. A Scoring method was developed after trial runs through the endometriosis slides. The trial run identified all the histological lesions. A subjective scoring of 0 to 4 was created. This designated the scores according to the intensity, severity and quantitative findings. In this effort a subjective and semi-quantitative basis of scoring was developed but there was no blinding to the type of treatment the baboon received had received. The scores were converted to numerical mean values which were used in statistical analysis using Kruskal Wallis test. The frequency of appearance of the histological changes were analysed using the CHI Square Fisher's Exact test.

3.2.5.1. Examination of the glandular epithelium features

The presence of haemorrhages in the lumen of glands or periglandular haemorrhages was recorded. The presence of hemosiderin in the lumen of glands or periglandular area and the presence of mucus in the lumen of glands was also noted.

The number of cross sections of glands was also scored, and any degenerative or necrotic changes on the glandular epithelium recorded. Erosions of the glandular epithelium were noted. The presence of acute inflammatory cells like neutrophils, eosinophils in the lumen of

inflammatory cells, such as lymphocytes in these areas were also noted. The checklist designed and used for scoring of lesion in glandular epithelium is attached in Appendix 4.

3.2.5.2. Examination of the interstitial tissue

The interstitial tissue is defined as all the tissue apart from the glands, endometrial stromal cells and peritoneum that is seen in each lesion. Usually the interstitium contains supporting structures such as connective tissue, blood vessels, smooth muscles etc.

The histological changes that were monitored in interstitial tissue included haemorrhages, haematomas and hemosiderin and other deposits. The presence of unique degenerative slits was noted and scored. These degenerative slits were defined as the presence of gaps or empty space, mainly narrow, long and spindle shaped following the grain of the connective tissue in interstitial tissues. These were associated with shrinking of interstitium after degeneration of stromal cells. Degeneration was more visible on the edges of the slits. Thus, presence of degeneration of interstitial endometriotic stromal cells (ESCs) was noted and scored. The presence of sworls, formation of arcs in the connective tissue was noted and scored as were the presence of acute inflammatory cells.

Presence of a diffuse ground substance (myxoid) in the connective tissue was noted and scored. Smooth muscle, the numbers of small or large blood vessel were also noted and scored. Other features like fat, oedema, amount of periglandular cuffing with endometriotic stromal cells (ESCs) were examined. The scatter and spread of ESCs was also scored as was hemosiderin and degenerated ESCs. The checklist designed and used for scoring of lesions

3.2.5.3. Examination of the peritoneal surface of lesions

In the biopsies where the peritoneum surface was visible, the same changes as seen in the glands and interstitial connective tissue were examined. Thus, the presence of haemorrhages, hemosiderin, lymphocytes, acute inflammatory cells, fibrin on and under the peritoneal surface were recorded. The above information was used to compare the features within the types of lesions and between the types of treatments. The checklist designed and used for scoring of peritoneal surface lesions is attached in Appendix 6.

3.3. STATISTICAL ANALYSIS

The data was managed through collection of the raw data in tabular format followed by systematic analysis and checking for any significant changes. The tests used for significance tests were the Paired Student t-tests within each group (to test the difference before and after treatment); the Univariate analysis of variance (ANOVA) followed by post hoc analyses like Tukey's or Least Significant Difference (LSD) (to test how they differ between the groups), and finally, the CHI Squared, Fisher's Exact test for frequencies of occurrence of changes in tissues.

The following parameters were subjected to the statistical tests;

1) Changes in surface area of lesions within animals and between groups in respect to changes before and after the treatments.

2) Changes in the types of lesions (red, typical, white fibrotic plaques or nodules, etc).

and then compared among the three treatment groups. This was done using the Kruskal Wallis One – way ANOVA test by ranks.

3) Haematological changes before and after treatment.

4) Clinical chemistry changes before and after treatment.

5) Scoring of histopathology findings of endometriotic lesions.

6) Histology changes in other body organs.

A relative change value was calculated for the surface area of each lesion using the following equation:

Surface area after treatment (laparoscopy 3) minus Surface area before (laparoscopy 2) divided by Surface area before (laparoscopy 2).

A computer package (INSTAT 3.42, Reading, England) was used to analyse the data. At first the One-way ANOVA was used to compare the means. The tabulated F- tabulated at α = 0.05 at degrees of freedom V1 = 2 and V2 = 9 was 2.785. The Chi Squared, Fisher's Exact test was performed using GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com. Copyright 1992-1998 GraphPad Software Inc. (www.graphpad.com).

CHAPTER FOUR

4.0. RESULTS

4.1. ENDOMETRIOSIS LESIONS

4.1.1. Gross appearance of endometriosis lesions assessed during laparoscopy

The presence of endometriosis was confirmed in all baboons. Representative *in vivo* laparoscopic images showing the macroscopic changes in the placebo, GnRH antagonist and rosiglitazone treated lesions are shown in Plates 1 to 9. Out of the several lesions in each animal and each treatment group, only a few were selected. In the placebo group, 3 out of 11 lesions are represented in Plate 1, 2 and 3 (PAN 2985, PAN 2912 and PAN 2991). In the GnRH group, 3 out of the 19 lesions are represented in Plate 4, 5 to 6 (two from PAN 2871 and one from PAN 2993). Finally from rosiglitazone group, 3 out of 18 lesions are represented in Plate 7, 8 to 9 (one from PAN 3032 and two PAN 3030). The selected lesions represent the trend of changes in lesions from pre-treatment to post-treatment for each treatment group.

4.1.1.1 Placebo group.

In the placebo group, some of the lesions progressed to more active lesions and some remained in the same status after the 25 days of treatment. A few also turned to the typical bluish-black lesions which is called typical because in women it is the commonly seen type at

(PAN 2985), which was initially a red hemorrhagic (Plate 1A) and remained the same after placebo treatment (Plate 1B). While at the same time, some lesions appeared typical bluish-black lesions in the placebo group at laparoscopy II and remained as such in laparoscopy III. Plate 2 shows endometriotic lesion N5 from a placebo animal (PAN 2912), which typical bluish black (Plate 2A), and did not change after placebo treatment (Plate 2B). Also the same was true in Plate 3, where the red lesion maintained the status quo. The endometriotic lesion N2 from PAN 2991, which was initially a red hemorrhagic vesicular one (Plate 3A), did not change its morphology after placebo treatment (Plate 3B).

Table 2, 3 and Figure 1 shows the increase in number of both typical and red lesions when compared between the pre and post treatment stage within the placebo group. Though the change was not significant, the trend was apparent where more active lesions appeared.

4.1.1.2 GnRH group

After 25 days of treatment, most lesions in the GnRH antagonist treated group appeared to change towards less active lesions i.e. typical bluish black or white lesions in a majority of cases. Plate 4 shows an endometriotic lesion (N5) from PAN 2871 which was initially a bluish-black lesion (Plate 4A) that changed to a white nodule after GnRH antagonist treatment (Plate 4B). Some lesions turned from red (active) to white nodules (inactive). This is exemplified by endometriotic lesion (N7), from PAN 2871, which was initially a gelatinous red hemorrhagic lesions (Plate 5A), which turned to a white nodule with pigmented spots after GnRH antagonist treatment (Plate 5B). And finally, Plate 6 shows

lesion (N11), from PAN 2993 which was initially a red nodule (Plate 6A) and changed to a pale orange yellow nodule after GnRH antagonist treatment (Plate 6B).

Table 2, 3 and Figure 1 show the increase in the number of typical lesions within the GnRH antagonist treated group when compared between the pre-treatment and post-treatment stage. Though the changes were not significant, the trend was clear where less active lesions appeared to increase at post treatment.

4.1.1.3 Rosiglitazone group.

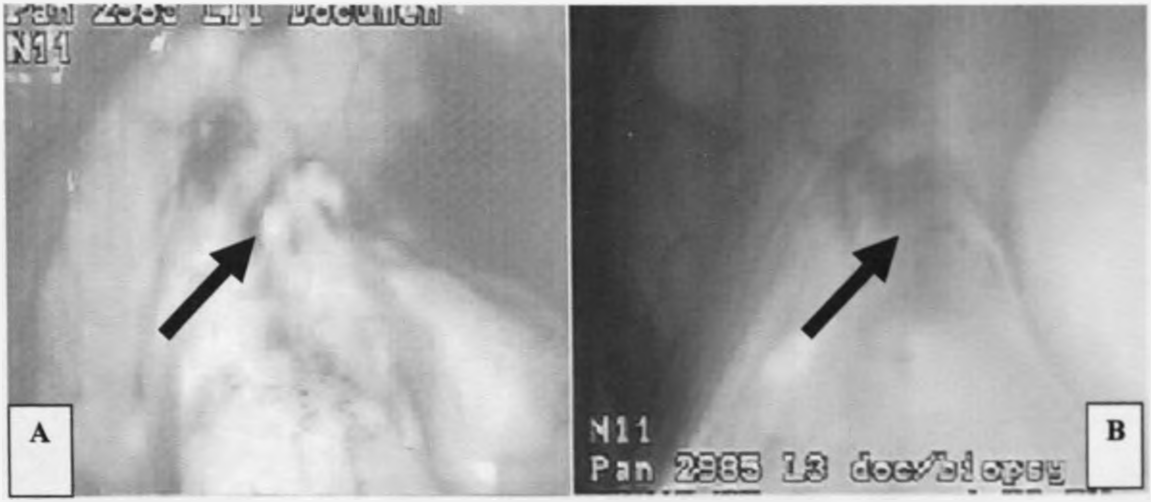
After 25 days of treatment, most lesions in the rosiglitazone group (like in GnRH antagonist group) appeared to change towards less active lesions i.e. typical bluish black or white lesions in a majority of cases. Examples of this transition are shown in Plate 7. The Plate shows an endometriotic lesion (N4) from PAN 3032 that was a red-white nodule (Plate 7A) that changed to a white plaque after rosiglitazone treatment (Plate7B). And also in Plate 8, it shows an endometriotic lesion (N8) from PAN 3030, which was red white hemorrhagic nodule (Plate 8A) that turned to a typical bluish black lesion after rosiglitazone treatment (Plate 8B). And finally Plate 9 shows an endometriotic lesion (N9) from PAN 3030 which was a red haemorrhagic nodule (Plate 9A) that turned to a white nodule after rosiglitazone treatment (Plate 9B).

From the results, the following statements can be made. The rosiglitazone and GnRH [illegible] showed healing or improvement of endometriotic lesions, as evidenced by

8 and 9). In these cases, the lesions mostly turned from red (active) to white nodule or typical types which are considered less active.

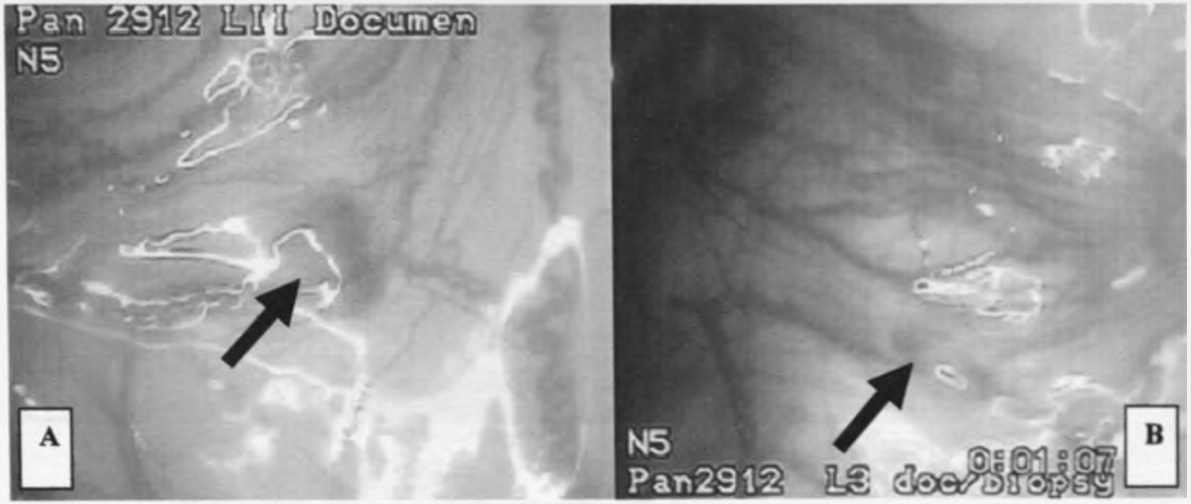
An analysis of the distribution of types of endometriosis lesions among the 3 treatment groups showed a general increase in the number of typical bluish black (less active) lesions in all the treatment groups (Table 2, 3 and Figure1). However, this increase in the less active lesion was more significant in rosiglitazone group than in both GnRH and placebo group.

Plate 1. Gross appearance of endometriotic lesion (N11) from a placebo treated baboon (PAN 2985)



Key:		
	Arrows	Indicate location of lesion.
	PAN 2985	Animal identification number.
	N11	Identification number of endometriosis lesion
	LII	Photo taken during second laparoscopy documentation
	L3	Photo taken during third laparoscopy documentation

Plate 2. Gross appearance of endometriotic lesion (N5) from a placebo treated baboon (PAN 2912)

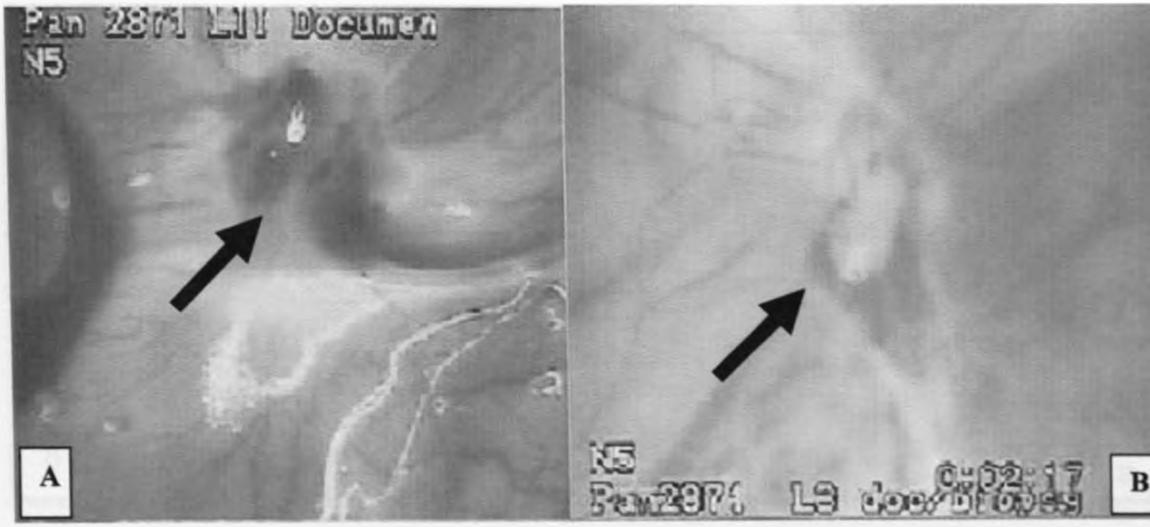


Key:		
	Arrows	Indicate location of lesion.
	PAN 2912	Animal identification number.
	N5	Identification number of endometriosis lesion
	LII	Photo taken during second laparoscopy documentation
	L3	Photo taken during third laparoscopy documentation

Plate 3: Gross appearance of endometriotic lesion (N2) from a placebo treated baboon (PAN 2991)

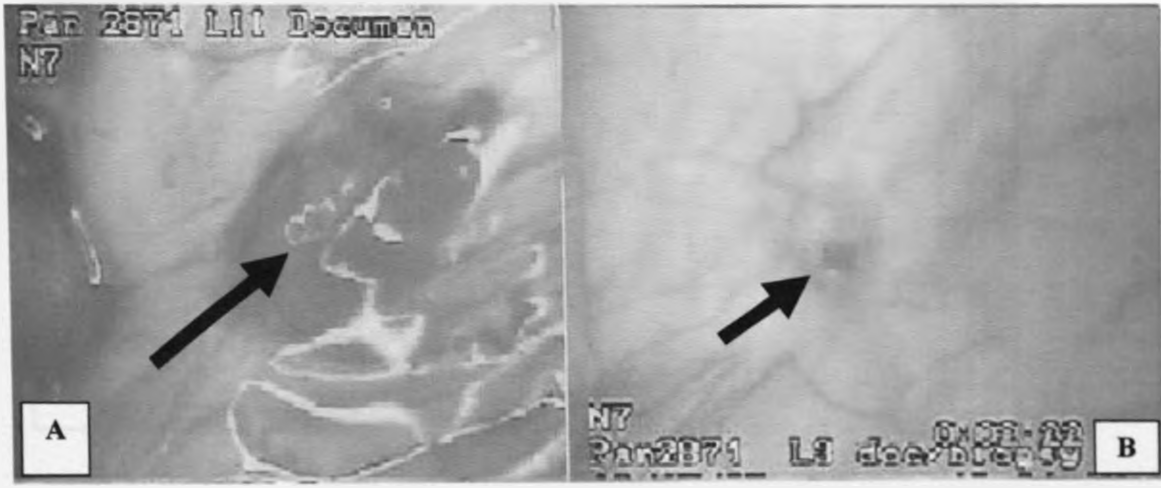
Key:	Arrows	Indicate location of lesion.
	PAN 2991	Animal identification number.
	N2	Identification number of endometriosis lesion
	LII	Photo taken during second laparoscopy documentation
	L3	Photo taken during third laparoscopy documentation

Plate 4: Gross appearance of endometriotic lesion (N5) from a GnRH treated baboon lesion (PAN



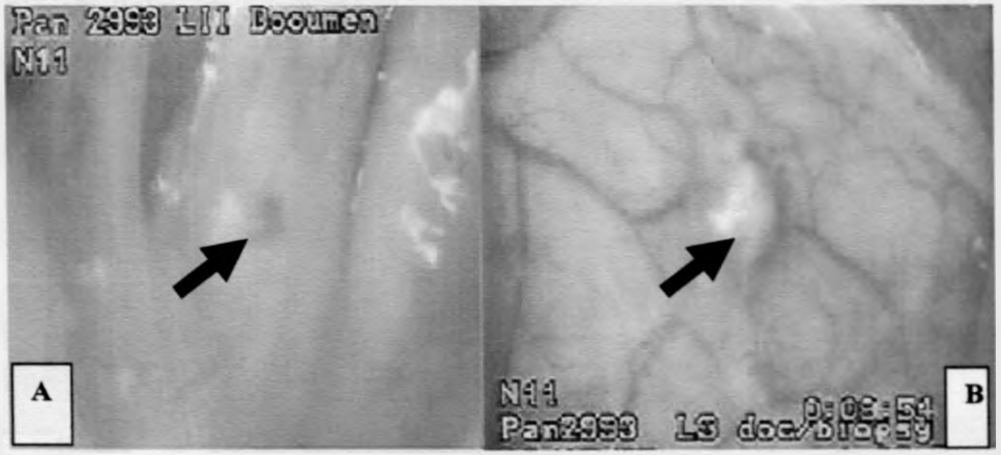
Key:		
	Arrows	Indicate location of lesion.
	PAN 2871	Animal identification number.
	N5	Identification number of endometriosis lesion
	LII	Photo taken during second laparoscopy documentation
	L3	Photo taken during third laparoscopy documentation

Plate 5: Gross appearance of endometriotic lesion (N7) from a GnRH treated baboon (PAN 2871)



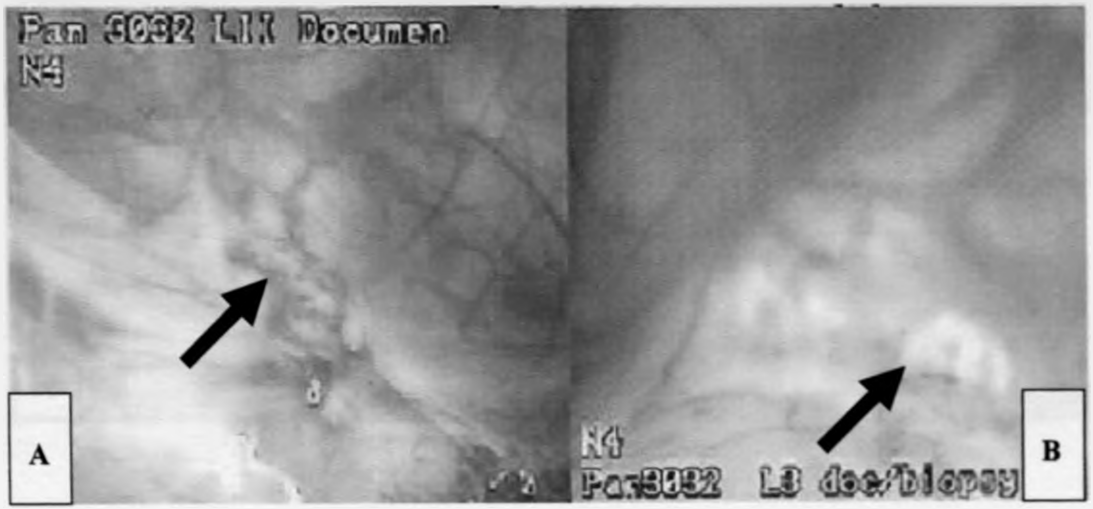
Key:		
	Arrows	Indicate location of lesion.
	PAN 2871	Animal identification number.
	N7	Identification number of endometriosis lesion
	LII	Photo taken during second laparoscopy documentation
	L3	Photo taken during third laparoscopy documentation

Plate 6: Gross appearance of endometriotic lesion (N11) from a GnRH treated baboon (PAN 2993)



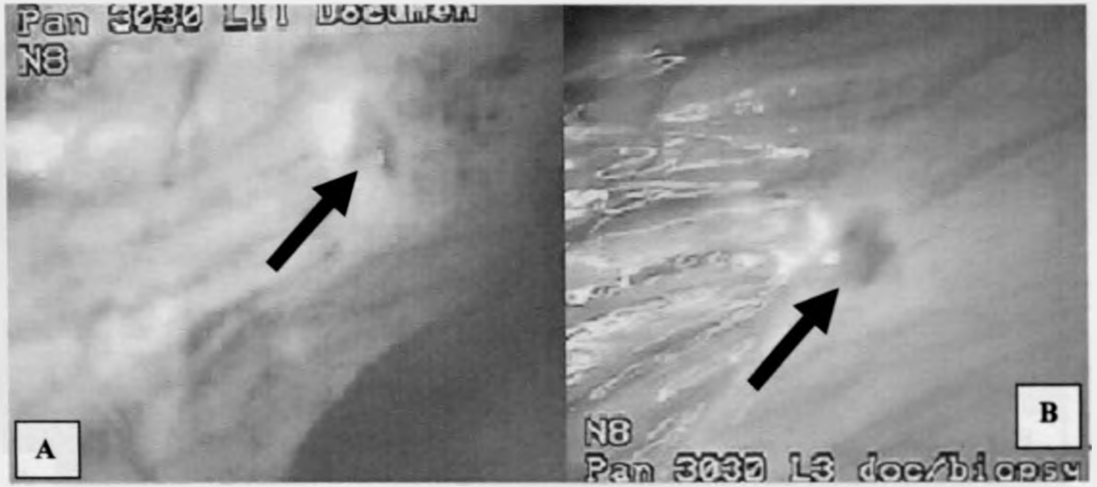
Key:		
	Arrows	Indicate location of lesion.
	PAN 2993	Animal identification number.
	N11	Identification number of endometriosis lesion
	LII	Photo taken during second laparoscopy documentation
	L3	Photo taken during third laparoscopy documentation

Plate 7: Gross appearance of endometriotic lesion (N4) from a rosiglitazone treated baboon (PAN 3032)



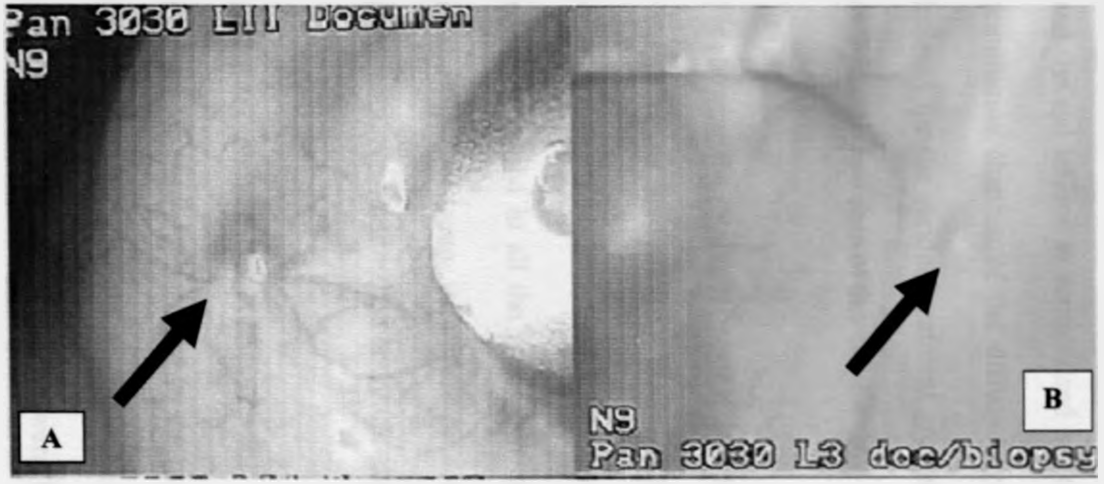
Key:		
	Arrows	Indicate location of lesion.
	PAN 3032	Animal identification number.
	N11	Identification number of endometriosis lesion
	LII	Photo taken during second laparoscopy documentation
	L3	Photo taken during third laparoscopy documentation

Plate 8: Gross appearance of endometriotic lesion (N8) from a rosiglitazone treated baboon (PAN 3030)



Key:		
	Arrows	Indicate location of lesion.
	PAN 3032	Animal identification number.
	N11	Identification number of endometriosis lesion
	LII	Photo taken during second laparoscopy documentation
	L3	Photo taken during third laparoscopy documentation

Plate 9: Gross appearance of endometriotic lesion (N9) from a rosiglitazone treated baboon (PAN 3030)



Key:		
	Arrows	Indicate location of lesion.
	PAN 3030	Animal identification number.
	N11	Identification number of endometriosis lesion
	LII	Photo taken during second laparoscopy documentation
	L3	Photo taken during third laparoscopy documentation

4.1.2. The distribution of the types of lesions

4.1.2.1. Observations on the Typical and Red lesions

Briefly, there was a change in the distribution and frequency of two types of lesions when the pre-treatment and post-treatment results were compared. These lesions were the typical bluish-black and the red lesions as summarised in Table 2, 3 and Fig. 1). Both Tables show the quantitative changes of the different types of endometriosis lesions (typical and red lesions) for the various treatment groups before and after the treatment month. Appendix 7 has the raw data of this results.

The plot graph (Figure 1) is showing the total number of lesions for each the two types of lesion. A Student t-paired test analysis in Placebo, GnRH–antagonist, and rosiglitazone groups revealed 0.41, 0.84 and 0.04, respectively. Although not significant, the typical lesions increased in all the groups. While the red number of lesions increased only for the placebo group, they were reduced in the GnRH and rosiglitazone though not significantly (p value = 0.38 and 0.08, respectively).

4.1.2.2. Observations on the total number of Red lesions

Briefly, the total number of red lesions was increased in the placebo group and was reduced in both the GnRH and rosiglitazone treated group though none were significant (Table 2 and 3; Figure 1 and 2). The paired t-tests were 0.31, 0.38 and 0.08, respectively.

Table 2: Data from lesions collected during the pre-and post-treatment laparoscopy of endometriotic baboons

Observation	Placebo		GnRH - antagonist		Rosiglitazone	
	Pre	Post	Pre	Post	Pre	Post
Mean surface Area (mm^2) with adhesions	180.8 ± 113.9	164.6 ± 80.6	315 ± 150.1 $	177.9 ± 141.2 $	240.3 ± 86.8 †	123.2 ± 78.3 †
Mean surface Area (mm^2) without adhesions	121.3 ± 77.2	89.6 ± 71.8	109.8 ± 17.7 *	55.9 ± 7.3 *	106.3 ± 69.2 @	(42.2 ± 10.8 @
Mean number of lesions	9.5 ± 2.6	9.3 ± 3.3	10.3 ± 3.2	10.8 ± 2.4	13.3 ± 4.6	12.5 ± 4.5
Mean number of Red lesions	1.8 ± 2.1	3 ± 2.4	4.3 ± 4.7	2.5 ± 1.7	4.3 ± 2.9 #	0.8 ± 1.3 #
Mean area of red lesions (mm^2).	21± 24.5	40.3 ± 49.6	8.5 ± 43.7	8.7 ± 5.3	62.9 ± 72.5 ©	4 ± 6.7 ©
Mean number of adhesions	2.8 ± 1.9	2.5 ± 1.9	2.8 ± 0.5	3.3 ± 0.5	3.5 ± 2.9	3.3 ± 2.5

KEY:

$ GnRH relative surface area reduction was significant with Dunnett t-test post hoc statistic (p = 0.044)

† Rosiglitazone group had significant reduction (F value = 5.7, Tukey p value = 0.033).

* GnRH surface area without adhesion, the reduction was significant (Student's test, t value = 4.89, p value = 0.0164)

@ Rosiglitazone group though reduced was not significant (Student t value = 2.14, p value = 0.1214).

Rosiglitazone group total number of red lesions was reduced though not significantly (p value = 0.08).

© Rosiglitazone group area of red lesions was reduced though not significantly (p value = 0.201)

Table 3: Quantitative changes of the different types of endometriosis lesions for the various treatment groups

Observation	Placebo		GnRH - antagonist		Rosiglitazone	
	Pre	Post	Pre	Post	Pre	Post
Typical blue black lesions	1 (0-5)	2.5 (0-7)	1.5 (0-6)	2.5 (1-4)	0.5 (0-5)	4 (1-11)
Red lesions	1.5 (0-4)	3 (0-6)	2.5 (0-1)	2 (1-5)	5.5 (0-6)	0.5 (0-2)
Typical and red lesions	4 (0-9)	5 (1-13)	5.5 (2-3)	6 (2-6)	6 (0-11)	4.5 (3-11)
Note: All data are presented as median (range).						

Key: Pre = Pre-treatments counts.
Post = Post treatment counts.

Figure 1: Changes in specific types and number of endometriotic lesions for the treatment groups

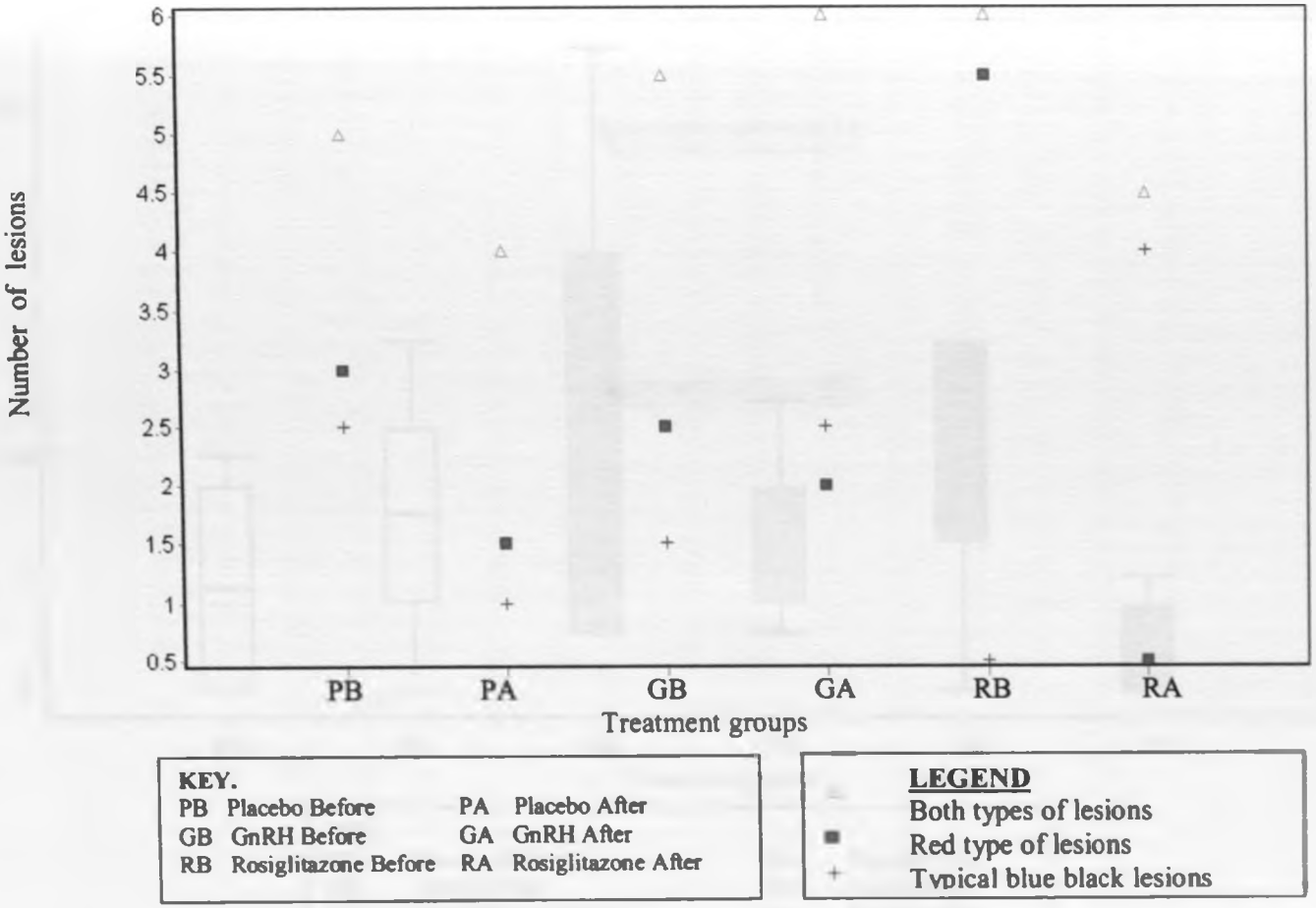
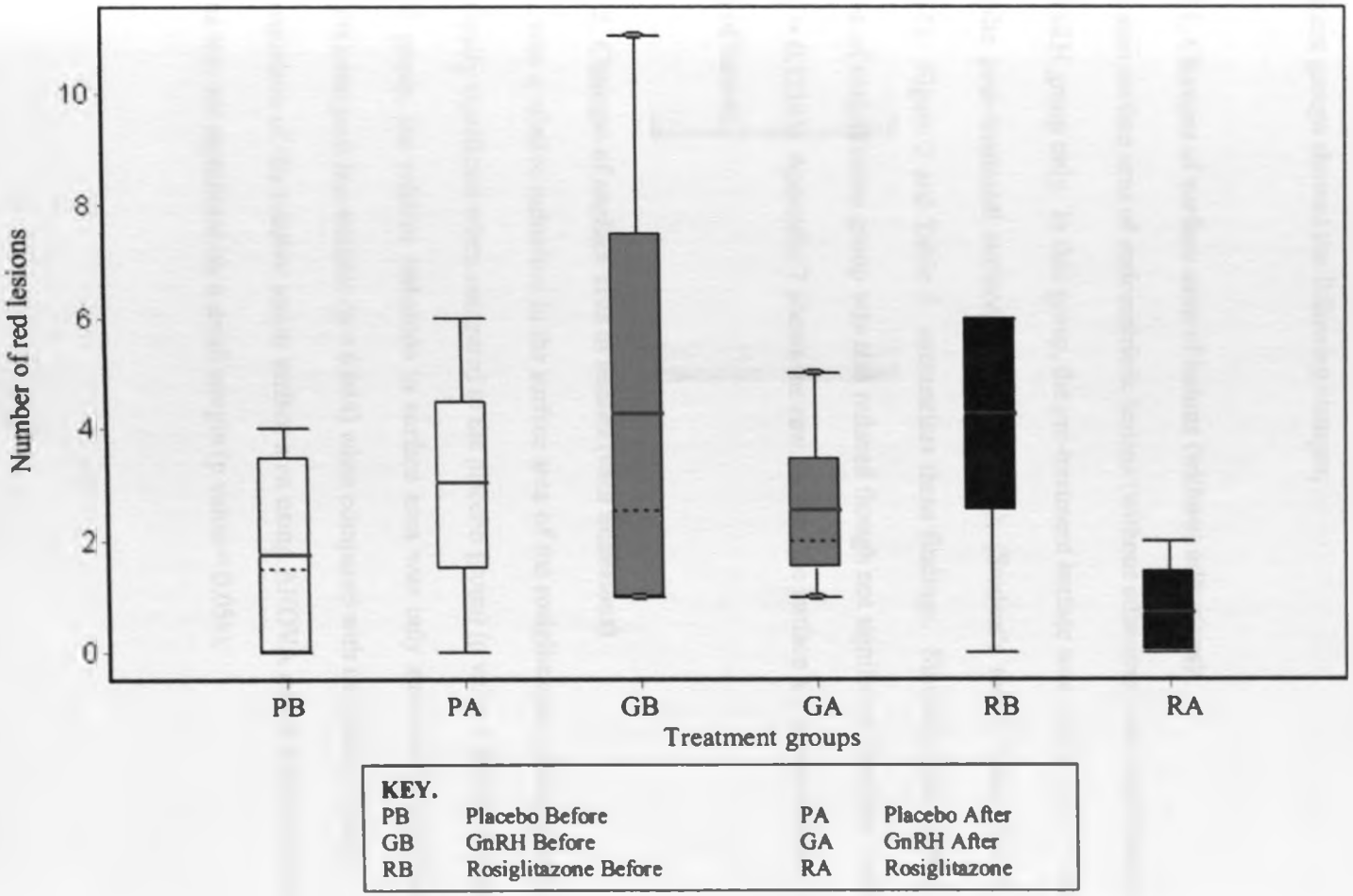


Figure 2: Change in total number of red lesions in endometriotic baboons



4.1.3 Surface area of endometriotic lesions

The sizes of the endometriosis lesions (with adhesions) or (without adhesions) were compared separately. An analysis of the changes in surface area of the endometriosis lesions in the three treatment groups showed the following changes;

4.1.3.1. Changes of surface area of lesions (without adhesions)

The mean surface area of endometriotic lesions (without adhesions) was significantly reduced in the GnRH group only. In this group, the pre-treatment surface area (109.8 ± 17.7) was compared with the post–treatment surface area (55.9 ± 7.3), (Student's test, t value = 4.89, p value = 0.0164). Figure 3 and Table 2 summarises these findings. Similarly, the surface area of the lesions of rosiglitazone group was also reduced though not significant (Student t value = 2.14, p value = 0.1214). Appendix 7 shows the raw data on the surface are measurements, number and types of lesions.

4.1.3.2. Changes of surface area of lesions (with adhesions)

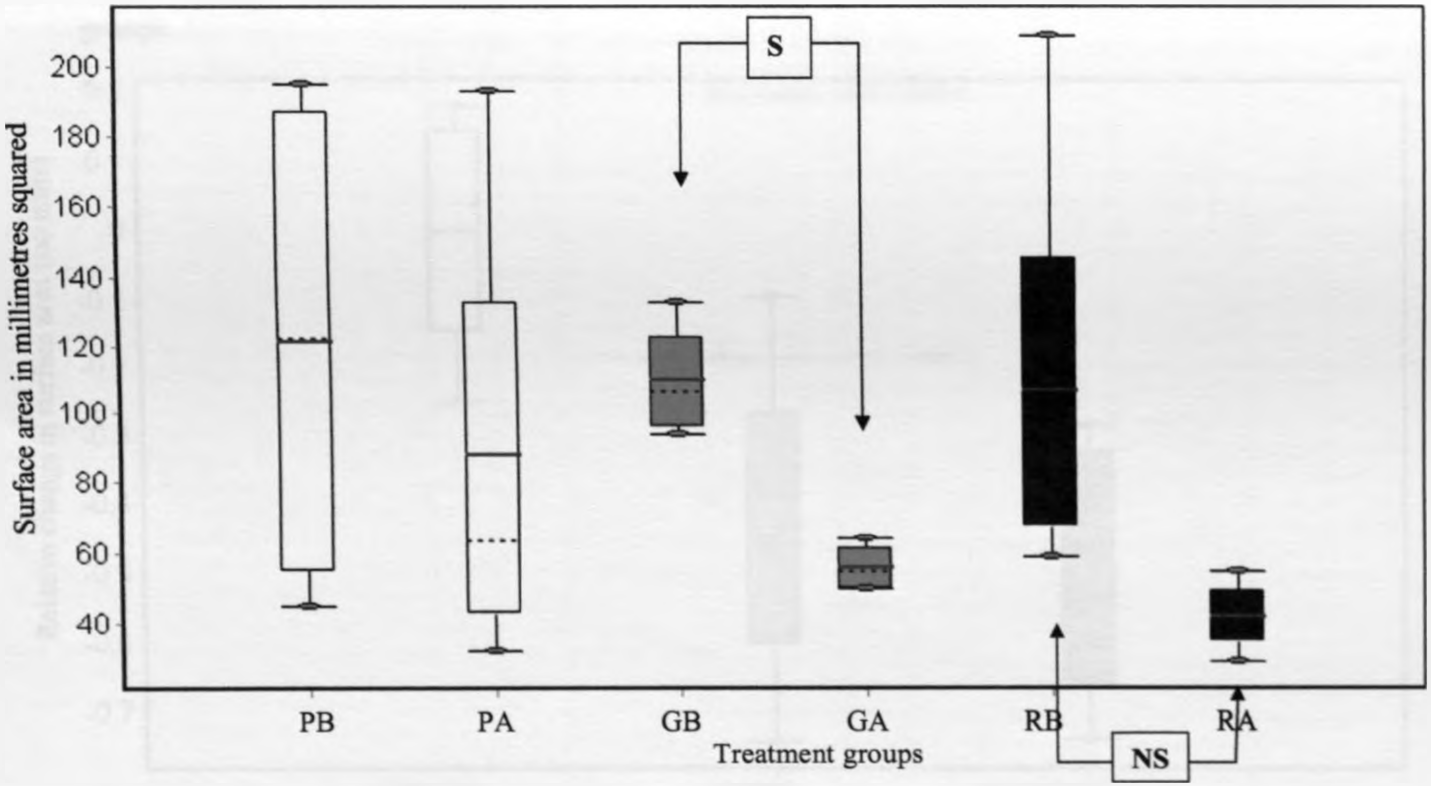
There was a relative reduction in the surface area of the rosiglitazone–treated group (which was statistically significant when compared to the placebo group) (p value = 0.033) (Figure 4). In the GnRH group, the relative reduction in surface area was only statistically significant with the Dunnett t-test post hoc statistic (p = 0.044) when compared with the placebo group.

A comparison of the relative lesion surface area using ANOVA of the means between the three

4.1.3.3 Changes in surface area of Red lesions

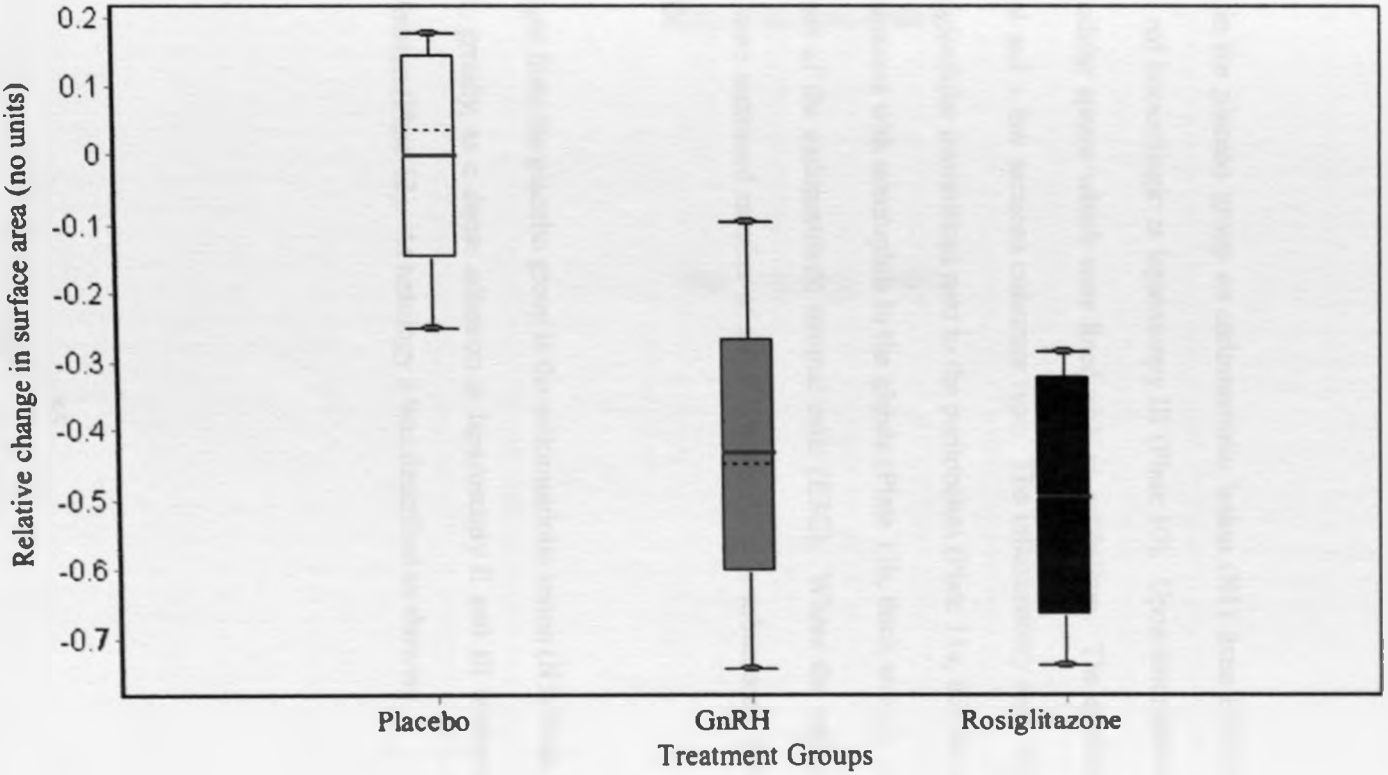
The reduction of the mean surface areas of red endometriotic lesions from pre-treatment to post-treatment was reduced though not significant within the rosiglitazone group i.e. from 5.5 (0-6) to 0.5 (0-2), p-value = 0.201, where the data is represented as median (range). See Table 2.

Figure 3: Changes in surface area of lesions (without adhesions) before & after treatment of endometriotic Baboons



KEY.					
PB	Placebo Before	PA	Placebo After	NS	Difference not significant
GB	GnRH Before	GA	GnRH After	S	Difference Significant
RB	Rosiglitazone Before	RA	Rosiglitazone After		

Figure 4: Relative change in surface area of lesions (with adhesions) in endometriotic baboons in the treatment groups



4.1.4 Gross appearance and histopathology of endometriosis lesions

A comparison of the gross and histopathology appearance was done. In this case the histopathology of each type of lesion was looked into and the following results were documented.

For example, in the placebo group an endometriotic lesion (N1) from PAN 2912 was grossly classified as a red hemorrhagic at laparoscopy III (Plate 10). Upon histopathology examination it showed glandular spaces which were lined with an epithelium. The epithelium was in most cases cuiboidal and a few sections columnar type. The inflammatory cells were both in lumen and in the periglandular interstitium next to the peritoneum (Plate 11a, thin arrows). There were hemorrhagic contents with neutrophils in the glands (Plate 11b, thick arrow). A few eosinophils occurred as part of the endometriotic stromal cells (ESC). Where the peritoneal surface was visible, there were increased number of small lymphocytes in submucosa, haemorrhages and a few neutrophils

Another example from the placebo group is the endometriotic lesion (N3) from PAN 2991 which was classified, grossly, as a dense adhesion at laparoscopy II and III implying the lesion was covered by adhesion (Plate 12). At histology it was described as showing

glandular structures lined by both simple cuiboidal and simple columnar epithelium type. Some haemorrhages were evident in the glandular lumen with desquamated epithelial cells mixed with proteinous exudate within the glandular lumen (Plate 13, thin arrow). There was focal and diffuse infiltration of lymphocytes & macrophages in the interstitial area (Plate 13, thick arrow). There were also haemorrhages and mild congestion in the connective tissue.

In the GnRH group, there were signs of degeneration and atrophy of the glandular epithelium and desquamation of epithelial cells. An example was the endometriotic lesion (N3) from PAN 2993 which was classified as both a white plaque with pigmented spots and flimsy adhesion (Plate 14) at laparoscopy III, after treatment. The histology showed an endometriotic lesion with a low simple cuiboidal epithelium a mild amount of endometrial stromal cells (Plate 15, thin arrow). The epithelium had degenerated cells (Plate 15, thick arrow). There was evidence of extensive desquamation of epithelial cells. Some of the surrounding endometriotic stromal cells were slightly degenerated (Plate 15, thin arrow). At times there were small amount of hemosiderin present around the endometriotic stromal cells.

Plate 16 shows another example of a GnRH treated endometriotic lesion (N9) from PAN 2993 which was classified as a white plaque with pigmented spots at laparoscopy II, and remained

the same at laparoscopy III (Plate 16, arrow). And at histology, the description of the endometriotic lesion showed simple cuiboidal and low pseudostratified epithelia. There was evidence of degenerated and desquamation of epithelium (Plate 17, arrow). The surrounding periglandular tissue had extensive connective tissue and small amounts of smooth muscles. There were mild haemorrhages and deposits of hemosiderin

Another endometriotic lesion (N5) from PAN 2998 (Plate 18), a rosiglitazone treated baboon, was classified as a white plaque at laparoscopy III. At histology, it showed necrosis (nuclei are pyknotic and karyorhexis) of glandular epithelium nuclei and desquamation of the epithelium into the lumen of the glandular structures (Plate19, arrow). The rest of the section showed excessive haemorrhage and a haematoma covered with extensive fibrin with moderate lymphocytic infiltrating.

The endometriotic lesion (N13) from PAN 3030, a rosiglitazone treated animal. The lesion was classified as a white plaque at laparoscopy III (Plate 20). At histology, it showed degeneration, atrophy and desquamated areas on the glandular epithelium. Degenerated epitheliums was visible (Plate 21, thick arrow). Interstitial area had mainly connective tissue and smooth muscles. Peritoneal surface (not in photomicrograph) had fibrin deposits that could explain a white plaque type of lesion. There was mild infiltration of lymphocytes around glands (Plate 21, thin arrow).

Below are a series of representative photomicrographs of histopathology of some endometriosis lesions from various treatment groups (See Plate 10 to 23). In all the plates, the photographs of the endometriotic lesions are followed by a photomicrograph of its histopathology. The plates were paired in the following manner; Plate 10 with plate 11; Plate 12 with plate 13; Plate 14 with plate 15; Plate 16 with plate 17; Plate 18 with plate 19 and Plate 20 with plate 21. The lesions were taken at laparoscopy III i.e. these were the lesions that were observed after treatment. In summary, rosiglitazone treated group showed more necrosis and degeneration compared to GnRh and Placebo animals. This also correlated with the reduction in size and changes in morphology seen in the groups. The placebo had fewer changes while GnRH had more changes and rosiglitazone most, in that order.

In this study there were 25 out of 34 cases (74%) of glandular lumen haemorrhages; 27 out of 34 cases (79 %) of periglandular haemorrhages; 35 out of 38 cases (92 %) of interstitial haemorrhages; 32 out of 37 cases (87%) of peritoneal haemorrhages across all the treatment groups.

In conclusion, a general description of the endometriosis lesions that was consistent in all 3 treatment groups is that: the epithelia of endometriotic glands at times appeared less uniform with varying heights in the same gland often showing varying degree of cytologic polymorphism. A loss of polarity of the epithelial cells was at times seen and cellular

times a mantle of fibrosis, which, to varying degrees replaced the specialised endometriosis stromal cells, frequently surrounded the endometriotic lesions. In some instances, the endometriotic tissue appeared to develop the menstrual bleeding phase pattern.

Plate 10: Photograph of endometriotic lesion (N1) from a placebo treated baboon at laparoscopy III (PAN 2912)

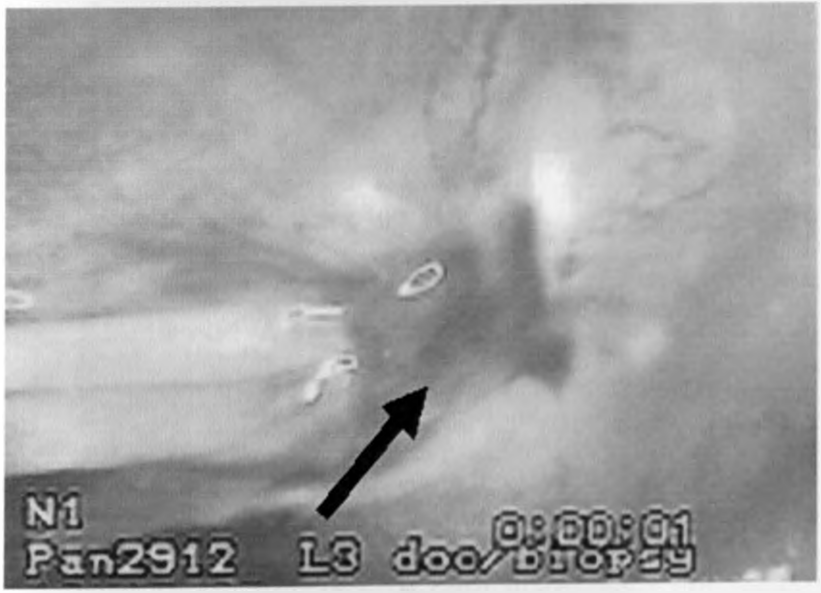
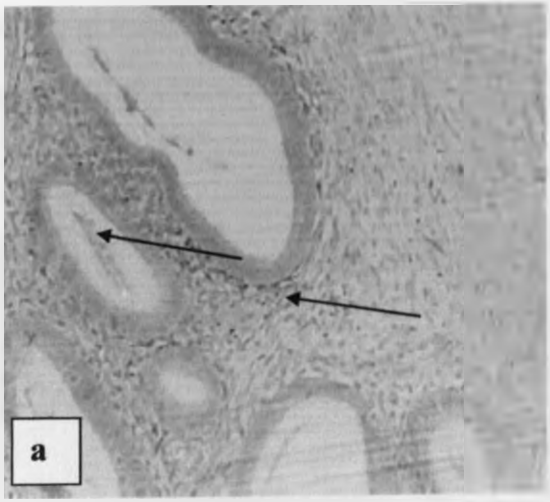
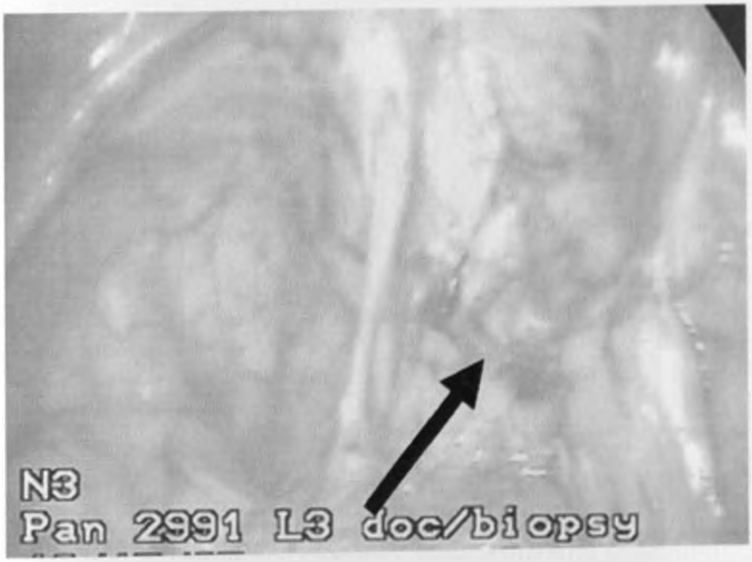


Plate 11: Photomicrograph of endometriotic lesion (N1) at laparoscopy III from a placebo treated baboon (PAN 2912)



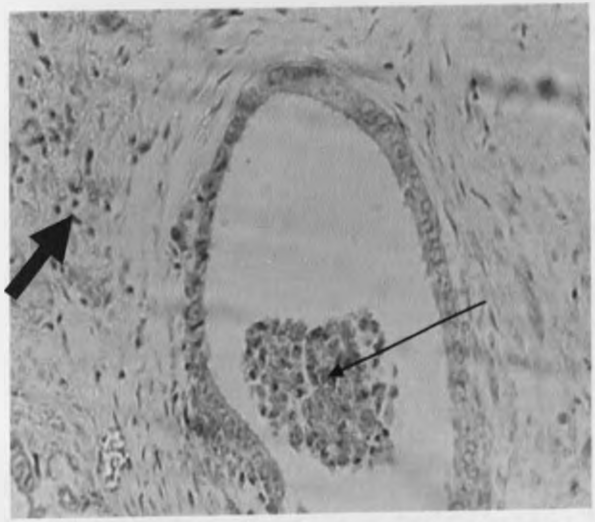
(Magnification x 100, H & E).

Plate 12: Photograph of endometriotic lesion (N3) from a placebo treated baboon at laparoscopy III (PAN 2991)



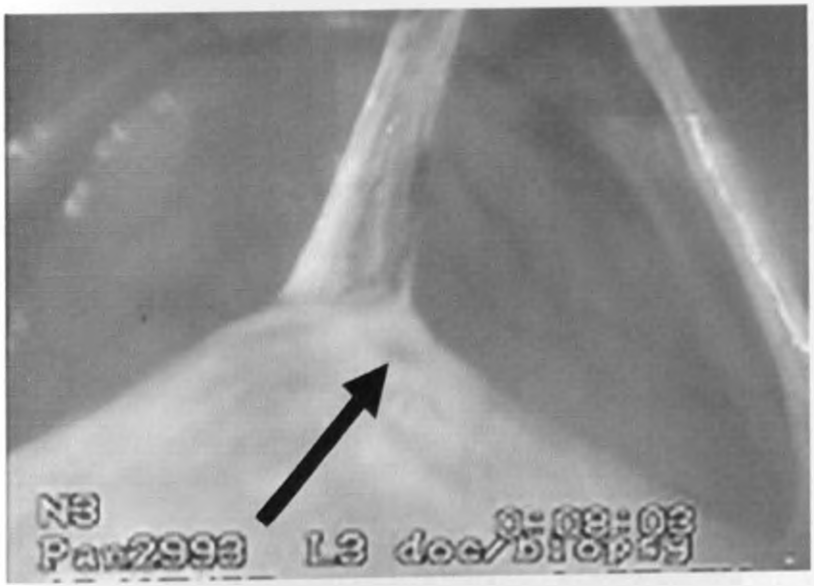
Key:	Arrows	Indicate location of lesion.
	PAN 2991	Animal identification number.
	N3	Identification number of endometriosis lesion
	L3	Photo taken during third laparoscopy documentation

Plate 13: Photomicrograph of endometriotic lesion (N3) from a placebo treated baboon at laparoscopy III (PAN 2991)



(Magnification x 100, H & E).

Plate 14: Photograph of endometriotic lesion (N3) from a GnRH treated baboon at laparoscopy III (PAN 2993)



...decomposition of endometriotic lesion

Plate 15: Photomicrograph of endometriotic lesion (N3) from a GnRH treated baboon at laparoscopy III (PAN2993)

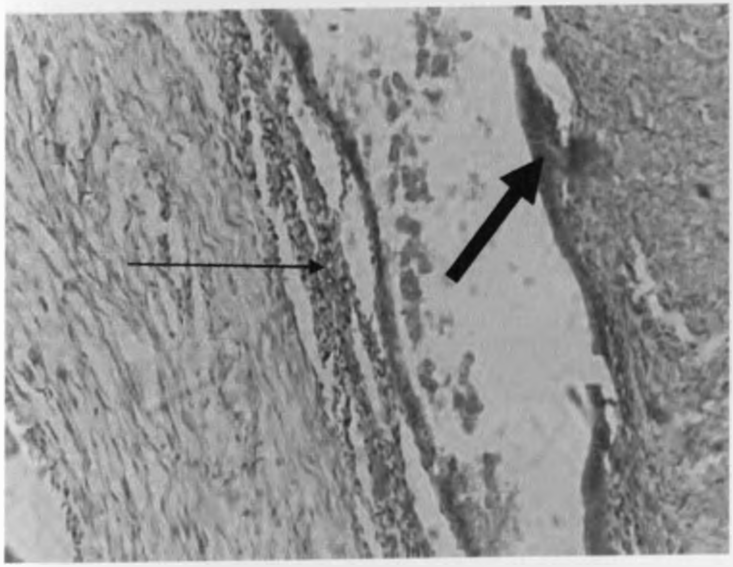
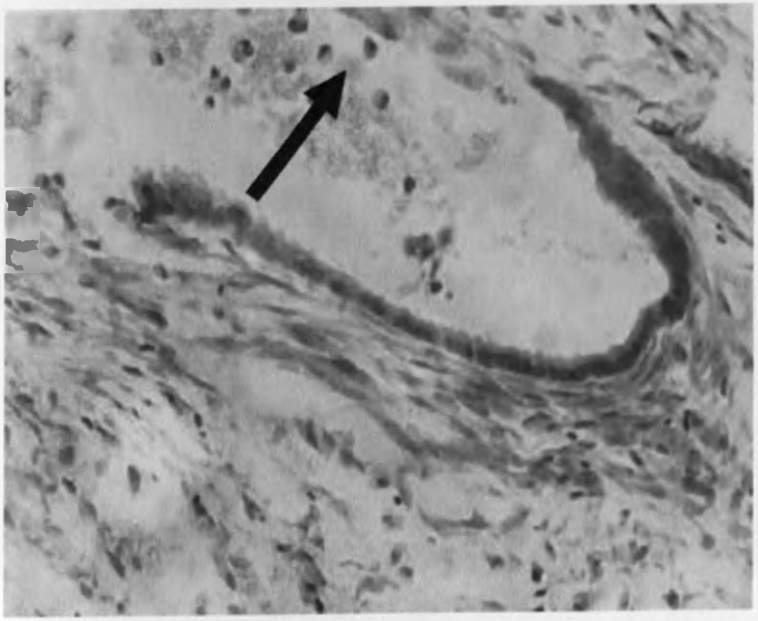


Plate 16: Photograph of endometriotic lesion (N9) from a GnRH treated baboon at laparoscopy III (PAN 2993)

Key: Arrow shows position of endometriotic lesion.

Plate 17: Photomicrograph of endometriotic lesion (N9) from a GnRH treated baboon at laparoscopy III (PAN 2993)

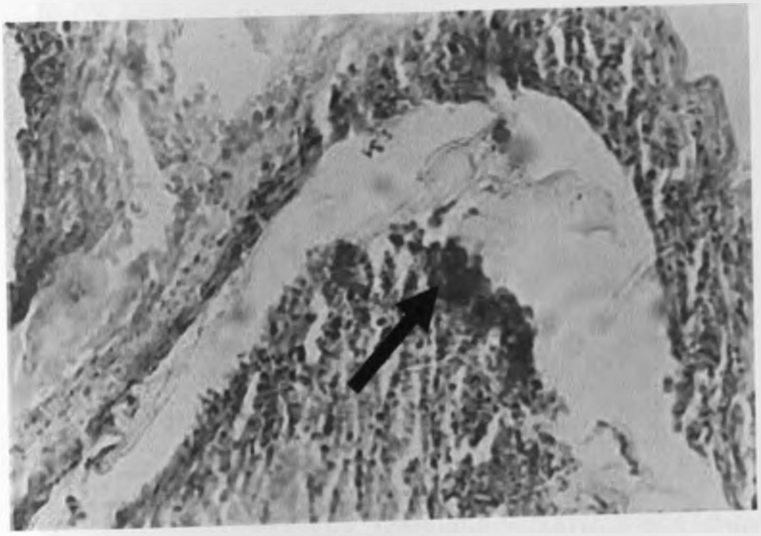


(Magnification x 250, H & E).

Plate 18: Photograph of endometriotic lesion (N5) from a rosiglitazone treated baboon at laparoscopy III (PAN 2998)

Key:		
	Arrow	shows position of endometriotic lesion.
	L3	Photo taken during third laparoscopy documentation
	PAN 2998	Animal identification number.
	N5	Identification number of endometriosis lesion

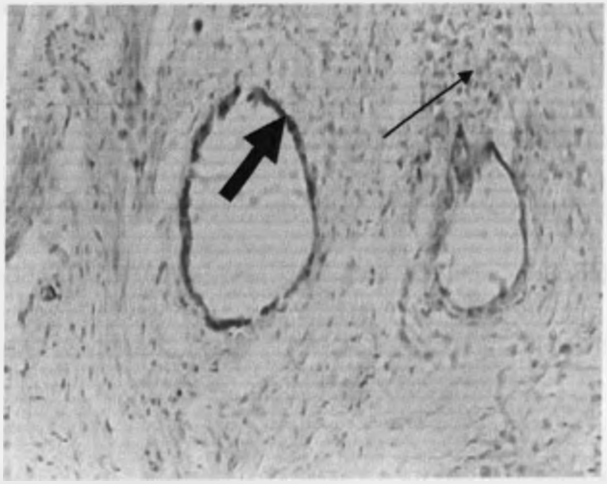
Plate 19: Photomicrograph of endometriotic lesion (N5) from a rosiglitazone treated baboon at laparoscopy III (PAN 2998)



(Magnification x 250, H & E).

Plate 20: Photograph of endometriotic lesion (N13) from PAN 3030 from a rosiglitazone treated baboon at Laparoscopy III

Plate 21: Photomicrograph of endometriotic lesion (N13) from a rosiglitazone treated animal at laparoscopy III (PAN3030)



(Magnification x 100, H & E).

4.1.5 Scoring of the histological changes in the endometriotic lesions

The histological scoring was done by checking sections of endometriotic tissue. While guided by and using the Appendices 4 to 6, the approximate range or nearest description fitting the microscope reading was recorded. A raw data chart was used to enter the scores collected. Each endometriotic lesion had a maximum of 5 slides, which after reading an average of the slides was retrieved and use for statistical analysis.

4.1.5.1 Scores among the types of treatment

Scores of the various histopathological changes were compared between the 3 treatment groups. The scoring on the slides was subjective and relied on the Appendix 4, 5 and 6. The design of the key was based on the fact that there were varying degrees of changes in all the 33 features identified. The lack of appearance of the feature was labelled zero (0) and the lowest intensity was 1 and highest 4. The number of slides made from a lesion were scored and an average given taken. The number of slides per lesion ranged from 1 to 5. The mean of all the scores from each lesion were used for statistical comparison of the treatment groups or type of lesions.

Tables 4 to 5 are the details of the synthesized scores for all the treatment groups. Table 6 shows a summary of the comparison of scores among the treatment groups of endometriotic baboons. Figures 5 to 10 are a graphical description of these changes among the 3 treatment groups.

data of the scores inserted in the tables are the average scores of all the slides examined from each lesion.

A comparison of the histopathological scores of the endometriosis lesions from the 3 treatment groups, showed significant differences in degeneration of glandular epithelium; and that of endometrial stromal cells, along with presence of peritoneum lymphocytes (Figures 5 to 7).

The rosiglitazone treated group had the highest amount of glandular degenerative changes among the 3 groups of endometriotic baboons, followed by GnRH – antagonist treated and the least the placebo treated group. The placebo group had an average score of 1, GnRH was 2.48 and rosiglitazone was 2.82. (See Table 4). The scoring was based on the level of degenerative or necrotic changes on the glandular epithelium. The variation in the scores glandular degenerative scores when compared between the 3 groups is graphically represented in Figure 5. (Wallis One – way ANOVA test by ranks, p value = 0.0043).

The ESCs degenerative scores when compared between the 3 groups showed both rosiglitazone and GnRH- antagonist groups had the highest scores and placebo group the least scores. The placebo group had an average score of 0.82, GnRH was 2.45 and rosiglitazone was 2.63 (See Table 5). A graphical representation of the variation is shown in Figure 6. (Wallis One – way ANOVA test by ranks, p value = 0.0002).

A graphical representation of the variation in the presence of peritoneum lymphocytes scores when compared between the 3 groups is shown if Figure 7. The rosiglitazone had the highest score followed by the placebo and the last was the GnRH- antagonist treated group. The placebo group had an average score of 1.54, GnRH was 1.33 and rosiglitazone was 2.05 (Wallis One – way ANOVA test by ranks, p value = 0.0224) (see Table 5). The presence of lymphocytes in the peritoneum adjacent to the endometriosis was significantly different among the treatment groups.

Figure 8 shows a graphical representation of the variation in the glandular luminal mucus scores when compared between the 3 groups. The GnRH-antagonist treated group had highest score followed by rosiglitazone and finally the placebo group. Although varied, the scores were not significant enough (Wallis One – way ANOVA test by ranks, p value = 0.0609).

Occurrence of degenerative slits was a unique feature in this study. This can be described as the presence of narrow spindle shaped gaps within the interstitial tissue and appearance of degenerated shrunk cells at their edges. These lesions could be linked with degeneration and possibly shrinking in the surrounding ESCs. Figure 9 shows a graphical representation of the variation in the interstitial degenerative scores when compared between the 3 groups. Though there were no significant differences between the three treatment groups, the rosiglitazone had the highest score followed by GnRH-antagonist treated group and the least was the placebo group. Although varied, the scores were not significant enough (Wallis One – way ANOVA test

Figure 10 shows a graphical representation of the variation in the presence of acute inflammat cells scores when compared between the 3 groups. Though there were no significant differer between the three treatment groups, the rosiglitazone group had the highest scores followed both GnRH- antagonist group and placebo group. Although varied, the variation among th groups was not significant (Wallis One – way ANOVA test by ranks, p value = 0.0636).

Table 4 : Summary of the histological scores for glands and interstitial tissue in all treated endometriotic baboons

	PLACEBO		GnRH		ROSIGLITAZONE	
Number of slides 7	**Mean scores**	**Frequency of feature**	**Mean scores**	**Frequency of feature**	**Mean scores**	**Frequency of feature**
Glandular tissue						
Luminal Haemorrhages	1.47	5/5	0.98	10/15	0.91	11/14
Hemosiderin	0	0/6	0.027	1/15	0.00	0/14
Periglandular Haemorrhages	1.45	5/5	0.75	10/15	1.16	12/14
Mucus	0.06	1/5	0.87	11/15	1.81	12/14
Cross sections glands	5.05	5/7	4.29	15/16	4.82	14/15
Degenerate Epithelium †	1	4/5	2.48	15/15	2.82	14/15
Eroded Epithelium	0.934	3/5	1.38	11/15	1.68	13/14
Acute Inflammatory cells	1.06	2/5	0.63	4/15	0.31	6/14
Chronic Inflammatory cells	0.694	4/5	0.68	10/15	0.98	11/14
	Interstitial tissue					
Haemorrhages	1.26	6/7	2.4	16/16	1.50	13/15
Hemosiderin	0	0/7	0.26	3/16	0.10	4/15
Degenerative Slits	0.94	5/7	1.66	15/16	1.92	15/15
Sworls, arcs	2.21	7/7	1.78	16/16	1.53	14/15
Acute inflammatory cells	0.75	4/7	0.178	3/16	0.44	6/15
Lymphocytes	1.85	7/7	1.58	16/16	1.95	15/15
Myxoid	1.57	6/7	1.42	14/16	1.71	15/15
Connective tissue	3.11	7/7	3.11	16/16	2.91	15/15
Smooth muscle	1.46	7/7	0.97	13/16	1.03	12/15
Number of blood vessels	2.44	7/7	2.06	15/16	2.09	15/15
Large blood vessels	1.71	7/7	1.06	12/16	1.29	11/15
Fat	0.6	2/7	0.55	4/16	0.57	4/15
Oedema	0.75	4/7	0.97	14/16	1.05	13/15

KEY † = Feature with significant differences when compared among the treatment groups.
Scores of scale 0 - 4

Table 5 : Summary of the histological scores for ESCs and peritoneum tissue in all treated endometriotic baboons

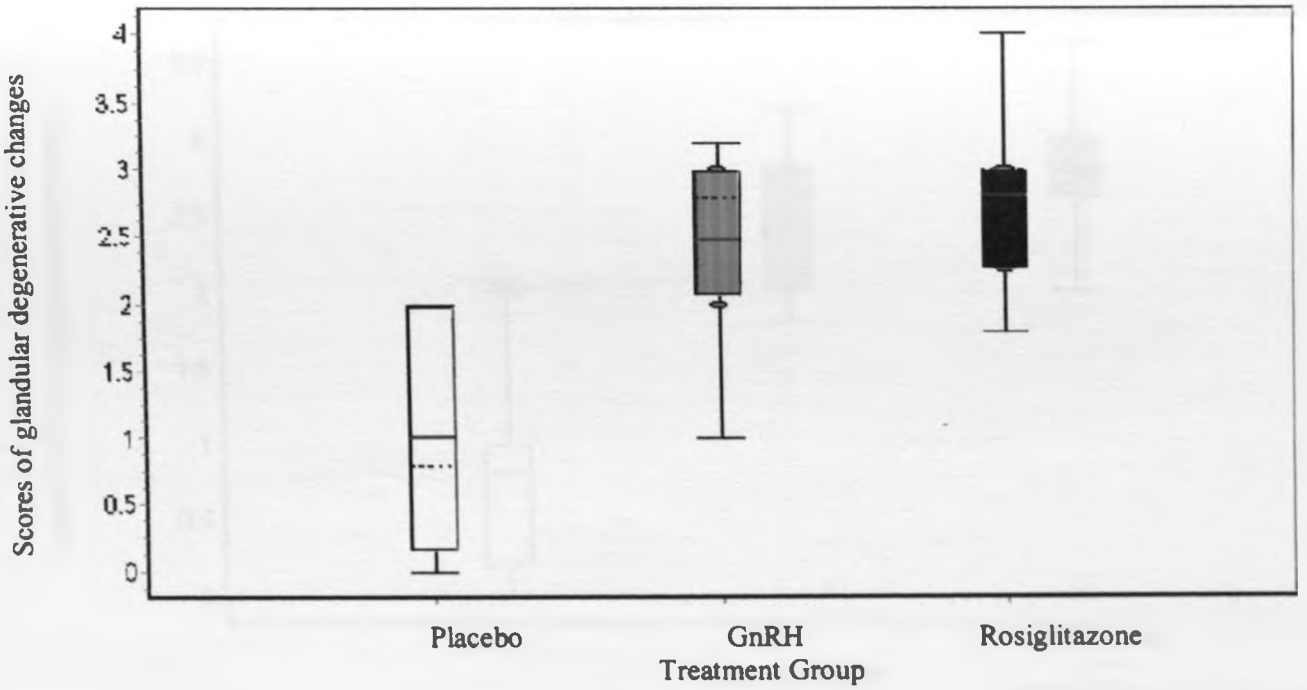
Number of slides 7	PLACEBO		GnRH		ROSIGLITAZONE	
	Mean scores	Frequency of feature	Mean scores	Frequency of feature	Mean scores	Frequency of feature
	ESCs					
Periglandular cuff	0.75	4/6	1.17	15/16	1.38	13/15
Hemosiderin	0	0/7	0	0/16	0.00	0/15
Degenerate stroma †	0.82	5/7	2.45	16/16	2.63	14/15
Scatter	2.47	7/7	1.66	16/16	1.92	14/15
	Peritoneum					
Haemorrhages	1.52	6/7	1.77	14/16	1.52	13/15
Hemosiderin	0	0/7	0.103	2/16	0.01	1/15
Lymphocytes †	1.54	6/7	1.33	16/16	2.05	15/15
Acute inflammatory cell	0.38	2/7	0.15		0.77	9/15
Fibrin	0.68	5/7	1.19	15/16	1.20	12/15
Myxoid	1.34	6/7	1.6	16/16	1.85	15/15

KEY: † = Feature with significant differences when compared among the Treatment groups.
Scores of scale 0 - 4

Table 6: Summary of comparison of scores among the treatment groups of endometriotic baboons

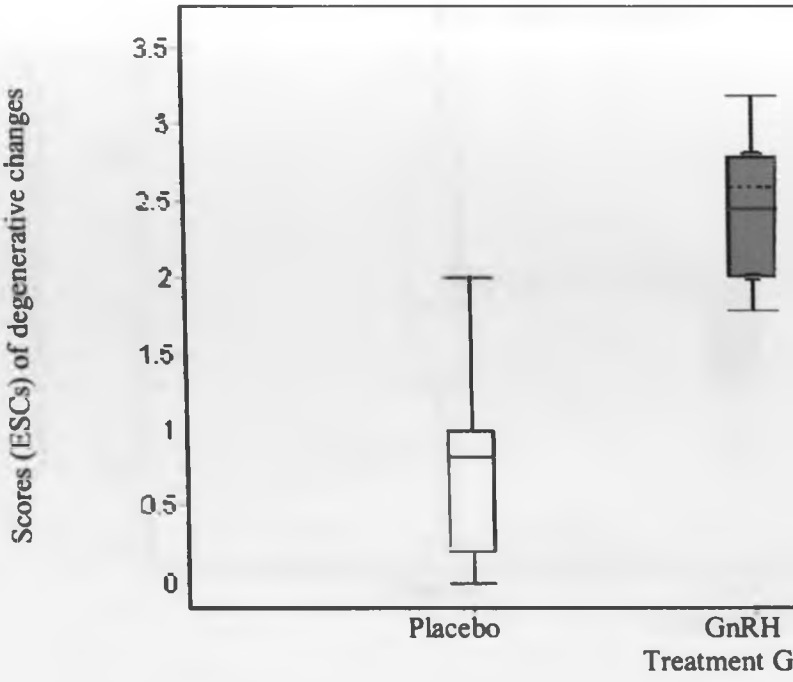
Figure number	Histological changes	P value	Statistical decision
1	Degenerated glandular epithelium	0.0043	Significant
2	Degenerate ESC	0.0002	Significant
3	Peritoneum Lymphocytes	0.0224	Significant
4	Glandular Luminal mucus	0.0609	Not Significant
5	Interstitial Degenerative slits	0.0701	Not Significant
6	Peritoneum Acute inflammatory cells	0.0636	Not Significant

Figure 5: Comparison of glandular degenerative changes among the 3 groups of endometriotic baboons



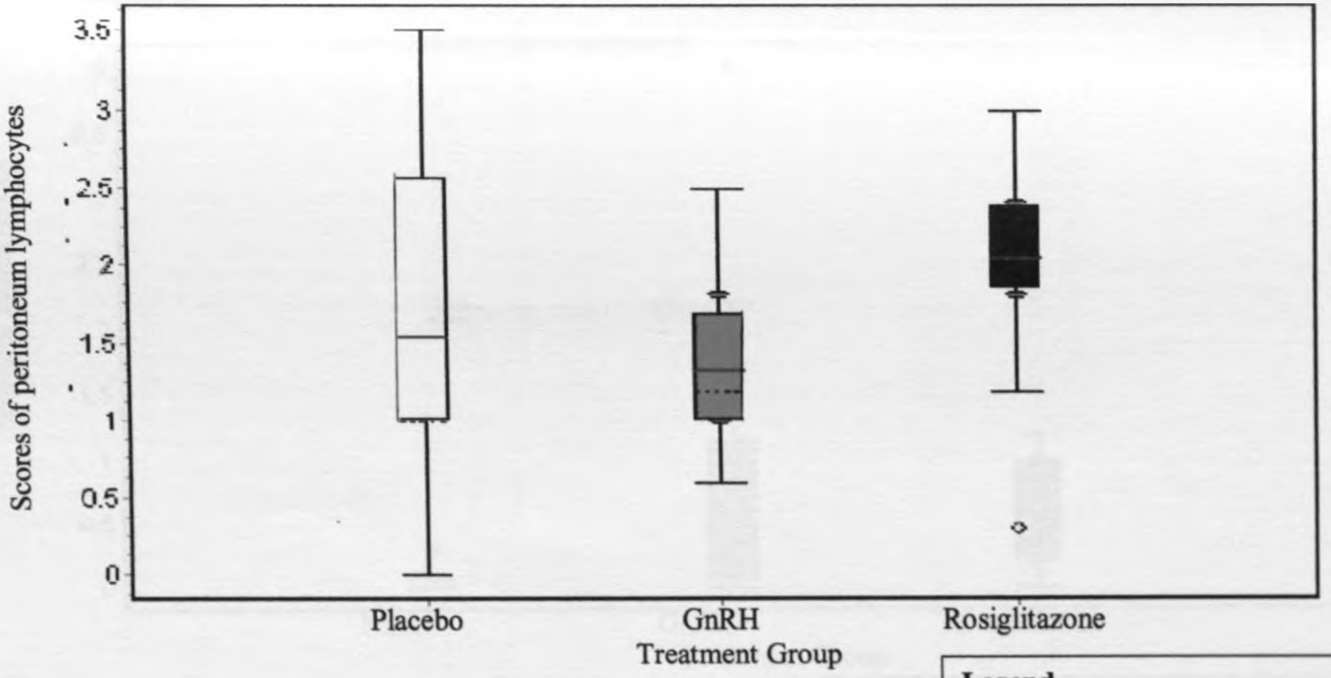
Key: GnRH = GnRH- antagonist treated group.
Score of 0 = Normal epithelium
1 = Traces of degeneration in some sections of the gland
2 = Mild degeneration with pyknotic nuclei with epithelial cell having more dark nuclei
3 = Moderate degeneration nucleus completely pyknotic nucleus. Epithelia cells with intact shape and organisation.
4 = Severe degeneration and karyorhexis and disintegration of the nucleus and epithelial cells i.e. epithelium necrotised.

Figure 6: Comparison of degenerative changes in endometrioti endometriotic baboons



Key: GnRH GnRH- antagonist treated group.
Score of 0 = No evidence of ESCs scatter or spread within interstitial tissue.
1 = Small to traces of amount of ESCs scatter or spread within interst
2 = Mild amount of ESCs scatter or spread within interstitial tissue.
3 = Moderate of ESCs scatter or spread within interstitial tissue.
4 = Wide spread amount of ESCs scatter or spread within interstitial t

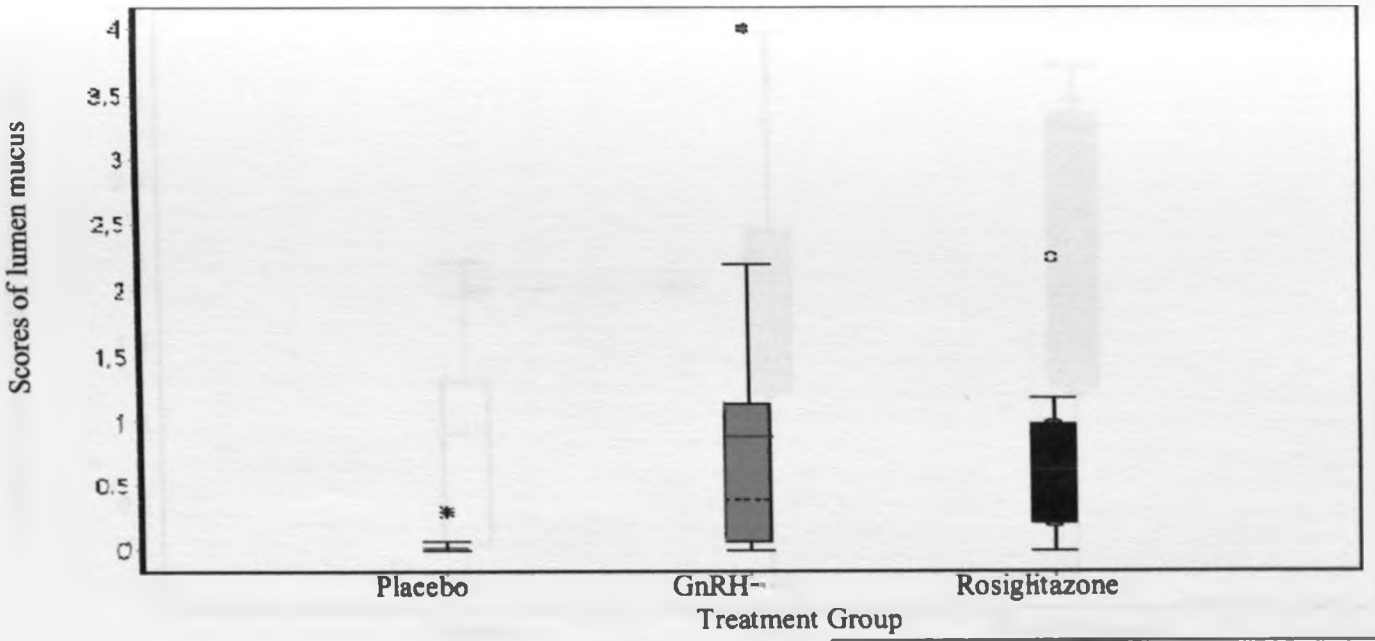
Figure 7: Comparison of peritoneum lymphocytes among the 3 treatment groups of endometriotic baboons



Key: GnRH= GnRH- antagonist treated group.

Score of 0= No lymphocytes within the surface and subepithelia tissue of peritoneum.
1= Small to traces of lymphocytes within the surface and subepithelia tissue of peritoneum.
2= Mild amount lymphocytes within the surface and subepithelia tissue of peritoneum.
3= Moderate amount of lymphocytes within the surface and subepithelia tissue of peritoneum.
4= Severe infiltration of lymphocytes within the surface and subepithelia tissue of peritoneum.

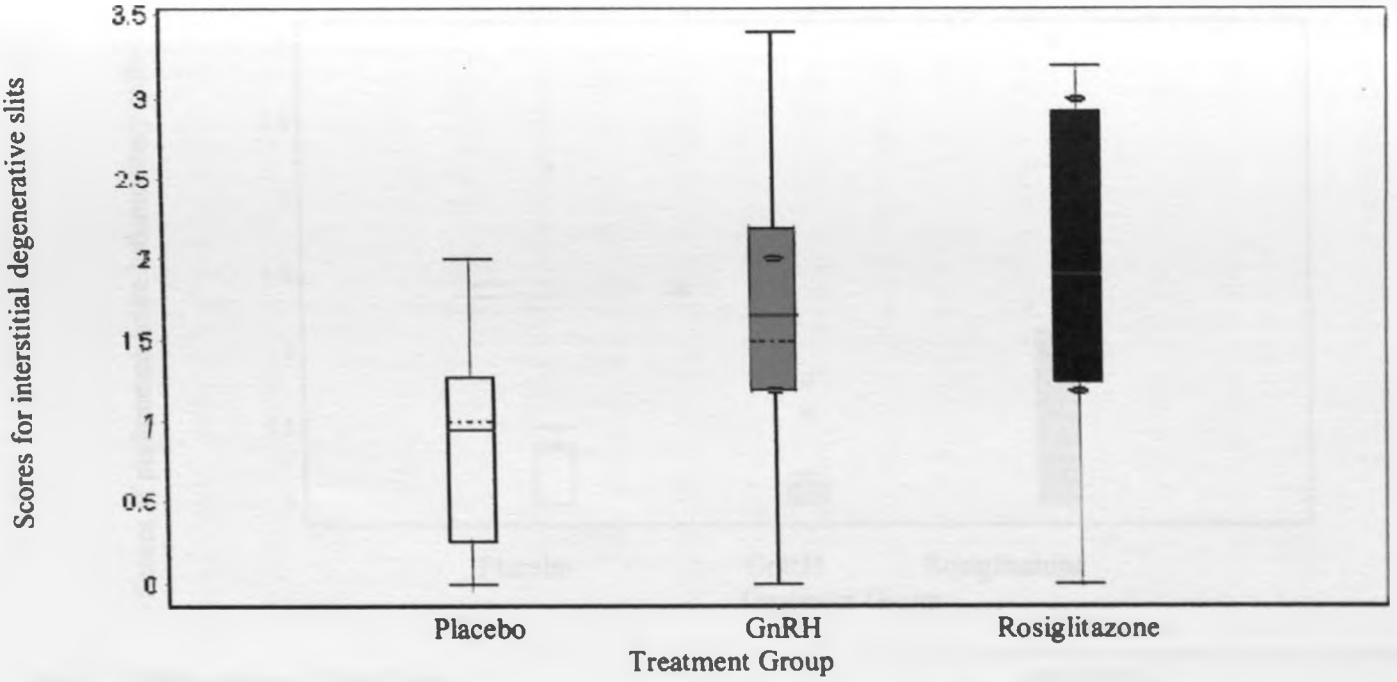
Figure 8: Comparison of glandular luminal mucus among the 3 treatment groups of endometriotic baboons



Key:
GnRH = GnRH- antagonist treated group.
Score of 0 = No mucus or proteinous exudate in lumen
1 = Small traces of amount of mucus or proteinous exudate in glandular lumen covering less than ¼ of lumen
2 = Glandular lumen ¼ to ½ half filled with mucus or proteinous exudate
3 = About ½ to ¾ glandular lumen full of mucus or proteinous exudate
4 = Whole Glandular lumen or cavity filled with mucus or proteinous exudate

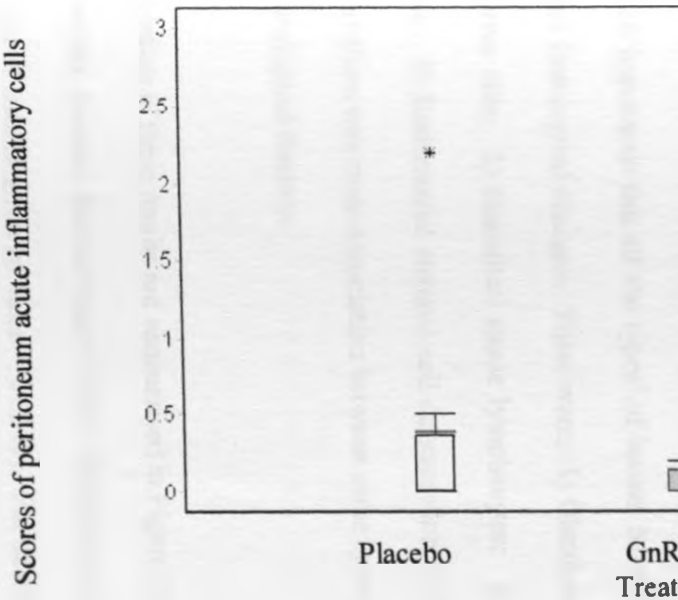
Legend	
*	Outlier lesion 2991N3, the only not having a 0 scores i.e 0.3
●	Outlier lesion 2871N10, highest score of 4
○	Outlier lesion lesion 2788N16, highest score

Figure 9: Comparison of interstitial degenerative slit scores among the 3 treatment groups of endometriotic baboons



Key: GnRH = GnRH- antagonist treated group.
Score of 0 = No degenerative slits
1 = Traces of degenerative slits
2 = Mild presence of degenerative slits
3 = Moderate amount of degenerative slits
4 = Severe or extensive presence of degenerative slits

Figure 10: Comparison of acute inflammatory cells within the baboons.



Key: GnRH = GnRH- antagonist treated group.
Score of 0 = No acute inflammatory cells present.
1 = Traces amount of acute inflammatory cells.
2 = Mild amount of acute inflammatory cells.
3 = Moderate amount of acute inflammatory cells.
4 = Severe infiltration of acute inflammatory cells.

4.1.5.2. Scores among the types of lesions

Histopathology on transformation of the lesions with treatment was comprehensively done in this study. The synthesized scoring of data collected from lesions was recorded as a mean of endometriosis from the slides examined and these are tabulated in Table 8 and 9. Table 10 is a summary of the statistical analysis showing the comparison of histological changes among the types of lesions in endometriotic baboons.

There were over 33 histological features or changes in different grades that were compared. Histopathological features of the different lesions were tested for association with type of lesion. A test on the null hypothesis that all the types' of lesions have an equal histological score was rejected for some histological changes. These were; 1) Glandular haemorrhages; 2) Interstitial tissue degenerative slits; 3) Interstitial tissue lymphocytes; 4) presence of Smooth muscles Interstitial tissue; 5) Endometrial stromal cell degeneration and 6) Peritoneal haemorrhages. This implied that there was cross-association between some types of endometriotic gross lesions with specific histological features.

A graphic description of these results are summarised in Figure 11 to 16. In Figure 11, the figure shows the glandular luminal haemorrhages had a significant variation in the scores/ amount between the types of lesions given (p value = 0.0249). For example it shows Type 1 lesion had the highest amount of glandular luminal haemorrhages than for example type 6 and 7. There was

classification of lesions (by color of lesion), where Type 1 was a Typical blue-black due to deoxygenated blood in the lumen.

In Figure 12, the findings are represented graphically and shows that the interstitial degenerative scores were significantly different among the types of lesions given Table 4 (p value = 0.025). For example its shows Type 2 had the highest amount of interstitial degeneration than for Type 7.

In Figure 13 the findings are represented graphically and show that there is a significant difference in the amount of interstitial lymphocytes among the types of lesions (p value = 0.0242). Types 1 and 2 appear to have a higher amount than type 6 and 7.

In Figure 14, the significant findings on the amount of smooth muscles are represented graphically. The figure shows the smooth muscles amount varied significantly between the types of lesions given Table 10 (p value = 0.0325). Type 7 had the highest amount of smooth muscles than type 6.

Briefly, the significant findings on ESCs degenerative changes are represented graphically in Figure 15. It shows that there was a significant difference in the scores among the different types of lesions Table 10 (p value = 0.0393). Type 2 had the highest changes together with type 6 and

Figure 16 shows the peritoneal haemorrhages had a significant variation in the scores/ amount between the types of lesions (p value = 0.0224). Type 1 and 2 had the highest amount of peritoneum haemorrhages than both type 6 and 7. This can be expected since the morphological classification of type of lesion depends on colour of lesions.

Table 7: Endometriosis lesions at necropsy.

Code for type of Lesion	Morphological Description of endometriotic lesion	Number of lesions		
		Placebo	**GnRH**	**Rosiglitazone**
1	Typical blue-black	4	5	2
2	White plaque with pigmented spots		4	2
3	Red vesicular			
4	Red haemorrhagic	1		
5	Red polyp			
6	White plaques		2	6
7	White nodules	1	6	1
8	White clear vesicle			
9	Red white plaque			
10	Peritoneal ulcer			
11	Flimsy adhesion			3
12	Dense adhesion	2	1	2
	Post-mortem findings	3	1	
	Not seen at Laparoscopy III but seen at Post Mortem	1		1
	Not found at 3rd laparoscopy			1
	TOTAL	**11**	**19**	**18**

Table 8: Summary of the histological scores for gl

	Histological feature	1		2	
		Mean scores	Frequency	Mean scores	
1	Luminal Haemorrhages †	1.85	6/9	1.18	
2	Hemosiderin	0	0	0	
3	Periglandular Haemorrhages	1.26	5/9	1.18	
4	Luminal Mucus	0.39	4/9	0.24	
5	Cross sections of glands	5.36	7/9	5.43	
6	Degenerate Epithelium	2.14	7/9	2.73	
7	Eroded Epithelium		7/9	1.68	
8	Acute Inflammatory cells	0.93	3/9	0.2	
9	Chronic Inflammatory cells	0.61	4/9	0.94	

KEY † = Feature with significant differences when
Type of lesion (1= Typical blue-black; 2 = White
4 = Red haemorrhagic; 6 = White
Scores of scale 0 – 4

Table 9: Summary of the histological scores for Interstitial tissue and Peritoneum in all types of endometriotic lesions

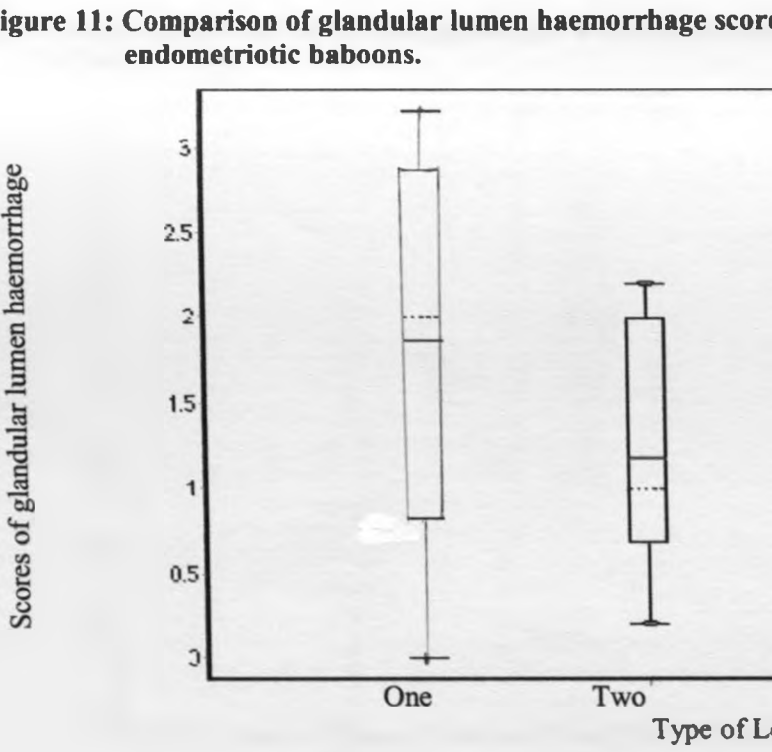
		Type of lesion									
		1		2		4		6		7	
Histological feature		Mean scores	Frequency	Mean scores	Frequency	Mean scores	Frequency	Mean scores	Frequency	Mean scores	Frequency
		Interstitial tissue									
10	Haemorrhages	1.49	9/9	1.63	6/6	0	-	1.2	2/2	0.64	6/8
11	Hemosiderin	0.14	2/9	0.33	4/6	0	-	0	0	0	0/8
12	Process Slits	1.68	9/9	2.25	6/6	2.4	-	2.4	2/2	1.52	8/8
13	Degenerative Slits †	1.17	9/9	2.32	6/6	1	-	2.3	2/2	0.98	6/8
14	Sworls, arcs	1.86	9/9	1.76	6/6	2.8	-	1.1	2/2	1.62	7/8
15	Acute inflammatory cells	0.43	3/9	0.34	2/6	0	-	0.6	1/2	0	0/8
16	Lymphocytes †	1.87	9/9	2.14	6/6	1.6	-	1.9	2/2	1.28	8/8
17	Myxoid	1.3	9/9	1.8	5/6	2	-	1.7	2/2	1.65	8/8
18	Connective tissue	3.09	9/9	2.83	6/6	2.8	-	3	2/2	3.33	8/8
19	Smooth muscles †	1	9/9	1.05	5/6	1.2	-	0	0	1.6	8/8
20	Number of blood vessels	2.21	8/9	2.27	6/6	2	-	1.8	2/2	2.36	8/8
21	Large blood vessels	1.5	8/9	1.43	5/6	1	-	0.5	1/2	1.38	7/8
22	Fat	0.8	4/9	0.23	2/6	0	-	0.7	1/2	0.43	2/8
23	Oedema	0.97	7/9	0.68	4/6	1	-	1.6	2/2	0.6	5/8
24	Periglandular cuffing ESCs	1.49	7/8	1.21	6/6	1.8	-	0.4	2/3	1.26	7/8
25	ESC Hemosiderin †	0	0/9	0	0/6	0	-	0	0	0	0/8
26	Degenerated ESC	2.01	8/9	2.77	6/6	1	-	2.4	3/3	2.25	7/8
27	Amount of Scatter of ESC	1.87	9/9	1.41	6/6	2.6	-	1.8	3/3	1.95	8/8
		Peritoneum									
28	Haemorrhages †	1.25	8/9	1.63	6/6	2.6	-	1	1/2	0.25	3/8
29	Hemosiderin	0.14	1/9	0.08	2/6	0	-	0	0	0	0/8
30	Lymphocytes	1.33	8/9	2.14	6/6	3	-	1.3	2/2	0.83	7/8
31	Acute inflammatory cells	0.07	1/9	0	0/6	2.2	-	0.3	1/2	0.1	3/8
32	Fibrin	0.87	8/9	0.5	3/6	1	-	1	2/2	0.75	7/8
33	Myxoid	1.38	8/9	2	6/6	2.4	-	1.9	2/2	1.6	7/8

KEY † = Feature with significant differences when compared among the types of lesions
Type of lesion (1= Typical blue black; 2 = White plaque with pigmented spots; 4 = Red

Table 10: Comparison of histological changes among th endometriotic baboons

Histological changes	p-va
Glandular Luminal haemorrhages	0.02
Interstitial tissue degenerative slits	0.02
Interstitial tissue lymphocytes	0.02
Interstitial tissue Smooth muscles	0.03
Endometrial stromal cells degeneration	0.0
Peritoneum haemorrhages	0.0

Figure 11: Comparison of glandular lumen haemorrhage scor endometriotic baboons.



Key for scoring of haemorrhages within the lumen of the glands.

0 = No blood in lumen

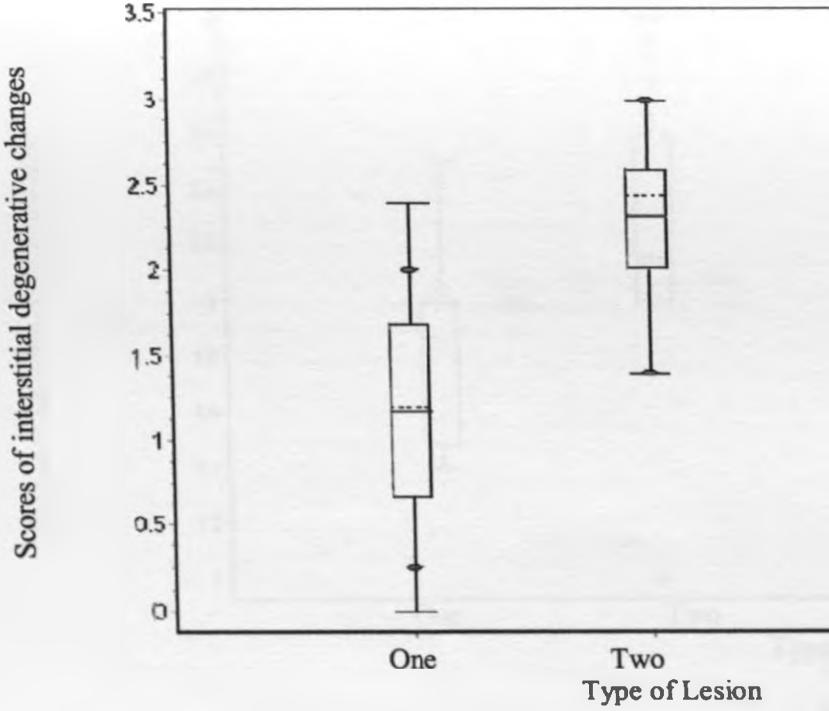
1 = Small to traces of extravasated blood in glandular lumen covering less than ¼ of lumen

2 = Glandular lumen or Cavity ¼ to ½ half filled with extravasated blood.

3 = About ½ to ¾ glandular lumen full of extravasated blood

4 = Whole Glandular lumen or cavity filled with extravasated blood.

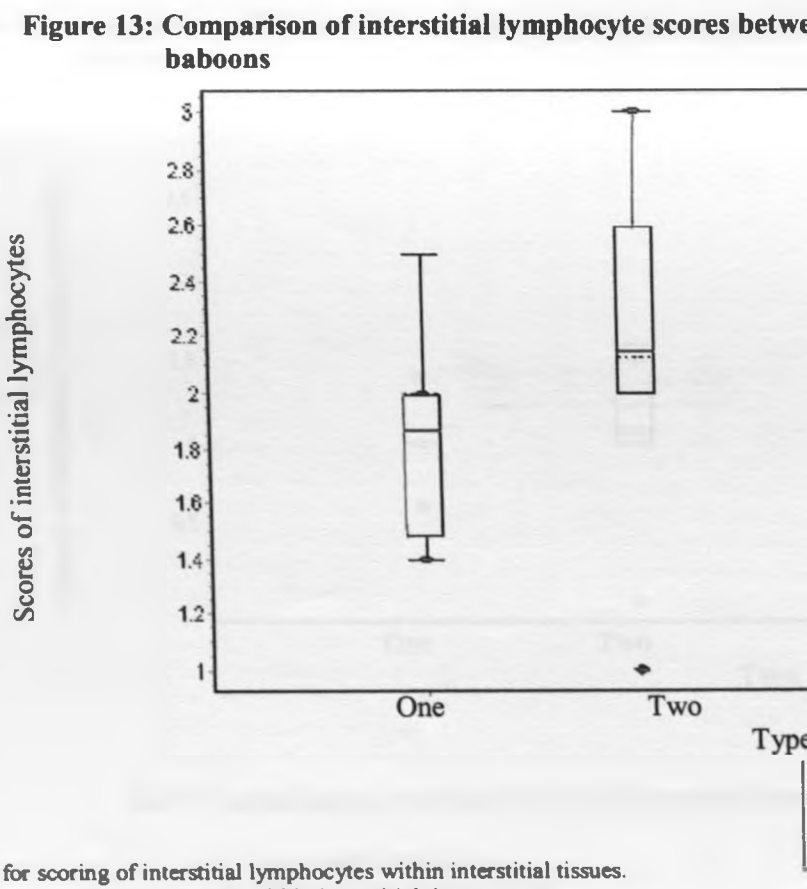
Figure 12: Comparison of interstitial degenerative slits scores betwee baboons



Key for scoring of degenerative slits within interstitial tissues.

0 = No degenerative slits
1 = Traces of degenerative slits
2 = Mild presence of degenerative slits
3 = Moderate amount of degenerative slits
4 = Severe or extensive presence of degenerative slits

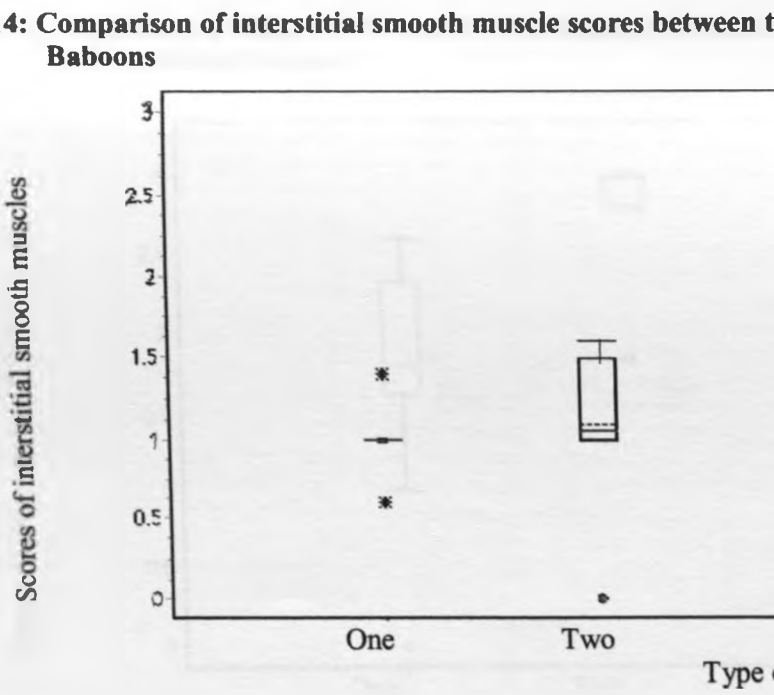
Figure 13: Comparison of interstitial lymphocyte scores betwe baboons



Key for scoring of interstitial lymphocytes within interstitial tissues.

0 = No lymphocytes within interstitial tissues.
1 = Traces of lymphocytes within interstitial tissues.
2 = Mild presence of lymphocytes within interstitial tissues.
3 = Moderate amount of lymphocytes within interstitial tissues.
4 = Severe or extensive presence of lymphocytes within interstitial tissues.

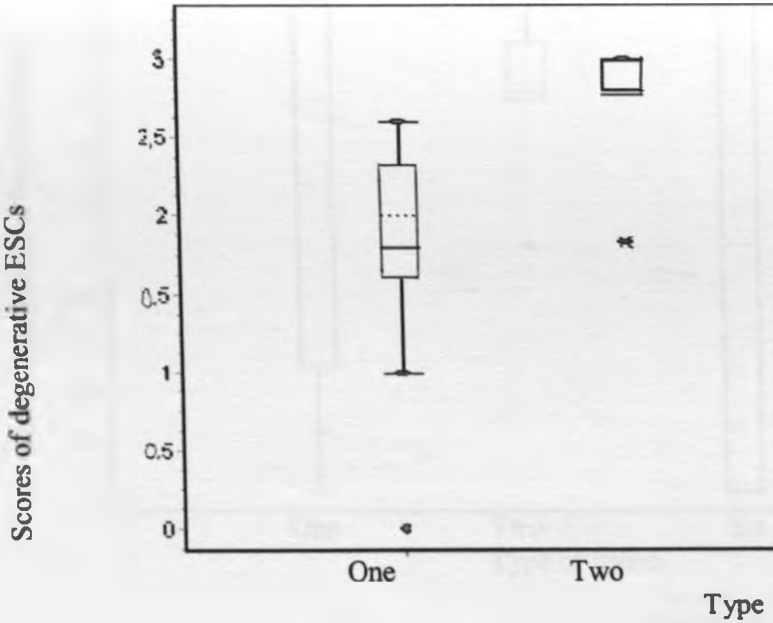
Figure 14: Comparison of interstitial smooth muscle scores between t
Baboons



Key for scoring of presence of smooth muscle in the interstitial tissu

0 = No smooth muscles seen
1 = Traces of smooth muscle seen.
2 = Mild presence of smooth muscle seen.
3 = Moderate amount of smooth muscle seen.
4 = Large amount of smooth muscle seen.

Figure 15: Comparison of degenerative scores of endometrial stroma endometriotic baboons



Key for scoring amount periglandular cuffing of glands with endometriotic stromal cells (ESCs)
0 = No ESCs surrounding glands.
1 = Small amount of ESCs surrounding glands.
2 = Mild amount of ESCs surrounding glands.
3 = Moderate amount of ESCs surrounding glands.
4 = Large amount of ESCs surrounding glands.

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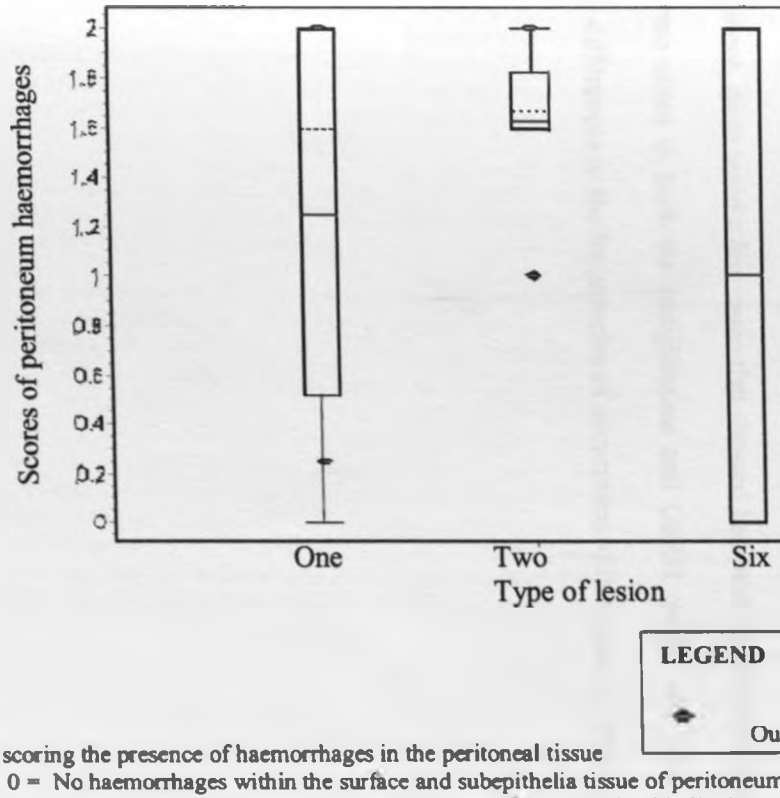
Figure 16: Comparison of haemorrhage scores in the peritoneum betw endometriotic baboons



Key for scoring the presence of haemorrhages in the peritoneal tissue

0 = No haemorrhages within the surface and subepithelia tissue of peritoneum

1 = Small to traces of haemorrhages within the surface and subepithelia tissue

2 = Mild haemorrhages within the surface and subepithelia tissue of peritoneu

3 = Moderate haemorrhages within the surface and subepithelia tissue of perit

4 = Severe haemorrhages within the surface and subepithelia tissue of peritone

4.1.6. Frequency of histological changes or features among the treatment groups

Briefly, Table 11 below summarises the results of frequency of occurrence of each histopathological change as compared among the treatment groups. This was done by using CHI Square Fisher's Exact test and only the significant changes are shown.

The frequency of histological feature was calculated from the entries within each group, as summarised in Table 4 and 5. To calculate the frequency the positive scores were divided by total number of lesions in the group.

Hemosiderin

In our experiment, there were a few cases that showed hemosiderin in the endometriotic tissues. There were two cases in both the rosiglitazone and GnRH groups and none in the placebo. However, the differences in the frequencies of occurrence of hemosiderin were not significant.

Table 11: Summary of frequency of occurrence and comparison of histological changes among the treatment groups using CHI Square Fisher's Exact Test of endometriotic baboons

Histological features	Frequency/ proportion in each group with changes			Results
	Placebo	GnRH	Rosiglitazone	
Glandular Lumen mucus	1/5	11/15	12/14	Rosiglitazone and GnRH show significantly more intense lesion than placebo (p =0.0173).
Degenerative slits in Interstitial tissue	5/7	15/16	15/15	Rosiglitazone and GnRH, though high in frequency do not show quite significant lesions than placebo (p =0.0909).

4.1.7. Presence of tissue eosinophils in endometriosis

One feature noted in endometriosis lesions was the presence of eosinophils in some of th lesions. The frequency of tissue eosinophils in the endometriosis lesions was as follows; in th rosiglitazone treated group the frequency was (4/15); the placebo group had a frequency of (1/7 and finally, the GnRH had a frequency of (1/15). There was no significant difference betwee the groups tested using CHI square Fisher's Exact test.

4.1.8. Presence of stromal endometriosis

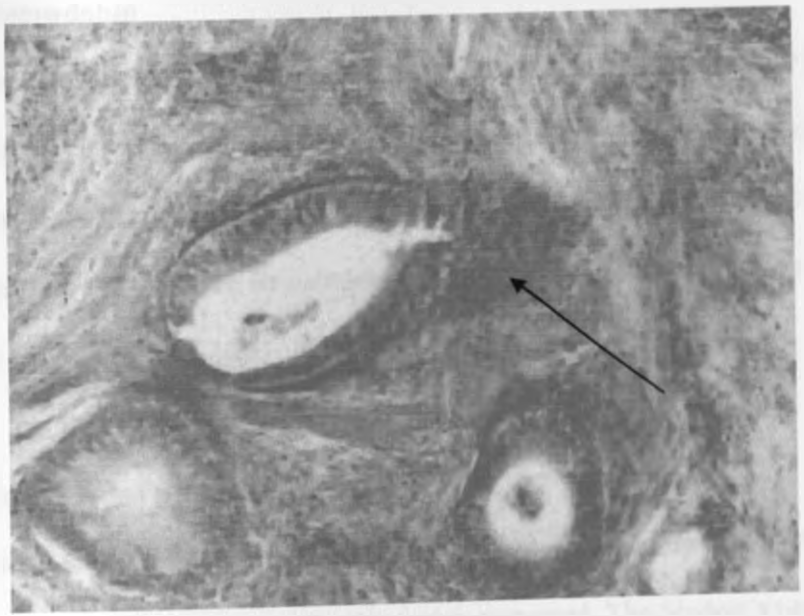
There was evidence of stromal endometriosis in some lesions. Stromal endometriosis is described as being characterised histological by small microscopic nodules or plaques o endometrioid-type stroma, sometimes with a whorled pattern and prominent vascularity and erythrocyte extravasation. These were found in 4 out of the 48 endometrial biopsies (8.3 %). In the placebo group there were 2 lesions out of 7 lesions (28.6%), and both lesions were from one animal (i.e. lesions 2912N5 and 2912N6). The GnRH group had 1 case out of 16 lesions (6.25 %), and Rosiglitazone group had also 1 case out of 16 lesions (6.25%).

4.1.9. Invasiveness of endometriosis.

Briefly, endometriosis has a property which can be termed as invasiveness. Plate 22 shows an area with this property indicated by an arrow. The photomicrograph shows an endometriotic

were immature, large with pleiomorphic nucleus and these do not rest on a clear basemen membrane. This is a characteristic that is associated with the invasiveness properties of th lesions. The gross lesion was an adhesion scar found at necropsy.

Plate 22: An endometriotic lesion from a baboon showing invasiveness property (PAN 3036).



Magnification x 250

4.2. HAEMATOLOGY.

Several parameters were checked and the significant changes are summarized in Appendix 8. The parameters are further described below.

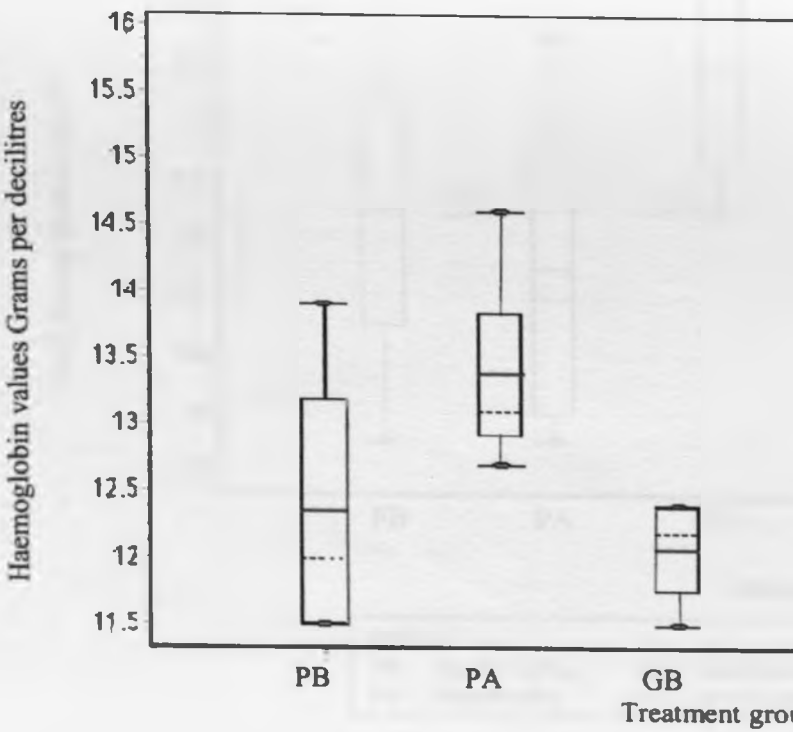
4.2.1 Haemoglobin

The haemoglobin mean values were found to have significantly increased in the Placebo and Rosiglitazone treatment groups, when the pre-treatment values and post treatment values were compared (Fig. 17). In the Placebo group the Student's test was -4.41 at a p value of 0.0216 for two-sided test. While in the Rosiglitazone group, the Student's paired t- test was -5, at a p value of 0.015 for 2 sided test.

4.2.2. Total plasma protein

There was a significant decrease in the Total protein values in the GnRH group, when the pre-treatment value and post-treatment values were compared. The Student's t-test was - 4.33, and p value of 0.023 for 2 sided test (Fig. 18).

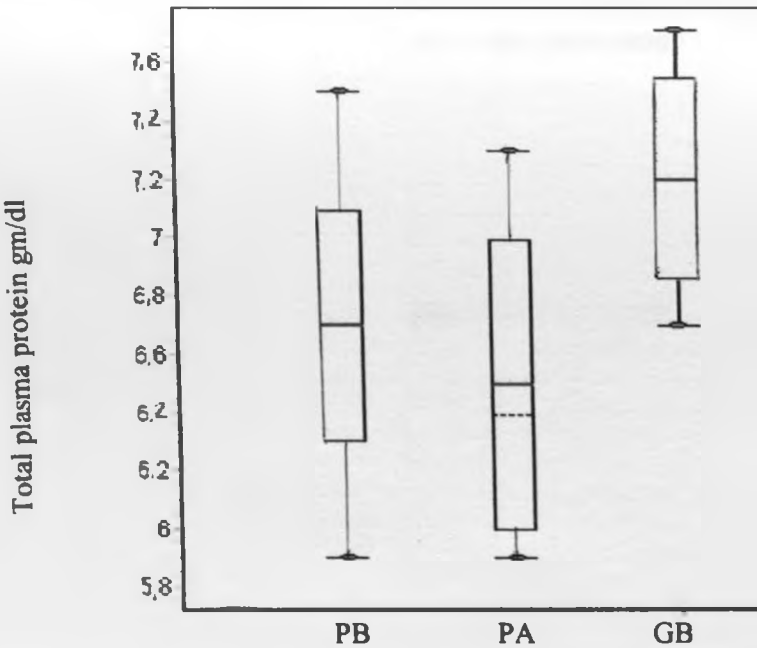
Figure 17: Haemoglobin changes before and after treatment of endo



KEY:

PB	Placebo before	GB	GnRH before
PA	Placebo after	GA	GnRH after

Figure 18: Inter group changes in total plasma in endometriot



KEY:			
PB	Placebo before	GB	GnRH bef
PA	Placebo after	GA	GnRH aft

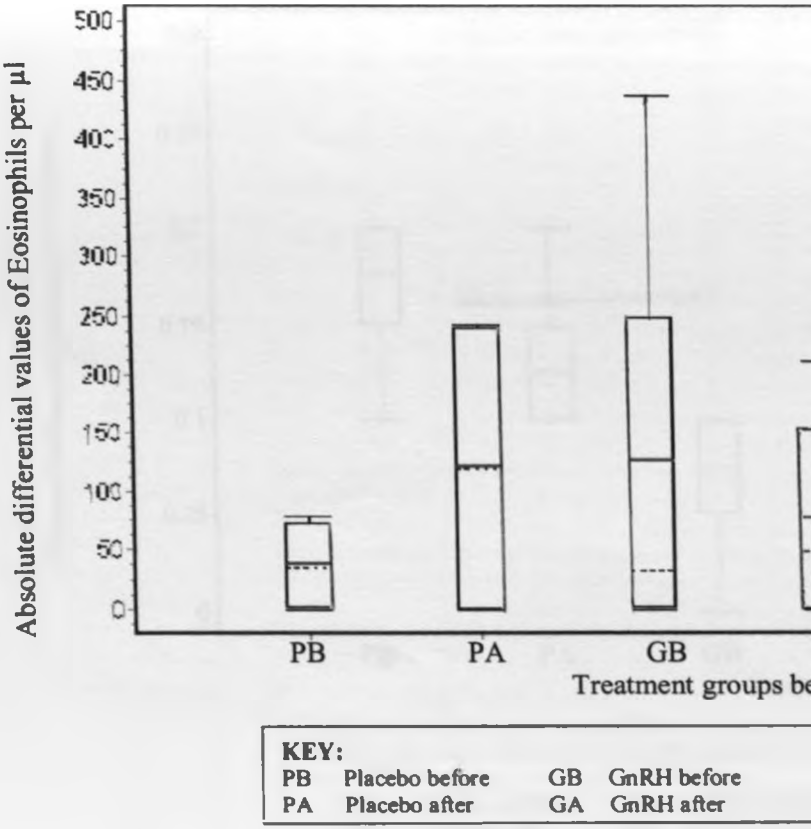
4.2.3. Eosinophils

Eosinophils values were significantly increased in the Rosiglitazone group when the pre-treatment and post treatment values were compared (Student's pair t-test = - 2.53 (Fig. 19)

4.2.4. Plasma fibrinogen

The plasma fibrinogen value was found to be significantly increased, within the GnRH group, when the pre- treatment values (0.075 ± 0.05) and the post treatment values (0.15 ± 0.06) were compared using Student's paired t- test = 3.0, p value 0.0577 (Fig. 20).

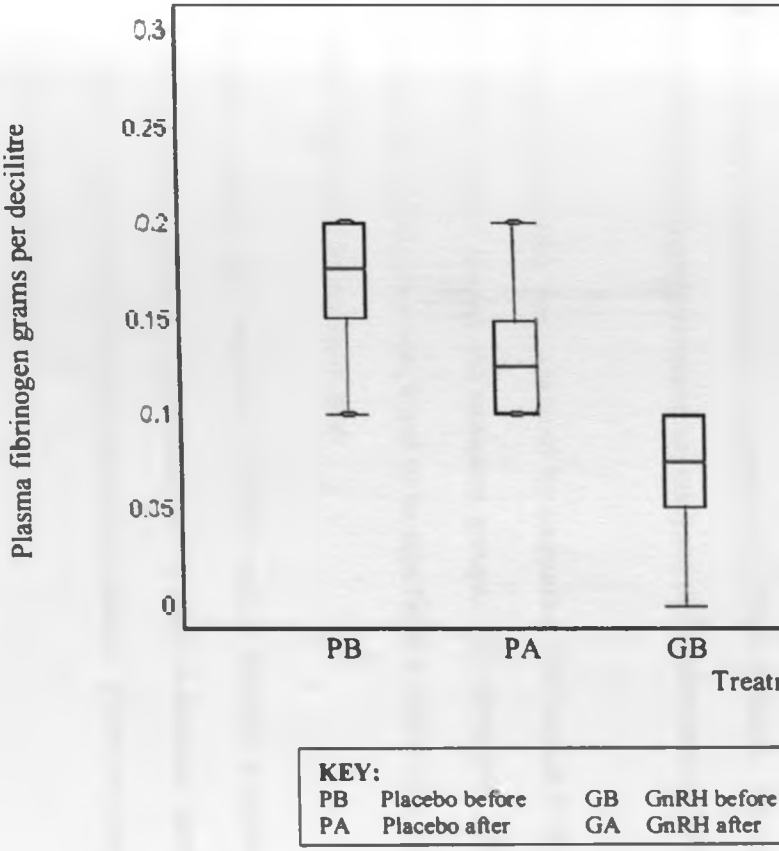
Figure 19: Eosinophils counts in endometriotic baboons



KEY:

PB	Placebo before	GB	GnRH before
PA	Placebo after	GA	GnRH after

Figure 20: Plasma fibrinogen levels in endometriotic ba



4.3. CLINICAL CHEMISTRY

Analysis using Student t tests and ANOVA showed no significant results to report.

See Appendix 11.

4.4. HISTOLOGY OF ORGANS OTHER THAN THOSE WITH ENDOMETRIOTIC LESIONS

All the individual slides examined and the organs with significant change are given in Appendix 9 to 10. A further analysis and comparison is summarised in Table 12 below. The table shows a summary of the significant changes (after statistical analysis) of the histopathology of the various other organs.

The systemic effects of treatment were analysed by comparing the frequency of histological changes in the various organs among the treatment groups. The frequency of histological changes in rosiglitazone treated group was found to be significantly higher than in the placebo and GnRH treated groups in the following organs:

i) Lung: Pulmonary oedema was moderate to severe and it occurred together with mild hemorrhages. The pulmonary oedema was characterised by mild to moderate accumulation of fluid in alveoli (Plate 23, arrow) in the rosiglitazone treated baboon. There was also presence of

ii) Liver: Bile canaliculi were surrounded or infiltration of mild amount of lymphocytes (Plate 24, arrow) around the bile duct wall and bile duct hypertrophy in the rosiglitazone treated animal.

Plates 23 to 26, shows some of the changes in the lungs and the liver. Pulmonary oedema was mild to moderate (Plate 23) in the rosiglitazone group.

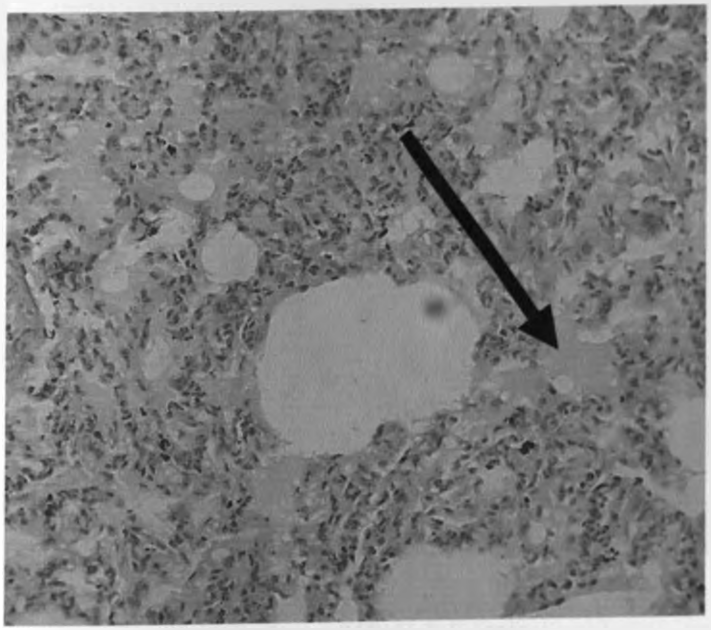
The changes in bile canaliculi changes are shown in plate 24 to 25. One section of the gall bladder showed heavy infiltration of lymphocytes in the wall, in a rosiglitazone treated baboon (Plate 25, arrow).

In our study, two animals in the rosiglitazone group showed fatty degeneration and myocardial infarcts in the heart although the frequency of the cases were not significantly high (CHI square Fisher's Exact test, p value = 0.4286). Plate 27 shows an area in the myocardium which was necrotic, infiltrated mononuclear cells, with collagen fibres, diffuse interstitial oedema, loss of myocytes and decreased striations (arrow).

Table 12: Summary and analysis of histologic findings in each

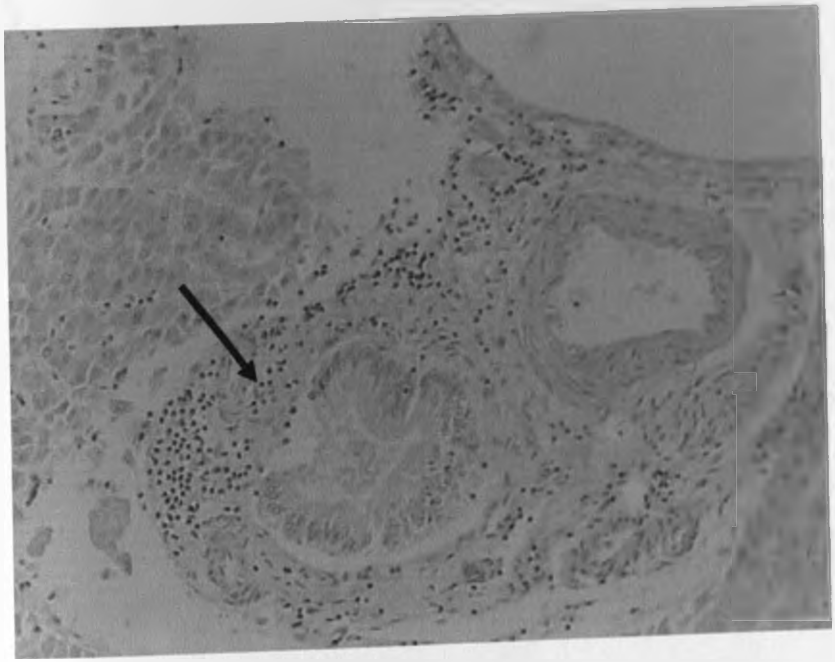
		Number of positive animals group with changes		
Organ	**Histological changes**	**Placebo**	**GnRH**	**Rosigl**
Lungs	Moderate to severe edema & hemorrhages. Emphysema with corresponding mild atelectasis	0/4	1/4	4
Liver	Bile ducts surrounded by lymphocytes.	0/4	1/4	4

Plate 23: Lung section from a rosiglitazone treated animal (PAN 2788)



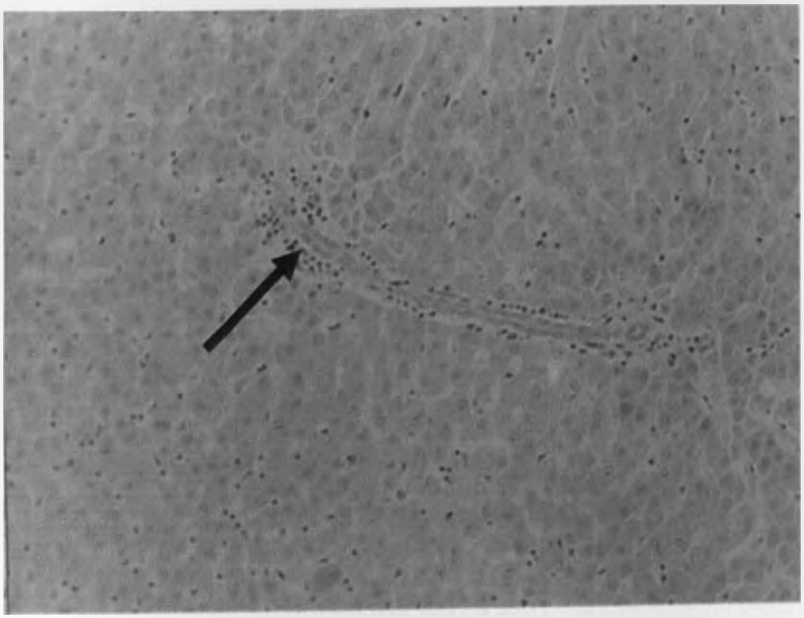
Magnification x 100, H & E Stain

Plate 24: Liver section from a rosiglitazone treated baboon (PAN 2998).



Magnification, x 100, H & E Stain

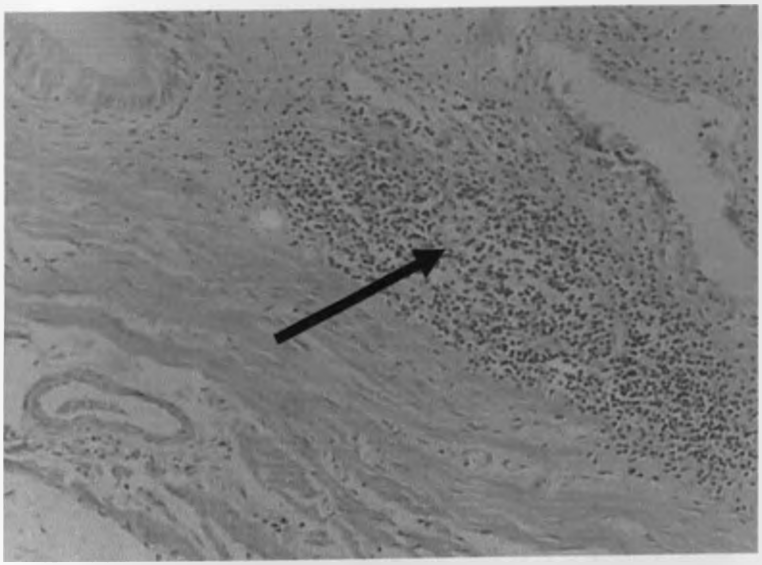
Plate 25: Liver section from a rosiglitazone treated animal (PAN 2998)



Magnification x 200, H & E Stain

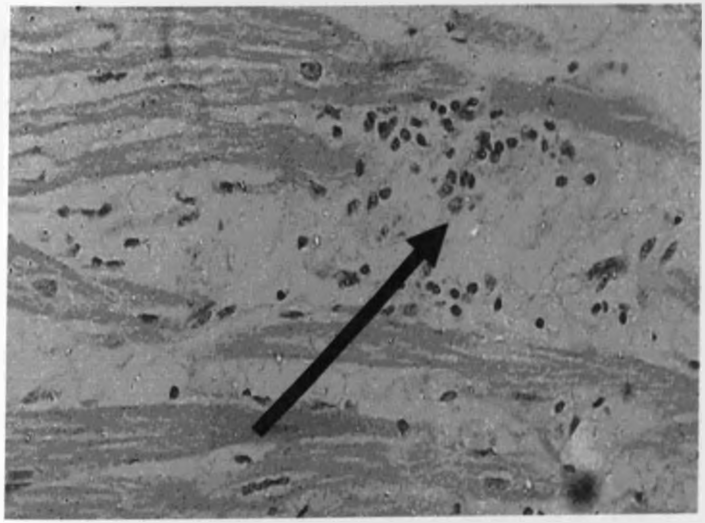
Plate 26: Section of gall bladder wall from a rosiglitazone treated animal

(PAN 2998)



Magnification x 100, H&E Stain

Plate 27: A photomicrograph showing a myocardial infarct in a rosiglitazone treated animal (PAN 2998)



Magnification x 250. H &E Stain

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. DISCUSSION

The broad objective of this study was to determine the effects of rosiglitazone on clinical feature and pathology of experimental endometriosis in a baboon model. The specific objectives were first, to determine the nature and extent of gross and histopathology changes in endometriosis lesions in baboons treated with rosiglitazone. Secondly, to document any pathological indicators of the side effects of the drug using serum chemistry, haematology and histology. And finally, to establish the target organs of rosiglitazone toxicity based on histopathology of different organs.

In summary, there was clinical evidence of therapeutic effects of rosiglitazone on the endometriosis lesions. The transformation of active lesions (red) to less active lesions (typical bluish-black or white lesions) was significantly more in rosiglitazone treatment when compared with placebo tablet treated group or (Ganirelix®) injection treated group. In this study, the placebo group appeared to worsen with time by progressing to the more active form that had typical bluish appearance; and the red lesion increased in number. This change in the placebo group is a natural progression and also occurs in untreated humans (Koninckx *et al.*, 1991) and baboons (D'Hooghe *et al.*, 1996a).

provided more information on this tissue degeneration process and corroborated the gross findings. In the present study, degenerated cells appeared with condensed pyknotic nuclei and dark staining. In all the rodent studies, changes in the ESCs were not reported (Lebovic *et al.*, 2004; Demirturk *et al.*, 2006; Aytan *et al.*, 2007).

In the present study, there was evidence of increased peritoneum lymphocytes in the rosiglitazone group. This is in contrast to Hornung *et al.*, (2003), who showed that TZDs inhibits peritoneal inflammation although comparison may not be fair, since Hornung *et al.*, 2003, used peritoneal fluid and in this study we used perineum tissue to assess the inflammatory response. In women, a preliminary report (Debrock *et al.*, 2000) described that the peritoneal fluid concentration of leukocytes and erythrocytes increased during menstruation in women with endometriosis.

In our study, it appears that the gross endometriotic lesions are associated with specific histopathological features, only in some cases. This is contrary to what some authors have documented that there is no correlation between histologic and visual findings at laparoscopy with histology (Dmowski 1984; Jansen and Russell 1986; Walter *et al.*, 2001).

To understand the effects of rosiglitazone on the endometriosis lesions, first we need to look at the pathophysiology of the disease. Inflammation, is a major feature of endometriotic lesion, it is associated with the overproduction of prostaglandins (Carli *et al.*, 2009)

cytokines such as interleukin-1β, interleukin-6 (Tseng *et al.*, 1996), and tumor necrosis factor among others (Osteen *et al.*, 1996; Giudice and Kao 2004; Bulun, 2009). Monocyte chemoattractant protein 1, interleukin-8, and RANTES attract the granulocytes, natural killer cells, and macrophages that are typical of endometriosis. The transcription nuclear factor- κB (NK- κB) appears to be activated (Guo 2006) and this activation is important in production of many cytokines (Lebovic *et al.*, 2001). Autoregulatory positive-feedback loops ensure further accumulation of these immune cells, cytokines, and chemokines in established lesions (Lebovic *et al.*, 2001). In patients with endometriosis, inflammatory and immune responses, angiogenesis, and apoptosis are altered in favour of the survival and replenishment of endometriotic tissue. These basic pathologic processes depend in part on oestrogen or progesterone (Bulun 2009). Both rosiglitazone (Yee *et al.*, 1999; Pritts *et al.*, 2002; Houston *et al.*, 2003; Wanichkul *et al.*, 2003) and GnRH-antagonists (Meresman *et al.*, 2003) have anti-inflammatory properties. And as observed in our study on endometriosis, it appears this anti-inflammatory aspect of the drug reduces lesion size and progression. Both TZD receptors have been identified in endometriotic lesions (Pritts *et al.*, 2002; Wanichkul *et al.*, 2003), and their role in the reduction of the sizes of the lesions though unknown, may be important. Earlier work on rats has documented that TZDs do cause epithelial changes in the endometriosis of rats (Lebovic *et al.*, 2004; Demirturk *et al.*, 2006; Aytan *et al.*, 2007).

The effect of GnRH-antagonist on the endometriotic glands is probably due to down regulation of LH and FSH in treated baboons (Rice 2002; Shalev 2003; Mahutte and Arici

(Donnez *et al.*, 1998; Mc Laren, 2000; Meresman *et al.*, 2003; Meresman *et al.*, 2005). VEGF may be involved in maintenance of endometriosis. GnRH receptors have been identified in ectopic endometrium (endometriosis) (Lebovic *et al.*, 2000). Oestrogens are considered more of pro-inflammatory factors, rather than anti-inflammatory (Josefsson *et al.*, 1992; Vegeto *et al.*, 2002). Different biological mechanisms underlying estrogen anti-inflammatory activity have been proposed (Vegeto *et al.*, 2002).

In our study, there was incidental induction of stromal or micronodular endometriosis. Stromal endometriosis has been described by Clement (2007), as a specific type of endometriosis with no glands. There are reports on the incidence of stromal endometriosis in humans and baboons. They range from 6.6% of human biopsies (Boyle and McCLuggage 2009), and 8% in baboons (Cornillie *et al.*, 1992). Stromal endometriosis tends to take a superficial position as a typical location on the peritoneum, intimately associated with surface mesothelium (Cornillie *et al.*, 1992; Boyle and McCLuggage 2009). After studying many stromal endometriosis tissues, Boyle and McCLuggage (2009) concluded that stromal endometriosis is induced when the menstrual tissue comes into contact with the peritoneum via retrograde menstruation. The superficial production of stromal endometriosis in this study, supports the theory that coeleomic metaplasia may be involved in pathogenesis of endometriosis. There are some suggestions that endometriosis originates or grows from the endometriotic stromal cells (Mai *et al.*, 1998). To support this theory, we have seen several incidences of histological evidence of invasiveness or continuity of glandular epithelium into

It is observed elsewhere that the endometriotic tissue epithelium may exhibit different phases in the same woman (Bergqvist *et al.*, 1984; Metzger *et al.*, 1988). Bergqvist *et al.* (1984), reported that 25 to 49 % of endometrial implants displayed some form of local haemorrhages irrespective of the timing of menstrual cycle. The presence of local haemorrhages, (either hemosiderin-laden macrophage or extravaseted red blood cells (RBCs) may indicate menstrual activity in the endometriotic glands (Bergqvist *et al.*, 1984; Metzger *et al.*, 1988). Since the baboons used in our experiment were not in the menstruation phase, these results may support this observation.

In his article, Clement (2007) discussed and emphasised on the problems faced by pathologists in diagnosis of endometriosis. Many diagnostic problems can arise as a result of alterations or absence of glandular or stromal components in the lesion. The diagnostic difficulty can be compounded by the limited tissue in a small biopsy specimen. Also histological diagnosis of endometriosis can also be challenging when the gross lesion occurs in an unusual or unexpected site. Clement (2007) mentions five such sites as on or near ovarian surface, superficial cervical endometriosis, vaginal endometriosis, tubal endometriosis, and intestinal endometriosis. Finally, endometriotic foci can occasionally be intimately admixed with another process, such as peritoneal leimyomatosis or gliomatosis, resulting in a potentially confusing histologic appearance (Clement 2007).

of a number of pathological conditions e.g. parasitic infections, local allergic reactions (such as asthma), rhinitis and eczema, and hypereosinophilic syndrome (Gleich *et al.*, 1993). In addition, eosinophils produce cytokines such as interleukin (IL-3, IL-5) and granulocyte-macrophage colony stimulating factor (GM-CSF) that contribute to their pro-inflammatory functions. The cationic proteins, major basic protein (MBP), eosinophil peroxidase (EPO) and eosinophil cationic protein (ECP) are toxic to airway epithelium and may be deposited over large areas of the bronchial epithelial lining in patients with asthma (Gleich *et al.*, 1993; Rabe *et al.*, 1994). The eosinophil MBP also causes the release of histamine from mast cells and basophils, activates neutrophils and alveolar macrophages, and is directly implicated in epithelial cell damage, exfoliation and bronchospasm in asthma.

The presence of eosinophils in endometriosis tissue might be associated with a physiological repair role (Birkland *et al.*, 1994), or a possible pathophysiology role in endometriosis (Nagata 2005). At sites of inflammation, multiple inflammatory cells including eosinophils, neutrophils and macrophages are capable of generating reactive oxygen species (ROS) which can contribute to development of various diseases, including endometriosis (Nagata 2005). Morphological and functional properties of endothelial cells may be altered by ROS, including permeability and adhesion molecule expression (Nagata 2005). In endometriosis, oxidative stress and antioxidant biomarkers are present in both serum and peritoneal fluid. Products of this process are exported to serum/plasma where the oxidised metabolites are incorporated into carriers, such as ox-LDL (Murphy *et al.*, 1998). Studies have

In this present experiment, haemoglobin levels increased within the placebo treated group and in the rosiglitazone treated baboons compared to the GnRH group. It is suggested that heme may be involved in the pathogenesis and/or development of endometriosis and that the immune system, might be insufficient to detoxify heme in women with endometriosis (Van Langendonckt *et al.*, 2002a; Van Langendonckt *et al.*, 2002b). Iron metabolism by macrophages seems to be enhanced in case of endometriosis. Also higher levels of iron, which is probably released after lysis of erythrocytes, have been found in the peritoneal fluid of patients with endometriosis (Arumugam 1994), and the concentration of iron is related to the severity of disease (Arumugam and Yip 1995). This is supported by the fact that siderophages, iron-storing macrophages that are heavily laden with hemosiderin, are considered to be indicators of endometriosis (Gaulier *et al.*, 1983; Stowell *et al.*, 1997). The presence of hemosiderin in the endometriotic lesions cannot though be extrapolated to the increase in peripheral haemoglobin levels in our experiment.

In rosiglitazone treated patients, haemoglobin has been reported to go down and in most cases fluid retention has been implicated through hemodilution (Actos 2000; Avandia 2000). It is reported that TZDs can cause weight gain and fluid retention in some patients. These changes in weight gain and blood profile are usually observed during the first weeks of therapy and plateau thereafter. In a review of several studies, Hollenberg (2003), showed that the decreases in haemoglobin and in hematocrit were highly correlated, providing evidence favouring hemodilution as the responsible mechanism. Plasma protein concentrations are

In one study, the frequency of decrease in plasma protein concentration was about half that observed for haemoglobin and hematocrit and did not correlate with either of these parameters (Hollenberg 2003). This observation is not in agreement with simple hemodilution, suggesting that additional studies are needed to explain these findings. Toxicity studies by Rothwell *et al.*, (2001) with troglitazone in nonhuman primates documented a reduction of erythrocyte counts, haemoglobin, and hematocrit of 8% to 16% in haemoglobin values of males at all doses used in their experiment. This was quite opposite with what we found in this study, where there was an increase in haemoglobin values from pre treatment to post treatment period in all groups.

The results on total plasma protein showed that a significant decrease occurred from the pre-treatment to post-treatment period in the GnRH treated group. From the literature it appears that there is a positive relationship between oestrogen and plasma protein (Nowaczynski *et al.*, 1978; Korsgaard, 1990). This could explain the decrease in total plasma protein, since oestrogen was decreased in GnRH antagonist treated group.

Although haematology in this study indicated changes only in eosinophils, total plasma and haemoglobin in the peripheral circulation, there are publications that show that endometriosis as a disease may have other subclinical systemic manifestations (Barbcri *et al.*, 1986; Pittaway and Fayez 1986; D'Hooghe *et al.*, 1996c; Mol *et al.*, 1998; Bedaiwy *et al.*, 2002; Somigliana *et al.*, 2004; Agic *et al.*, 2006; Cho *et al.*, 2007; Seeber *et al.*, 2008).

Factor α (TNF-α) (Dmowski 1995), Monocyte Chemotactic Factor – 1 (MCP-1) (Akoum *et al.*, 2002), Interleukin -6 (IL-6) (Tseng *et al.*, 1996), Interleukin- 8 (IL-8) (Bedaiwy *et al.*, 2002) and Chemokine (C-C motif) receptor 1 (CCR1) (Zhang *et al.*, 1999), in the peripheral blood of patients with endometriosis (Agic *et al.*, 2006). CD44+ and CD14+ monocytes are significantly increased, while CD3+ T lymphocytes and CD20+ B lymphocytes show modest, but significant decrease in peripheral blood of women with endometriosis. Some previous work at the IPR, has shown that the percentage of CD4+ and IL2R subsets of white blood cells, was increased in baboons with the stage II-IV spontaneous endometriosis (D'Hooghe *et al.*, 1996c).

The histology on the various tissues other than the endometriotic lesions showed varying changes in some organs. The lungs had mild, moderate to severe pulmonary oedema which was significantly more in rosiglitazone treated group than in the placebo group. Pulmonary oedema has been reported by clinicians as an important side effect of rosiglitazone in treatment of diabetes (Idris *et al.*, 2003; Çekmen *et al.*, 2006). From the literature, the use of TZDs is associated with a triad of fluid retention, oedema, and weight gain (Niemeyer and Janney 2002; Kermani and Garg 2003; Nesto *et al.*, 2003; Idris *et al.*, 2003; Mudaliar *et al.*, 2003). The incidence of oedema ranges from 2% to 5% in diabetic patients under rosiglitazone therapy (Kermani and Garg 2003). If combined with insulin, this ratio increases to 14.7% (Çekmen *et al.*, 2006). Fluid retention generally is considered mild and

weight gains were not significantly different between pre-treatment and post-treatment period within group; or between the 3 treatment groups.

Rosiglitazone may have the risks of congestive heart failure (CHF) and Myocardial infarcts (MI) (Wooltorton 2002; Nissen and Wolski 2007; http://www.nytimes.com. 22 June 2007). The theory behind the CHF is a cumulative effect of fluid overload described above. Recently, warnings about possible myocardial ischemia and MI have been issued by Food, Drugs Administration in the USA (http://www.fda.gov 21st May 2007; http://www.fda.gov 19th November 2007). The interim report on Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of glycemia in Diabetes (RECORD) trial has also raised substantial uncertainty about the complete cardiovascular safety of the use of this drug, at least in the diabetic population (Home *et al.*, 2007). Therefore, TZDs should be used cautiously in patients with heart failure symptoms.

Toxicity studies by Rothwell *et al.*, (2001) with troglitazone documented an increase in absolute and relative liver weights at all doses in cynomolgus monkeys of both sexes. The doses used ranged from approximately 3, 6 to 10 times those given to humans. Humans are given 400 mg daily. Minimal or mild bile duct hypertrophy was present at all doses and both incidence and severity increased with increasing doses (Rothwell *et al.*, 2001). This finding confirms with reports on changes on gall bladder and bile ducts.

The hepatotoxicity of troglitazone does not seem to translate across the class of TZDs (Scheen 2001; Van Gaal and Scheen 2002). Reversible liver injury and hepatic failure have occurred in a few patients with both drugs (Ravinuthala and Nori 2000; May *et al.*, 2002) but cause and effect have not been established (Lebovitz *et al.*, 2002). With either drug, the FDA recommends monitoring of liver function every 2 months for the first year and less frequently thereafter. In our study, the biochemical tests did not reveal any significant changes among the 3 treatment groups. The absence of any change many exonerated rosiglitazone from hepatotoxicity.

The other known effects of TZDs are as follows; they may slightly increase LDL cholesterol levels, but they also change the small, dense cholesterol particles, thought to increase risky effects to more the buoyant ones, thought to be safer. These drugs increase HDL by about 10 -20%, decrease triglycerides, decrease PAI-I activity and improve endothelial function (Nesto *et al.*, 2003). They also are thought to increase subcutaneous but not visceral adiposity (Parulkar *et al.*, 2001). Pioglitazone may have a greater favourable effect than rosiglitazone on serum lipids (Khan *et al.*, 2002). There are "TZDS class effects" and "TZD-specific effects" that has differentiated the use of rosiglitazone vis a vis troglitazone. For this reason there is need to study other TZDs so as to know more on the other "TZD – specific effects" which may be different from Rosiglitazone. Class effects include fluid

In conclusion, this study presents the first subhuman primate evidence that treatment with a TZD can reduce the surface area (~50% decrease in relative change compared with placebo) of peritoneal endometriosis in baboons with established disease. This work shows lesions such as extensive glandular epithelium necrosis and degeneration of glandular epithelium of endometriotic lesions. Degenerative changes of endometriotic stromal cells (ESCs) were also a significant finding. Histopathology has also confirmed that the gross laparoscopy appearance of endometriosis conforms to some histological features. Total plasma protein concentration increased in GnRH treatment group. The eosinophil count increased in rosiglitazone treatment group, and plasma fibrinogen also increased in GnRH treatment group. There was also an incidental induction of stromal or micronodular endometriosis. The most consistent changes were moderate to severe pulmonary oedema & hemorrhages; and bile ducts that were surrounded by inflammatory cells. Presence of pulmonary oedema and bile duct changes was significantly more prominent in rosiglitazone group.

This study shows that rosiglitazone as a thiazolidinedione is a helpful anti-inflammatory and growth inhibitor agent in the treatment of the disease. It reduced the extent of endometriosis in the baboon model.

5.2. CONCLUSIONS

1) The present study successfully showed that rosiglitazone could significantly diminish

reduction of volume and surface area of the endometriosis lesions is concerned. It also provides more detailed explanation of the necrosis in the epithelial glands.

2) The baboon model can be used to test new drugs for treatment or prevention of endometriosis and it is therefore important in testing general and reproductive safety of new anti-endometriosis drugs.

3) An ideal therapy option for treating endometriosis should possess minimal side effects, low cost burden, and proven efficacy at diminishing pelvic pain. It should spare fertility potential during treatment. Rosiglitazone is therefore a potential candidate for this therapy.

5.3. RECOMMENDATIONS

1) Clinical trials would be helpful in determining the utility of this novel class of compounds for women with endometriosis.

2) More of the TZDs class of drugs should be investigated to determine other "TZD – specific effects" which may be different from those of rosiglitazone.

3) Currently no available drugs resolve pain due to endometriosis. The baboon can be developed as an animal model for endometriosis related pain-alleviating drugs, since pain is a major problem in endometriosis.

4) Research on biomarkers for diagnosis of endometriosis is an important area of future research. Currently there are no non-invasive tests for diagnosis of endometriosis. The

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7.0 APPENDICES

Appendix 1: Sampling Form

PRE TREATMENT/ POST TREATMENT LAPAROSCOPY SAMPLING FORM

Animal No: Pan ..... Date: ...../...../.....

Weight At Surgery: ..... kgs.

Description of Surgery: Classical laparascopy.
Remarks:

Peritoneal Fluid: Present Yes/No.

If Yes: Volume:

Color: Red/Brown/Pink/Yellow/Clear.

Endometrial Biopsy.

Technique: Transcervical Laparascopic control:
Chai Technique:
Verress needle technique.

Weight (mg):

Volume (ml):

Documentation:

Photo: .....

Video: .....

Appendix 2: Staging laparoscopy form

Rosiglitazone project

Animal Number; Pan ..... Date: ...../...../.....

No	Type of lesion	Location of lesion	Size of lesion	Comments
1			x x mm	
2			x x mm	
3			x x mm	
4			x x mm	
5			x x mm	
6			x x mm	
7			x x mm	
8			x x mm	
9			x x mm	
10			x x mm	
11			x x mm	
12			x x mm	
13			x x mm	
14			x x mm	
15			x x mm	
16			x x mm	
17			x x mm	
18			x x mm	
19			x x mm	
20			x x mm	
21			x x mm	
22			x x mm	
23			x x mm	
24			x x mm	
25			x x mm	
26			x x mm	
27			x x mm	
28			x x mm	

	KEY for type of lesion
1	Typical blue-black
2	White plaque with pigmented spots
3	Red vesicular
4	Red hemorrhagic
5	Red polyploid
6	White plaques
7	White nodules
8	White clear vesicles
9	Red-white plaque
10	Peritoneal ulcer
11	Filmy adhesion
12	Dense adhesion

	Key for location of lesion.
1	Anterior fundus
2	Posterior fundus
3	Uterovesical fold
4	Anterior broad ligament
5	Posterior broad ligament
6	Bladder
7	Round ligament
8	Fallopian tube
9	Pouch of Douglas
10	Pelvic wall
11	Posterior cervix
12	Anterior cervix
13	Guttural pouch
14	Ovarian bursa
15	Rectum
16	Anterior peritoneal wall
17	Sacrouterine ligament wall

Side of location
1 Left

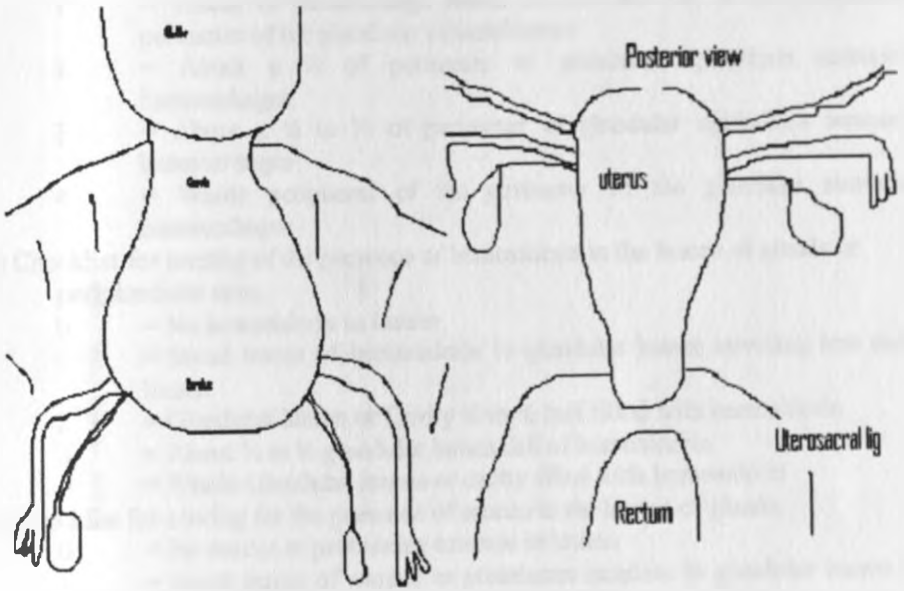
Appendix 3. Pelvic anatomic map

Induction Laparoscopy (Lap 1)/ Post-treatment staging Laparoscopy (Lap 2).

Animal Number: Pan ..... Date .....

Cycle Stage/ Day, ...

Localization of lesions and Adhesions.



Appendix 4. Checklist for scoring of the histological features in glandular epithelium features

i) Checklist for scoring for haemorrhages within the lumen of the glands.

0 = No blood in lumen

1 = Small traces of extravasated blood in glandular lumen covering less than ¼ of lumen

2 = Glandular lumen or Cavity ¼ to ½ half filled with extravasated blood.

3 = About ½ to ¾ glandular lumen full of extravasated blood

4 = Whole Glandular lumen or cavity filled with extravasated blood.

ii) Checklist for scoring of the Periglandular haemorrhages.

Scoring for presence and amount haemorrhages around the immediate vicinity of glands within the interstitial tissue.

0 = No haemorrhage

1 = Traces of haemorrhage found on less the 1/4 of the peripheral of the perimeter of the glandular circumference

2 = About a ½ of perimeter of glandular epithelium surrounded by haemorrhages

3 = About a ½ to ¾ of perimeter of glandular epithelium surrounded by haemorrhages

4 = Whole peripheral of the perimeter of the glandular surrounded by haemorrhages

iii) Checklist for scoring of the presence of hemosiderin in the lumen of glands or periglandular area.

0 = No hemosiderin in lumen

1 = Small traces of hemosiderin in glandular lumen covering less than ¼ of lumen

2 = Glandular lumen or Cavity ¼ to ½ half filled with hemosiderin

3 = About ½ to ¾ glandular lumen full of hemosiderin

4 = Whole Glandular lumen or cavity filled with hemosiderin

iv) Checklist for scoring for the presence of mucus in the lumen of glands.

0 = No mucus or proteinous exudate in lumen

1 = Small traces of mucus or proteinous exudate in glandular lumen covering less than ¼ of lumen

2 = Glandular lumen ¼ to ½ half filled with mucus or proteinous exudate

3 = About ½ to ¾ glandular lumen full of mucus or proteinous exudate

4 = Whole Glandular lumen or cavity filled with mucus or proteinous exudate

iv) Checklist for scoring of the number of cross sections of glands.

The numbers of cross sections of glands were counted and the average noted

v) Checklist for scoring of the degenerative or necrotic changes on the glandular epithelium.

0 = Normal epithelium

1 = Traces of degeneration in some sections of the gland as characterised by a compact nuclei and cytoplasm with a deep staining.

2 = Mild degeneration with pykynotic nuclei with epithelial cell having more dark stain within the whole glandular epithelium.

3 = Moderate degeneration nucleus completely pykynotic nucleus epithelia cells with intact shape and organisation.

4 = Severe degeneration and karyorhexis and disintegration of the nucleus and epithelial cells i.e. epithelium necrotised.

vi) Checklist for scoring of the erosion of the glandular epithelium.

0 = Normal epithelium.

1 = Trace or early signs of areas with epithelial separation/absent from basement membrane. Cells also with degeneration as characterised by a compact nuclei and cytoplasm with a deep staining nuclei.

2 = About ¼ of epithelium desquamated/ absent with remaining cells characterised by a compact nuclei and cytoplasm with a deep staining nuclei.

3 = Moderate about ½ to ¾ of epithelium in glands separated/absent with remaining cells characterised by a compact nuclei and cytoplasm with a deep staining nuclei.

4 = Whole gland has no epithelium

vii) Checklist for scoring of the presence of acute inflammatory cells like neutrophils in the lumen of glands.

0 = No acute inflammatory cells in lumen

1 = Small number of inflammatory cells in glandular lumen covering less than ¼ of lumen

2 = Glandular lumen ¼ to ½ filled with acute inflammatory cells

3 = About ½ to ¾ glandular lumen filled with acute inflammatory cells.

4 = Whole Glandular lumen filled with acute inflammatory cells.

viii) Checklist for scoring of the chronic inflammatory cells, i.e. mononuclear cell, lymphocytes in the glands.

0 = No chronic inflammatory cells in lumen

1 = Small number of chronic inflammatory cells in glandular lumen covering less than ¼ of lumen

2 = Glandular lumen ¼ to ½ filled with chronic inflammatory cells

3 = About ½ to ¾ glandular lumen filled with chronic inflammatory cells.

4 = Whole Glandular lumen filled with chronic inflammatory cells.

Appendix 5. Checklist of scoring of the histologic features in interstitial tissue.

i) Checklist for scoring of haemorrhages,

0 = No haemorrhages within interstitial tissue.
1 = Traces of haemorrhages within interstitial tissue.
2 = Mild haemorrhages within interstitial tissue.
3 = Moderate haemorrhages within interstitial tissue.
4 = Severe haemorrhages within interstitial tissue.

ii) Checklist for scoring of hemosiderin deposits in the interstitial tissues.

0 = No hemosiderin
1 = Traces of hemosiderin
2 = Mild amount of hemosiderin
3 = Moderate amount of hemosiderin
4 = Severe hemosideriosis

iii) Checklist for scoring the degenerative slits within interstitial tissues.

0 = No degenerative slits
1 = Traces of degenerative slits
2 = Mild presence of degenerative slits
3 = Moderate amount of degenerative slits
4 = Severe or extensive presence of degenerative slits

iv) Checklist for the amount of degeneration of endometriotic stromal cells (ESCs) within the interstitial

0 = No degeneration of ESCs
1 = Traces of degeneration in some ESCs as characterised by a compact nuclei and cytoplasm with a deep staining nuclei.
2 = Mild number of ESCs with degeneration.
3 = Moderate number of ESCs with degeneration.
4 = Most of ESCs with degeneration.

v) Checklist for scoring for the presence of sworls, arcs formation of the connective tissue.

0 = No presence of sworls, arcs formation of the connective tissue.
1 = Traces of sworls, arcs formation of the connective tissue.
2 = Mild presence of sworls, arcs formation of the connective tissue.
3 = Moderate presence of sworls, arcs formation of the connective tissue.
4 = Extensive presence of sworls, arcs formation of the connective tissue.

vi) Checklist for scoring of the presence of acute inflammatory cells in the interstitial tissue.

0 = No acute inflammatory cells in interstitial tissue
1 = Small number of inflammatory cells in interstitial tissue
2 = Mild presence of acute inflammatory cells in interstitial tissue.
3 = Moderate presence of acute inflammatory cells in interstitial tissue
4 = Severe presence of acute inflammatory cells in interstitial tissue.

vi) Checklist for scoring for the presence of a diffuse ground substance, myxoid, in the interstitial tissue.

0 = No homogenous ground substance in interstitial tissue
1 = Traces or scant amount of homogenous ground substance in interstitial tissue.
2 = Mild presence homogenous ground substance in interstitial tissue
3 = Moderate presence homogenous ground substance in interstitial tissue
4 = Severe presence homogenous ground substance in interstitial tissue

vii) Checklist for scoring of presence of Smooth muscle in the interstitial tissue.

0 = No smooth muscles seen
1 = Traces of smooth muscle seen.
2 = Mild presence of smooth muscle seen.
3 = Moderate amount of smooth muscle seen.
4 = Large amount of smooth muscle seen.

viii) Checklist for scoring for the numbers of blood vessel i.e. both small or large blood vessels.

0 = No arterioles or arteries present.
1 = Small number of arterioles and arteries present
2 = Mild number of arterioles and arteries present
3 = Moderate number of arterioles and arteries present
4 = Arterioles and arteries spread evenly all over the interstitial space.

vix) Checklist for scoring amount of fat.

0 = No fat
1 = Traces of fat seen
2 = Mild amount of smooth muscle seen.
3 = Moderate amount of smooth muscle seen.
4 = Large amount of smooth muscle seen.

x) Checklist for scoring for interstitial oedema as evidence by spatial separation of interstitial structures by empty spaces.

0 = No evidence of oedema seen.
1 = Traces of oedema seen.
2 = Mild evidence of oedema seen.
3 = Moderate evidence of oedema seen.
4 = Large separation of structures with empty spaces.

xi) Checklist for scoring amount periglandular cuffing of glands with endometriotic stromal cells (ESCs) were checked.

0 = No ESCs surrounding glands
1 = Small amount of ESCs surrounding glands
2 = Mild amount of ESCs surrounding glands
3 = Moderate amount of ESCs surrounding glands.
4 = Large amount of ESCs surrounding glands

xii) Checklist for scoring the scatter and spread of ESCs within the interstitial tissue.

0 = No evidence of ESCs scatter or spread within interstitial tissue.
1 = Small to traces of amount of ESCs scatter or spread within interstitial tissue.
2 = Mild amount of ESCs scatter or spread within interstitial tissue.
3 = Moderate of ESCs scatter or spread within interstitial tissue.
4 = Wide spread amount of ESCs scatter or spread within interstitial tissue.

xiii) Checklist for scoring the amount of hemosiderin in the interstitial tissue.

0 = No amount of hemosiderin in the interstitial tissue.
1 = Small to traces of amount of hemosiderin in the interstitial tissue.
2 = Mild amount of hemosiderin in the interstitial tissue.
3 = Moderate amount of hemosiderin in the interstitial tissue.
4 = Severe hemosiderosis.

ix) Key for scoring of interstitial lymphocytes within interstitial tissues.

0 = No lymphocytes within interstitial tissues.
1 = Traces of lymphocytes within interstitial tissues.
2 = Mild presence of lymphocytes within interstitial tissues.
3 = Moderate amount of lymphocytes within interstitial tissues.
4 = Severe or extensive presence of lymphocytes within interstitial tissues.

Appendix 6. Checklist of scoring for the histologic features in peritoneum histologic features.

i) Checklist for scoring the presence of haemorrhages in the peritoneal tissue

0 = No haemorrhages within the surface and subepithelia tissue of peritoneum.
1 = Small to traces of haemorrhages within the surface and subepithelia tissue of peritoneum.
2 = Mild haemorrhages within the surface and subepithelia tissue of peritoneum.
3 = Moderate haemorrhages within the surface and subepithelia tissue of peritoneum.
4 = Severe haemorrhages within the surface and subepithelia tissue of peritoneum.

ii) Checklist for scoring for the presence of hemosiderin within the surface and subepithelia tissue of peritoneum.

0 = No hemosiderin within the surface and subepithelia tissue of peritoneum tissue of peritoneum.
2 = Mild hemosiderin within the surface and subepithelia tissue of peritoneum.
3 = Moderate hemosiderosis within the surface and subepithelia tissue of peritoneum.
4 = Severe hemosiderosis.

iii) Checklist for scoring the presence of lymphocytes within the surface and subepithelium tissue of peritoneum.

0 = No lymphocytes within the surface and subepithelia tissue of peritoneum.
1 = Small to traces of lymphocytes within the surface and subepithelia tissue of peritoneum.
2 = Mild amount lymphocytes within the surface and subepithelia tissue of peritoneum.
3 = Moderate amount of lymphocytes within the surface and subepithelia tissue of peritoneum.
4 = Severe infiltration of lymphocytes within the surface and subepithelia tissue of peritoneum.

iv) Checklist for scoring for the presence of acute inflammatory cells within the surface and subepithelia tissue of peritoneum

0 = No acute inflammatory cells present.
1 = Traces amount of acute inflammatory cells.
2 = Mild amount of acute inflammatory cells.
3 = Moderate amount of acute inflammatory cells.
4 = Severe infiltration of acute inflammatory cells.

v) Checklist for scoring for the presence of fibrin on and under the peritoneal surface if present.

0 = No evidence of fibrin deposits on surface of peritoneum.
1 = Trace evidence of fibrin deposits on surface of peritoneum.
2 = Mild fibrin deposits on surface of peritoneum.
3 = Moderate fibrin deposits on surface of peritoneum.
4 = Large fibrin deposits on surface of peritoneum.

Appendix 7: Data from lesions collected during the pre-and post-treatment laparoscopy of individual endometriotic baboons (Mean ± standard deviation)

	Placebo		GnRH - antagonist		Rosiglitazone	
	Pre	Post	Pre	Post	Pre	Post
Mean surface area (mm^2)with Adhesions	145,219,314,45. (180.8 ± 113.9)	162,209,235,53. (164.6 ± 80.6)	191,459,430,180 (315 ± 150.1) $	109,118,389,96. (177.9 ± 141.2) $	209,328,134,291) (240.3 ± 86.8) †	55,235,87,116 (123.2 ± 78.3) †
Mean surface area (mm^2) without Adhesions	65, 195, 180,45. (121.3 ± 77.2)	32, 193, 74, 53. (89.6 ± 71.8)	133, 94, 114, 98. (109.8 ± 17.7) *	50, 64, 60, 50. (55.9 ± 7.3) *	209, 83, 59, 75 (106.3 ± 69.2) @	55,45, 29, 40. (42.2 ± 10.8) @
Mean number of lesions	8,13,7,10 (9.5 ± 2.6)	7,13,6,11 (9.3 ± 3.3)	9,9,8,15 (10.3 ± 3.2)	9,9,11,14 (10.8 ± 2.4)	15,18,7,13 (13.3 ± 4.6)	15, 16, 6, 13 12.5 ± 4.5)
Mean number of Red lesions	4, 3, 0, 0 (1.8 ± 2.1)	3, 6, 0, 3. (3 ± 2.4)	4,1, 1, 11. (4.3 ± 4.7)	1, 2, 2, 5. (2.5 ± 1.7)	6, 6, 5,0. (4.3 ± 2.9)	1, 0, 2, 0. (0.8 ± 1.3)
Mean area of red lesions (mm^2).	38, 46, 0, 0. (21± 24.5)	17, 112, 0, 32. (40.3 ± 49.6)	69, 1, 1, 83. (8.5 ± 43.7)	9,3,7,16. (8.7 ± 5.3)	166,29,57,0 (62.9 ± 72.5)	2,0,14, 0 (4 ± 6.7)
Mean number of adhesions	3,4,4,0. (2.8 ± 1.9)	4,2,4,0. (2.5 ± 1.9)	2,3,3,3. (2.8 ± 0.5)	3,4,3,3. (3.3 ± 0.5)	0,4,7,3. (3.5 ± 2.9)	0,3,6,4. (3.3 ± 2.5)

Appendix 8: Haematology data of individual endometriotic baboons (Mean ± standard deviation)

	Placebo		GnRH – antagonist		Rosiglitazone	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**
White Blood Cells $x10^3/\mu l$	9.4,7.9,6.9,9.8 (8.5±1.344)	8,6.6,12.2, 11.4 (9.55±2.68)	21.9,10.8, 11.6, 6.5 (12.7±6.53)	21.2, 11.8, 9.7, 9.8 (13.3±5.47)	16.6, 6, 10.7, 6.7 (10±4.86)	16.4, 7,12.4, 9 (11.2±4.12)
Red Blood Cells $x10^6/\mu l$	4.01,4.12,4.83,4.41 (4.34±0.37)	4.16, 4.5, 4.6, 4.66 (4.48±0.22)	4.61,4.84, 4.39, 4.02 (4.47±0.35)	4.88, 4.71, 5.16, 4.22 (4.74±0.40)	5.31,4.2, 5.11, 4.36 (4.75±0.55)	4.78, 4.8, 5.25,4.18 (4.75±0.44)
Haemoglobin x gm/dl	**11.5,11.5,13.9,12.5 (12.35±1.14)** *	**13.1,12.7, 14.6, 13.1 (13.38±0.84)** *	12.4, 12, 12.4, 11.5 (12.01±0.43)	13.2, 13.5, 15.7, 12.2 (13.65±1.48)	**12.7, 12.3, 14.4,12.2 (12.9±1.02)** $	**13.7, 12.8, 15.9, 13.4 (13.95±1.35)** $
Total Plasma Protein gm/dl	6.7, 5.9, 7.5, 6.7 (6.7±0.65)	6.1, 5.9, 7.3, 6.7 (6.5±0.63)	**7.7, 7, 6.7, 7.4 (7.2±0.44)** ≠	**6.5, 5.8,6.4, 6.2 (6.23±0.31)** ≠	5.9, 6.4, 6.4, 7 (6.43±0.45)	6.3, 6.2, 6.7, 7.2 (6.6±0.46)
Plasma fibrinogen gm/dl	0.2, 0.2, 0.2, 0.1 (0.175±0.05)	0.1, 0.1, 0.2, 0.1 (0.125±0.05)	**0.1,0, 0.1, 0.1 (0.075±0.05)** †	**0.2,0.1,0.2, 0.1 (0.15±0.06)** †	0.2, 0.1, 0.2, 0.2 (0.18±0.05)	0.2, 0.1, 0.2, 0.3 (0.2±0.08)
Band Neutrophils per µl	94, 79, 0, 0 (43.25±50.32)	0, 0, 244, 0 (61±122)	219, 108, 116,0 (110.75±89.48)	424, 118,0, 98 (160±183.4)	0,0,0, 67 (16.75±33.5)	492,0,0,90 (145.5±234.86)
Segmented Neutrophils per µl	2726, 4898, 3243, 5292 (4039.75±1247.06)	3360, 2772, 7564, 4322 (4504.5±2137.39)	11388, 5508, 8468, 2945 (7077.25±3653.98)	15624, 6962, 4656, 4998 (8060± 5144)	11620, 2460, 5350, 2479 (5477.3±4314.4)	9020, 3360, 6200, 3780 (5590±2606.8)
Lymphocytes per µl	6486, 2686, 3381, 3999 (4138±1654.67)	4160, 3498, 3782, 6840 (4570±1537.44)	9636, 5076, 2900, 3445 (5264.25±1177.11)	5088, 4248, 4753, 4312 (4600.3±395.14)	4814, 3480, 5136, 4020 (4362.5±752.39)	6532, 3430, 5580, 4590 (5033±1330.7)
Monocytes per µl	94, 237, 207, 279 (204.25±79.21)	240, 330, 366, 228 (291±67.62)	219, 108, 116, 65 (127±65.29)	212, 472, 291, 29 (251±183.71)	166, 60, 214, 134 (143.5±64.65)	164, 140, 496, 270 (267.5±162.47)
Eosinophils per µl	0, 79, 69,0 (37±42.92)	240, 0, 244, 0 (121±139.73)	438,0,0, 65 (125.75±210.4)	212,0,0, 98 (77.5±100.87	**0,0,0,0 (0±0)** *	**492, 70, 124, 270 (239±188.64)** *

$ Haemoglobin changed significantly in Rosiglitazone group.
* Eosinophils changed significantly in Rosiglitazone group
† Plasmafibrinogen changed significantly in GnRH group
≠ Total Plasma Protein reduced

Appendix 9: Histopathology of lungs in endometriotic baboons

Findings	Animal identification Number in Placebo group	Frequency of occurrence in placebo group	Animal identification Number in GnRH	Frequency of occurrence in GnRH group	Animal identification Number in Rosiglitazone	Frequency of occurrence in Rosiglitazone group
Normal	2631, 2912 2991, 2985	4/4	2993, 3036 3014	3/4		1/4
Edema moderate to severe & hemorrhages. Emphysema with corresponding atelectasis mild		0/4	2871	1/4	3032, 2788 2998, 3030	4/4
Focal emphysema and corresponding atelectasis		0/4		0/4	2998, 2788	2/4
Congestion & mild hemosiderin		0/4	2871	2/4		0/4

Appendix 10: Histopathology of liver in endometriotic baboons

Findings	Animal identification Number in Placebo group	Frequency of occurrence in placebo group	Animal identification Number in GnRH	Frequency of occurrence in GnRH group	Animal identification Number in Rosiglitazone	Frequency of occurrence in Rosiglitazone group
Normal	2912	1/4		0		0
Bile ducts surrounded by inflammatory cells.			3036	1/4	2998, 3032 3030, 2788	4/4
Focal infiltration of lymphocytes	2912 2631	2/4	3014,3036, 2993	3/4		0
Both focal Focal infiltration of lymphocytes and bile duct surrounded lymphocytes	2991	1/4	3014, 2871 2993	3/4	2788	1/4
Bile duct hyperplasia and surrounded by lymphocytes					3023	1/4

Appendix 11: Summary of Serum chemistry values at pre-induction and post-
(Mean ± standard deviation)

	Placebo		GnRH	
Serum test	**Pre-induction**	**Post-induction**	**Pre-induction**	**Post-i**
Total protein	7.18, 7.98, 6.38, 8.38 (7.48±0.89)	6.58, 7.78, 9.38, 9.38 (8.28±1.36)	10.77, 7.78, 7.18, 8.98 (8.68±1.58)	13.3 7.7 (8.6
Albumin	4.76, 5.79, 5.04, 5.93 (5.38±0.57)	5.66, 5.66, 5.80, 6.90 (6.0±0.60)	7.79, 6.28, 6.69, 9.31 (7.52±1.36)	8.62, 4 (6.2
Urea	33.33, 59.26, 77.78, 44.44 (53.70±19.25)	57.41, 68.52, 46.30, 48.15 (55.09±10.19)	48.15, 70.37, 37.04, 105.56 (65.28±30.22)	74.0 37.0 (45.3
Alkaline Phosphatase	107.89, 177.74, 239.12, 142.63	136.01, 224.24, 390.39, 161.01	116.90, 196.85, 215.41, 154.39	226.4 391.4
GOT/ASAT	10.74, 25.71, 9.59, 16.11 (15.54±7.35)	19.18, 54.10, 32.61, 12.27 (29.54±18.42)	16.50, 17.27, 19.81, 16.11 (17.42±1.66)	52.1 72.8 (26.3
GPT/ALAT	9.98, 4.60, 4.22, 6.14 (6.235±2.63)	9.21, 23.02, 6.14, 5.37 (6.91±8.23)	5.76, 11.51, 7.67, 8.06 (8.25±2.39)	6.9 23. (6.7
Bilirubin	0.26, 0, 0, 0	0, 1.04, 0,0	0, 0, 0.52, 0	0,0
Creatinine	1, 1.2, 1.4, 1.4 (1.25)	1.2, 1.8, 1.2, 2.2 (1.6)	1.8,1.2, 1.2, 1.2 (1.35)	1.4, 1