EFFECTS OF CYLOTRAXIN-B TREATMENT ON ENDOMETRIOTIC LESION SURVIVAL IN AN IMMUNOCOMPROMISED MOUSE MODEL

Effects of cyclotraxin-b treatment on endometriotic lesion survival in an immunocompromised mouse model

By:

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LAY ABSTRACT

Endometriosis is a chronic pain condition affecting more than 176 million women worldwide. Despite its commonality and severity its gold standard diagnosis is performed through invasive laparoscopy and there is no cure resulting in low quality of both physical and mental health of the affected individuals. Levels of a neurotrophic factor - BDNF and its high affinity receptor TrkB have been found increased in women with endometriosis and thus, BDNF-TrkB signaling cascade appears to be a promising therapeutic target. This study investigated an effect of TrkB inhibitor – cyclotraxin-b on reduction of endometriotic lesion number and volume within an immunocompromised model. Replication of results and increase of sample size may provide more details about cyclotraxin-b clinical relevance for treatment of endometriosis.

ABSTRACT

Endometriosis is a steroid-dependent common gynecological condition characterized by the growth of endometrial epithelial and stromal cells outside of its cavity. Estrogen dependence, progesterone resistance, in situ estrogen production, increased inflammation, resistance to apoptosis, active cell growth and proliferation, and overall disease's heterogeneity makes it challenging to treat patients without compromising fertility. BDNF and its high affinity receptor TrkB has been shown to be dysregulated in women with endometriosis suggesting their role in disease progression. Immunocompromised mice (Rag2γc) (n=25) underwent an implantation surgery during which a cell suspension consisting of human endometrioma cells was injected into the animals' peritoneal cavity. Upon lesion stablishment all animals were divided into five groups and treated with either high (7.5mg/kg/day), medium (5mg/kg/day) or low (2.5mg/kg/day) dose of TrkB inhibitor cyclotraxin-b, negative control (saline) or 0.04mg/kg/day letrozole. After four weeks of treatment all animals were sacrificed, all major organs were collected and assessed with routine histology for potential adverse effects of the treatment, all endometriotic implants were analyzed with routine histology and IHC for a panel of markers: BDNF, TrkB, anti-human mitochondrial protein, CD31, VEGFa, VEGFR1, VEGFR2. Treatment did not cause any significant side effects. There was a dose-dependent trend in reduction of endometriotic implants' volume and number. IHC confirmed expression of the angiogenic markers within endometriotic implants. A larger study may be required to replicate results and advance the search for novel non-hormonal endometriosis treatment.

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TABLE OF CONTENTS

Lay Abstract **iv**

Abstract **v**

Acknowledgements **vi**

Table of Contents **viii**

List of Figures **xi**

List of Abbreviations and Symbols **xiii**

Declaration of Academic Achievement **xviii**

CHAPTER ONE: INTRODUCTION 1

1.1. Background 1

1.2. Hallmarks of Endometriosis 2

1.3. Etiology of endometriosis 4

1.3. Diagnosis 5

1.4. Biomarkers 7

1.5. Neurotrophins 12

1.6. Treatment 13

1.6.1. NSAIDs 13

1.6.2. COCs and Progestin only 14

1.6.3. GnRH receptor analogues 16

1.6.4. Aromatase inhibitors 18

1.6.5. SPRMs and SERMs 19

1.6.6. Alternative therapies 20

1.6.7. Limitations of current treatment therapies 21

1.6.8. Attenuation of BDNF and TrkB signaling with Cyclotraxin-b 22

1.7. Study animal model 23

1.8. Hypothesis 25

1.9. Objectives 26

CHAPTER TWO: MATERIALS AND METHOD 27

2.1. Animals 27

2.2. Human Participants 28

2.3 Xenotransplantation 29

2.4. Treatment 30

2.5. Post-Treatment Endometriosis Assessment 32

2.6. Statistical analyses 35

CHAPTER THREE: RESULTS 36

3.1. Systemic effects 36

3.2. Effects of the Treatment on major organ mass 38

3.3. Histopathology 39

3.4. Effects of Cyclotraxin-b Treatment on Endometriotic lesions 42

3.5. Localization of BDNF, anti-human mitochondrial protein, TrkB, CD31, VEGF, VEGFR1 and VEGFR2 in endometriotic lesions 47

CHAPTER FOUR: DISCUSSION..... 52

4.1. Summary of findings..... 52

4.2. Reproductive health of study animals..... 54

4.3. Strength and limitations..... 54

4.4. Conclusions..... 55

4.5. Future directions..... 56

CHAPTER FIVE: REFERENCES..... 57

LIST OF FIGURES

Figure 1. Processes, proteins, and genes involved in the development of endometriosis 8

Figure 2: Study design of the project 28

Figure 3: Surgical induction of endometriosis in rag2γ(c) mouse 30

Figure 4. The Mouse Grimace Scale 32

Figure 5: Weight of animals before and after treatments 37

Figure 6: Average organ mass at necropsy after 4 weeks of treatment 38

Figure 7: Histological findings in the liver of the treated mice 40

Figure 8: Histological findings in the kidney of the treated mice 41

Figure 9: Follicular characterization and count 43

Figure 10: Lesions at the surgical site 44

Figure 11: Effect of 4-week treatments on endometriotic implant volume 45

Figure 12: Effect of 4-week treatments on endometriotic implant number 46

Figure 13: Immunostained tissue sections (anti-human mitochondrial protein and anti-BDNF) 47

Figure 14: IHC results 48

Figure 15: Effect of a four-week treatment on BDNF staining 49

Figure 16: Effect of a four-week treatment on TrkB staining 49

Figure 17: Effect of a four-week treatment on CD31 staining 50

Figure 18: Effect of a four-week treatment on VEGFa staining 50

Figure 19: Effect of a four-week treatment on VEGFR1 staining 51

Figure 20: Effect of a four-week treatment on VEGFR2 staining 51

LIST OF ABBREVIATIONS

3-βHSD: 3β-hydroxysteroid dehydrogenase

17-βHSD: 17β-hydroxysteroid dehydrogenase

b-HIVS: b-hydroxyisovalerylshikonin

AI: aromatase inhibitor

AREB: Animal Research Ethics Board

AUP: animal utilization protocol

BDNF: brain-derived neurotrophic factor

BMD: bone mineral density

CCDC170: gene encoding for Coiled-Coil Domain-Containing Protein 170

CD31: cluster of differentiation 31 also known as PECAM-1: Platelet endothelial cell adhesion molecule

CHC: combined hormonal contraceptive

CLI: chloroindazole

CNS: central nervous system

COC: combined oral contraceptive

COX-2: cyclooxygenase-2

CPA: cyproterone acetate

CYP2C19: gene encoding for Cytochrome P450 Family 2 Subfamily C Member 19

DAB: diaminobenzidine tetrahydrochloride

DIE: deep-infiltrating endometriosis

DNG: dienogest

DSG: desogestrel

E1: estrone

E2: estradiol

EDC: endocrine disrupting chemical

EE: ethinylestradiol

ECS: endocannabinoid system

ELISA: enzyme-linked immunosorbent assay

ESR1: estrogen receptor 1

ESR2: estrogen receptor 2

FSH: follicular stimulating hormone

ESR1: gene encoding for estrogen receptor 1

ESR2: gene encoding for estrogen receptor 2

FBS: fetal bovine serum

Foxn1: forkhead box N1 gene

FSHB: gene encoding for Follicle Stimulating Hormone Subunit Beta

GnRH: gonadotropin-releasing hormone

GnRHa: gonadotropin-releasing hormone agonist

GREB1: gene encoding for Growth Regulating Estrogen Receptor Binding 1

GWAS: genome-wide association study

HBSS: Hanks Balanced Salt Solution

H&E: heamatoxylin and eosin

ICAM-1: intercellular adhesion molecule-1

IFN-γ-2b: interferon gamma

IHC: immunohistochemistry

IL: interleukin

IP: intraperitoneal

IUD: intrauterine device

LH: luteinizing hormone

LNG: levonorgestrel

lngRNA: long noncoding RNA

MAPK: mitogen-activated protein kinase

miRNA: microRNA

mRMA: messenger RNA

MT: metallothionein

MMP: matrix metalloproteinase

MRI: magnetic resonance imaging

NETA: norethisterone acetate

NGF: nerve growth factor

NGL: norgestrel

NK: natural killer cell

NOMAC: nomegesterol acetate

NT: neurotrophin

NSAID: nonsteroidal anti-inflammatory drug

OBHS: oxabicychloheptene sulfonate

PBS: phosphate-buffered saline

PEA: palmitoylethanolamine

PEDF: pigment epithelium-derived factor

PGE2: prostaglandin E2

PLC: phospholipase C

POCs: progestin-only contraceptives

PR-A: progesterone receptor-A

PR-B: progesterone receptor-B

PRAR: proliferator-activated receptor

qPCR: quantitative polymerase chain reaction

rag2γ(c): Recombinant Activating Gene 2/common cytokine receptor γ chain (γc) double null

SCID: Severe Combined Immunodeficient

SD: standard deviation

SERMs: selective estrogen receptor modulators

Shc: Src homology and collagen

SOP: Standard Operating Procedure

SPRM: selective progesterone receptor modulator

SQ: subcutaneous

SYNE1: gene encoding for Spectrin Repeat Containing Nuclear Envelope Protein 1

TIMP: tissue inhibitor of matrix metalloproteinase

TF: tissue factor

TIAR: tissue injury and repair mechanism

TNF-α: tumour necrosis factor alpha

TrkB: tropomyosin receptor kinase B

VCAM-1: vascular cell adhesion molecule-1

VDA: vascular-disrupting agent

VEGF: vascular endothelial growth factor

VEGFR1: vascular endothelial growth factor receptor 1

VEGFR2: vascular endothelial growth factor receptor 2

WNT4: gene encoding for wingless-related integration site 4 protein

DECLARATION OF ACADEMIC ACHIEVEMENT

All described experiments were performed by me.

CHAPTER ONE: INTRODUCTION

1.1. Background

It is estimated that approximately ten percent of women of reproductive age are affected by endometriosis[1]. This chronic estrogen-dependent gynecological condition is characterized by endometrial cells (epithelial and stroma) growing outside of the uterus[2]. Endometriosis signs and symptoms vary based on the location of the lesions and the stage of the disease[3]. Generally, endometriotic lesions are located in the pelvic cavity as with ovarian endometriosis (endometrioma), superficial peritoneal endometriosis, abdominal endometriosis and deep infiltrating endometriosis (DIE). However, the lesions have been found in other organs including the lungs and brain[4]. The staging of the condition is based on the spread and the depth of the lesions. There are four stages ranging from stage I being minimal endometriosis with a few small lesions to stage IV characterized by deep lesions, thick adhesion and large cysts[5]. Importantly, while the stage of the condition does not correlate with the pain symptoms, it is the location, depth and innervation of the lesions that contributes to pain symptoms[6–8]. The most common symptoms reported by patients are chronic pelvic pain, painful bowel movements, dysmenorrhea, dyspareunia, and in many cases infertility[9]. Endometriosis can result in severe and in some cases debilitating pain that can impact all aspects of one's life and lead to social dysfunction[10].

In addition to the significant physiological and psychological impacts of this disorder, epidemiologic studies have shown the hospital cost of care for individuals with endometriosis in Canada was USD $147.79 million per year and CaD $3143 per-case[11]. This represents the direct costs of the disorder, which includes the diagnosis and

medication, making endometriosis management cost similar to such chronic diseases as heart disease, diabetes, migraines, asthma and Crohn's disease. It has been found that the cost of the disease varies greatly among countries such that in 2014 it ranged from USD $1,109 per patient per year in total direct cost in Canada to USD $12,118 per patient per year in the USA. Indirect costs to patients, employers and society due to loss of productivity, absence from work and time lost on travelling to appointments ranges from a low of USD $3,314 per patient annually in Austria to a high of USD $15,737 per patient annually in the USA[12].

1.2. Hallmarks of Endometriosis

Although endometriosis is a common gynecological disorder with important negative impacts on one's health and social interactions, the etiology remains unclear. Hallmarks of endometriosis include estrogen dependence, progesterone resistance, genetic predisposition, and inflammation. Endometriosis is dependent on the most biologically active estrogen, estradiol (E2) for development and growth. Although eutopic endometrium and endometriotic tissue are similar histologically, they vary greatly in steroid biosynthesis, response to progesterone and synthetic progestins, as well as expression of aromatase and 17β-hydroxysteroid dehydrogenase (17-βHSD), and production of cytokines and prostaglandins[13–15]. Both aromatase and 17-βHSD are actively investigated as therapeutic targets for endometriosis due to their major roles in estrogen biosynthesis and metabolism. Aromatase catalyzes conversion (aromatization) of androgens into estrogens: androstenedione and testosterone into estrone (E1). E1 is then converted into E2 by the

enzyme 17-βHSD mainly in the ovarian granulosa cells. Endometriotic lesions were found not only to be dependent on the E2 produced by the ovaries, but also to produce E2 locally within the tissue. Moreover, levels of aromatase and prostaglandin E2 (PGE2) were found increased in women with endometriosis demonstrating a positive feedback loop leading to continuous local production of estrogen and prostaglandins favouring the proliferative and inflammatory characteristics of endometriosis. Furthermore, E2 and PGE2 together induce expression of vascular endothelial growth factor (VEGF), the crucial angiogenesis regulator shown to be dysregulated in women with endometriosis[13,16].

In addition to being a steroid-dependent condition, endometriosis is also a hereditary disorder and studies investigating familial clustering, concordance in monozygotic twins, as well as high prevalence of the disease in first degree relatives suggest genetic predisposition for endometriosis[17–20]. Many biochemical signatures of endometriosis correlate with aberrant molecular patterns of gene expression as well as with microRNA (miRNA) and long noncoding RNA (lncRNA) expression[21,22]. Inheritance mechanisms and predisposition to endometriosis remains to be an area of active research and there have been 15 Genome-wide association studies (GWAS) performed to date(23–36). 34 genes were reported to survive the genome-wide significance threshold in endometriosis suggesting their role in the disease genomic basis. The genetic risk of endometriosis was found to be associated with genes involved in uterine development and stem cell function (wingless-related integration site 4- *WNT4*), ovulatory function (estrogen Receptor 1- *ESR1*, Follicle Stimulating Hormone Subunit Beta- *FSHB*), as well as regulation of estrogen activity and estradiol biosynthesis (Growth Regulating Estrogen

Receptor Binding 1- *GREB1*, Spectrin Repeat Containing Nuclear Envelope Protein 1- *SYNE1*, Cytochrome P450 Family 2 Subfamily C Member 19 - *CYP2C19*, Coiled-Coil Domain-Containing Protein 170 - *CCDC170*)[37–40].

1.3. Etiology of endometriosis

Once proposed in 1927, Sampson's theory of retrograde menstruation still remains the most widely accepted theory of endometriosis origin[2]. Retrograde menstruation is the process during which blood with endometrial lining partially flows through the fallopian tubes into the peritoneal cavity. However, retrograde menstruation happens in 90% of women of reproductive age, but only 10% develop disease[41]. Therefore, this theory cannot account for cases of DIE and extrapelvic deposits, as well as for cases of endometriosis in individuals with Müllerian agenesis (Mayer-Rokitansky-Kuster-Hauser syndrome)[42] and in men underdoing high-estrogen treatment for prostate cancer[43]. Furthermore, immunological disruption[44] in affected women, as well as the hereditary predisposition[45–47] to endometriosis are currently investigated as other important factors. Therefore, other theories have been suggested to better account for the full spectrum of endometriosis.

The benign metastasis theory proposes that ectopic endometriotic implants could result from endometrial cells being disseminated through lymphatic or vascular system[2]. Coelomic metaplasia is a theory supporting non-uterine origin of the disease[48]. It is based on the idea of extra-uterine stem cells differentiation from bone marrow into endometriotic tissue. This theory suggests a possible transformation of mesothelial cells within the tissues of pleural and peritoneal surfaces into endometrial stroma and glands[49]. Currently, cell-

candidates investigated for this theory are bone marrow mesenchymal stem cell and endothelial cell progenitors. Among factors that could be responsible for such cell differentiation are endocrine disrupting chemicals (EDCs)[50] as well as various hormonal and immunologic factors[51,52].

Müllerianosis is another theory according to which, during embryogenesis the remnants of the Müllerian duct (the embryonic structure which gives rise to the female reproductive tract) migrate to and are deposited within other organs, where they further establish endometriotic lesions under the influence of estrogen at the onset of puberty[53]. A recent report suggest that DIE may arise from this process[54].

Another theory proposes a polygenetic/polyepigenetic origin of endometriosis. In this theory it is suggested that a combination of inherited genetic and acquired epigenetic defects act on a cell despite its origin to enhance disease predisposition. Further, stable and transmittable changes are required for development of subtle and microscopic lesions; while typical, cystic, deep, and other lesions form under the influence of additional genetic and epigenetic changes[55]. As described, endometriosis is a heterogeneous and multifactorial disease, which makes its diagnosis challenging.

1.3. Diagnosis

Misinterpretation of symptoms, the delay in seeking medical care by patients, and many other reasons lead to an 8-10-year diagnostic delay[56]. The techniques employed in the diagnosis of endometriosis include pelvic exam, ultrasonography, and magnetic

resonance imaging (MRI). While these techniques are semi-invasive and provide reliable first-line diagnosis, identification of endometriosis with these methods is challenging[57,58].

The current gold standard for the diagnosis of endometriosis is laparoscopy followed by histopathological analysis. In order to confirm a diagnosis of endometriosis histopathology of surgical specimens is needed in which, it is necessary to demonstrate two of three of the following structures within tissue sections: endometrial stroma, endometrial-type epithelium, and hemosiderin-laden macrophages[59]. Both laparoscopic and histopathological findings may vary based on the extend of the training specialisation and experience of the health care provider, type of lesion, and stage of the disease. Laparoscopy alone has 97.7% sensitivity and 79.2% specificity[60], but the findings are not always supported by the histopathological analysis. For instance, depending on the lesion type and location the biopsy specimen of peritoneal lesions suspected to be endometriosis during laparoscopy are confirmed histologically in 3.1% to 100% of cases[61].

Despite being the gold standard diagnostic procedure, laparoscopy is currently recommended as the last resort as a diagnostic measure and is usually only performed when a direct therapeutic benefit is expected. In addition to direct visualization of lesions, laparoscopy enables surgical removal of the lesions and cysts and thus, decreases pain. Constantly advancing surgical techniques include excision or removal of endometrial implants, electrocautery or laser treatment, presacral neurectomy, hysterectomy, salpingooophorectomy, and robot-assisted laparoscopy[62]. Generally, the surgical intervention has a 50–80% success rate in reducing symptoms; however, recurrence rates of 5-15% even after hysterectomy and bilateral oophorectomy have been reported[63].

Moreover, repetitive surgeries can lead to further adhesions formation, chronic pain, and affect the ovarian reserve and thus alternatives to laparoscopy are desired[64]. Many professional societies now advocate for non-surgical interventions[56] and therefore, the search of non-invasive biomarkers for the diagnosis of endometriosis has emerged as a high priority research need[65].

1.4. Biomarkers

While the etiology of endometriosis remains unknown; however, it is widely appreciated that the establishment and growth of endometriotic lesions requires dysregulation of cell adhesion, proliferation, inflammation, tissue remodeling, angiogenesis, and resistance to apoptosis[66]. Moreover, endometriosis shares some characteristics with malignant tumours as the endometrial epithelial and stromal cells have to implant on the ectopic site, establish blood and nerve supply, proliferate and resist apoptosis. Therefore, for each step required for disease progression, multiple potential biomarkers have been identified (Fig.1.). Some of the molecules have now been recognized not only as potential biomarkers, but also as promising therapeutic targets.

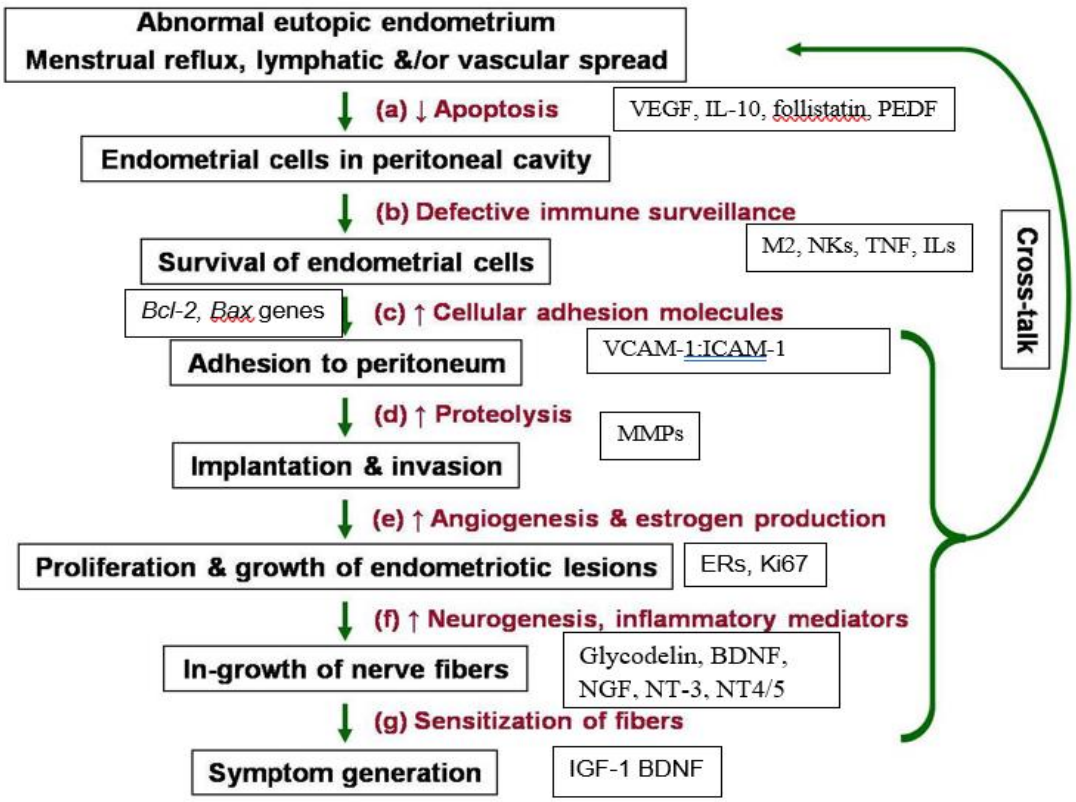

Figure 1. Processes, proteins, and genes involved in the development of endometriosis. (Modified from Hey-Cunningham, A. J., Peters, K. M., Zevallos, H. B., Berbic, M., Markham, R., & Fraser, I. S. (2013). Angiogenesis, lymphangiogenesis and neurogenesis in endometriosis. Front Biosci (Elite Ed), 5, 1033-56.)

Considering the classic theory of retrograde menstruation[2], the refluxed cells can resist apoptosis both throughout dissemination to target tissues, as well as during initiation of lesions until a new blood supply can be established. Ectopic implants have been found to overexpress an antiapoptotic gene *Bcl*-2, and inhibit proapoptotic gene *Bax*[67–71]. Current experimental agents have shown effects in reducing the number of viable cells, induction of p53-mediated apoptosis, and inhibition of DNA synthesis are bufalin and b-hydroxyisovalerylshikonin (b-HIVS) as well as the popular natural anti-inflammatory agent curcumin[72,73]. After surviving through the initial cell clearance mechanisms, the cells are typically not recognized by the host organism's immune system. Inflammation is

crucial for endometriosis establishment and dysregulation of macrophages (M2), natural killer cells (NKs), inflammatory cytokines such as tumour necrosis factor (TNF-α)[74], interleukins: IL-1β[75], IL-2, IL-4[76], IL-6[77], IL-8[78], IL-11, IL-12, IL-13, IL-15, IL-17, IL-18, IL-23[79], IL-25[80], IL-32[81], IL-33[82], IL-37[83] have all been documented. However, an inflammatory biomarker with high sensitivity and specificity has yet to be discovered[82].

Various immunomodulatory agents have been proposed as currently experimental treatments of endometriosis including cytokines IL-12 and interferon gamma (IFN-γ-2b), leflunomide and imiquimod, rapamycin (Sirolimus), romidepsin, quinalizarin, peroxisome proliferator-activated receptors (PPAR) g-ligands, and TNF-α blockers[72,84–88]. Preliminary data suggests effectiveness of the selected compounds in animal models, but either lack of effectiveness or absence of testing and/or replication in humans.

Assuming cell survival, the sluffed endometrial cells migrate to the site of implant establishment. In order to establish an ectopic lesion, endometrial cells must attach, adhere, establish a blood supply, proliferate and invade the tissue by breaking its integrity for which matrix metalloproteinases (MMPs) are responsible(89). Specifically, MMP-9 and metallothionein (MT) were shown altered in women with endometriosis[90,91]. Preliminary data suggests efficacy of tissue inhibitor of matrix metalloproteinase (TIMP) such as ONO-4817, statins, melatonin, mitogen-activated protein kinase (MAPK) inhibitors, and metformin. However, while interfering with endometriosis progression, excessive TIMP activity is associated with adverse effects in reproductive system[72,92–94].

In addition to MMPs cell adhesion molecules and their activation have been found dysregulated in endometriosis. The most researched cell adhesion specific biomarkers include vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1)[95–97]. mRNA levels of the markers have been found increased in both eutopic and ectopic tissues of women with endometriosis. mRNA of the markers were also detected dysregulated in circulation of the affected women suggesting a potential of the molecules in further research for non-invasive biomarkers.

Endometrial cell proliferation is regulated by the gonadal steroids: estrogen and progesterone. Additionally, the levels of nuclear estrogen receptors (ERs) and progesterone receptors (PR) were used as proliferation signatures in endometriosis[98]. Estrogen and progesterone act through specific receptors: estrogen receptor 1 (EsR1) and estrogen receptor 2 (EsR2) (previously termed ERα and ERβ, respectively), progesterone receptor-A (PR-A), and progesterone receptor-B (PR-B). ESR1 mRNA levels are higher than ESR2 in normal endometrium[99]. As for the endometriotic lesions, ESR1 mRNA expression is about sevenfold lower than in eutopic endometrial stromal cells, while ESR2 mRNA expression is 40- to 140-fold higher[100,101]. The ratio of the receptors is significantly higher at both mRNA and protein levels in endometriotic stromal cells when compared to healthy endometrial stromal cells[102]. This may be critical for disease establishment and progressions, since in endometriosis ESR2 suppresses ESR1; consequently, low ESR1 levels may result in reduced PR levels leading to progesterone resistance[103]. Extremely high detected levels of ESR2 have been linked to endometriotic cell proliferation, inflammation, as well as resistance to apoptosis, and hyperalgesia[102].

Progesterone receptors also vary between women with and without endometriosis. PR-B is a dominant transcriptional activator of progesterone-responsive promoter, while PR-A is a dominant repressor of PR-B and other steroid receptors[104]. There are high levels of PR-A and very low protein levels of PR-B in both endometriotic stromal cells and eutopic endometrium of diagnosed women[105,106]. Furthermore, there is low expression of progesterone-responsive genes, leading to the progesterone resistance[107,108]. These receptors also act as targets for novel therapies discussed in the next section.

Endometriotic lesions are known for establishing their own blood and nerve supply through processes of angiogenesis and neovascularization. Angiogenesis is associated with dysregulation of VEGF[109–113], IL-8, IL-10[114], follistatin[115], placental growth factor, and pigment epithelium-derived factor (PEDF)[116]. These proangiogenic agents have been found in peritoneal fluid of women with endometriosis and are suggested to be secreted by the lesions themselves or peritoneal macrophages. Glycodelin, a progesterone-regulated endometrial protein facilitating cell proliferation and neovascularization, was found to be overexpressed in women with endometriosis and was added to a panel for the diagnosis of ultrasound-negative endometriosis[117–119]. VEGF is a crucial angiogenic factor and both VEGF and its receptor VEGF-R2 were found to be overexpressed in early-stage (red) endometriotic lesions as well as in highly innervated DIE lesions. Antiangiogenic treatments are being actively investigated and several candidates have been preliminarily shown promising in decreasing the vascular density of the lesions, decreasing the implant size, and pain symptoms. Among such experimental agents are bevacizumab (Avastin), dopamine and its agonist cabergoline, vascular-disrupting agents (VDAs), tissue factor

(TF), selective cyclooxygenase-2 (COX-2) inhibitors (i.e. parecoxib, Rofecoxib, Celecoxib), and angiogenesis inhibitors as endostatin, romidepsin-a histone deacetylase inhibitor, and anginex(120–130). In addition to the mentioned proangiogenic agents above, several members of the neurotrophin family contribute to neovascularization of endometriotic lesions and may participate in promoting and maintenance of lesions innervation. Thus, the neurotrophin family may play a central role in endometriosis progression and pain perception.

1.5. Neurotrophins

The neurotrophin family is comprised of several proteins including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT4/5). The neurotrophins have been extensively studied in the context of the central nervous system (CNS), but their role in the reproductive system, endometrial pathologies, and endometriosis is just beginning to be elucidated. These proteins are growth factors that facilitate endometriosis pain generation through process as nerve growth, survival[131,132], and differentiation[133]. Pain is the leading reason of endometriosis patients' hospital admittance and that is why the involved molecules and pathways are actively investigated.

BDNF and its high affinity receptor tropomyosin receptor kinase B (TrkB) is involved in ovarian development[134], follicular development[135], and oocyte survival[136]. While BDNF is crucial for endometrial stem cell neurogenesis[137] and normal placental development[138], their overexpression has been linked to such reproductive pathologies as

premature ovarian failure[136], endometrial cancer[139], and endometriosis[140–142]. BDNF and TrkB specifically participate in endometriosis development by facilitating cell adhesion[143–145], angiogenesis[146,147], proliferation[148], and resistance to apoptosis[144,149].

Circulating levels of BDNF are reported to be greater in individuals with endometriosis compared to women without this disease[150]. Furthermore, our laboratory found BDNF and TrkB to be over-expressed in women with surgically confirmed endometriosis compared to symptomatic controls[151]. We have also shown that miRNA (miR-155-5p) downregulating BDNF expression is lower in women with endometriosis compared to controls, consequently leading to higher circulating levels of BDNF (Ricci et al., In preparation). According to a previous study, BDNF can be used as an effective non-invasive biomarker of stage I-II endometriosis with 91.7% sensitivity and 69.4% specificity[151]. BDNF has also been shown to positively correlate with pain in women with endometriosis suggesting its role in pathogenesis of endometriosis pain[152]. Therefore, BDNF signalling pathway is thought to be a potential target for the management of endometriosis.

1.6. Treatment

1.6.1. NSAIDs and opioids

Currently there is no cure for endometriosis. Generally, existing treatment strategies involve pain management, hormone treatments and surgical interventions[153]. The first-line medical treatment for endometriosis is focused on pain management and thus, prescription of nonsteroidal anti-inflammatory drugs (NSAIDs)[154]. They might be useful in treating primary dysmenorrhea by providing general analgesic and anti-inflammatory effect[155,156].

However, they are not endometriosis specific and there is a lack of high-quality evidence supporting NSAIDs' (such as naproxen) efficiency in suppressing endometriosis pain when compared to placebo[157].

Diagnosed women are more likely to be prescribed opioids, than women without endometriosis[158]. The patients are usually prescribed opioids as their post-surgery therapy or to manage their chronic pain when other therapies fail, when the pain is no longer tolerable and/or NSAID hypersensitivity is developed.

1.6.2. CHCs and Progestin only

The second line of endometriosis treatment is usually the prescription of hormonal medications. These medical treatments include combined hormonal contraceptive medications (CHCs)[159], progestins[160], gonadotropin-releasing hormone agonist (GnRHa)[161] or antagonist therapy, followed by experimental treatments as selective progesterone receptor modulators (SPRMs)[162]. The goal of the most current medications is to achieve a hypo-estrogenic state since establishment of lesions and development of disease is estrogen-regulated. The most common end-result of such treatments is interference with the pituitary-gonadal stimulation leading to anovulation, reduction, or suppression of menstrual flow.

Due to the known hormonal background of endometriosis, a long-term therapy reducing menstruation and ovulation is considered. CHCs is the most prescribed therapy, which is used as initial treatment and, in some practices, diagnostic measure. CHCs come in different forms and concentrations but are generally a complex of low-dose estrogen and

synthetic progestin. Functioning of CHCs in endometriosis is similar to the general contraceptive mechanism of action. Therefore, it functions through estrogen-mediated inhibition of ovulation caused by inhibition of luteinizing hormone (LH) and follicular stimulating hormones (FSH) for suppression of ovarian folliculogenesis. This therapy can be prescribed as a daily pill, vaginal rings or transdermal patches[163]. There are many formulations available on the market and the choice is often made according to the personal preference and responsiveness, price, and availability of the medication. Ethinylestradiol (EE)/norethisterone acetate (NETA) formulation is one of the most common combined oral contraceptives (COCs) prescribed, followed by EE/norgestrel (NGL), EE/levonorgestrel (LNG), EE/desogestrel (DSG), EE/cyproterone acetate (CPA), EE/gestodene and many other[164]. Thus, this therapy may be convenient due to availability of multiple routes of administration and formulations, relative long-term safety due to low rates of associated adverse effects as well as its contraceptive effect. However, only about one-third of the treated patients responds to the CHCs, not all endometriosis symptoms improve with CHC administration, and there is a rapid symptom recurrence with discontinuation of the therapy[165–168].

While progestins can be prescribed in combination with EE, they can also be administered alone (progestin-only, POCs). Common medications include nomegesterol acetate (NOMAC), NETA, LNG, and dienogest (DNG)[169]. Sometimes POCs are prescribed before the COC therapy in order to avoid a potential risk of lesion growth progression caused by estrogen administration. They are available in various forms such as oral, subcutaneous and intramuscular injection, and intrauterine device (IUD). LNG-IUD

is a widely prescribed progestin treatment and when used postoperatively demonstrates slower symptom recurrence rate[170]. Overall, POCs demonstrate efficacy in managing dysmenorrhea and menstrual-related symptoms, as well as gastrointestinal and urinary symptoms in patients with colorectal and bladder endometriosis, respectively[171–175]. However, progestin medications are usually more expensive than when sold in combination with EE and require supplementation measures for patients desiring contraceptive effect. This type of therapy is also associated with several adverse effects as spotting, breakthrough bleeding, breast tenderness, fluid retention, serum cholesterol lipoprotein disruption, and decrease in bone mineral density (BMD)[176].

1.6.3. GnRH receptor analogues

Next commonly prescribed endometriosis treatment is a class of GnRH analogues. GnRHa used to be thought effective solely due to downregulation of pituitary GnRH secretion consequently leading to estrogen suppression. However, it is now known that GnRHa also inhibit uterine contractions, in particular, during menstruation, thus inhibiting activation of tissue injury and repair mechanism (TIAR), which is suspected to play a role in endometriosis development[177,178]. Furthermore, inhibition of ovulation and menstruation is associated with lower exposure of endometriotic lesions to inflammation[177]. GnRHa therapy was also found to result in decrease of cell proliferation and increase of apoptotic rate *in-vitro*[179–182]. This therapy has also been found to be effective in inhibiting angiogenesis by reducing the production of VEGF and IL-1β *in-vitro*[182]. Therefore, GnRHa therapy was shown to be effective not only by altering the

pituitary-ovarian cascade, but also through its direct effect on the endometrial cells within the lesions[183].

Administration of GnRHa therapy is followed by a "flare-up" effect due to initial upregulation of LH and FSH. Thus, patients must be warned about the potential side-effects and initial potential worsening of the symptoms. Patients undergoing such treatment need to be closely monitored to avoid significant decrease in BMD, hot flashes, inconsistent inhibition of ovulation, insomnia, vaginal dryness and loss of libido[184]. GnRHa therapy cannot be administered for longer than six months without hormone-replacement therapy due to a significant reduction (5%) in BMD, thus limiting its long-term potential. Add-back therapy consisting of an estrogen-progesterone replacement therapy is often suggested to limit the side effects from developed hypoestrogenism[185]. The most commonly prescribed GnRHa medication is Lupron[186]. Lupron depot (leuprolide acetate) treatment is administered intramuscularly once a month or once every three months. In addition to the general physiological drawbacks significantly reducing patients' quality of life, there have been reports of Lupron-induced manic episodes[187] triggered by hormonal imbalance.

More recent class of medication is GnRH receptor antagonists which act by competitive binding to GnRH receptors leading to a rapid blockage of the receptors and thus, reduction of ovarian estradiol production. Due to the antagonistic mode of action, this therapy does not provoke "flare-up" side effects and acts faster than the GnRHa leading to an immediate therapeutic effect[188]. Several GnRH antagonists are at the pre-clinical/clinical experimental phases, but elagolox is the first approved oral GnRH receptor antagonist used for treatment of moderate and severe endometriosis-associate pain[189–191].

While providing a rapid improvement in dysmenorrhea and nonmenstrual pelvic pain, elagolix therapy may lead to a dose-dependent progression of adverse effects such as the common vasomotor symptoms (vaginal dryness, night sweats, hot flashes) and decreased in BMD caused by hypoestrogenic state, which in some cases leads to the therapy's discontinuation[192,193].

Danazol, a testosterone derivative, is used for endometriosis treatment due to such effects as inhibition of gonadotropin release, immunological modulation, suppression of cell proliferation as well as competitive inhibition of steroidogenic enzymes[194–197]. Danazol can bind to androgen, progesterone, and glucocorticoid receptors[198]. Danazol inhibits 3β-hydroxysteroid dehydrogenase (3-βHSD), 17-βHSD, and aromatase activities[199]. Despite beneficial effects in some patients, danazol can cause severe adverse effects including hepatic injury, thromboembolism, deepening voice, acne, weight gain, seborrhea (a form of dermatitis and eczema, commonly known as dandruff if affects the scalp), vaginal atrophy and other androgenic adverse effects which discourage patients from the therapy[200,201].

1.6.4. Aromatase inhibitors

Endometriosis is a highly steroid-dependent condition, which does not only rely on the circulating E2, but also secretes its own supply of estrogen[202]. Consequently, GnRH therapy might not be effective as it targets estrogen production in the ovaries and not in the endometriotic lesions. Therefore, alteration of aromatase activity accomplished by aromatase inhibitors (AIs) might be considered, when the classical therapies are

insufficient[203]. While initially AIs were prescribed for treatment of estrogen receptor-positive (ER+) breast cancer in postmenopausal women, they are now suggested as treatment of endometriosis[204].

The most researched AI therapy for endometriosis is Letrozole, which was shown to significantly reduce pain symptoms and lesion volume, as well as to decrease rate of disease recurrence after surgery[205–208]. Due to such adverse effects as significant BMD loss and development of functional cysts, AI therapy is advised to be prescribed together with an add-back therapy and to be discontinued after six months of administration. Due to its use in clinical practice, efficacy, and mode of action Letrozole was chosen as the positive control treatment for this study.

1.6.5. SPRMs and SERMs

Another class of medication is SPRMs, which interact with progesterone receptors to inhibit endometrial cell proliferation[209]. They also induce suppression of uterine bleeding and synthesis of prostaglandins[210]. This therapy has shown undesirable effects, as some patients developed further endometrial hyperplasia due to the anti-progestogenic activity of such medication. Ulipristal acetate was approved by FDA in 2017 and moved forward as potentially effective and safe treatment for endometriosis in USA. In Canada the drug was not approved for treatment of endometriosis but was prescribed as a therapy for uterine fibroids. However, in September of this year (2020) Health Canada withdrawn the drug due to the reports of an associated major liver injury.

Selective estrogen receptor modulators (SERMs) such as chloroindazole (CLI) and oxabicychloheptene sulfonate (OBHS) which trigger ESR2 and ESR1, respectively, are currently actively investigated ligands. This intervention is suggested to inhibit cell proliferation and lesion growth, block neuroangiogenesis, suppress inflammation, and result in regression of established endometriotic lesions[211,212]. The ideal future SERM is expected to function on the targeted endometriotic tissues specifically, while not compromising circulating levels of estrogen and retaining its effect on the BMD, cardiovascular and nervous systems[213].

1.6.6. Alternative therapies

Since the conservative strategies are not always effective, there is an ongoing search for alternative therapies. While waiting for development of the ultimate cure people with endometriosis are trying to find strategies that would be helpful to carry on with their daily activities and lessen the painful symptoms. Among such strategies are physical methods: massage, acupuncture, yoga, pilates and other forms of exercising and stretching; psychological methods including meditation, breathing, and psychological therapies and counselling; and lifestyle interventions such as changes in sleeping and eating habits, alterations of alcohol and substance consumption as well as vitamin/minerals and probiotic intake. According to the self-reported data, cannabis is so far the most effective alternative treatment strategy[214].

Endocannabinoid system (ECS) has gained attention as a potential target for pain management of endometriosis due to its involvement in several physiological processes

central to endometriosis including: inflammation, cell proliferation, and cell survival[215]. Following the findings of cannabinoid antiproliferative, antifibrotic, and anti-inflammatory effects in cancer[216–219] ECS is being currently researched in the context of endometriosis. It has been implemented in studies demonstrating the efficacy of cannabinoid agonist both *in vitro* and *in vivo* within an animal model[220]. Clinical studies analyzed the effects of *N*-palmitoylethanolamine (PEA), a congener of cannabinoid receptor ligand (AEA), and reported a decrease in chronic pelvic pain, dysmenorrhea and dyspareunia symptoms which was comparable with NSAIDs; PEA was associated with fewer potential long-term use adverse effects and contraindications[221]. Although there is still no consensus about the safety and efficacy of cannabis products and their required formulation as endometriosis treatment, they are being actively used by people suffering from severe symptoms. According to the results of the self-management cannabis strategy in Australian women, those using cannabis experience pain and nausea reduction, improvements in sleep, and 56% of users reported a decrease in other pharmaceutical medications intake by at least a half[222].

1.6.7. Limitations of current treatment therapies

Currently available medications for the management of endometriosis allow only a temporal benefit in providing some control of the disease progression rather than a cure. If an effective hormonal treatment is found and the patient responds with decreasing pain symptoms, those effects are only seen during the medication administration, as no currently available treatment facilitates enduring effects after treatment discontinuation. Moreover,

women taking hormonal medications are at a greater risk of osteoporosis due to the decreased BMD occurring in response to induced hypoestrogenism. Those patients also tend to suffer from cardiovascular episodes, and other side effects including mood swings, weight gain, decreased libido, and painful intercourse – symptoms affecting the quality of life and leading to therapy discontinuation and as a result progression of the disease and worsening of the symptoms. Therefore, while there is no medication that can selectively target the ectopic endometrium, rather than the whole system, alternatives are needed.

Based on the currently available treatments, women are often faced with the option of managing their pain at the expense of pursuing pregnancy. Since it has been shown that BDNF can be used as a diagnostic marker for endometriosis, targeting the BDNF-TrkB signaling pathway could be a potential non-hormonal treatment strategy.

1.6.8. Attenuation of BDNF and TrkB signaling with Cyclotraxin-b

BDNF and its signaling pathways was initially linked to many neuropsychiatric disorders[148,223–225] and a search for an effective and safe potential inhibitor has been well-established. Cylotraxin-b is a TrkB antagonist that was brought forward in the literature as a promising therapeutic agent for neuropsychiatric disorders[226]. It works through a non-competitive allosteric binding to two active sites on TrkB, both different from the BDNF binding sites. This results in reduction of receptor's activation capacity and avoidance of an effect of full inhibition. This is important due to the recognized roles of BDNF-TrkB interaction in normal physiological processes. Upon binding to two sites on the receptor:

phospholipase C (PLC) site and Src homology and collagen (Shc) site, cyclotraxin-b decreases synaptic plasticity and prevents nerve growth and maturation.

Cyclotraxin-b has not previously been investigated as a potential therapeutic option for endometriosis. Therefore, this study was designed to test the effects of cyclotraxin-b on endometriotic implant survival and growth.

1.7. Study animal model

Endometriosis rarely occurs in non-menstrual species making its *in-vivo* studies challenging. Besides non human primates such as baboons and rhesus monkeys which can naturally develop the disease rabbits, rats, and mice are used more in endometriosis studies. The rabbit was the first animal model of experimental endometriosis when Victor Jacobson autotransplanted endometrial tissue in the rabbit in 1922[(227)]. While being the pioneer of animal models, the rabbit is not used too often nowadays since its reproductive maturity onsets at around 6 months of age depending on the breed and this makes the rabbit model not very cost-effective. Moreover, it does not have a regular estrus cycle and is considered an “induced ovulating” species which ovulate after coital stimulation.

Unlike humans whose reproductive cycle (menstrual cycle) lasts approximately 28 days, rodents have estrous cycle lasting 4-6 days. Both types of cycles are divided into similarly regulated stages: proliferative, secretory and menstrual for the menstrual cycle and proestrus, estrus, metestrus, and diestrus for estrous cycle. At the end of estrous cycle, the endometrium is reabsorbed and unlike menstruating mammals is not shed. Similar to

humans, cyclicity is dependent on the function of hypothalamus-pituitary-gonads axis, thus GnRH stimulation of LH and FSH and ovarian production of E2[228].

Rats are frequently used in studies investigating the effects of treatment and not pathophysiology of the disease, as induction of endometriosis in these models is possible only through surgical suturing of autologous tissues. Injections with endometrial cell suspensions are not effective for disease propagation as the cells do not attach and do not form endometriotic lesions[229].

Similarly mice do not develop endometriosis spontaneously without surgical induction, but are cost effective, have short gestation periods and are genetically manipulatable[230]. Depending on the objectives of the study, either an autologous (syngenic) or heterologous (xenotransplant) murine model is used. The first model utilizes donor mouse tissues for induction of endometriosis in the rest of the animals. Previously used for endometriosis research strains are BALB/C(130,231) and C57BL/6[232]. These models are superior to the immunocompromised mice due to their intact immune system regulation which is known to be involved in disease establishment and progression. But they are limited by their inability to receive human xenotransplants.

Heterologous model employs xenontransplantation of human tissues into the mice donors. It allows studying disease progression as well as testing novel therapeutics without developing an immune response to the foreign tissues. Immunocompromised strains that are currently used for endometriosis research are athymic (nude) mice, Severe Combined Immunodeficient (SCID) mice and rag2γ(c) (Recombinant Activating Gene 2/common cytokine receptor γ chain (γc) double null) mice[230].

Nude mice strain has a targeted mutation leading to underdeveloped thymus and declined T cells population, which further prevents B cell maturation. SCID mice have a mutation leading to limited activity of a DNA repair enzyme (Prkdc or "protein kinase, DNA activated, catalytic polypeptide"), which results in dysfunctional T and B lymphocytes. These two models develop extrathymic immunity over time and therefore experiments have to be completed before three months of the animals' age. However, rag2γ(c) model overcomes this issue. These mice are more immunosuppressed in comparison to other mouse models[230]. They lack functional receptors for such interleukins as IL-2, IL-4, IL-7, IL-9, and IL-15 compromising maturation of both B and T cells. Rag2γ(c) mice have been also shown to lack functional NK cells. This strain accepts human immune cells without rejection giving an opportunity to study the role of transplanted human immune cells in endometriosis. Moreover, while lacking age-related immunity, they are more disease resistant than SCID mice.

Studies utilizing murine models can leave the animals intact or perform ovariectomy depending on the aim of the investigation. However, for mimicking the growth-promoting environment of the human proliferative phase, either estrogen subcutaneous (SQ) injections or slow-releasing capsules may be used.

1.8. Hypothesis

As shown above, there is currently no cure for endometriosis. As described, BDNF is suggested to play a central role in the development of endometriosis and establishment of new lesions by triggering the BDNF-TrkB signaling pathway. Therefore, the main

objective of this project was to determine the effect of cyclotraxin-b treatment on endometriotic implant survival and growth. It was proposed that inhibition of the BDNF-TrkB signaling pathway would attenuate implantation, survival and growth of endometriotic implants.

It was hypothesized that systemic administration of cyclotraxin-b would dose-dependently attenuate the progression of endometriosis. During the present project, the following objectives were pursued.

1.9. Objectives

First, the effects of cyclotraxin-b treatment were evaluated based on the endometriotic implant number, volume, and BDNF localization using an established immune compromised mouse xenograft model.

The second aim assessed off-target effects of cyclotraxin-b by monitoring treated mice as well as by histopathological assessment of all major organs.

Overall, this study allowed testing the efficacy and safety of cyclotraxin-b as a potential therapeutic intervention for management of endometriosis. This novel therapy was suggested to give an opportunity to combine the disease treatment with family planning.

CHAPTER TWO: MATERIALS AND METHOD

2.1. Animals

Figure 2 summarizes experimental design. In order to address the objectives stated above, this pre-clinical study used a mouse xenograft model of endometriosis. Specifically, reproductively mature younger adult female mice Rag2$^{-/-}$ γc$^{-/-}$ with targeted knockout mutations on chromosome 2: gene *Rag2* and X chromosome: *Il2rg* replaced with a floxed PGK-neomycin cassette and a floxed human ubiquitin promoter-driver hygromycin cassette, respectively. Animals were purchased from a local commercial supplier (Jackson Laboratories; 014593) and allowed to acclimate for one week before inducing endometriosis. The animal study (AUP # 18-02-11) was approved by Animal Research Ethics Board (AREB). Animals were randomized according to mean weight per cage after arrival and housed according to the treatment group: 2.5mg/kg/day cyclotraxin-b (n = 5), 5.0mg/kg/day cycloraxin-b (n = 5), 7.5mg/kg/day (n = 4), saline (n = 5), 0.04mg/kg/day letrozole (n = 6). All mice were under standard conditions: three to five animals per cage supplemented with environment enrichment in form of nesting material as well as food and water available ad libitum and were maintained on a 12-h light/dark cycle.

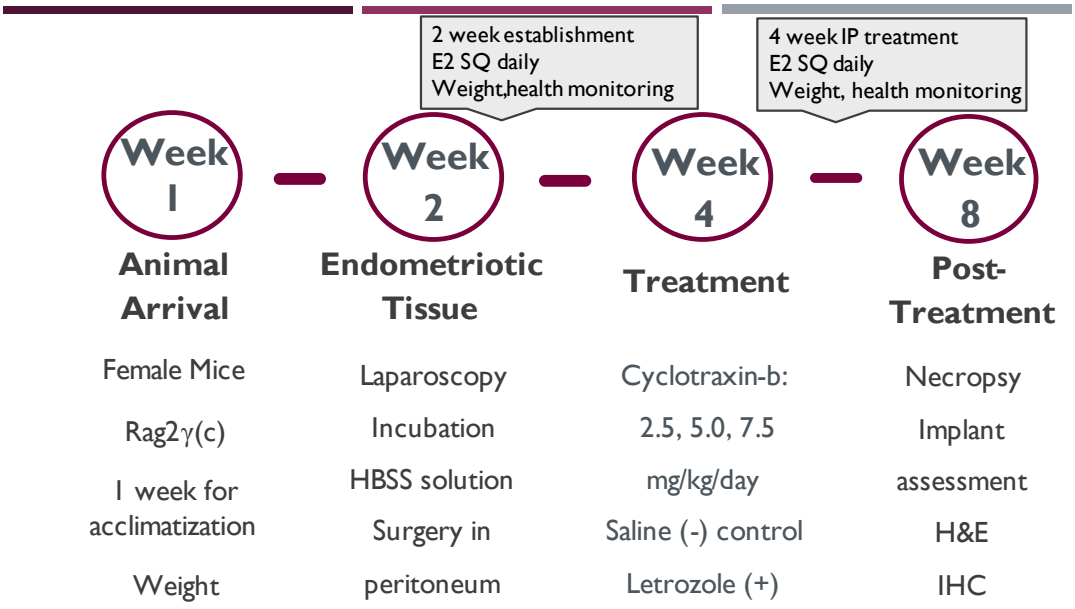

Figure 2: Study design of the project. Total n of animals = 25; 2.5mg/kg/day cyclotraxin-b (n = 5), 5.0mg/kg/day cycloraxin-b (n = 5), 7.5mg/kg/day (n = 4), saline (n = 5), 0.04mg/kg/day letrozole (n = 6).

2.2. Human Participants

Women (non-smokers) undergoing laparoscopy for treatment of endometriosis were invited to participate in this study approved by the Hamilton Integrated Research Ethics Board (12-083T). All participants provided written informed consent before entering the study. The tissues used for endometriosis induction in mice were endometriotic lesions collected at laparoscopy. Endometriosis diagnosis of patients was further confirmed upon availability of the pathology report. Tissue specimens were collected in a sterile falcon tube with 2% penicillin-streptomycin (ThermoFisher, Hamilton, ON)/ Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich, Oakville, ON), placed on ice and transferred to the lab within one hour after collection.

2.3 Xenotransplantation

In the lab, the collected endometriotic tissue (one per human participant) underwent three washes in Hank's-2%penicillin-streptomycin solution and centrifugation (1358.37 x g, 5 min). The tissue was then minced and incubated with collagenase (type I, ThermoFisher, 17100017) (2 mg/ml) solution at 37 ˚C water bath with agitation for two hours. Collagenase was dissolved in modified Eagle's medium (DMEM F-12) (ThermoFisher) containing 10% fetal bovine serum (FBS) (ThermoFisher), 2% penicillin/streptomycin mix and 0.1% ITS (liquid media supplement containing 1.0 mg/ml recombinant human insulin, 0.55 mg/ml human transferrin, and 0.5 μg/ml sodium selenite at the 100x concentration) (Sigma-Aldrich). Followed by two rounds of centrifugation, the cell-dense pellet was mixed in sterile Dulbecco's phosphate-buffered saline (PBS) (Sigma-Aldrich).

Mice were anaesthetised with isoflurane (Fresenius Kabi, Toronto, ON) for the surgical induction of endometriosis. The cell suspension consisting of a combination of stromal and epithelial cells (n total = 1 million cells/animal) obtained from one patient was distributed into mice from different treatment groups, including the control group. Once the surgical plane of anaesthesia was achieved, mice were shaved, and the abdominal area was cleaned with 7.5% Povidone-Iodine detergent solution (Labarotoire Atlas Inc., Toronto. ON), 70% Ethanol, 10% Povidone-Iodine solution (Labaratoire Atlas Inc.). The skin and peritoneal wall was cut, and the cell suspension (100 μl) was infused into the abdominal cavity using a pipette (Fig.1). After suturing, each mouse received 1ml fluids,

Anafen (5mg/kg; CDMV, Merial Canada Inc., Baie-D'Urfe, QC) and estradiol (5 μg/animal; 17β-estradiol, Sigma-Aldrich) injections. Mice were allowed to recover and were monitored daily for two weeks after the surgery before the start of the treatments.

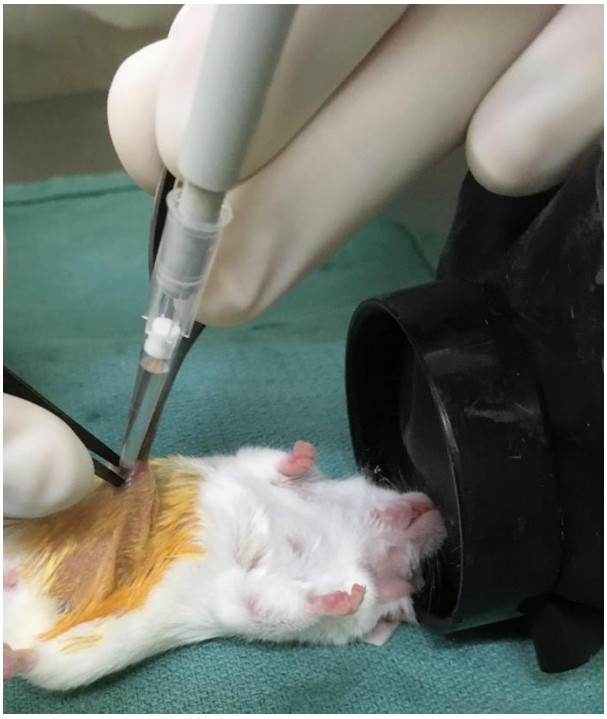

Figure 3: Surgical Induction of Endometriosis in rag2γ(c) mouse: Infusion of cell suspension prepared from the human endometriotic lesion into abdominal cavity.

2.4. Treatment

E2 stock solution (1mg/ml) was prepared by dissolving 17β-estradiol (Sigma-Aldrich) in ethanol and stored at +5°C. The required amount of E2 based on the number of animals in experiment was evaporated from the stock solution, resuspended in heated sterile PBS, filter sterilized and packaged under sterile conditions of biosafety cabinet environment to be further transferred to the animal facility.

Cyclotraxin-b solutions were prepared from 1mg supplied stock powder (Sigma-Aldrich). Powder was dissolved in sterile PBS, filter sterilized, aliquoted and store at -20°C.

Letrozole solutions were prepared from a supplied stock powder (Sigma-Aldrich). The required mass of the chemical was weighed according to the number of animals to be treated in experiment, dissolved in sterile PBS, filter sterilized, aliquoted and stored +5°C.

All mice received an estrogen SQ injection daily (5 μg/animal; 17β-estradiol, Sigma-Aldrich) from the day of the implantation surgery to promote endometriotic implant adherence, attachment, and growth in the peritoneal space. Mice were monitored daily for signs of discomfort and body weight changes until post operation day 14 to allow endometriotic implants to form. All animals were randomly divided into groups and were treated with cyclotraxin-b: 2.5, 5.0, 7.5 or 10 mg/kg animal weight/day, PBS (100 μL) as a vehicle or Letrozol (0.04 mg/kg/day (Sigma-Aldrich) as a positive control. Mice were administered treatments by intraperitoneal injections (IP) five days a week for four weeks. Monitoring of health of animals was performed by regular (once a week) weighing as well as by assessing for any signs of distress based on facial expressions as outlined in the scale (orbital tightening, nose bulge, cheek bulge, ear position, whicker change) in the Grimace scale (Fig.3) and endpoint monitoring form in which animal's appearance (coat condition, pigmentation, animal position, hunching), physical signs (respiration, stool condition), behaviour (signs of depression, isolation, and hyperactivity), and body condition (signs of emaciation or obesity) was assessed.

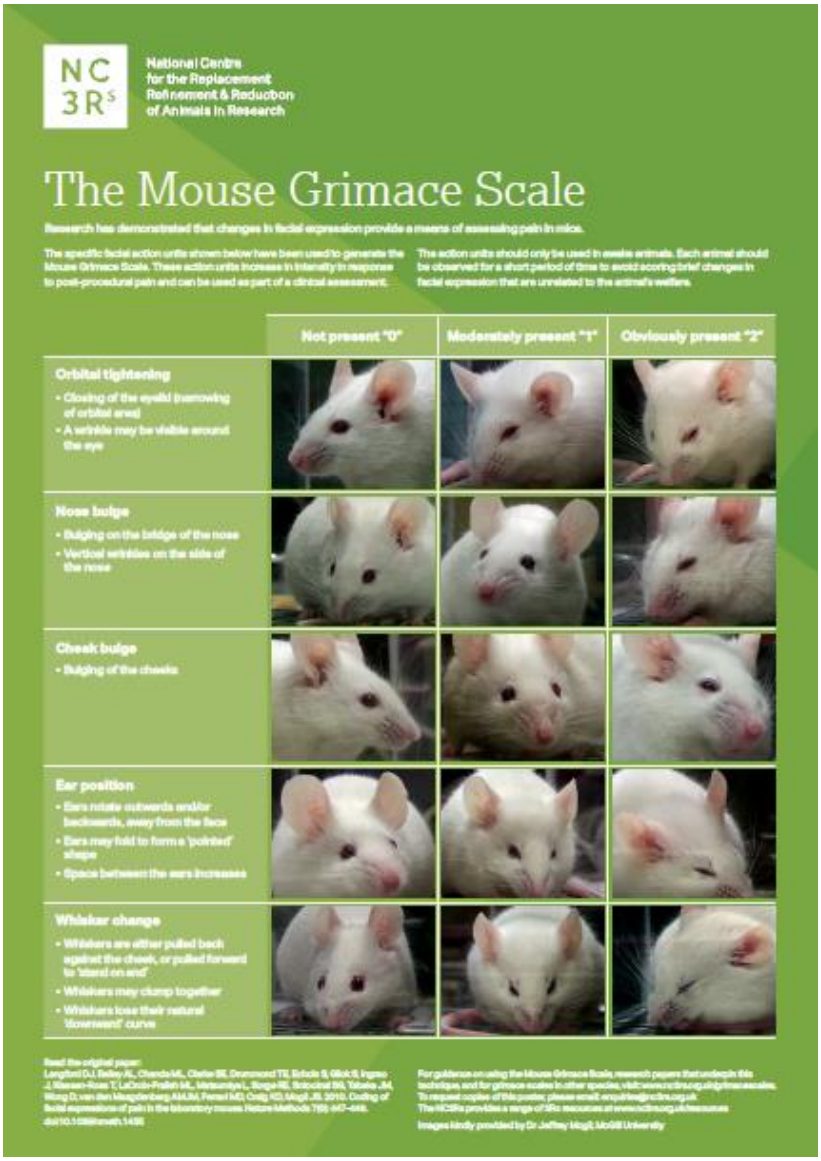

Figure 4. The Mouse Grimace Scale used to assess well-being and pain symptoms in treated animals.

2.5. Post-Treatment Endometriosis Assessment

After the 4-week treatment, all mice underwent a complete necropsy. Briefly, mice were euthanized by carbon dioxide inhalation as per McMaster Central Animal Facility

Standard Operating Procedure (SOP# ROD755). The effects of the treatment were evaluated by quantifying the number of endometriotic implants, implant volume measured by a micrometer to the nearest 0.5 mm (length x width x depth), major organ and endometriotic implant histology.

All major organs (heart, liver, kidneys, spleen, adrenal glands, uterus, and ovaries) were dissected, weighed, preserved in 10% buffered formalin for 48 hours and transferred to 70% ethanol for storage. Tissues were trimmed, processed through a graded ethanol and Xylene series, embedded in paraffin, and sectioned at 4 μm. One section (obtained from approximately the same section position) per organ and endometriotic implants were stained with the routine heamatoxylin and eosin (H&E) to determine histology. One ovary per mouse was sectioned at 4 μm for the total of ten random sections. Slides were stained with H&E, follicles were classified as primordial, transitioning (when both flattened and cuboidal granulosa cells were present), primary, secondary, pre-ovulatory, and atretic (when at least ten pyknotic nuclei were present). Number of corpora lutea was also recorded and compared among the treatment groups with Two Way Analysis of Variance.

Endometriotic implants were subjected to immunohistochemistry (IHC) for staining with anti- BDNF (ab203573; Abcam, Toronto, ON), -TrkB (ab187041, Abcam), -CD31 (ab182981, Abcam), VEGF (ab232858, Abcam), -VEGF receptor 1 (ab32152, Abcam), and -VEGF receptor 2 (ab39256, Abcam). The implants were also stained for anti- human mitochondrial protein (MAB1273, Sigma-Aldrich) which specifically binds to the surface of 65kD nonglycosylated protein of mitochondria from human cells. This was performed to confirm human origin of BDNF protein. During the IHC the implant slides were

deparaffinized in three 5-minute washes of Xylene (9800-1-40, Caledon), followed by two 2 minutes washes in each 100%, 90% and 70% Ethanol solutions (Commercial Alcohols, Brampton, ON), and two 5-minute washes in PBS. The endogenous peroxidase was inhibited by 30% hydrogen peroxide (Thermo-Fisher Chemical) solution. After two 10-minute PBS washes the slides were blocked for one hour with normal horse serum (Vectastain; Mouse IgG ABC Kit, Abcam) in a humidified light-protected chamber at room temperature. After two 10-minute PBS washes antigen retrieval was performed using citrate buffer (Sigma-Aldrich) for 30 minutes. The slides were then washed twice with PBS and incubated overnight in a humidified light-protected chamber at 5°C with the primary antibody: anti-mouse BDNF IgG (1:200), anti-human mitochondrial protein (1:100), anti-TrkB (1:1000), anti-cluster of differentiation 31 (anti-CD31) (1:2000), anti-VEGF (0.1ug/ml), anti-VEGF receptor 1 (1:250), and anti-VEGF receptor 2 (1:250). On the second day the primary antibody was washed off with two 5-minute PBS washes and the slides coated with anti-mouse BDNF were then incubated with biotynilated anti-mouse IgG (Vectastain; Mouse IgG ABC Kit, Abcam), and the rest of the slides were incubated with biotynilated anti-rabbit IgG (Vectastain; Elite ABC-Peroxidase Kit, Rabbit IgG, VECTPK6101) for two hours in a humidified light-protected chamber at room temperature. The slides were then washed twice for 5 minutes in PBS and incubated with Avidin-Biotin peroxidase complex (Vectastain; Mouse IgG ABC Kit, Abcam/ Vectastain; Elite ABC-Peroxidase Kit, Rabbit IgG, VECTPK6101) for two hours in a humidified light-protected chamber at room temperature. Once the complex was washed off by PBS, the slides were stained by 3,3'-Diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich), washed and

counterstained with Hematoxylin (Sigma-Aldrich). The slides were then washed, dehydrated in a graduated series of Ethanol washes, cover slipped and analyzed.

2.6. Statistical analyses

Data were presented as the mean ± standard deviation (SD) for treatment groups. Data were checked for normal distribution and equal variance by Sigma Plot 11.0. Treatment effects were determined by one-way ANOVA and an appropriate post-hoc test. A $p < 0.05$ was considered statistically significant.

CHAPTER THREE: RESULTS

3.1. Systemic effects

At the start of the project animals were divided into six groups including the 10mg/kg/day cyclotraxin-b group. This group was immediately discontinued after the first test animal's pre-term necropsy. The animal presented lethargic after initiation of the cyclotraxin-b treatment suggesting its adverse effects. According to the necropsy finding, the animal suffered from subcutaneous granuloma. Further all animals (n = 25) were randomly divided into 5 groups: saline negative control (n = 5), 2.5mg/kg/day cyclotraxin-b (n = 5), 5.0mg/kg/day cyclotraxin-b (n = 5), 7.5mg/kg/day cyclotraxin-b (n = 4), and 0.04mg/kg/day letrozole (n = 6). All mice survived the surgery and remained healthy throughout the study. There were no detectable changes in their behavior or appearance. Briefly, no signs of lethargy, increased respiration, poor grooming and stary coat were noted. Moreover, none of the facial expressions of pain were present during the experiment (orbital tightening, nose bulge, cheek bulge, ear position, whicker change) (Mouse Grimace Scale [233], Fig. 2).

Weight of animals was recorded and compared among the groups at the start of the experiment and at necropsy to assess general health outcome of the animals. None of the test treatment dosages resulted in significantly different results. The weights of all animals were consistent at the start and end of the experiment as well as comparable among all treatment groups (Fig. 5).

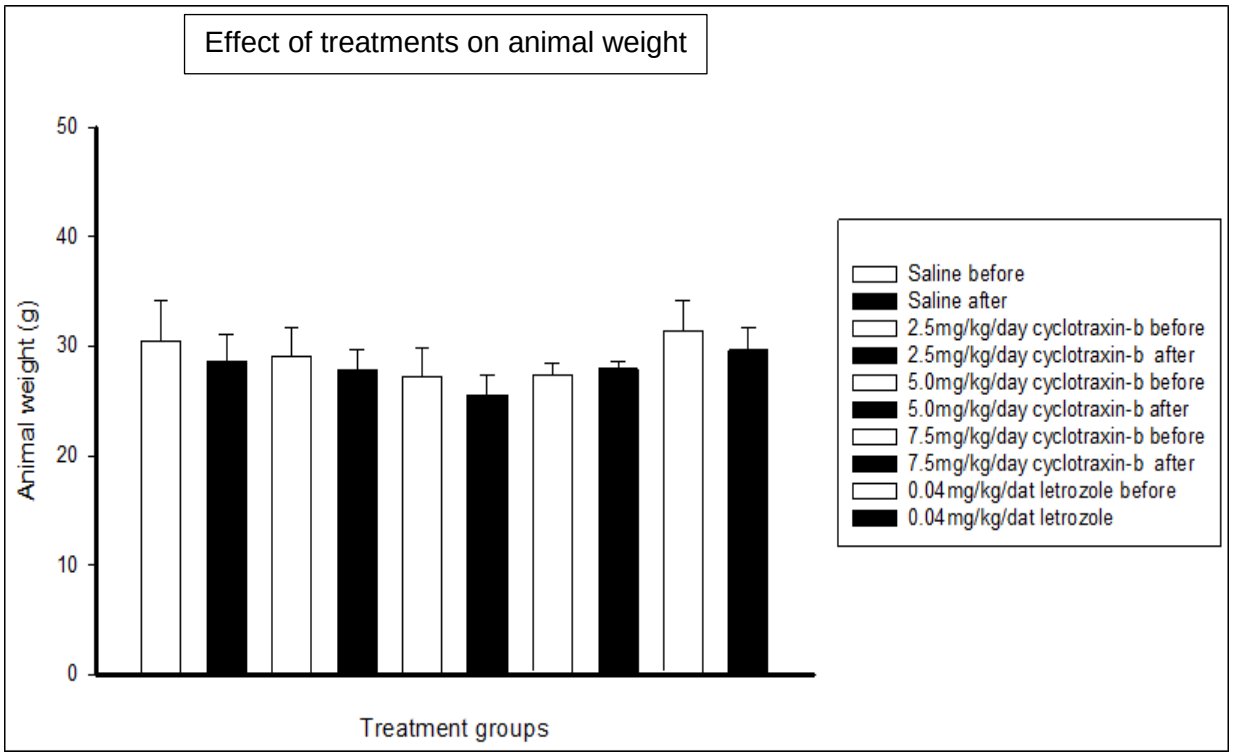

Figure 5: Mice Body Weight Before and After Treatments: No significant difference in weight was detected during the study. The weights were assessed weekly. The black bars represent the mean animal weight ± SD before the treatment and white bars demonstrate the mean animal weight ± SD at necropsy after four weeks of treatment. Total n of animals = 25; saline (n = 5), 2.5mg/kg/day cyclotraxin-b (n = 5), 5.0mg/kg/day cyclotraxin-b (n = 5), 7.5mg/kg/day cyclotraxin-b (n = 4), and 0.04mg/kg/day letrozole (n = 6)

3.2. Effects of the Treatment on major organ mass

All major organs: heart, liver, both kidneys, both adrenal glands, spleen, ovaries and uterus were collected and weighed. None of the treatments had significant effect on the mass of major organs (Fig. 6).

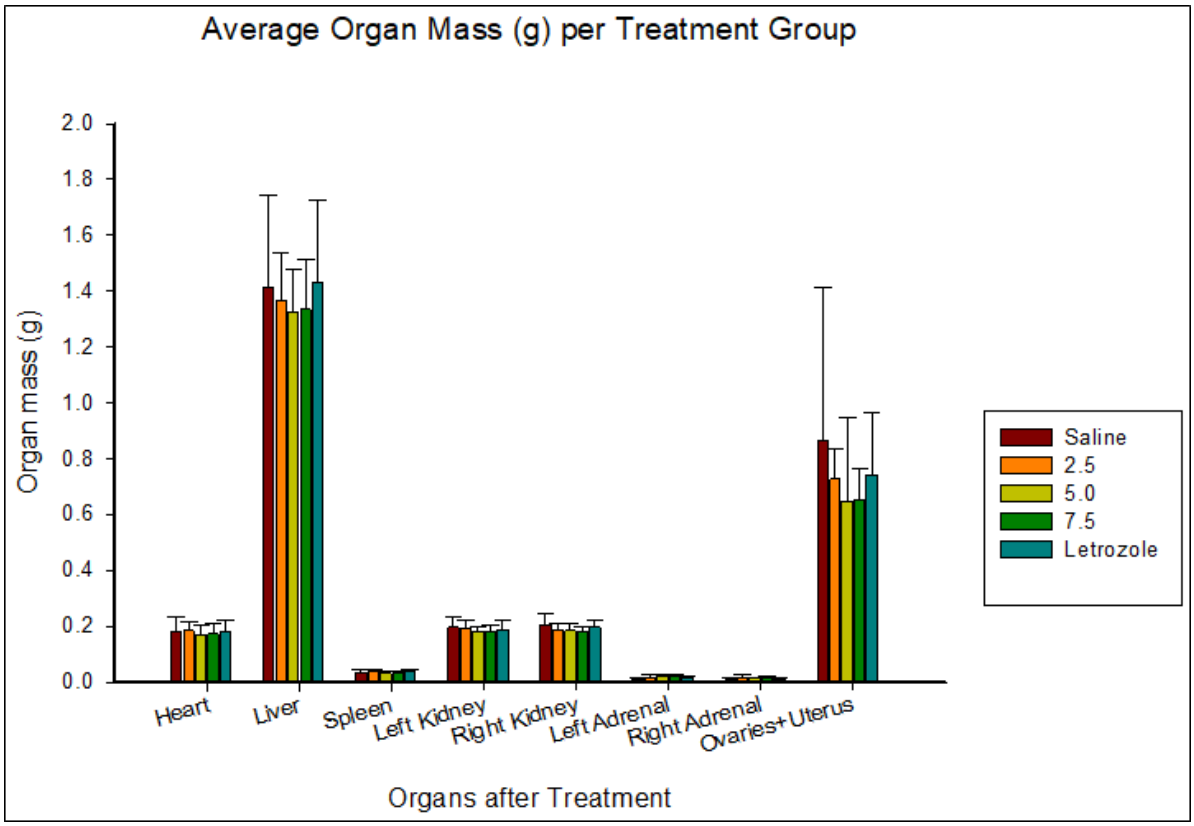

Figure 6: Average Organ mass at necropsy after 4 weeks of treatment. The collected at necropsy organs were heart, liver, spleen, left and right kidneys, left and right adrenal glands, and ovaries and uterus together. Red colour represents: 2.5mg/kg/day cyclotraxin-b (n = 5), Orange: 5.0mg/kg/day cyclotraxin-b (n = 5), Yellow: 7.5mg/kg/day cyclotraxin-b (n = 4), Light blue: Saline negative control (n = 5), and Dark Blue: 0.04mg/kg/day Letrozole positive control (n = 6).

3.3. Histology

All major organs were subjected to routine histology. Heart, spleen, adrenal glands, uterus and ovaries presented healthy with no abnormalities.

Heart sections from all animals showed regular cell distribution and normal myocardium architecture, illustrating the variable diameter of the fibers and the central position of nuclei.

Spleen sections from all animals had normal organ architecture with distinct red and while pulp structures.

Adrenal glands sections from all animals presented healthy organ architecture with distinct cortex and medulla.

Uterus sections from all animals had normal architecture with distinct columnar epithelium and endometrial glands.

Ovary sections from all animals presented normal stroma and interstitial glands.

Liver sections of mice had normal organ composition with normal hepatic cells each with well-defined cytoplasm, prominent nucleus, nucleolus and central vein. Liver vacuolization (Fig.7) was observed in 1 animal treated with high dose (7.5mg/kg/day) Cyclotraxin-b.

Kidney sections from animals presented normal renal corpuscles, each consisting of a glomerulus and an intact Bowman's capsule with adjacent proximal and distal convoluted tubules. There were noted hemosiderin deposits in kidneys of animals treated with medium (5.0mg/kg/day) and high (7.5mg/kg/day) cyclotraxin-b (Fig.8).

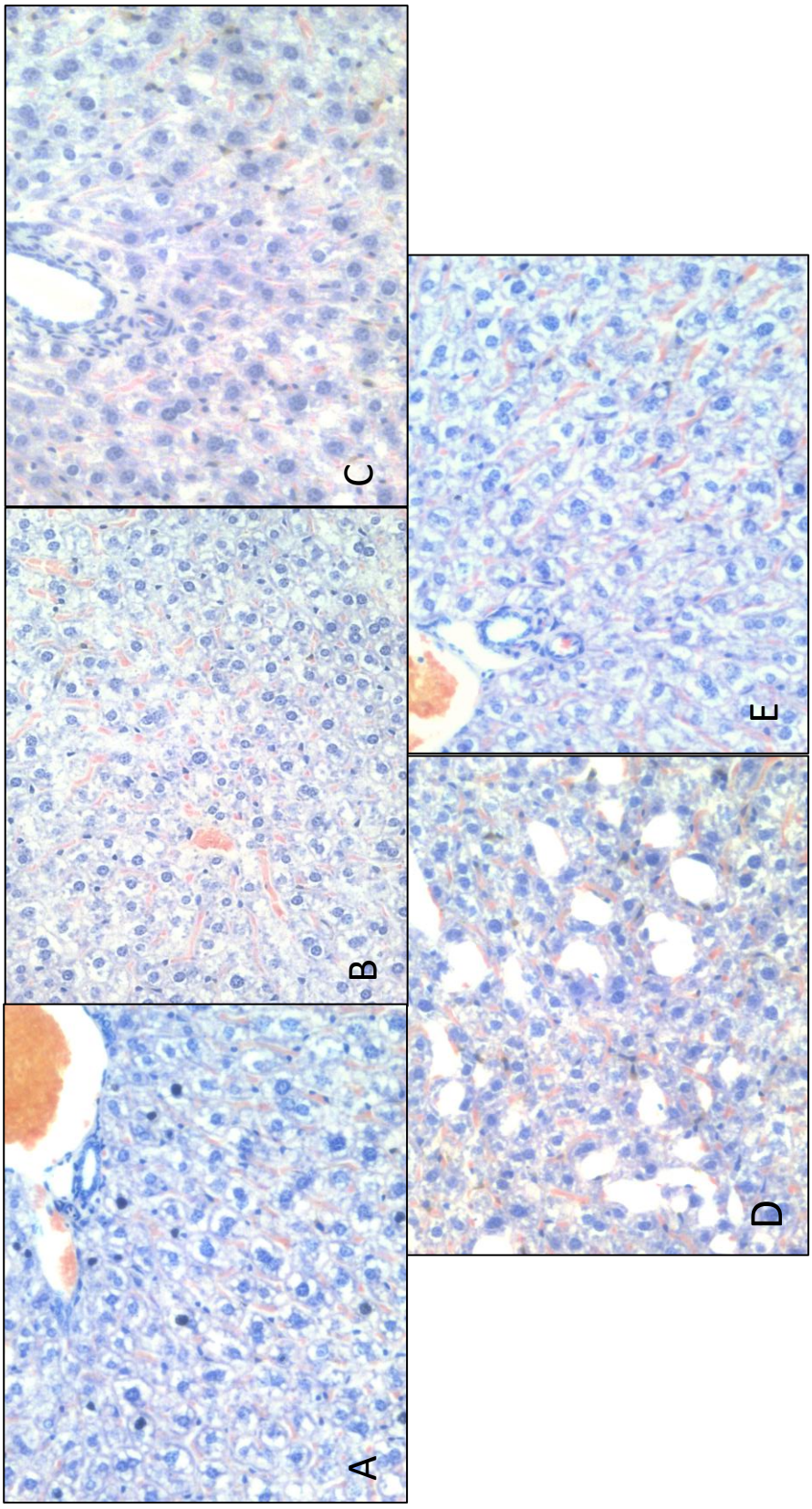

Figure 7: Histopathological findings (x20) in the liver of the treated mice. A: Negative control (saline, n = 5). B: 2.5mg/kg/day cyclotraxin-b (n = 5) C: 5mg/kg/day cyclotraxin-b (n = 5). D: 7.5mg/kg/day cyclotraxin-b (n = 4). E: 0.04mg/kg/day letrozole (n = 6).

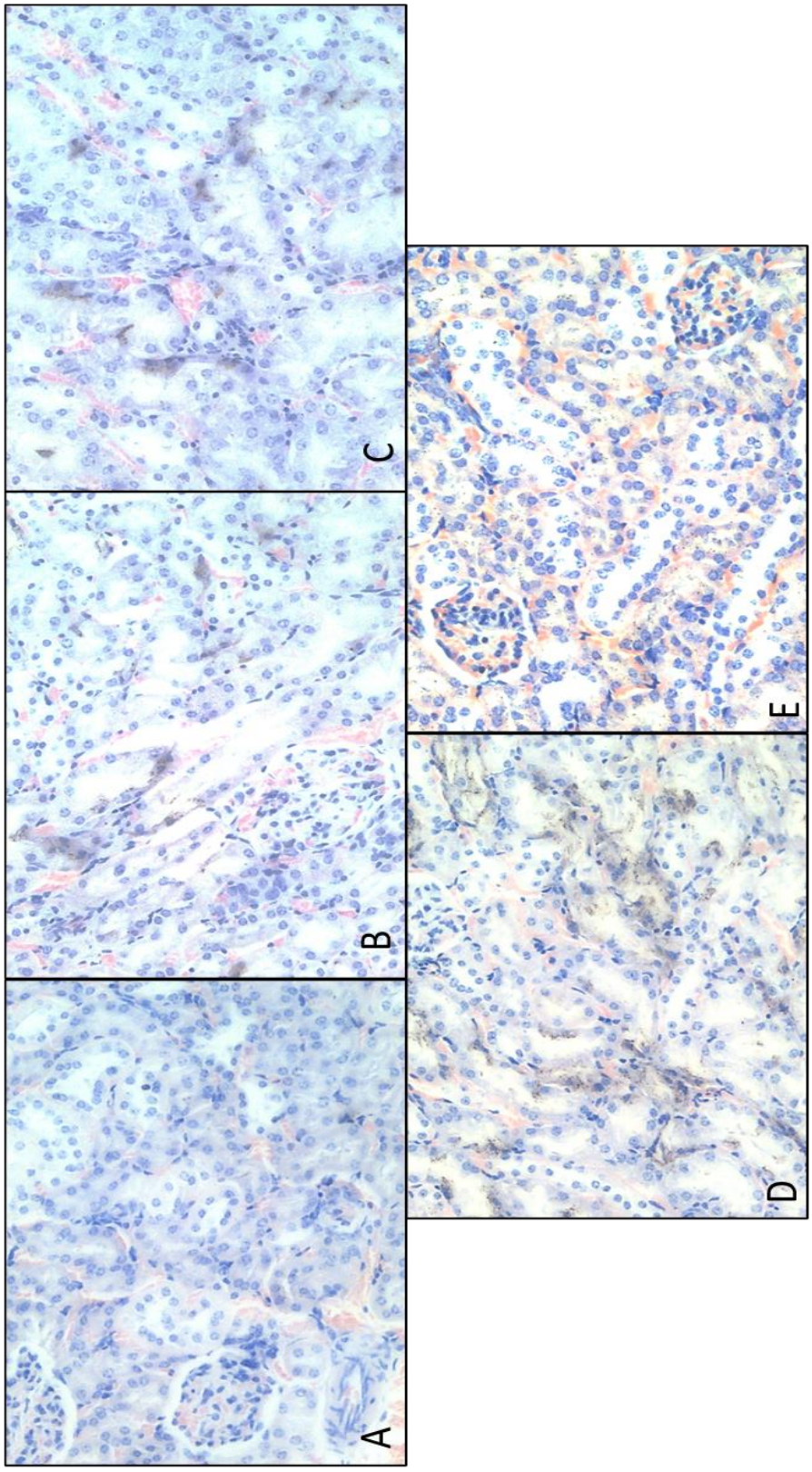

Figure 8: Histopathological findings (x20) in the kidney of the treated mice. A: Negative control (saline, n = 5). B: 2.5mg/kg/day cyclotraxin-b (n = 5). C: 5mg/kg/day cyclotraxin-b (n = 5). D: 7.5mg/kg/day cyclotraxin-b (n = 4). E: 0.04mg/kg/day letrozole (n = 6).

3.4. Effects of Treatment on Follicle count

Fig.9 represents the results of follicular characterization and count performed in 10 sections per mouse according to the tissue availability (4 mice per group with exception of letrozole group comprised of 5 animals). There was no statistically significant difference in the number of follicles of any developmental stage among the treatment groups.

There were statistically significant differences among mice within the same treatment groups. Negative control mice (2/4) varied in the number of primordial follicles (P = 0.07), transitionary follicles (P = 0.07), and secondary follicles (with the lowest P = <0.001). Two mice treated with 2.5mg/kg/day cyclotraxin-b varied in the number of primordial follicles (P = 0.003). Mice treated with 5.0mg/kg/day cyclotraxin-b varied in the number of primordial follicles (P = 0.001, P = 0.001, P = 0.007), primary follicles (P = <0.001), and secondary follicles (P = <0.001 and P = 0.002). One mouse treated with 7.5mg/kg/day cyclotraxin-b differed in the number of secondary follicles (P = <0.001 and P = 0.005). One mouse treated with letrozole differed from three other animals of the same group in the number of primordial follicles (P = <0.001, P = 0.006, P = 0.005) as well as in the number of secondary follicles (P = <0.001). The second mouse treated with letrozole significantly differed in the number of secondary follicles (P = <0.008, P = 0.002, P = <0.001).

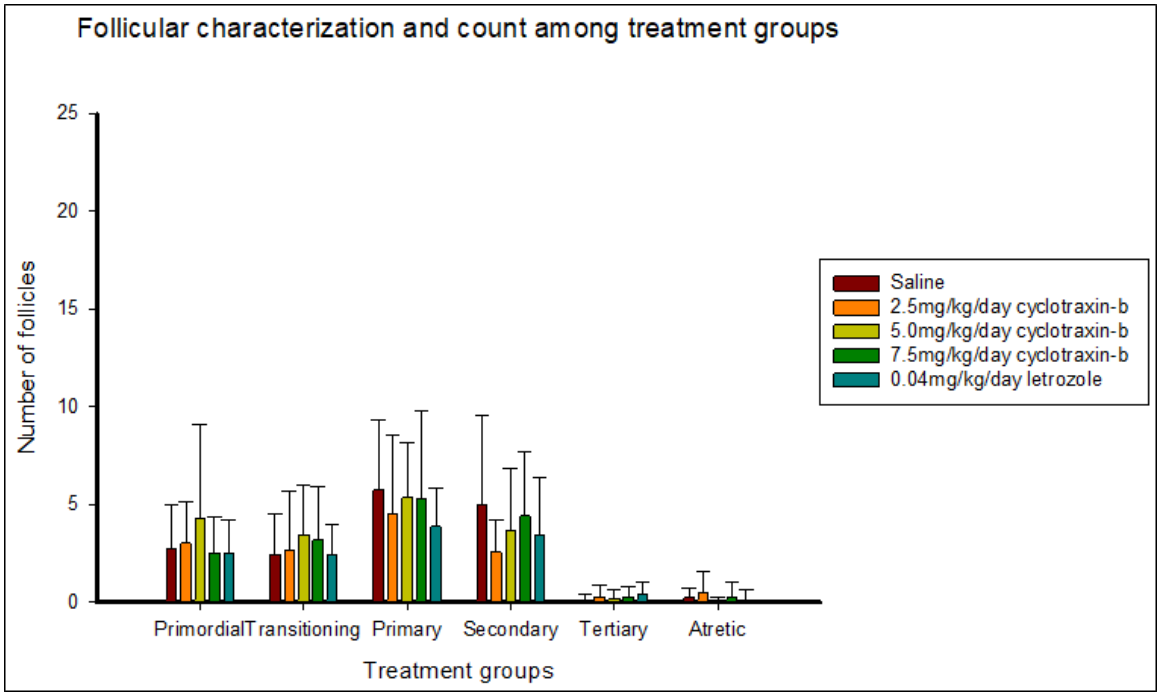

Figure 9: Follicular characterization (performed at 20x magnification) and follicular count among the animals treated with saline (n = 5), 2.5mg/kg/day (n =5), 5.0mg/kg/day (n = 5), 7.5mg/kg/day cyclotraxin-b (n = 4) and 0.04mg/kg/day letrozole (n = 6).

3.5 Effects of Cyclotraxin-b Treatment on Endometriotic lesions.

During necropsy sphere-shaped lesions were found on the peritoneal wall lining. The implants were red and generally brighter, more distinct and more invading at lower dose cyclotraxin-b treatment. Some lesions collected from high-dose cyclotraxin-b treated animals presented as superficial clear cysts at the site of surgical incision (Fig.10). The diagnosis was confirmed by histological examination of endometriotic tissues (presence of stromal and epithelial cells).

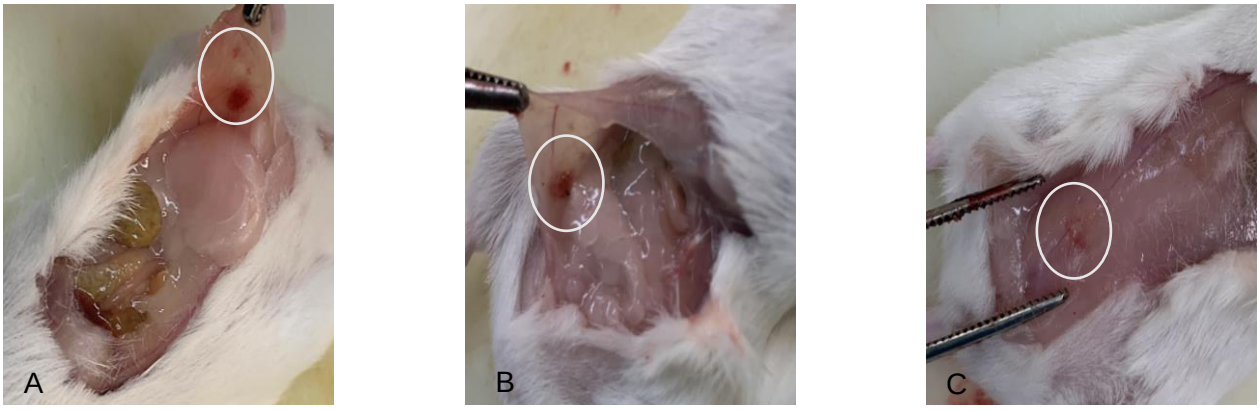

Figure 10: Lesion at the surgical site, after a 4-week treatment. A: 2.5mg/kg/day cyclotraxin-b. B: 5mg/kg/day cyclotraxin-b. C: 7.5mg/kg/day cyclotraxin-b. Circular lesion found on the peritoneal wall at the site of the surgical incision.

The volume and number of endometriotic implants was found to be inversely proportional to the concentration of cyclotraxin-b. Mice treated with low concentration of cyclotraxin-b (2.5 mg/kg/day) developed larger lesions (average volume = 21.24 mm^3), than mice treated with high dose (7.5 mg/kg/day) cyclotraxin-b (average volume = 1.18 mm^3) (Fig. 11). Due to the failed normality test, the data was analyzed with Kruskal-Wallis one-way analysis of variance test and, according to the Dunn's post hoc test, statistically significant difference was detected between a low-dose cyclotraxin-b group and letrozole treatment (p = 0.014).

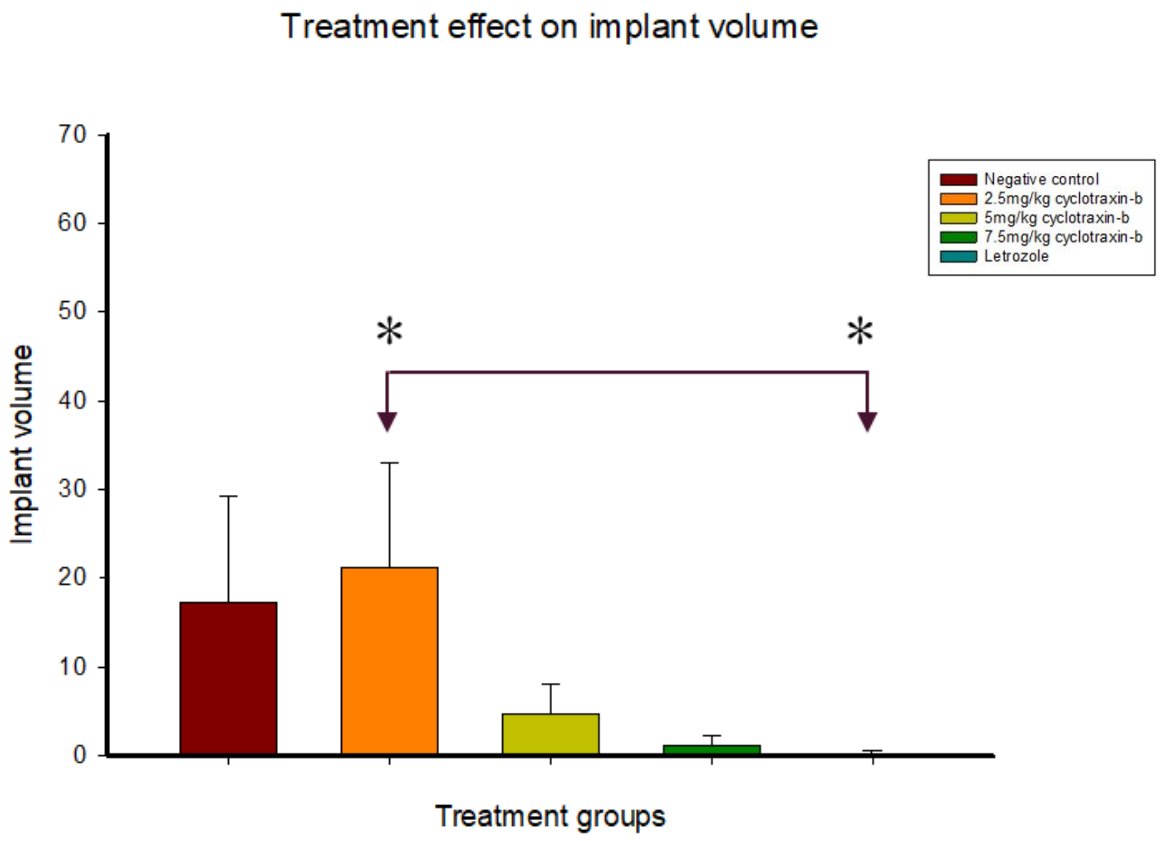

Figure 11: Effect of 4-week treatments on endometriotic implant volume.Total n of animals = 25; saline (n = 5), 2.5mg/kg/day cyclotraxin-b (n = 5), 5.0mg/kg/day cyclotraxin-b (n = 5), 7.5mg/kg/day cyclotraxin-b (n = 4), and 0.04mg/kg/day letrozole (n = 6)

Number of endometriotic lesion dose-dependently decreased although statistical significance could not be demonstrated. According to the Kruskal-Wallis test, there was a statistically significant variation among the treatments' effect on the number of the formed endometriotic lesions. Animals in the low dose group developed on average more lesions, than in the high concentration group (n= 1.2 vs. n= 0.75). (Fig. 12).

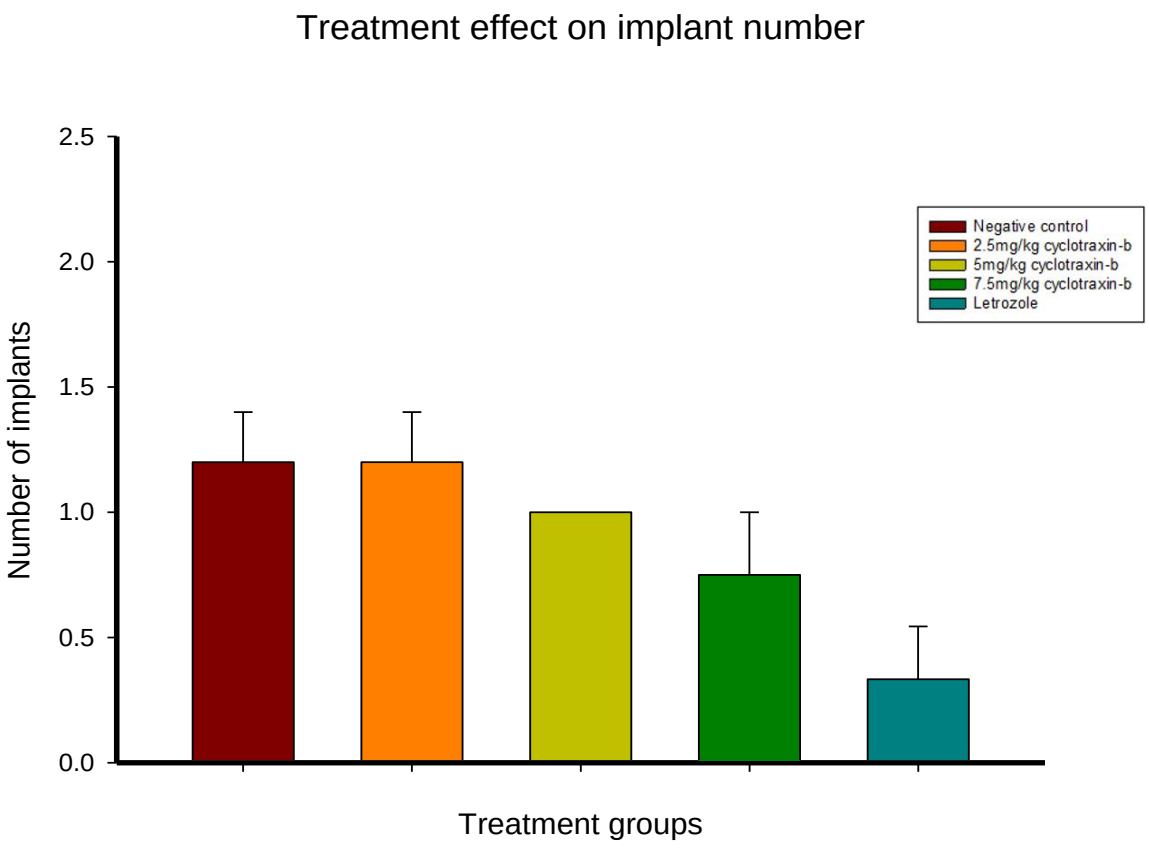

Figure 12: Effect of treatment on the endometriotic implant number.Total n of animals = 25; saline (n = 5), 2.5mg/kg/day cyclotraxin-b (n = 5), 5.0mg/kg/day cyclotraxin-b (n = 5), 7.5mg/kg/day cyclotraxin-b (n = 4), and 0.04mg/kg/day letrozole (n = 6).

3.5. Localization of BDNF, anti-human mitochondrial protein, TrkB, CD31, VEGF, VEGFR1 and VEGFR2 in endometriotic lesions

The protein localization of BDNF, anti-human mitochondrial protein, TrkB, CD31, VEGF, VEGFR1, and VEGFR2 in the formed endometriotic tissue specimens was evaluated by immunochemical staining with the corresponding antibodies. Slides immunostained against human mitochondrial protein and BDNF revealed co-localization of the two proteins in the endometriotic implants. (Fig. 13).

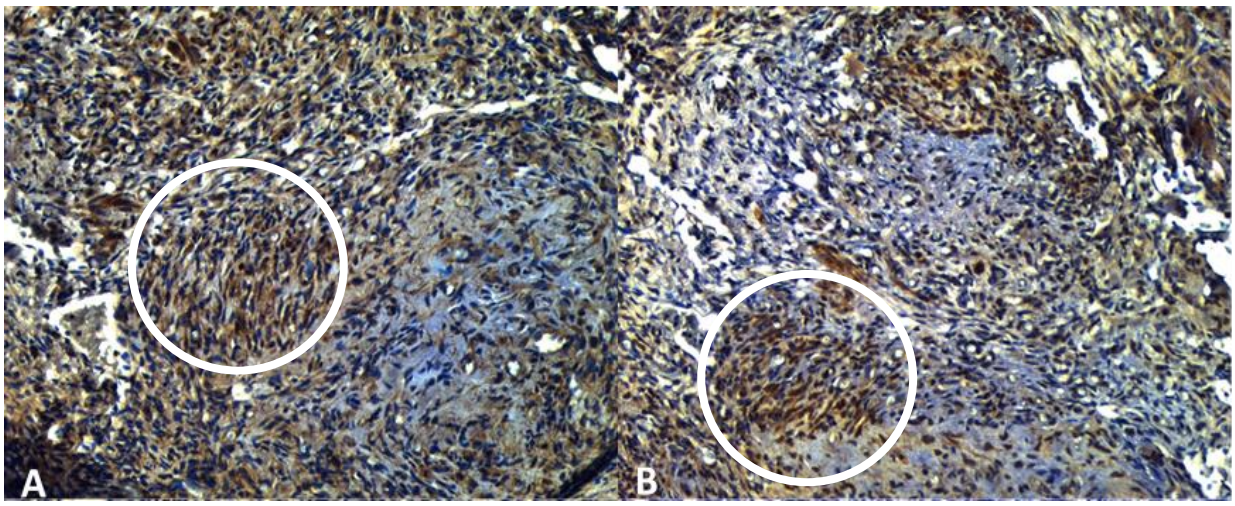

Figure 13: Immunostained tissue sections from 7.5mg/kg/day cyclotraxin-b treated mouse lesion. A: Anti-human nonglycosylated protein (1:100). B: Anti-BDNF (1:200).

BDNF, VEGF, and VEGFR1 were detected mostly secreted or cell membrane-associated. CD31, TrkB, and VEGFR2 were localized within cytoplasm. There was no linear dose dependent correlation between the treatments and protein detection (Fig. 14-20). The only statistically significant difference was found between the VEGFR2

positively-stained cells of negative control and 5.0mg/kg/day cyclotraxin-b with positive rates of 58.7% and 34.23%, respectively (p <0.05).

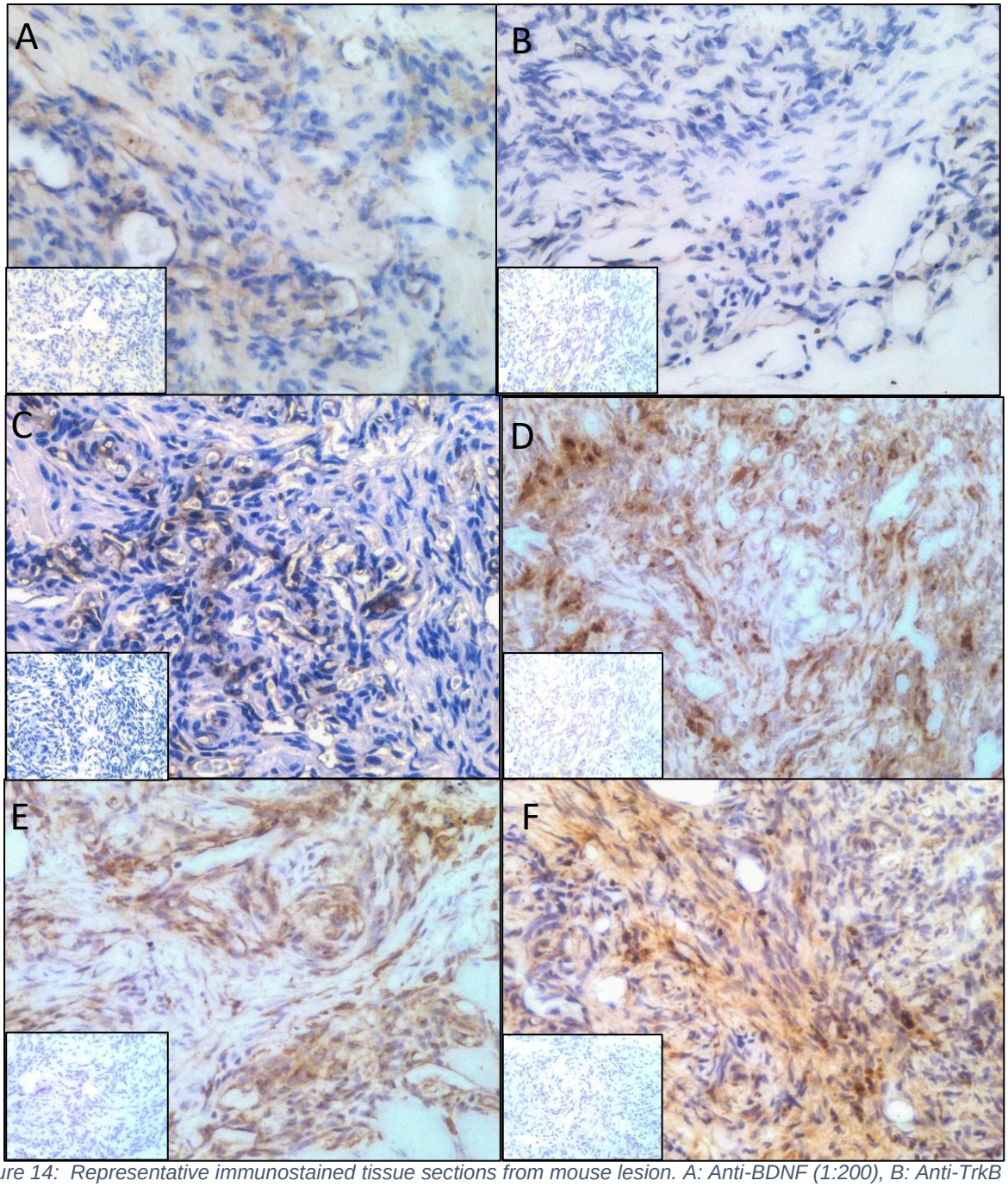

Figure 14: Representative immunostained tissue sections from mouse lesion. A: Anti-BDNF (1:200), B: Anti-TrkB (1:1000), C: Anti CD31 (1:2000), D: Anti-VEGFa (1:10000), E: Anti-VEGFR1 (1:250), F: Anti-VEGFR2 (1:250). All slides are counterstained with Harris hematoxylin. All microphotographs were made under 20x magnification. Left lower corner images correspond to negative control slides of the same tissue (no primary antibody).

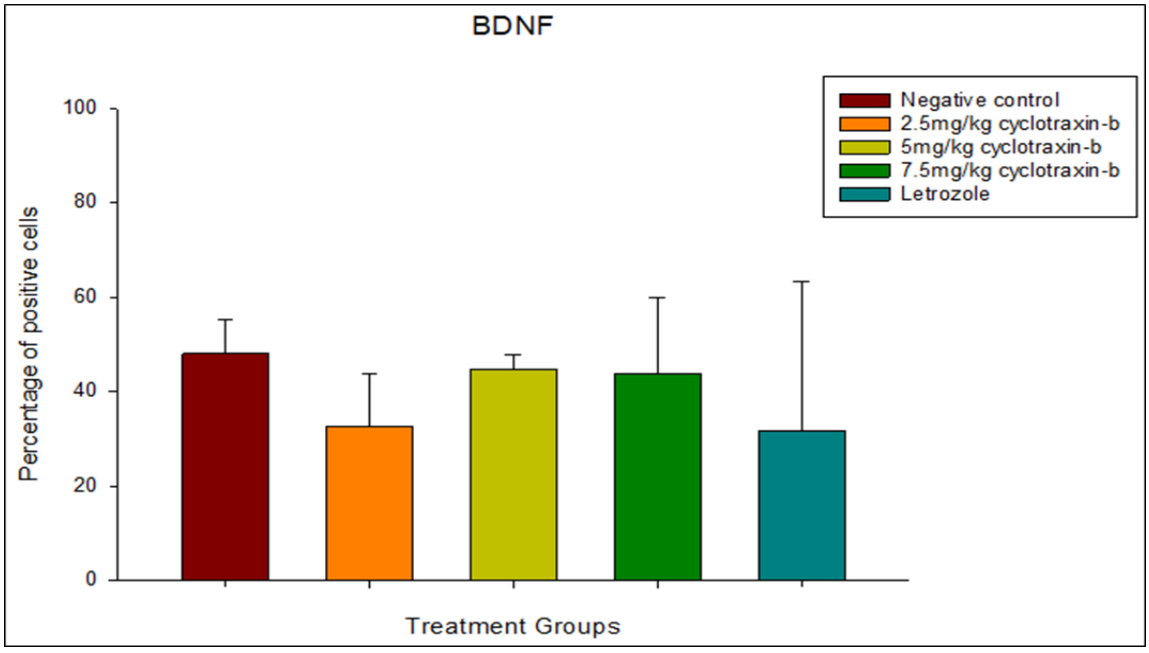

Figure 15: Effect of a four-week treatment on BDNF staining (IHC: Anti-BDNF 1:200). Percentage of positive cells based on the three quadrants method via Image J analysis.Total n of animals = 25; saline (n = 5), 2.5mg/kg/day cyclotraxin-b (n = 5), 5.0mg/kg/day cyclotraxin-b (n = 5), 7.5mg/kg/day cyclotraxin-b (n = 4), and 0.04mg/kg/day letrozole (n = 6).

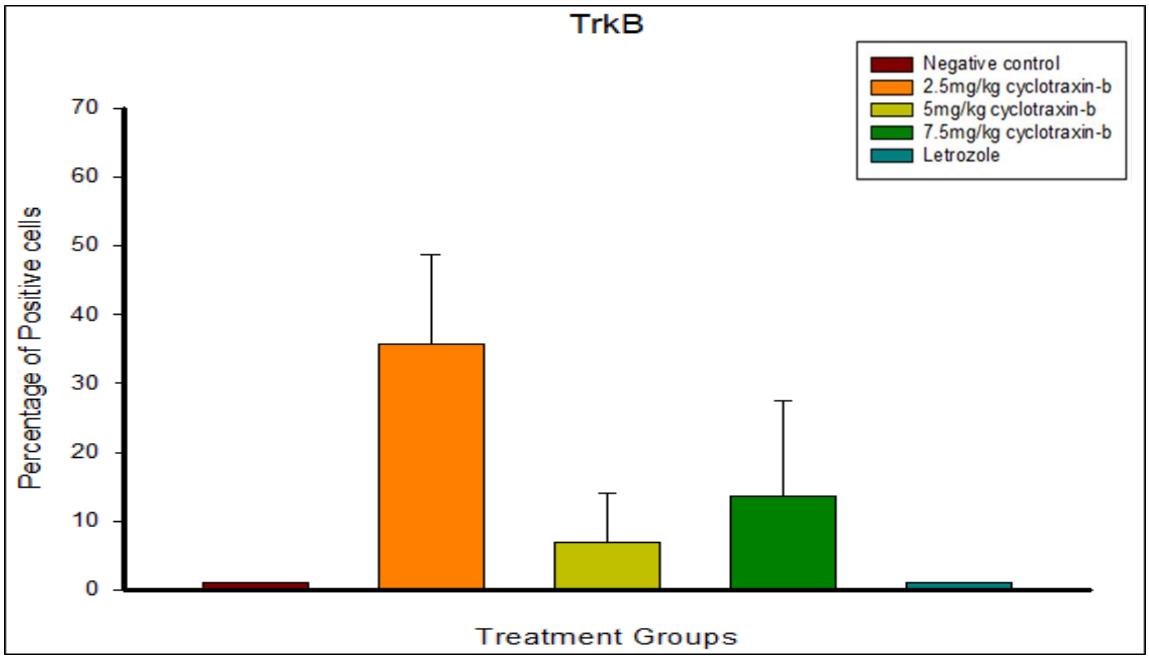

Figure 16: Effect of a four-week treatment on TrkB staining (IHC: Anti-TrkB 1:1000). Percentage of positive cells based on the three quadrants method via Image J analysis. Total n of animals = 25; saline (n = 5), 2.5mg/kg/day cyclotraxin-b (n = 5), 5.0mg/kg/day cyclotraxin-b (n = 5), 7.5mg/kg/day cyclotraxin-b (n = 4), and 0.04mg/kg/day letrozole (n = 6).

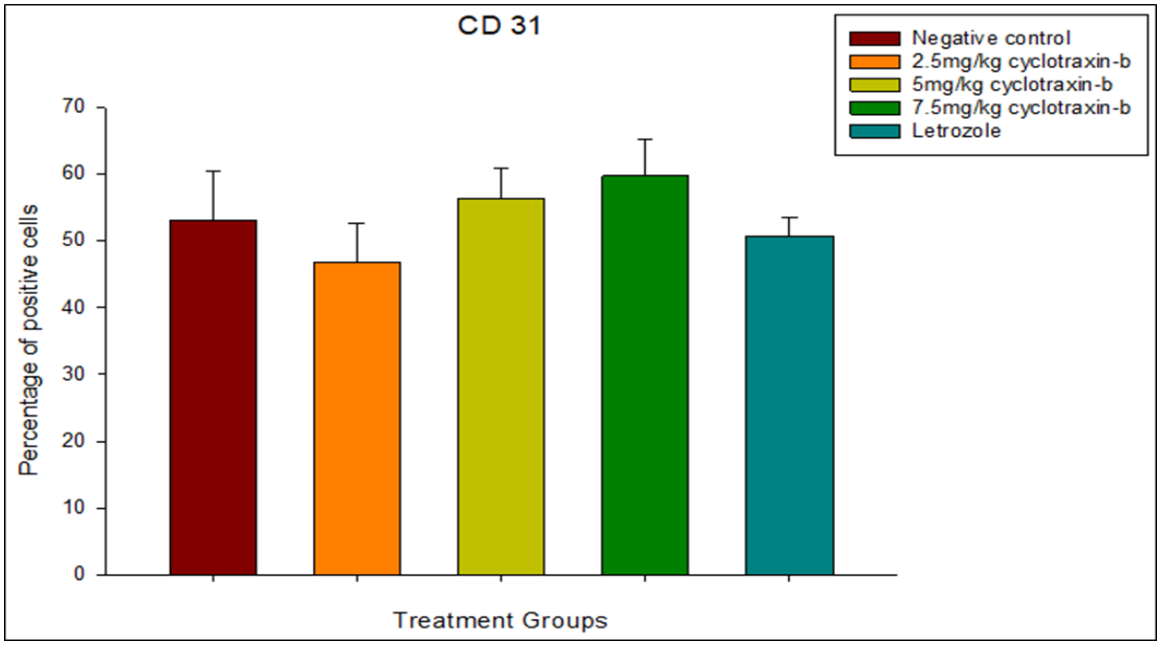

Figure 17: Effect of a four-week treatment on CD31 staining (IHC: Anti-CD31 1:2000). Percentage of positive cells based on the three quadrants method via Image J analysis. *Total n of animals = 25; saline (n = 5), 2.5mg/kg/day cyclotraxin-b (n = 5), 5.0mg/kg/day cyclotraxin-b (n = 5), 7.5mg/kg/day cyclotraxin-b (n = 4), and 0.04mg/kg/day letrozole (n = 6).*

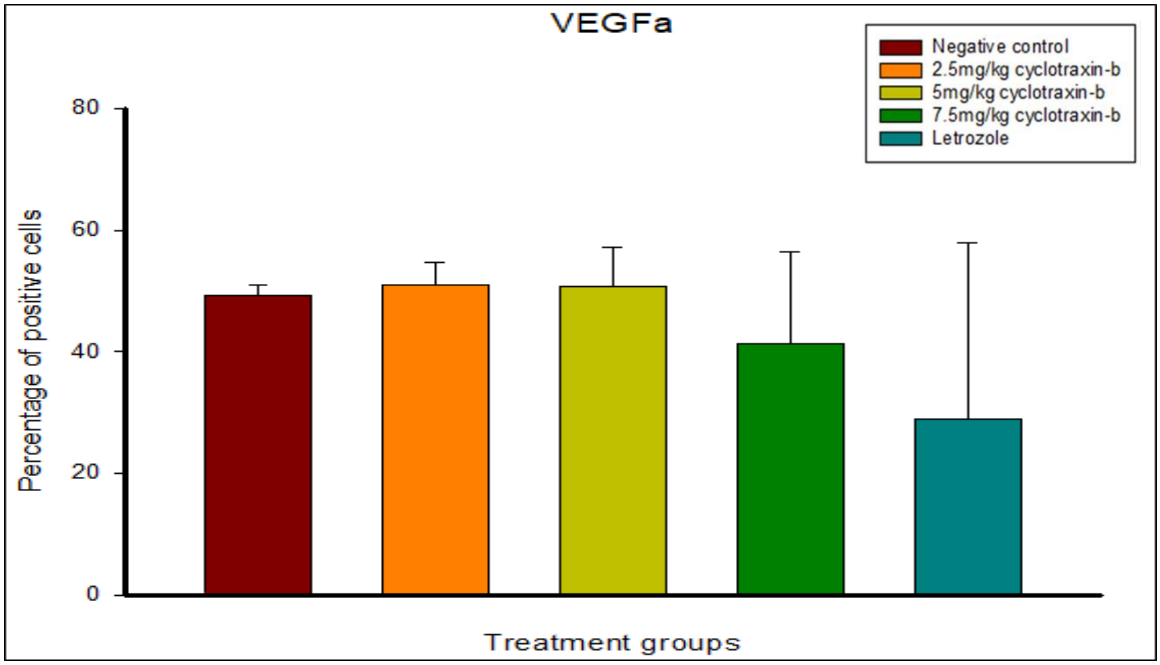

Figure 18: Effect of a four-week treatment on VEGFa staining (IHC: Anti-VEGFa 1:10000). Percentage of positive cells based on the three quadrants method via Image J analysis. Total n of animals = 25; saline (n = 5), 2.5mg/kg/day cyclotraxin-b (n = 5), 5.0mg/kg/day cyclotraxin-b (n = 5), 7.5mg/kg/day cyclotraxin-b (n = 4), and 0.04mg/kg/day letrozole (n = 6).

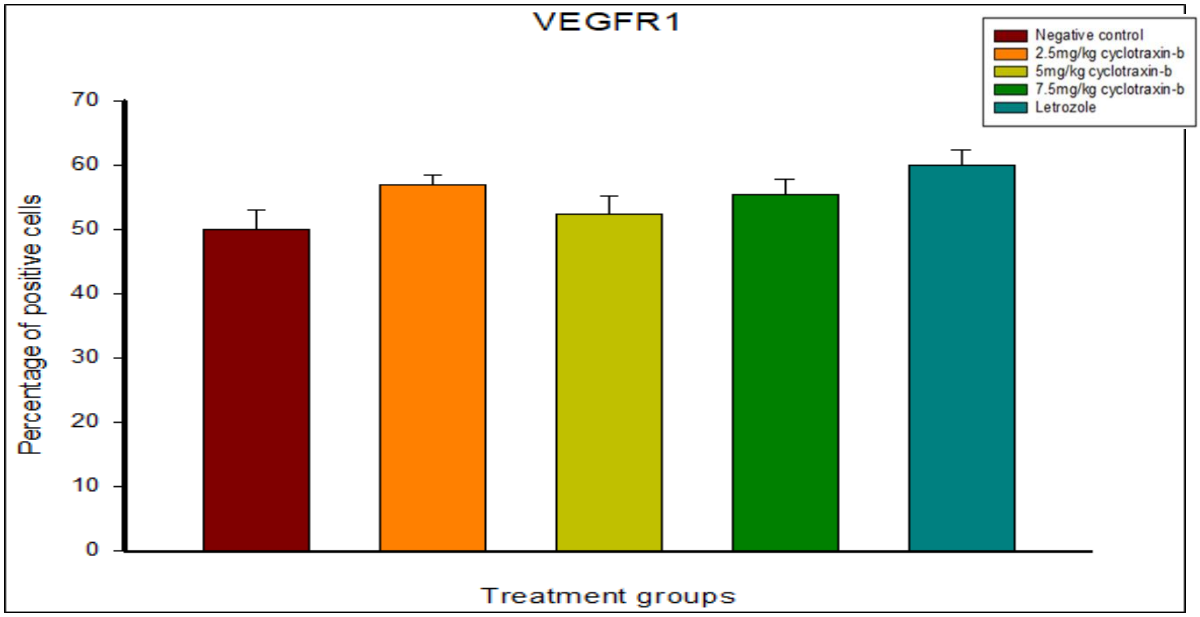

Figure 19: Effect of a four-week treatment on VEGFR1 staining (IHC: Anti-VEGFR1 1:250). Percentage of positive cells based on the three quadrants method via Image J analysis. Total n of animals = 25; saline (n = 5), 2.5mg/kg/day cyclotraxin-b (n = 5), 5.0mg/kg/day cyclotraxin-b (n = 5), 7.5mg/kg/day cyclotraxin-b (n = 4), and 0.04mg/kg/day letrozole (n = 6).

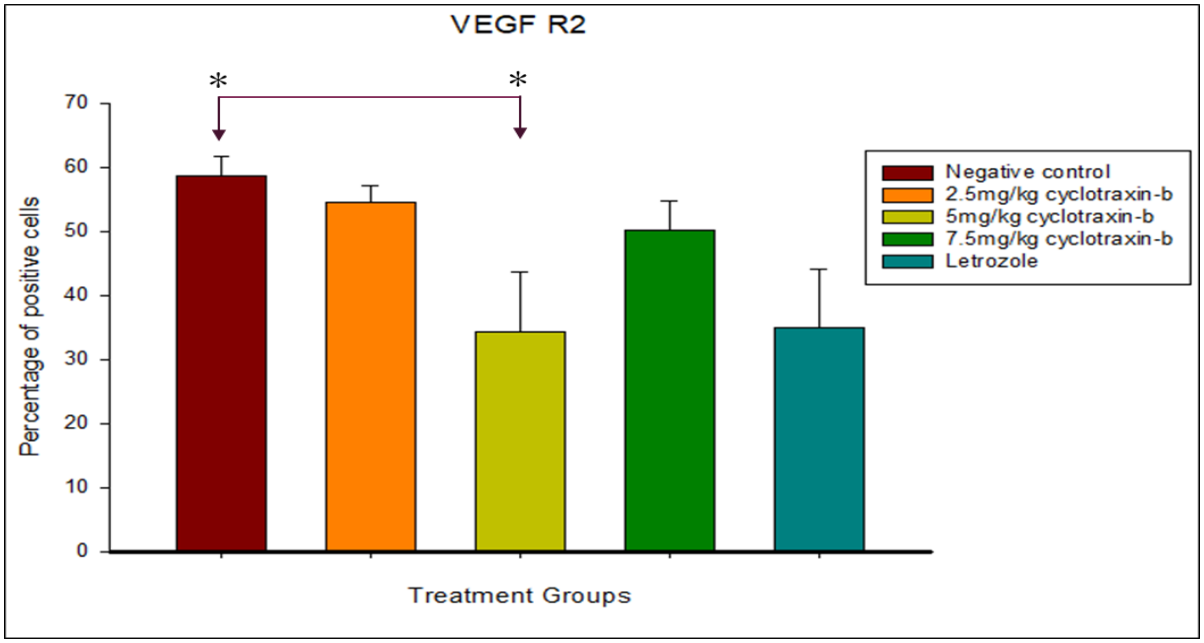

Figure 20: Effect of a four-week treatment on VEGFR2 staining (IHC: Anti-VEGFR2 1:250). Percentage of positive cells based on the three quadrants method via Image J analysis. Total n of animals = 25; saline (n = 5), 2.5mg/kg/day cyclotraxin-b (n = 5), 5.0mg/kg/day cyclotraxin-b (n = 5), 7.5.mg/kg/day cyclotraxin-b (n = 4), and 0.04mg/kg/day letrozole (n = 6)

CHAPTER FOUR: DISCUSSION

4.1. Summary of findings

The aim of the study was to investigate the effects of cyclotraxin-b treatment potential toxicity and its effect on endometriotic implant survival and growth. According to the results, the proposed cyclotraxin-b therapy may cause adverse effects at a 10mg/kg/day concentration, however, replication of the results is required. Overall, there were no significant differences in body weight or major organ mass of animals across treatment groups. Moreover, signs of general toxicity such as erythema and edema were not observed. The histopathology revealing some abnormalities in the liver and kidney organization may suggest potential toxicity of high dose cyclotraxin-b. Medium- and high-dose cyclotraxin-b treatment was shown to be trending effective in limiting implant survival and growth making it a presumably promising therapeutic agent.

IHC staining confirmed BDNF staining in the area of human protein signaling, suggesting the human origin of BDNF. This could be an important detail taking into consideration that the therapy was effective in reducing endometriotic lesion number and volume. Thus, although the lesions were xenografts within an animal model, they demonstrated to maintain human cell profile, which got suppressed by the high dose therapy.

Results of BDNF and TrkB IHC analysis revealed no particular dose-dependent relationship between the treatment dose and staining. According to the staining patterns, staining intensity of BDNF decreased after treatments. These results are in accordance with a study that found decreasing concentrations of free (circulating) BDNF after melatonin

treatment[234]. As for the BDNF receptor TrkB, the percentage of positive cells increased in cyclotraxin-b treatment groups, but not in letrozole which was similar to the negative control. These results are contradicting with the results of GnRHa and continuous progesterone therapeutic intervention after which the expression of BDNF receptor was decreased[235]. While no statistical difference was detected, decreased staining score of BDNF was correlated with increased staining of TrkB (most obvious at 2.5mg/kg/day cyclotraxin-b group). This observation may suggest an underlying compensation mechanism.

According to the IHC results of the anti-VEGFa staining, lesions treated with high dose cyclotraxin-b demonstrated fewer antigen targets. These results were only lower in mice treated with letrozole. While a technique as the Western Blot could give a better quantifiable result, IHC revealed the localization of the marker. Preliminary quantification based on the percentage of positive cells demonstrated the trending effect of the current treatment on VEGFa. As VEGFa is a crucial regulator of angiogenesis and pathological neovascularization, decrease of its expression is an important therapeutic target.

VEGFR1 is generally a negative regulator of angiogenesis and studies employing VEGFR1 null mice demonstrated overgrowth and dysregulation of blood vessels[236]. On the other hand, VEGFR2 is essential for development of vascular system and VEGFR2 null mice failed to develop blood vessels[237]. Therefore, it can be suggested, that ideal therapy would aim to increase VEGFR1 expression, receptor activation or VEGF affinity for this receptor, while limiting activity of the VEGFR2. According to the IHC results, there was statistically significant reduction of VEGR2 positively stained cells in 5.0mg/kg/day

cyclotraxin-b group. Other doses of cyclotraxin-b and letrozole while not significant but also affected percentage of antigen targets. On the contrary, implants formed in animals form all treatment groups had greater percentage of VEGFR1 positively stained cells. Therefore, it is suggested that cyclotraxin-b therapy may be effective in altering angiogenesis, the crucial process for endometriosis lesion establishment and progression.

4.2. Reproductive health of study animals

Follicular characterization and quantification were performed. Based on the results there were no significant differences among the treatment groups. Absence of statistical difference between negative and cyclotraxin-b groups suggests potential safety of the therapy on reproductive health of the animals. However, the variation within the treatment groups makes it challenging to draw any definite conclusions from the data. Increasing the sample size (n = 10 animals per group) and performing the count within serial sections could benefit reliability of this preliminary data.

4.3. Strength and limitations

The main strength of this study was employment of immunocompromised xenograft mouse model which allowed to test the efficacy of cyclotraxin-b on human tissue *in vivo*. While this model advantageously allowed implantation of the human tissue without an immune response, it did not account of the complete immunological niche associated with the disease progression which may potentially affect the efficacy of cyclotraxin-b. Another limitation of this study was a limited sample size (total n = 25 vs. the desired n = 50), which

was caused by the inability to receive patient tissues due to cancelation of all elective surgeries during SARS CoV-2 associated pandemic.

Usually there is a poor mouse-to-human research results translation and the majority of suggested medications are not as effective in human population as they are in murine models, which can be explained by anatomical, physiological, and gene expression differences. While the model employed in this study partially overcame this challenge by using the human tissue, extensive translational studies would be required for further evaluation of the efficacy of the proposed therapy. The employed doses of cylotraxin-b were chosen according to the previously conducted study[238], however, extensive pharmacological studies will be required to determine a NOAEL (no observed adverse effect level).

Results of the IHC staining showed the localization of the selected markers and provided semi-quantitative results of the proteins' expression. Further analysis of tissues and potentially serum of mice with techniques as the Western blot, enzyme-linked immunosorbent assay (ELISA) and KIRA-ELISA (kinase receptor activation) and quantitative polymerase chain reaction (qPCR) could provide more accurate quantifiable results.

4.4. Conclusions

In conclusion, this study was conducted to investigate the potential effects of cyclotraxin-b treatment on endometriotic implant survival and growth. It was hypothesized that the treatment will ameliorate the lesion growth and progression. The collected data

supports the hypothesis. Importantly, no adverse health effects were detected after the four-week treatment with cyclotraxin-b. Moreover, cyclotraxin-b treatment-induced a dose-dependent trend towards a decrease in endometriotic implant survival and growth. Therefore, I conclude that cyclotrain-b and inhibition of BDNF signaling is a promising treatment strategy deserving further investigation.

4.5. Future directions

Although limited by sample size owing to COVID-19 disruption of the study, these results demonstrated a potential benefit of cyclotraxin-b treatment. Moving forward, it would be beneficial to further investigate the outcomes of cyclotaxin-b administration by employing a larger sample size, performing Western blot to quantify the angiogenic proteins, as well as to analyze mRNA for the selected markers in animals' circulation through qPCR. Furthermore, while currently available endometriosis therapies interfere with family planning, it would be beneficial to subject a subgroup of mice to breeding in order to confirm safety of cyclotraxin-b treatment on reproductive function as well as to test the risk of adverse effects in the second generation of animals. Current therapy administration was performed through IP injections which could limit it use and thus alternative routes of administration should be evaluated.

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