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Amd3100 Administration For The Treatment Of Asherman's Syndrome In A Murine Model

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AMD3100 ADMINISTRATION FOR THE TREATMENT OF ASHERMAN'S SYNDROME IN A MURINE MODEL

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Introduction

Asherman's syndrome (AS) is a uterine disorder characterized by intrauterine adhesions. These adhesions, also known as synechiae, result from loss of the regenerative cell population residing in the basalis layer of the endometrium[1]. Under normal physiologic conditions, these cells clonally expand to replace the functionalis layer of the uterus after menses or parturition[2]. Without a properly functioning basalis layer, the highly vascular functionalis layer of the uterus is replaced by dense fibrotic tissue which spans the spans the endometrial walls, and in severe cases, can completely obliterate the uterine cavity.

In Asherman's Syndrome, the presence of these intrauterine adhesions precludes normal functioning of the endometrial lining. A healthy functionalis layer is required for implantation and regular menstruation, which may be hindered or halted altogether in AS. The lack of a functioning endometrial lining often leads to symptoms of hypomenorrhea or amenorrhea. Implantation may be impaired as fewer viable sites are available on the endometrial surface. Adhesions may also block the movement of either sperm or fertilized ova to the site of fertilization or implantation, respectively. Infertility often results; however, if pregnancy is achieved in females with AS, there is an increased risk of abnormal placentation and pregnancy loss as the fibrous synechiae

impair normal gestational development[3]. In addition to infertility, the consequences of AS include recurrent pregnancy loss, and in pregnant women, an increased risk of preterm premature rupture of membranes, placental abruption, and fetal malpresentation.

There are multiple etiologies for AS. Asherman's Syndrome occurs most commonly after uterine instrumentation. Dilation and curettage is the most frequently implicated procedure as vigorous scraping can lead to removal or damage of the deeper basal layer of the endometrium, where stem cells reside. Women are at particularly high risk in the post-partum period when the endometrium is relatively thin and estrogen levels are low. Indeed, curettage for retained products of conception and post-partum hemorrhage are frequent mechanisms for the development of AS[3]. Other etiologies of AS include severe infection of the endometrium, particularly by *Mycobacterium tuberculosis,* and radiation exposure in the setting of oncologic treatment[4].

Precursors of this disease are not uncommon. One study found the presence of intrauterine adhesions in 10% of women after one episode of curettage, and in 30% of women with two or more curettage events. It has been estimated that 4.6% of infertile females are affected by AS[3].

Several different modalities may be used for the diagnosis of AS. Hysterosalpingography can detect synechiae as filling defects when contrast is

introduced into the uterine cavity. This method has a high false positive rate as air bubbles and debris may be misinterpreted as intrauterine adhesions. Another method of diagnosis is transvaginal ultrasound. One hallmark seen on ultrasound is a thin uterine lining, but this is a nonspecific finding. Visualization of synechiae can be enhanced using saline to expand the uterine cavity, providing further contrast between the echogenic adhesions and anechoic fluid. Interpretation of sonographic testing is limited by the skill of the sonographer and the patient's anatomy. Magnetic resonance imaging can be useful in cases of complete obstruction of the internal cervical os by adhesions, precluding other methods of diagnosis. The gold standard of diagnosis in AS is hysteroscopy where adhesions can be directly visualized and, if appropriate, intervened upon surgically[5].

Hysteroscopy is often required for clinical staging of AS. Although many systems of AS classification exist, none are universally implemented. The European Society of Hysteroscopy released its classification of intrauterine adhesions in 1989. This system consists of four separate grades. Grade I AS involves only thin, filmy adhesions, with no disruption of the cornual regions. Grade II disease is defined by the presence of a singular firm adhesion, which cannot be ruptured by the hysteroscope alone, but does not obstruct visualization of both tubal ostia. Grade III AS involves multiple firm adhesions, with unilateral obliteration of the tubal ostia. Grade IV is reserved for extensive disease with coaptation of the uterine walls or blockage of both tubal ostia[6].

A more recent system was created by Donnez and Nisolle in 1994. This system includes three degrees of classification of intrauterine adhesions based primarily on adhesion location. Degree I is designated for cases with only centrally located adhesions. I(a) denotes thin filmy adhesions, in contrast to I(b) adhesions which are myofibrous in character. Degree II intrauterine adhesions are marginal in location and subdivided into II(a): wedge-like projections and II(b): adhesions with blockage of a uterine horn. Degree III is noted for cases where the uterine cavity is absent on hysterosalpingography due to either occlusion of the internal os, III(a), or extensive coaptation throughout the lumen, III(b)[6].

Limited treatment options exist for AS. Hysteroscopic adhesiolysis is the standard for treatment and involves direct visualization and removal of synechiae using a hysteroscope via blunt dissection, surgical scissors, or thermal energy. Some providers supplement this intervention with post operative placement of an intra-uterine balloon and one month of oral estrogen supplementation to help promote endometrial healing, facilitate regeneration, and to prevent reformation of adhesions. Despite these interventions, recurrence rates of synechiae can be as high as 41.9% in high grade disease, necessitating second, third, or even fourth look hysteroscopic procedures. Fertility outcomes after this procedure are remarkably poor. A systematic review in 2018 found a pregnancy rate of only 50.4% after surgical treatment of Asherman's Syndrome[7]. Often, women with recurrent AS must resort to surrogacy or adoption in order to have a child.

Recently, there has been increasing interest in bone marrow derived stem cells (BMDSC) as a potential therapeutic approach to AS. Engraftment of stem cells to the uterus has been shown incidentally in uterine biopsies of patients receiving allogenic bone marrow transplants for leukemia[8]. Mouse studies have replicated this phenomenon of uterine engraftment after bone marrow transplant and intravenous injection of bone marrow derived stem cells[9]. Bone marrow-derived stem give rise to various non-hematopoietic endometrial cells including epithelial, stromal and endothelial cells, suggesting that BMDSCs may serve as a source of progenitor cells for endometrial regeneration.

Clinically, therapeutic injury to the endometrium has been used in an attempt to improve implantation success in women with infertility resulting from endometrial pathology. Indeed, it has been shown that ischemia/ reperfusion injury causes a stimulus for homing and engraftment of BMDSCs into the uterus. This may represent one mechanism by which therapeutic injury to the uterus improves endometrial receptivity. Studies have also shown that administration of bone marrow after uterine injury (Asherman's Syndrome induction surgery) improves endometrial function and increases the rate of successful pregnancy. Similarly introducing stem cell chemoattractant in a mouse model of Asherman's Syndrome improved uterine recovery and pregnancy rates[9].

BMDSC's are used routinely in the treatment of hematologic malignancies such as lymphomas, leukemias, and multiple myeloma where these transplanted cells replace the patients absent or faulty bone marrow cells[10]. Experimental use of BMDSC's is also

being explored as a means of regenerating articular cartilage in degenerative joint disease and cardiomyocyte function post myocardial infarction[11, 12]. These successes have generated further interest in exploring this treatment modality in AS[13]. Indeed, improved fertility outcomes have been shown in a murine model of AS with tail vein injection of BMDSC's[14].

In patients with AS, there is thought to be insufficient recruitment of one's own native stem cells. Stem cell therapy has been piloted in several studies as a means to improve infertility and menstrual symptoms in patients with AS. Methodologies include mobilization and peripheral collection of stem cells with the mobilizing agent granulocyte colony stimulating factor (G-CSF). Other studies utilized stem cells obtained directly via bone marrow aspiration. Endometrial mesenchymal stem cells demonstrate reduced clonogenic potential in patients experiencing recurrent pregnancy loss. This strongly suggests functional stem cell deficiency in these patients. Thus, supplying bone marrow derived stem cells to the endometrium may serve as a means by which to replenish stem cells in the endometrium, and effectively treat AS by allowing for normal endometrial regeneration and replenishment of essential endometrial stem cells.

There have been several case reports demonstrating success with stem cell use in human subjects with AS. In 2011, a 33-year-old woman with AS underwent successful implantation with in vitro fertilization five months after introduction of autologous stem cells into the uterine cavity[15]. A case series in 2014 of six women with severe AS looked at sub-endometrial zone injection of stem cells via ultrasound

guidance. Five out of the six women achieved menstruation after previously being amenorrheic[16]. Santamaria et. al. injected stem cells into the spiral arterioles of eleven women with refractory AS. All eleven subjects experienced an increase in endometrial thickness, three had spontaneous pregnancies, and three achieved pregnancy with embryo transfer[17].

These studies have used apheresis and subsequent injection as a means of introducing stem cells into the damaged endometrium. Peripheral mobilization is another method of harnessing BMDSC's. This involves using pharmacologic agents to release stem cells from the bone marrow into the systemic circulation. Once in the serum, stem cells can home to sites of injury and exact their regenerative effects. This method is more efficient and less invasive as it involves a single administration of a medication and avoids the discomfort and potential complications of bone marrow aspiration and peripheral blood draws.

Historically mobilization was achieved using G-CSF alone. However, a subset of patients fails to mobilize BMDSCs effectively with administration of this singular agent. Mobilization via 1,1'-[1,4-phenylenebis-(methylene)]-bis-1,4,8,11-tetraazacyclotetradecane (AMD3100) was approved in 2008 by the FDA. Utilization of AMD3100 and GCSF for mobilization of stem cells for transplantation results in successful engraftment in nearly all patients. In one trial utilizing healthy donors, a single dose was effective in 75% of individuals. Common side effects include erythema or stinging at the injection site, peri-oral paresthesia, nausea, diarrhea, and headache[18,]

[19]. No significant changes in serum values of electrolytes, blood urea nitrogen, creatinine, liver function markers, bilirubin, or alkaline phosphatase has been reported with this agent. AMD3100's dosing is weight based, and one subcutaneous administration, on average, allows for mobilization of 2 x10^6 stem cells[20].

AMD3100 functions by antagonizing interactions between the C-X-C Motif Chemokine Receptor 4 (CXCR4) and the chemokine Stromal Cell-Derived Factor-1 (SDF-1), also known as C-X-C Motif Chemokine Ligand 12 (CXCL12). The CXCR4 receptor is located on BMDSC's and binds CXCL12 via powerful disulfide bones, which are responsible for securing the cells' location within the bone marrow[21]. By disrupting these bonds, AMD3100 allows BMDSC's to travel through the systemic vasculature and then enter inflamed tissues. Currently, AMD3100 is used to augment peripheral stem cell collection in patients who are poor mobilizers.

The benefits of AMD3100 use without peripheral cell collection have been postulated in several disease states[22]. In Asherman's Syndrome specifically, this agent could be used after hysteroscopic lysis of intrauterine adhesions to facilitate proper healing and reduce the risk of synechiae recurrence. Yet, the ideal timing for administration of AMD3100 for this purpose is unknown.

Recently, it has been shown that CXCL12 is one of the predominate mediators of stem cell engraftment to the uterus after injury[23]. Stem cells follow a gradient of CXCL12 released from inflamed tissues to ultimately home to sites of damage. It has also been shown that daily administration of AMD3100 can block engraftment of stem

cell to the uterus, by precluding the formation of interactions necessary to stabilize residence in the uterine stroma. To optimize the restorative effects of AMD3100, its peak mobilizing effects should coincide temporally with peak concentrations of CXCL12 located in the target organ. Furthermore, consideration must be paid to its half-life, allowing adequate time for this medication to be processed and excreted, thus permitting formation of the new receptor interactions necessary for proper engraftment at sites of injury. In contrast, if AMD3100's antagonistic effects on the CXCR4 receptor remain throughout a target organs receptive window, recruitment may never occur, and optimal healing may not be achieved.

AMD3100 has a half-life of twenty minutes and a terminal half-life between three and five hours. Peak mobilization of BMDSC's with AMD3100 occurs six to nine hours after administration in human subjects[22]. Post injury CXCL12 levels have been studied in various tissues as well. One study found a peak in CXCL12 concentration in the cardiomyocytes of rats one hour after myocardial infarction[24]. Peak concentrations in mouse kidney occurs ninety minutes after ischemia-reperfusion injury[25]. CXCL12 concentrations in mouse femur peaks three days after fracture[26]. To date, there are no studies analyzing peak CXCL12 levels in the uterus after injury. Thus, I hypothesize that systemic delivery of BMDSCs to the uteri of mice with surgically induced Asherman's Syndrome through the use of AMD3100 would have a beneficial effect on the endometrium—subsequently restoring fertility— and is dependent on timing of peak CXCL12 levels in the uteri.

Statement of Purpose

1. Optimize a murine model of Asherman's Syndrome.

2. Assess fertility outcomes in mild Asherman's Syndrome model mice treated with AMD3100.

3. Determine timing of peak serum and uterine levels of CXCL12 after AS induction.

4. Identify an optimal time point for AMD3100 administration in AS.

Materials and Methods

Student Contributions: Surgeries were taught by Dr. Flores and performed by the author. Statistical analysis was performed under the guidance of Dr. Flores. Mouse blood collection was performed by Ramanaiah Mamillapalli, Ph.D. Protein extraction reagents were mixed and aliquoted by George Zhou. All other tasks were performed by the author.

Ethics statement: This research was performed with the utmost consideration toward academic integrity and ethical treatment of research animals.

Mice

C57BL/6 wild-type mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed and maintained (four to five per cage) in a room (21 ± 1°C) with a 12-h light/dark cycle (7:00 a.m. to 7:00 p.m.) with ad libitum access to food and water, in the Yale Animal Resources Center (YARC) at Yale School of Medicine. All animal experiments were conducted in accordance with an approval protocol from the Institutional Animal Care and Use Committee (IACUC) appointed by Yale University[22]. All experiments were performed on mice eight to twelve weeks of age.

Determination of the estrus phase was essential for ensuring consistency in endometrial susceptibility to surgical injury and assessing timed-pregnancy measures. The estrous cycle refers to the reproductive cycle in rodents, analogous to the menstrual cycle in humans. The estrus cycle consists of four phases, proestrus, estrus, metestrus and diestrus. The cycle in its lasts 4 to 5 days in its entirety. The reproductive period and estrous cycle of mice commences around day 26 of life and aligns with the opening of the vagina. Vaginal secretions include three uniquely identifiable cell types and vary with estrus phase. These cell types are leukocytes, cornified epithelial cells and nucleated epithelial cells. Determination of estrus phase is most commonly based on the proportion of these cell types in aspirates of the vaginal secretions[27].

A 100μL pipette was filled with 40μL of normal saline. The pipette was placed within the vaginal opening. The vaginal canal was washed and aspirated with the normal saline four times sequentially. The final aspirate was dispensed on a slide and observed under a light microscope. The diestrus phase was identified as having numerous leukocytes and an absence of epithelial cells[23]. Surgeries were exclusively performed on mice in the diestrus phase of the cycle. Mice that were found to be in proestrus, estrus, or metestrus phase were resampled after twenty-four hours.

Asherman's Syndrome Mouse Model- Mild

Induction of the mild variant of the murine Asherman's Syndrome model is based on a previously validated technique[14]. AS induction is only performed on mice in

the diestrus stage of the estrus cycle. Mice were anesthetized using a mixture of isoflurane at 2.5 L/min and oxygen at 3.0 L/min, and subsequently placed supine on a sterile operative field. Maintenance anesthesia was provided via the same mixture delivered by a specially designed rostral tube. A vertical incision was made in the skin three millimeters above the vaginal margin and extended one centimeter cranially. After careful dissection of the peritoneum from the skin and abdominal musculature, an identical incision was made in the peritoneal membrane. The visceral fat and bladder were mobilized allowing for identification of the left and right uterine horns. A one centimeter, twenty-seven gauge needle was inserted into the lumen of the uterine horn just proximal to its junction with the uterine corpus. The needle was rotated within the lumen of the horn while simultaneously being withdrawn, causing scraping of the needle's bevel along the length of the endometrial surface. This procedure was repeated four times per horn. The visceral tissues were replaced within the peritoneal cavity. The peritoneum was sutured in a running fashion with 5.0 vicryl suture. The abdominal wall was sutured in a running, locked fashion with 4.0 vicryl suture. Finally, the mouse was given a unique ear tag for identification purposes then transferred to a warmed recovery chamber.

AMD3100 Trial- Mild AS

Mice were divided into three experimental groups: subcutaneous injection of 10mg/kg of AMD3100 eight hours before AS induction (n=7), at time of AS induction (n=7), or seventy-two hours after AS induction (n=7). A control group received Asherman's Syndrome induction and subcutaneous injection of normal saline at time of surgery (n=7).

Asherman's Syndrome mice were stored, five to a cage, in the YARC for three estrus cycles or twelve days after AS induction to allow for adequate recovery time before mating. Mice were then transferred to a separate cage with a proven male, verified to be fertile, with a ratio of three females to one male. The females were weighed daily to assess for pregnancy. Upon reaching a weight of twenty-eight grams, females were transferred to an individual cage and noted as pregnant. Upon delivery, date of birth, litter size, and number of live pups was recorded. Mice that did not achieve pregnancy within seven estrus cycles (twenty-eight days) were excluded from the statistical analysis.

Optimization of Asherman's Syndrome Induction Technique

I compared several different techniques for inducing Asherman's Syndrome. Mice were divided into one of four experimental groups: AS induction with a twenty-seven gauge needle (n=4), AS induction with a twenty gauge needle (n=5), AS induction with a twenty-seven gauge needle and micro scissor incision (n=5), and AS

induction with a twenty gauge needle and micro scissor incision (n=5). A control group underwent a sham surgery, in which the abdominal wall and peritoneum were incised, the uterine horns were exposed, and the mouse was subsequently closed without direct trauma to the uterine horns (n=4).

After twelve days of post operative recovery, mice were mated with proven males at a ratio of three females to one male. The mice were monitored and weighed in the fashion previously described. Litter size, live birth fraction (number of live born pups divided by litter size), and number of miscarriages were recorded. Upon reaching the weight of 31 grams if a mouse subsequently lost weight without delivering pups, this was designated as a miscarriage.

Asherman's Syndrome Mouse Model- Severe

After determining AS induction with a twenty-gauge needle and micro scissor incision to be the preferred method for severe injury, the protocol was further modified to be more replicable and more uniform in the trauma delivered of the uterine horns.

Mice were anesthetized, incised, and the uterine horns identified in the previously described fashion. Using micro scissors, an incision just large enough to accommodate the blade of the micro scissors was made immediately proximal to the

junction of the uterine horn and uterine corpus. Then a one centimeter, twenty-gauge needle was inserted into the lumen and passed along its length up to the terminus of the horn. The bevel of the needle was pressed against the anterior wall of the cavity as the needle was slowly withdrawn from the horn, scraping the entire length of the lumen. The needle was once again inserted and withdrawn, and this procedure was repeated for a total of ten passes. The needle was then rotated ninety degrees along its short axis, such that its bevel faced the medial wall of the uterine horn cavity. Ten passes were completed once again in this orientation. This was repeated for the posterior and lateral walls of the uterine horn cavity as well. An identical procedure was performed on the opposite horn. The mouse was then closed in the fashion previously described and transferred to a recovery chamber.

Determination of CXCL12 Levels in Blood and Serum

Mice underwent severe Asherman's Syndrome induction and were divided into four groups: blood draw and uterine collection twelve hours after induction surgery (n=3), blood draw and uterine collection twenty-four hours after induction surgery (n=3), blood draw and uterine collection forty-eight hours after induction surgery (n=3), and blood draw and uterine collection seventy-two hours after induction surgery (n=3). A control group was established for each timepoint by performing the sham surgery previously described (n=3 per time point).

Blood Collection

Blood was collected through retro orbital apheresis and cardiac puncture. The end of a 100μL curette was pressed firmly against the mouse's orbit and twisted against its inferior margin to collect blood from the venous sinus. Blood was transferred from the curette to a 1.5mL Eppendorf tube. This procedure was then repeated on the opposite side. Next, a twenty-seven gauge needle was inserted through the ventral chest wall to puncture the heart. Blood was aspirated from the cardiac chambers and added to the Eppendorf tube containing the previously collected blood. The sample was then centrifuged at 12,000 relative centrifugal force for 30 minutes. The resultant supernatant was collected and stored in Eppendorf tubes at -80°C.

Uterine Collection and Processing

Immediately after blood collection, mice were euthanized via carbon dioxide inhalation with cervical dislocation and uterine samples were collected. The abdominal wall and peritoneum were incised vertically, and uterine horns identified. Peritoneal fat was stripped from the uterine horns and discarded. The horns were collected by making an incision proximal to their connection with ovary and at the junction of the horn and corpus. The horns were snap frozen using liquid nitrogen and stored in Eppendorf tubes at -80°C.

Protein was extracted from the tissue samples using a standardized protocol. The horns were weighed using an analytical scale and minced using a #21 scalpel blade. The minced samples were then transferred to a round bottom tube containing a mixture of non-denaturing lysis buffer, proteinase inhibitor cocktail (Sigma-Aldrich), and phenylmethylsulfonyl fluoride. The samples were then homogenized for 15 seconds three times. Samples were allowed to cool on ice for 15 seconds in between homogenization events. The samples were then aliquoted into Eppendorf tubes and sonicated for 10 seconds three times. The samples were allowed to cool on ice for 15 seconds in between sonication events. The tubes were centrifuged at 12,000 relative centrifugal force for 30 minutes. The resultant supernatant was collected and used for further analysis.

Measurement of CXCL12 levels

CXCL12 levels in the serum and uterine horns were determined using a Quantikine ELISA kit (R&D Systems). The kit protocol was followed without deviations. Optical densities were measured at 450nm on an iMark microplate reader (BIO RAD).

The Quantikine ELISA kit was validated on homogenized tissue by performing standardized curves with the kit-provided calibrator diluent and the previously described non-denaturing lysis buffer mixture. The curves were analyzed and found to

be identical. This indicated that the reagents used to extract protein from the samples did not interfere with the ELISA kit's reagents, and the resulting optical densities were reliable. The calibrator diluent was replaced with the lysis buffer mixture, otherwise the kit protocol was followed without deviations. Optical densities were measured at 450nm on an iMark microplate reader (BIO RAD).

Statistical Analysis

Student's t-test and Kaplan-Meier survival curve were used for statistical analysis, and a p value of ≤ 0.05 was considered significant. Comparisons were made between treatment mice and controls. All error bars represent standard error of the mean.

Results

Mild Asherman's Syndrome Fertility Outcomes

Administration of AMD3100 three days after surgery was associated with a significant decrease in time to pregnancy by 7 estrus cycles compared to normal saline injection: 15.0 days (SEM=1.4) vs. 20.7 (SEM=2.0) days, (p=0.03) (Figure 1). Administration of AMD3100 at time of surgery and eight hours before surgery were

both associated with a nonsignificant decrease in time to pregnancy compared to normal saline injection: 17.8 days and 18 days, respectively (Figure 1). The likelihood of pregnancy by 7 estrus cycles was higher in the 3 days post-surgery group compared to the control group (p=0.004) (Figure 2). All 21 treatment mice delivered by 13 estrus cycles. 1 of the 7 control mice never gave birth despite reaching a maximal weight of 31 grams.

There was no significant difference in average litter size between the groups: 7.5 (± 1.9) pups in the control group, 7.4 (± 1.9) pups in the eight hours pre surgery group, 7.0 (± 1.4) pups in the time of surgery group, and 7.7 (± 1.8) pups in the three days post surgery group (Figure 3). There was no significant difference in percentage of live pups at birth between the groups: 76 (± 30) % in the control group, 83 (± 35) % in the eight hours pre surgery group, 83 (± 18) % in the time of surgery group, and 86 (± 22) % in the three days post surgery group (Figure 4).

Asherman's Induction Technique Comparison

Mice underwent Asherman's Syndrome induction via one of four techniques: use of a twenty-seven gauge needle (n=4), a twenty gauge needle (n=5), a twenty-seven gauge needle and micro scissor incision (n=5), or a twenty gauge needle and micro scissor incision (n=5). A control group underwent sham surgery with no direct uterine trauma (n=4).

The 20G with incision group experienced significantly longer time to delivery compared to the control group, 46.5±13.7 days vs. 28.0±5.6 days (p=0.045) (Figure 5). Time to delivery for the 27G needle group, 20G needle group, and 27G needle with incision group were 25.8±4.0, 35.8±17.0, and 27.2±8.6, respectively (Figure 5). One mouse in both the 20G needle and 20G needle with incision groups never delivered.

The incision groups both had significantly lower litter sizes than the control group with 5.8±1.3 pups (p=0.011) for the 27G needle with incision group and 3.5±0.5 pups (p=0.0007) for the 20G needle with incision group (Figure 6). The litter sizes for the 27G needle group, 20G needle group, and control group were 7.3±2.2, 7.8±2.3, and 8.5±0.5 pups respectively (Figure 6). The 20G needle with incision group had a live birth fraction (number of live pups divided by total litter size) of 0. The live birth fraction of the 27G needle group, 20G needle group, 27G needle with incision group, and control group were 0.32±0.41, 0.80±0.23, 0.53±0.41, and 0.93±0.11, respectively (Figure 6). Two mice in both the 20G needle and 27G needle with incision groups experienced miscarriages. The 20G needle group experienced 0.4±0.5 miscarriages per mouse. The 20G needle with incision group experienced 0.6±0.8 miscarriages per mouse (Figure 6).

Serum CXCL12 Concentrations

Serum CXCL12 concentrations were determined using ELISA at four different time points after surgery: twelve hours post surgery, twenty-four hours post surgery, forty-eight hours post surgery, and seventy-two hours post surgery (n=3/time point). The serum CXCL12 concentrations for the sham surgery were 6.72 ($\pm$ 5.05) ng/mL twelve hours post surgery, 6.88 ($\pm$ 1.66) ng/mL twenty-four hours post surgery, 10.34 ($\pm$ 2.72) ng/mL forty-eight hours post surgery, and 9.07 ($\pm$ 1.29) ng/mL seventy-hours post surgery (Firgure 7). The serum CXCL12 concentrations for the treatment surgery were 6.26 ($\pm$ 2.99) ng/mL twelve hours post surgery, 8.97 ($\pm$ 1.01) ng/mL twenty-four hours post surgery, 7.39 ($\pm$ 1.46) ng/mL forty-eight hours post surgery, and 6.43 ($\pm$ 0.18) ng/mL seventy-two hours post surgery (Firgure 7). There were no signifcant differences in serum CXCL12 levels between AS induction mice compared to controls mice.

Uterine CXCL12 Concentrations

Tissue CXCL12 concentrations in the uterus were determined using ELISA at four different time points after surgery: twelve hours post surgery, twenty-four hours post surgery, forty-eight hours post surgery, and seventy-two hours post surgery. The uterine CXCL12 concentrations for the sham surgery were 0.20 ($\pm$ 0.12) ng/mL twelve hours post surgery, 0.36 ($\pm$ 0.17) ng/mL twenty-four hours post surgery, 0.27 ($\pm$ 0.09)

ng/mL forty-eight hours post surgery, and 0.36 (± 0.03) ng/mL seventy-two hours post

surgery (Firgure 8). The uterine CXCL12 concentrations for the treatment surgery were

0.23 (± 0.08) ng/mL twelve hours post surgery, 0.36 (± 0.08) ng/mL twenty-four hours

post surgery, 0.58 (± 0.10) ng/mL forty-eight hours post surgery, and 0.16 (± 0.12)

ng/mL seventy-two hours post surgery (Figure 8). Uterine CXCL12 concentrations

were significantly increased in the treatment group forty-eight hours post surgery

compared to the control group (p=0.03) (Figure 8).

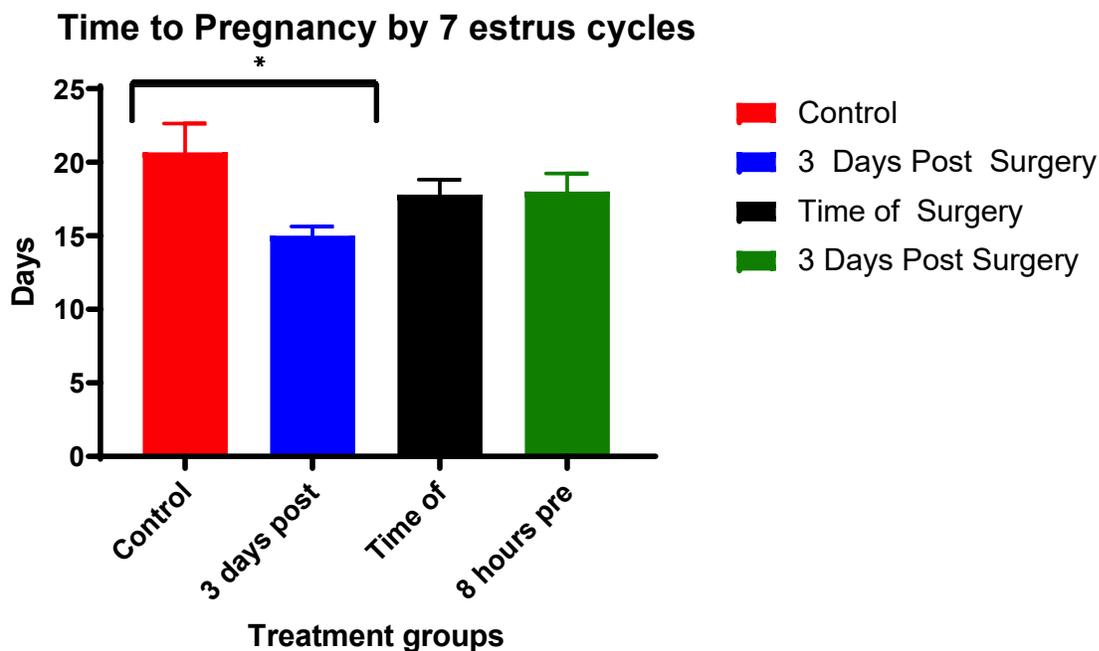## Time to Pregnancy by 7 estrus cycles

Figure 1: AMD3100 Trial- Time to Pregnancy Bar Chart: Mice underwent mild AS induction surgery and given a 10mg/kg subcutaneous AMD3100 injection eight hours before surgery (n=7), at time of surgery (n=7), or three days post-surgery (n=7). The control group underwent AS induction and received a subcutaneous normal saline injection at time of surgery (n=7). Mice were subsequently mated and followed for time to pregnancy for seven estrus cycles. The three-day postoperative injection group had a significantly lower time to pregnancy compared to the control group: 15.0 days vs. 20.7 days, *p=0.03.
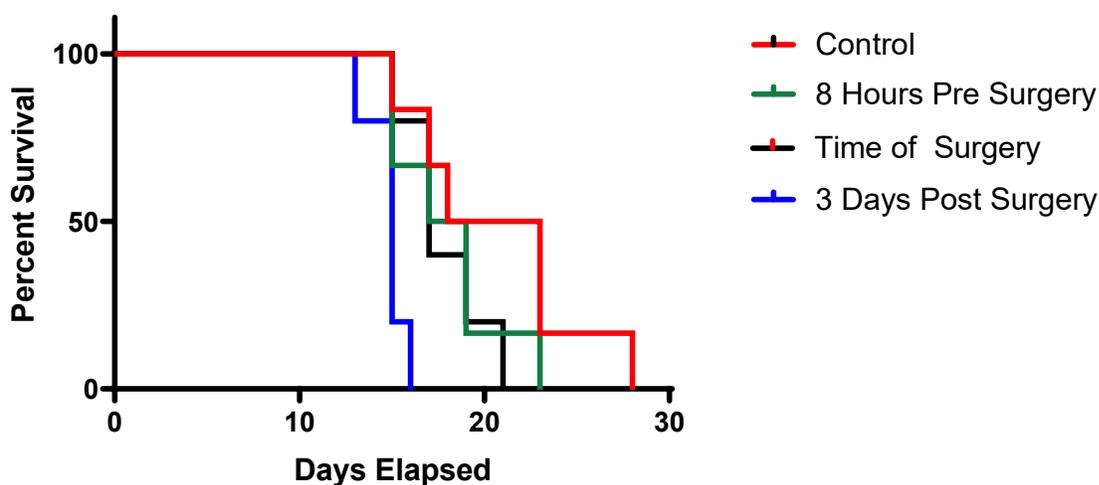

Figure 2: AMD3100 Trial- Time to Pregnancy Kaplan–Meier Curve: Fertility data from the AMD3100 trial is represented here as a Kaplan-Meier Curve. The three-day postoperative group achieved pregnancy significantly sooner than the control group (p=0.004).
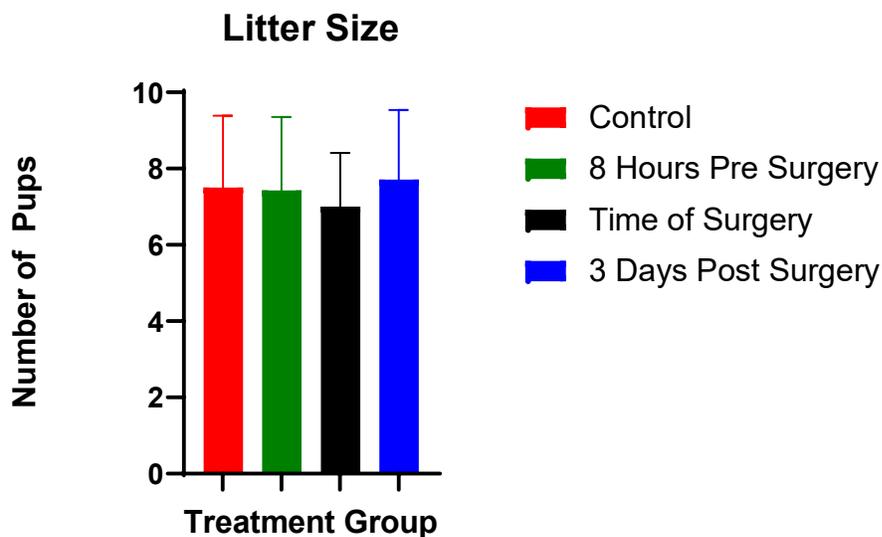
Litter Size

Figure 3: AMD3100 Trial- Litter Size: Litter size was recorded for all four groups. While litter sizes varied among the groups (range from 4 to 10 pups), there was no significant difference in litter size between the groups.
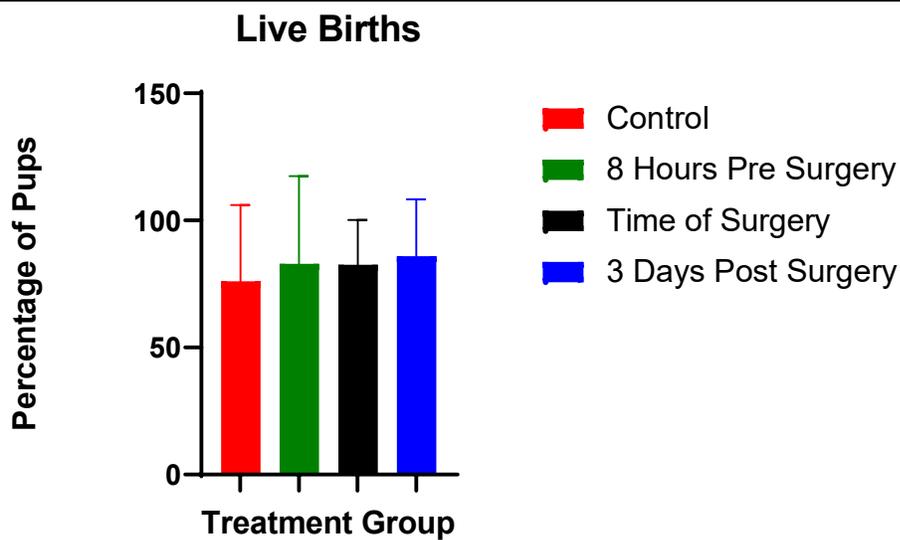
Live Births

Figure 4: AMD3100 Trial- Live Births: The number of live pups was recorded for all four groups. The percentage of pups that were alive immediately postpartum is shown for each group. There was no significant difference in percentage of live pups between the groups.
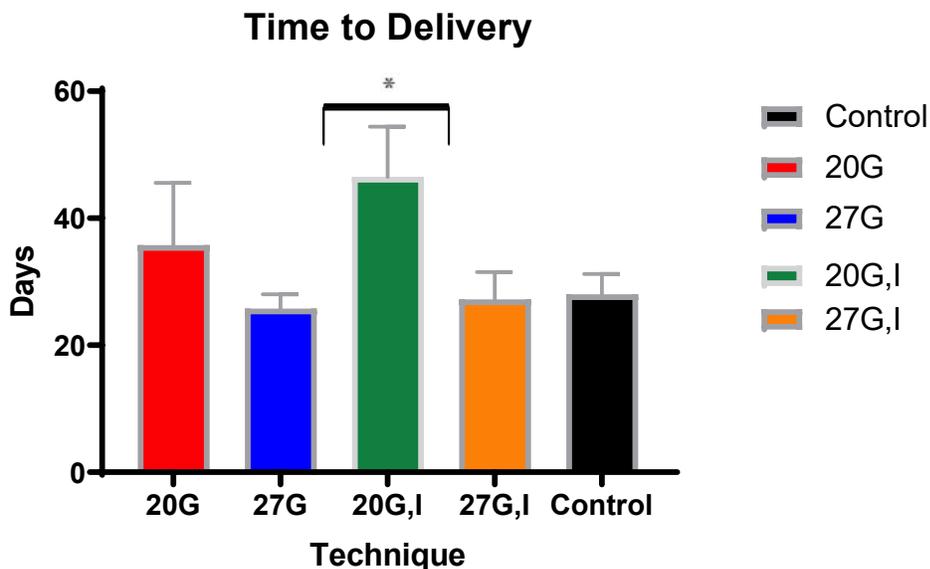
Time to Delivery

Figure 5: Technique Comparison- Delivery Time: Mice underwent Asherman's Syndrome induction via one of four techniques: 20G needle (n=4), 27G needle (n=5), 20G needle with incision (n=5), or 27G needle with incision (n=5). Control mice underwent sham surgery without uterine trauma. The 20G needle with incision group experienced significantly increased time to delivery compared to the control group: 46.5 ± 13.7 vs. 28.0 ± 5.6 days (*p=0.045).
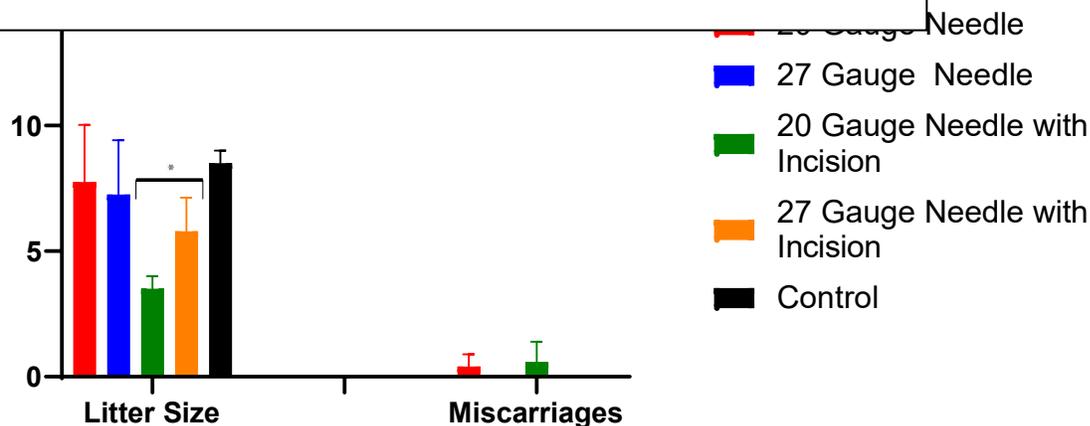

Figure 6: Technique Comparison- Pregnancy Outcomes: Mean litter size and miscarriages were recorded for each group. The 20G needle with incision and 27G needle with incision groups had significantly smaller litter sizes compared to the control group: 3.5 ± 0.5 pups (p=0.0007) and 5.8 ± 1.3 pups (p=0.011), respectively. Only the 20G needle and 20G needle with incision groups experienced miscarriages. The 20G needle with incision group had the smallest mean litter size and most miscarriages.
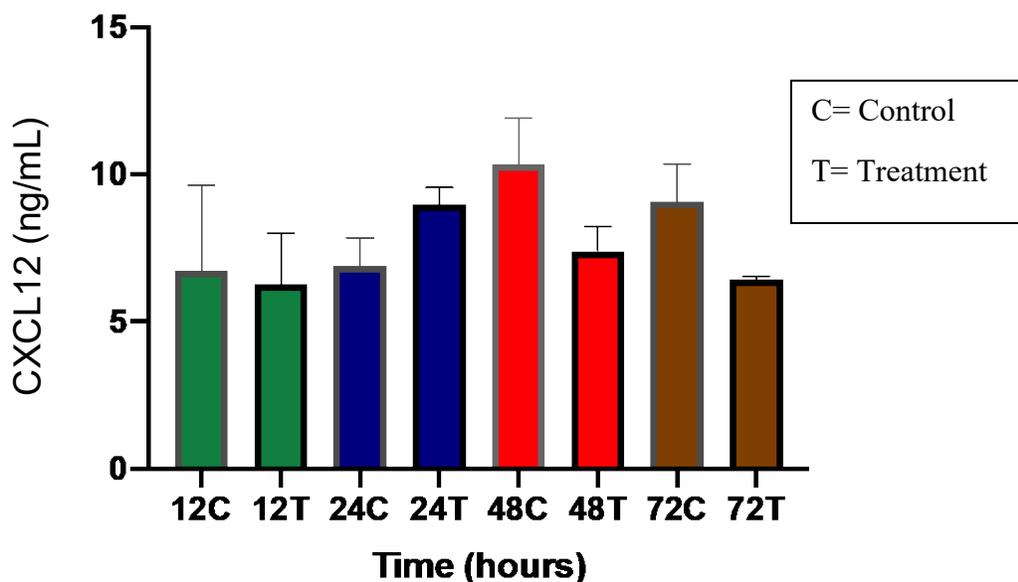
AS & Sham Post- Surgery Serum Time Points

Figure 7: Serum Concentration CXCL12: Relative serum concentrations of CXCL12 for four different time points after severe Asherman's Syndrome induction or sham surgery. There was no significant difference in serum CXCL12 levels between treatment and control mice. n=3 for each time point.
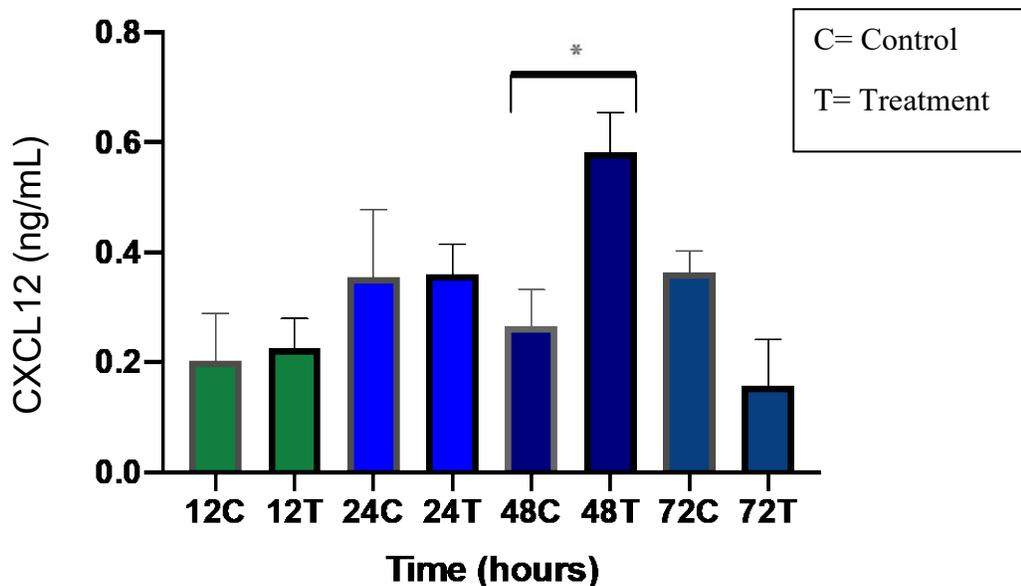
AS & Sham Post- Surgery Uterine Time Points

Figure 8: Relative Uterine CXCL12 Concentration: Relative uterine concentrations of CXCL12 for four different time points after severe Asherman's Syndrome induction or sham surgery. A statistically significant peak of 0.58 ng/mL occurred at 48 hours in the severe AS mice compared to the sham group (*p=0.03)

Discussion

 I set out to observe the effects of AMD3100 administration on fertility outcomes in a murine model of AS. Subsequently, I developed a technique for induction of a more severe AS phenotype that better recapitulates what occurs in women struggling with infertility resulting from AS. Furthermore, I determined for the first time the levels of CXCL12 in serum and uteri following injury, which allowed for the determination of the optimal timing for AMD3100 administration to help restore normally functioning endometrium following induction of AS.

Mild Asherman's Syndrome Fertility Outcomes

 The trial in the mild AS model showed that AMD3100 injection three days post induction surgery yielded the fastest resumption of fertility. This suggests that stem cells were best mobilized and able to enact their regenerative effects on the uterus at this timepoint. This may be due to the timing of inflammation within the uterine tissue. Injury caused by trauma releases reactive cytokines which facilitate tissue healing as well as recruitment of immune cells. One cytokine of particular importance is CXCL12/SDF-1 which has been shown to attract bone marrow derived stem cells[23].

 There was no significant difference in litter size or percentage of live pups in the litter between the four groups. This suggests that the effects of stem cell mobilization

between the different time points, while having a significant effect on the rate of

regeneration of the uterus, did not have an observable effect on its ability to ultimately

provide a suitable environment for implantation and development of murine pups. This

may indicate that the mild model did not induce trauma severe enough to manifest

changes in these outcomes. It must be noted that the cohort size, n=28, may not be

large enough to detect differences in these parameters.

Although litter size was recorded, no data was recorded on pup birth weight. It

is possible that scar tissue formation would affect nutrient delivery in utero, and

ultimately pup health and weight. A subsequent experiment could be performed where

pups are weighed at or near time of birth, and their average mass compared between

mice receiving AMD3100 after AS induction and mice receiving normal saline.

After 7 estrus cycles, all but one mouse became pregnant. As such, it was felt

that the mild AS model was not the best method to recapitulate AS in humans.

Therefore, I decided to conduct additional experiments aimed at finding a severity of

AS induction that better represented the condition in humans.

Optimization of a Murine Model of Asherman's Syndrome

Four different techniques were tested for inducing Asherman's Syndrome in mice. Micro scissor incision at the junction of the uterine horn and uterine corpus resulted in significantly smaller litter sizes as previously described[23]. Use of a larger, 20G needle resulted in miscarriages, where use of a 27G needle did not, suggesting severity of injury is correlated with the diameter of the needle. The 20G with incision technique led to the lowest litter size, no live births, most miscarriages, and longest time to delivery. This disease state most closely mimics patients affected by severe Asherman's Syndrome, the population most in need of new treatment modalities.

The severe Asherman's Syndrome technique described above has the benefit of uniformly traumatizing the lumen of the uterine horns by means that can be easily replicated. This model may be used to further study Asherman's Syndrome, local uterine inflammation, and the effects of stem cell mobilization with AMD3100. Our trial of AMD3100 use in mild AS showed post operative AMD3100 injection to be superior to pre operative or time of surgery administration, however, as I continued to follow mice pregnancies after 7 estrus cycles, I noted that most mice became pregnant. As such I developed a more severe AS model and aimed to determine the ideal timepoint for AMD 3100 administration in this model by utilizing CXCL12 measurements as a maker for peak stem cell mobilization.

CXCL12 Concentrations

There was no significant difference in serum CXCL12 concentrations between the different time points. CXCL12 concentrations while physiologically consequential in areas of local inflammation, may not cause drastic variations in cell function in the systemic circulation. BMDSC's follow a gradient of CXCL12 chemotactically to reach target organs[28]. Perhaps it is the differential in chemokine concentration at the injury site compared to the circulation that leads to homing and engraftment of regenerative cell types. If this was the case, the average serum concentration of such chemokines would be inconsequential, and instead the concentration at the capillary and tissue level that drives wound healing via stem cell engraftment. To explore this idea, I measured CXCL12 concentrations within uterine samples of severe Asherman's Syndrome model mice post operatively.

There was a statistically significant peak in uterine CXCL12 concentrations in severe AS model mice forty-eight hours post surgery. This peak was significantly higher than the concentration measured in sham mice. This supports the idea that direct trauma to tissues, for example the uterus, facilitates the transcription of chemokines like CXCL12, which play an invaluable role in stem cell recruitment. The difference in uterine CXCL12 concentrations between sham and AS induction surgery gives further insight into past observations that local inflammation increases stem cell engraftment to the uterus[13].

The data also shows a clear pattern of increasing uterine CXCL12 concentrations in the first forty-eight hours after surgery before falling off abruptly at seventy-two hours. This suggests there is an optimal window for stem cell mobilization with AMD3100. It is still unclear if the true peak lies at forty-eight hours, between twenty-four and forty-eight hours, or between forty-eight and seventy-two hours. Further experiments will be needed, ideally with a larger cohort, to elucidate a more precise timing of peak CXCL12 concentration in the healing uterus.

Forty-eight hours after surgery may represents a state of peak inflammation in the healing uterus. Theoretically this translates to maximum intensity in stem cell recruitment as BMDSC's passing through the capillaries perfusing the endometrium are drawn to leave the circulation and enter the injured tissues. Once in the endometrium, these cells can assist with tissue regeneration by releasing trophic factors and recruiting additional immune cells[29].

Mouse uterine samples were diluted using a lysis buffer solution and no effort was made to compare the measured CXCL12 concentration to the original dry protein weights of the samples. Thus, it should be noted that the concentrations measured in the uterus in the above experiment can only communicate relative peaks in CXCL12 values rather than the absolute tissue concentrations after surgery.

Timing of AMD3100 Injection

The fertility outcomes suggest that post surgical injection of AMD3100 is superior to administration at time of surgery or in the preoperative period. As indicated by tissue CXCL12 concentrations, the uterus is in a particularly inflammatory state and signaling for stem cell recruitment is at its peak forty-eight hours post surgery. To ensure maximal availability of BMDSC's in the peripheral circulation to act upon this signal, an appropriate dose of AMD3100 would need to be administered six to nine hours before this target inflammatory state. This translates to an ideal administration time of thirty-nine to forty-two hours after surgical intervention to facilitate maximal healing and restoration of reproductive function. Given that peak uterine CXCL12 concentrations could lie several hours before or after the forty-eight hour peak observed in the above experiment, a goal of AMD3100 administration between thirty-three and forty-eight hours after surgery would be reasonable.

Special Considerations of Research During the COVID-19 Pandemic

The COVID-19 pandemic has caused disruptions in virtually all global sectors including telecommunications, information technology, finance, and healthcare. Scientific research was not unaffected by this historic world event.

Conducting scientific research during a pandemic creates its own list of unique consequences and inconveniences. For instance, the typical channels for the exchange of information and ideas were not operating in their usual fashion. Laboratory meetings were temporarily canceled before being moved to an entirely virtual platform. This has

the effect of stifling communication between laboratory team members, an essential component of scientific discussion and inquiry.

The pandemic also had the effect of delaying the acquisition of certain essential research supplies. This caused deviations in the planned timeline of experimentation and created further difficulty in coordinating joint tasks between research members. While certain reagents and equipment are kept in excess within the lab space, others have a definitive shelf life making timely access to additional quantities essential to their successful use.

Overall, the pandemic created an air of uncertainty and tumultuousness which complicated the research process. Ultimately, through the consistent support of research mentors and colleagues, I was able to complete this project despite these challenges and without compromising the scientific quality or academic integrity of my work.

Future Directions and Next Steps

Asherman's Syndrome is a rare disease state characterized by intrauterine synechiae which can cause life changing reproductive consequences, most notably infertility. Standard of care treatments for Asherman's Syndrome result in high recurrence rates and low probabilities of successful pregnancy. Stem cell technologies are a promising avenue for augmenting current treatment methods. AMD3100, in particular, has the unique quality of offering measurable improvements in fertility outcomes with a single-dose injection.

Stem cell mobilization can ideally be used in conjunction with surgical treatment of AS to prevent recurrence of intrauterine adhesions and facilitate restoration of normal endometrial function. This would reduce the need for repeat hysteroscopies, a common occurrence in this disease state, and increase the probability of successful implantation for individuals seeking future pregnancy. A trial of AMD3100 in human subjects is an essential next step is exploring this therapeutic option. Overall, this data is promising and may represent a novel treatment option in women suffering from infertility due to Asherman's Syndrome.

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