University of Alberta

DEVELOPMENT OF A SIROLIMUS-ELUTING MESH TO REDUCE INTRA-ABDOMINAL ADHESIONS

by

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Dedication

This thesis is dedicated to my parents, whom I thank for their sacrifices, support, and love.

Abstract

Postoperative intra-abdominal adhesions are a significant clinical problem. This project addresses the potential novel role for a sirolimus-eluting hydrogel in the setting of postoperative adhesions caused by polypropylene hernia repair mesh. We review recent literature on adhesion pathogenesis, and examine the progress in development of anti-adhesion agents and strategies. We hypothesize an antiproliferative and antifibrotic agent such as sirolimus may minimize or prevent the process at critical steps in the pathway.

A mouse model was developed using a 1x1 cm^2 polypropylene mesh implanted into the peritoneal cavity. This study revealed that the addition of an agarose hydrogel to the adhesiogenic mesh significantly reduced adhesion incidence, severity and tenacity, and adding sirolimus to the hydrogel further significantly reduced adhesion surface area. Sirolimus has been used in many novel clinical settings for its antifibrotic properties; this work supports its consideration with agarose hydrogel as a prophylactic against postoperative intra-abdominal adhesions.

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LIST OF ABBREVIATIONS

ARD	Adhesion Related Disorder
bFGF	basic fibroblast growth factor
BMI	Body Mass Index
cc	cubic centimeter
CCL1	CC family chemokine ligand 1
CCR8	CC chemokine receptor 8
CD4+	cluster of differentiation 4 positive
FKBP	FK binding protein
GAG	glycosaminoglycan
IL	interleukin
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
NHS	National Health Service
PA	plasminogen activator
PAI	plasminogen activator inhibitor
PG	proteoglycan
PGE_2	Prostaglandin E2
PLGA	polylactic coglycolic acid
SAPL	surface active phospholipid
SCAR	Surgical and Clinical Adhesions Research
sIL-6R	soluble interleukin-6 receptor
SRL	sirolimus
TGF-β	Transforming Growth Factor Beta
TIMP	tissue inhibitor of matrix metalloproteinase
TNF-α	Tumour Necrosis Factor Alpha
tPA	tissue plasminogen activator
UK	United Kingdom
uPA	urokinase-like plasminogen activator
VEGF	Vascular Endothelial Growth Factor

Chapter 1

Understanding Pathogenesis and Developing New Strategies for Prevention of Intra-abdominal Adhesions

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Abstract

Postoperative intra-abdominal adhesions are a complication of healing after tissue trauma, and represent a significant clinical problem. This review summarizes the recent literature on estimates of disease burden and the sequelae of adhesions, such as risk involved in subsequent reoperation, female infertility and chronic pain. The history of our understanding of the development of adhesions is explored, and the pathogenesis of adhesion formation is summarized based on new findings including the role of fibrin and cellular mediators. Given the increasing burden of surgical disease in the world population, and the frequency of reoperation, prevention of adhesion formation will continue to be an important goal in surgical research. Many methods of prophylaxis are discussed in detail, not limited to barrier devices and pharmacological therapy but also summarizing surgeon factors which influence adhesiogenesis.

I: Introduction

Enduring as a surgical footprint, peritoneal adhesions present a challenging problem for surgeons and their patients. They develop following abdominal and pelvic surgery as a natural response to tissue trauma to visceral or parietal peritoneal surfaces. They are despairingly common, and a near certainty on re-operation: in a prospective analysis of 210 patients undergoing a laparotomy with one or more previous abdominal operations, 93% had adhesions, compared with 10.4% in first-time laparotomy patients (1). In a 1993 survey of UK surgeons, it was felt that adhesions and their sequelae represent a large portion of the general surgical workload, though treatment and prevention practice varies widely (2).

The phenomenon of pathological, persistent connections that form between peritoneum and intraperitoneal organs following surgery or other tissue trauma has several terms in the literature: post-operative adhesions, peritoneal adhesions, intra-abdominal adhesions, or simply adhesions. For the purposes of discussion, it is understood that these fibrous connections are not limited to contacts with peritoneal surfaces, but may form between organs as well.

In this review, we examine the morbidity and cost of adhesion-related complications, the spectrum of disease, and review the work that has been done to elucidate the pathophysiology of adhesion development. Methods of prophylaxis against abdominopelvic adhesions will be discussed as well as the development of future techniques and devices.

Estimates of Morbidity

Familiar to any general surgeon, there is a spectrum of morbidity, and mortality, associated with adhesions (**Figure1-1**). Stanciu and Menzies suggested adhesions should be considered to be the most common complication of abdominopelvic surgery, and "for any other disease or operation, with a recognized complication with such a high incidence, a prophylactic therapy or preventive strategy would be recommended if not mandatory." (3) As the incidence of abdominal surgery increases worldwide, so does the incidence of adhesions; as longevity increases, so does the incidence of relaparotomy and relaparoscopy. The issue is only becoming more relevant, however despite this, adhesion-related complications are rarely documented in a patients consent for surgery (4).

The Surgical and Clinical Adhesions Research (SCAR) group was formed in the UK to address the issue, using the Scottish NHS Medical records database, with the main purpose to follow a cohort of abdominal-surgery

patients for a defined period of time. In one retrospective SCAR study, 29,970 patients undergoing open abdominal or pelvic surgery were tracked through this database. In the ten years since their initial surgery, 34.6% were readmitted a mean of 2.1 times for a disorder directly or possibly related to adhesions. Of these readmissions, 22.1% happened within the first year of surgery (5). The SCAR group also examined differences in readmission rates by site of operation, age, concomitant disease (peritonitis, malignancy, Crohn's) and whether patients had surgery in the previous 5 years. They found that lower abdominal surgery carried the highest risk of readmission due to adhesions, with panproctocolectomy (15.4%), ileostomy surgery (10.6%) and total colectomy (8.8%) associated with the worst rates. There was a higher risk of readmission in patients <60 years of age, and in those with concomitant peritonitis at their initial surgery. Crohn's disease and malignancy were not associated with readmission due to adhesions (6).

Adhesive intestinal obstruction

The most serious complication of adhesions is intestinal obstruction, accounting for more than 40% of all cases of obstruction and 60-70% of small bowel obstructions (7). Studying a 25-year period, Menzies and Ellis determined 261 of 28,297 adult general surgical admissions were for intestinal obstruction from adhesions (0.9%), and of 4,502 laparotomies,

148 were for adhesive obstruction (3.3%)(1). In the first cohort study on this topic, laparotomies were followed up for an average of 14.5 months. Of the 2,708 laparotomies, 26 developed intestinal obstruction due to postoperative adhesions within 1 year of surgery (1%) and 14 within one month of surgery (0.5%) (1). This surgical emergency carries a mortality rate of 3-10% for simple obstruction, rising to 30% if bowel becomes necrotic or perforated (7).

A retrospective cohort review based on Medicare records in the US by Beck et al. in 1999 confirmed the SCAR findings of adhesion-related complications as a significant clinical burden. Within two years of surgery, small bowel obstruction was a problem in 14.3% of patients who had a small bowel operation, 17% of colonic operations, 15.3% of rectal operations, and 12.4% of other abdominal operations. Those requiring adhesiolysis for small bowel obstruction in these groups were 2.6%, 3.1%, 5.1% and 2.3% respectively (8).

Reoperation

Any subsequent laparotomy or laparoscopy adds potential risk for the patient. Adhesions between omentum and the anterior abdominal wall often make difficult work of entry into the abdomen, increases the risk of intestinal perforation and fistula formation, and can make safe insertion of

trocars and insufflation impossible. Adhesions surrounding normal anatomy can challenge one's ability to safely remove the pathology in several ways – including preventing adequate surgical exposure and safe mobilization or dissection.

It has been demonstrated that the presence of adhesions significantly increases operative time, not only by preventing access to the peritoneal cavity but also dissection at the site of the pathology (9). Adhesiolysis has well-known risks of bleeding, inadvertent enterotomy and damage to organs such as the liver, spleen, bladder and ureter. Despite this understanding, there is a paucity of literature on this aspect of the topic; perhaps due to underreporting. As Van Goor suggests, "One might speculate that surgeons consider a liver laceration, splenic bleeding or bladder injury caused by adhesiolysis, which are repaired intraoperatively, as an unpleasant but normal part of a reoperation not worthwhile reporting or not even worth informing the patient." (10)

Most studies on adhesions are retrospective in nature. In one such review of 270 reoperations, inadvertent enterotomy occurred in 52 (19%), with independent risk factors including increasing age and having more than three previous laparotomies. Increasing BMI was also associated with enterotomy on univariate analysis. Those patients with enterotomies suffered with more complications postoperatively as well, with significantly

more numbers of urgent relaparotomies, admissions to ICU, and parenteral nutrition use (11). The presence of an enterotomy adds considerable risk to incisional hernia repair, especially when foreign-body mesh material is required to reconstruct the abdominal wall.

Adhesions are a common reason for conversion from laparoscopy to laparotomy, and thereby may result in longer hospital stays and other morbidity associated with conversion.

Female reproductive issues

Adnexal adhesions are a cause of secondary female infertility and dyspareunia. In a 1994 analysis of a US national hospital discharge database by Ray, adhesions were responsible for an estimated 15-20% of female infertility cases (12). They may also be associated with ureteral obstruction and voiding dysfunction (13).

Chronic pain

The relationship between chronic abdominal or pelvic pain and adhesions is controversial. Many patients with chronic abdominal or pelvic pain have adhesions, and adhesions are a common finding in patients without chronic pain, making it difficult to establish a causal relationship.

Randomized trials have shown that adhesiolysis in patients with chronic pain is ineffective, possibly because adhesions are known to reform. It has been proposed that adhesions may cause pain when they interfere with normal anatomical relationships of structures, and during activity when peritoneum is pulled at attachment sites (14). There has been recent mention in the literature of defining Adhesion Related Disorder (ARD) and progression to a larger entity, Complex Abdominopelvic and Pain Syndrome (15).

Demco et al have discussed the relationship between adhesions and pain. A trial with 30 chronic pain patients undergoing an awake laparoscopy found that touching and moving adhesions with a stereotactic probe could elicit a pain sensation. Additionally, filmy adhesions that permitted more movement between structures caused more pain on a visual analog scale than denser, fibrous ones, and the worst pain was noted when the adhesion was between a mobile organ (ovary, bladder, small bowel) and the parietal peritoneum (16-17). Pathological studies of adhesion tissue have also identified the presence of sensory nerves in these structures (18).

Estimates of socioeconomic burden

In 1994, Ray et al. analyzed the economic impact of adhesions in the US by using a national hospital discharge database. In one year, adhesiolysis was responsible for 303,836 hospitalizations, primarily for procedures on the digestive and female reproductive systems. This accounted for 846,415 days of inpatient care and US$1.3 billion in hospitalization and surgeon costs (12). An estimate from Sweden suggested that adhesive bowel obstruction alone may cause 2,330 hospitalizations annually in that country, with an estimated direct cost of US$13 million (19). Costs estimated for Finland were over US$12 million (20). This topic was recently reviewed by Wilson (21), and a model was constructed using the SCAR data. The cumulative direct costs of adhesion-related readmissions due to lower abdominal surgery alone are estimated to be over €908 million for a 10-year period.

Though data is sparse, there has been one review from the UK by Ellis and Crowe, of medico-legal claims regarding adhesion-related complications. In 1995-2007, 57 claims related to abdominopelvic adhesions were closed, 29 of these successful with a settlement totaling over £3.3 million (22).

It follows that as surgeons create more surgical patients, and surgical patients live longer lives, the burden of abdominopelvic adhesions will continue to grow. This burden will manifest itself in a multitude of ways including increased patient morbidity and mortality, increasing need for emergent surgical treatment for obstructions, readmissions, lost productivity, lost operative time that would be allocated to other procedures, and overall health care costs. This problem will persist and progress, placing further strain on an already strained system.

II: Etiology and Pathogenesis

Historical Context

The earliest records of adhesions were observations made at autopsy, and were rare in the days before anaesthesia and antisepsis made laparotomy a more common procedure. Baille and others described fibrinous adhesions found in the abdomen of patients who had died of acute peritonitis, and the supposition was that these originated from coagulated lymph (23). The earliest report of postoperative adhesions may be attributed to Bryant in 1872 (24), who wrote of a fatal small bowel obstruction resulting from an adhesion passing to a ligature from an

ovarian cystectomy. Adhesions were identified as a significant cause of small bowel obstruction soon after this; in a review by Gibson of 1000 patients in 1888-1898, intraperitoneal adhesions caused 18 (6%) of the acute intestinal obstructions (25).

Observations of adhesions were made in the operating theatre and at autopsy, and surgeons began to take interest in the phenomenon towards the end of the 19th century. The role of denuded peritoneum and serosal surfaces was theorized to be important, as was the fibrinous exudate and whether or not it was reabsorbed or became organized.

The Peritoneum

Traversing peritoneal endothelium is an inevitable part of abdominal surgery, whether by hand or by instrument. Elucidation of the mechanisms of peritoneal tissue healing is integral to understanding adhesion formation, and is an area which has received increasing attention over the past 20 years. The biology of peritoneal repair is now known to involve a concert of chemical mediators, cytokines, cell types, degradation products, and proteases to produce a final result - which may ultimately form any place along a spectrum; from a cordlike adhesion to scarcely any artefact of the trauma.

The lining of the peritoneal cavity extends as parietal and visceral peritoneum; visceral peritoneum is present as mesothelial lining cells reflected onto the viscera (26). Histologically, the peritoneum consists of two layers: a mesothelium, one cell layer thick, and a connective tissue layer (27). The mesothelial layer is fragile and regenerates from injury by a simultaneous and rapid differentiation over the surface, rather than from centripetal migration from epithelial cells as is seen with healing skin (28). The implication is that large defects heal as rapidly as small ones. Interestingly, the mesothelial layer of both visceral and parietal peritoneum is coated with a surfactant-like agent, surface-active phospholipid (SAPL) which is theorised to serve as a “non-stick coating” (29).

Normal peritoneal fluid also contains many of the plasma proteins (in about 50% of the quantity of serum) including fibrinogen. There are also many active proteins and chemical mediators present, such as interleukins, interferon-γ, TNF-α, TGF-β, and VEGF (30). Additionally there are circulating, free macrophages and other immune cells present in the peritoneal fluid (31), which bathes the potential space in a small quantity (physiological mean 10cc). Molecules may enter and exit the fluid by means of transudation or exudation.

Healing following surgical injury to peritoneum follows one of two algorithms, as proposed by Duron in his 2007 review on the subject (30).

The first algorithm consists of the proliferation and regeneration of the mesothelial cell layer from an origin that has yet to be clearly identified; likely candidates include totipotent underlying mesenchymal cells, or migration of cells from another site (periphery of injury, nearby sites, or via transformation of cells in peritoneal fluid) (30). The second algorithm is centred on the alteration of fibrinolysis, producing a "peritoneal scar," and is of interest in the context of adhesion formation.

The Role of Fibrin

Many believe a pivotal tipping point in adhesion formation is how a healing body locally balances fibrin production and fibrinolysis. The inciting event of a surgical trauma or inflammation of the peritoneum results in a denuded surface, submesothelial damage and injury to blood vessels thereby invoking an inflammatory response (32). There is simultaneous activation of the coagulation cascade and fibrin deposition at the site (33), which is additive with any bleeding from the site. Mediators such as histamine and PGE_2 cause increased permeability of the blood vessels in the traumatized area, and a serosanguinous exudate rich in inflammatory cells pours forth – coagulating in as little as 3 hours (32, 34).

The exudate also contains substrates such as fibronectin, hyaluronic acid, various glycosaminoglycans (GAGs), and proteoglycans (PGs) (33). The

inactive fibrinogen turns to a tacky fibrin matrix gel, which may be distributed between two unrelated structures. Under normal conditions, the majority of fibrinous connections are lysed within a few days of their formation (34), by locally released proteases of the fibrinolytic system. It is theorized that if they persist, fibroblasts may proliferate within the substrate matrix, and establish these connections as permanent.

The physiologic fibrinolytic sequence is normally initiated by plasmin. Plasmin is a fully active serine protease which is made from plasminogen by the action of plasminogen activators (PAs) (30). One PA in particular, tissue plasminogen activator (tPA), is responsible for producing 95% of the plasmin generated in the response to peritoneal injury (35). After surgery, tPA knockout mice seem to be more susceptible to adhesion formation (36).

In a pathological state, plasminogen activator inhibitors (PAIs) interfere with the action of PAs and the production of plasmin, ultimately leading to an altered ability to degrade fibrin split products (fibrinolysis). It has been discovered that in the setting of peritoneal inflammation and injury, there are two types of PAI produced: PAI-1 (the main fibrinolytic inhibitor) and PAI-2 (37-38). PAI-1 specifically prevents the formation of plasmin by binding to and inhibiting the activities of tPA and uPA (urokinase-like plasminogen activator). These two serine proteases are the main

activators of fibrinolysis, and activation of PAI-1prevents the degradation of fibrin. Surgery dramatically diminishes fibrinolytic activity, by increasing levels of PAIs and by reducing tissue oxygenation (39). Eventually, in the absence of an effective fibrinolytic response, there exists a fibrin gel matrix which may serve as the scaffolding for development of a mature adhesion.

Cellular Players

Following the inciting trauma, the cells present are predominantly neutrophils, with a shift to mostly macrophages at 24h. Interestingly, macrophages at the peritoneal injury site after surgery have been found to differ from resident macrophages. These post-surgical macrophages secrete substances such as cyclooxygenase and lipoxygenase metabolites, plasminogen activator (PA), plasminogen activator inhibitor (PAI), collagenase, elastase, interleukins 1 and 6, tumour necrosis factor (TNF), leukotriene B_4, and prostaglandin E_2 (32, 40). These macrophages have the ability to recruit new mesothelial cells to the site of the injury (41).

Peritoneal macrophages in particular have been implicated as key players in the immune response triggering adhesion formation. They have a unique autocrine activation system whereby a chemokine (CCL1) and its receptor (CCR8) are released in response to tissue damage. Migration of peritoneal macrophages (and the development of adhesions) has been

interrupted and adhesion incidence reduced by abrogating the CCL1/CCR8 interaction (42).

There is some evidence to suggest that fibroblasts are the main cellular players in promoting the maturation of an adhesion. Fibroblast content increases in the second week post-trauma, followed by the inclusion of vessel structures and connective tissue elements (43-44). At three weeks, the development of the adhesion becomes quite prominent (45). Rout et al. isolated fibroblasts from normal peritoneum and adhesions, and found that they differed markedly in their phenotype (46). The expression of genes with products responsible for cell proliferation, differentiation, signalling molecules, transcription and translation factors, proteolysis and cytokines were seen to be differently expressed between normal fibroblast phenotype and the adhesion fibroblast phenotype. There were also marked effects of hypoxia and TGF-β on the expression of some of these products in the fibroblasts, suggesting some, but not exclusive, regulatory influence of these on the pathway (46).

Higher levels of degranulated mast cells have also been found in the presence of adhesions in rats (47), and the early event of release of VEGF by mast cells has been suggested to be central to the adhesion development (48). Further analysis of the cellular elements of adhesions by Binnebosel et al. has shown infiltrates of macrophages and T-cells, in

consistent quantities regardless of the maturity of the adhesion (49). This characterization of a state of chronic inflammation suggests T-cells may play a role in signalling pathways that maintain adhesions, and prompts consideration of adhesions as a dynamic process in remodelling tissue (49). Certainly it is clear that adhesions can re-form after adhesiolysis (50-53).

The infiltration of T-cells and the perpetuation of chronic inflammation in the peritoneum proceed under the influence of many signals, including the promoting effects of IL-6 (54) and other cytokines. T-cell depletion and adoptive transfer experiments have confirmed that adhesion formation requires the presence of CD4+ alpha beta T cells, and the production of some proinflammatory cytokines are dependent on T cells (IL-17 and others) (55). Since many fibrotic tissue disorders share a common etiology of T cell-mediated abnormalities in host defense, adhesions have been considered as another example of this.

Morphologically, the appearance of ongoing organization of scar tissue may be characterized by the formation of vascular capillaries and the disappearance of granulocytes from the adhesions. More organized adhesions have regular strands of collagen in connective tissue and the disappearance of activated fibroblasts and vascular capillaries (56). The mature adhesions are often covered with mesothelium (52).

These observations have all contributed to the recognition of adhesions as “dynamic regenerating structures that may be perpetuated by a distinct cellular and inflammatory response (49).”

Humoral mediators

Immune responses, including cell trafficking, are accomplished at the cellular level by the orchestrated release of cytokines and chemokines. Whether adhesion formation represents an abnormal or disordered version of peritoneal healing may be considered by examining the roles of various signalling molecules that appear along the course of events.

i) TGF-beta

Transforming growth factor beta (TGF-β) is the most studied cytokine in the pathophysiology of adhesion development, and has been suggested as the principal profibrotic mediator of the process (57). In an animal model, it has been shown that intraperitoneal application of TGF-β to surgical adhesions resulted in worsening of the adhesions compared with controls not given TGF-β (58) and animals given TGF-β-neutralizing antibody have shown reduced adhesion formation (59). In humans, the relationship is less clear. In vitro, TGF-β reduces peritoneal fibrinolytic capacity, an important step in disbanding of early adhesions (60). Interestingly, it has been observed in human peritoneal tissue that TGF-β

expression co varied with PAI-1, the main fibrinolytic inhibitor. Patients with more extensive adhesions had higher peritoneal concentrations of TGF-β (61).

ii) VEGF

Vascular endothelial growth factor (VEGF) is known as a potent angiogenic factor and may have a role in adhesion development (62-63). It is also directly involved in early inflammatory processes and wound healing by effects on fibroblast function (64). In an animal model, intraperitoneal treatment with an antibody to VEGF has resulted in a lower incidence of advanced adhesions (65).

iii) Interleukins

Other mediators, such as interleukins, are receiving attention for their role in adhesion development. Comparing serum and peritoneal fluid levels of interleukin-1 (IL-1), post-operative patients have a significantly higher level in the peritoneum, suggesting a possible local action of this interleukin (66). Rats treated pre-operatively with anti-IL-1 had fewer adhesions postoperatively than controls (67). Based on these and other observations, a likely mode of action of IL-1 is to promote adhesion formation by increasing fibrin deposits, reducing fibrinolysis, and stimulating mesothelialization of the structure (31).

Interleukin-6, interleukin-8 and interleukin-10 are all theorized to participate in modulation of the cellular response to peritoneal injury, however the roles are still not clear (31). Mesothelial cells are the principal IL-6 secreting cells in the peritoneal cavity, and on challenge by inflammation they produce large amounts of this cytokine (68). Interleukin-6 is known for both pro- and anti-inflammatory effects. When complexed with its receptor (IL-6/sIL-6R) found on invading neutrophils, it influences a shift from leukocyte recruitment in the acute phase of inflammation to an influx of sustained mononuclear leukocytes (69). Interestingly, a recent study in mice showed a reduction in postoperative adhesions in animals receiving an intra-abdominal infusion of cold saline postoperatively, a finding which also correlated with lower serum levels of IL-6 and higher serum levels of anti-inflammatory IL-10 (70). Intraperitoneal injection of exogenous IL-10 has been shown to reduce postoperative adhesion formation in a mouse model (71).

iv) Tumor necrosis factor-alpha

Tumor necrosis factor-alpha (TNF-α) is able to promote production of interleukins by mesothelial cells (72), and high levels of TNF-α in peritoneal fluid and serum postoperatively have been shown to be correlated with severity of adhesions in a rat model (73). However, administration of neutralizing anti-TNF-α antibodies failed to reduce

adhesion formation in this model (67) and while involved in the process, determining its role requires further study.

v) MMPS and TIMPS

Still other mediators deserve mention for their potential effects on adhesion development. Matrix metalloproteinase enzymes (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) are present in differing quantities throughout body tissues. Analysis of peritoneal fluid and serum in patients with and without postsurgical adhesions has shown that affected patients had lower serum TIMP-2 levels, which may have potential to serve as a marker for individuals who will form adhesions with greater severity (74).

A pictorial representation summarizing some of the proposed mechanisms of postsurgical adhesion formation is presented in **Figure 1-2**.

III. Prevention

Given that postoperative adhesions are common, costly, and cause significant burden to patients, there continues to be a growing interest in developing ways to reduce their incidence. An enormous body of work has been carried out both in the laboratory and the operating theatre to find ways of preventing or reducing adhesion formation. The most

common and modern means of approaching the problem has been the use of a barrier between or over damaged surfaces, administration of pharmacologics, or a combination of these. Additional factors such as technique are also relevant to the discussion.

Objective comparison of different methods of adhesion reduction remains problematic; there have been many small animal models developed for study, and accordingly many variations of standardized scoring systems. As well, animals sacrificed at different time points may prevent cross-comparison of studies. Clinical studies have the inherent problem of determining the extent of adhesions post-surgery and post-intervention as there is no planned opportunity for re-look in the majority of surgical patients. This is likely only one of the reasons that there is no method or technique of adhesion prevention that surgeons currently agree upon (2).

Classical approaches

Given the importance of fibrin in the adhesion formation pathway, there have been many attempts at reducing its presence at the scene. Removal of already-formed fibrin using trypsin, pepsin, papain, as well as mechanical removal by lavage and by hand has produced inconsistent and anecdotal results. Given the inevitability of adhesions, surgeons have attempted plication of the intestine or peritoneum to minimize the incidence of adhesions causing obstruction; the intestine has also been

‘splinted’ with a long intestinal tube to this end (75). Surgeons often mobilize the omentum to areas such as the wound closure site to protect the bowel from adhesion to areas of tissue trauma (2). Even so, the only method available to treat adhesions that have already formed is with surgical adhesiolysis.

Barrier methods

Preventing contact of two traumatized surfaces has been a recurring theme in efforts to reduce adhesions. Historically this has included trials of distending the abdomen with oxygen, or filling it with saline, Ringer’s lactate, gelatine, paraffin, olive oil, lanolin, dextran, polysiloxanes, or even amniotic fluid (75). As recently as 2009, aloe vera gel (76) and soybean oil (77) have been found to reduce adhesions if applied before, but not after, peritoneal trauma in an animal model. Octyl methoxycinnamate, a viscous biologically inert liquid, has also been studied by this group, with similarly positive outcomes (78). Icodextrin, a colloid used in peritoneal dialysis solutions, has also been instilled in the abdomen during gynaecological procedures to reduce adhesion formation, and its tolerance among general surgery patients was studied by creating a multicentre registry (79-80). Data supporting its use as an antiadhesive agent in general surgery patients is sparse. In reality, the use of some of

these substances has been uncomfortable for patients, causing wound leakage, and pulmonary and perineal edema(81).

Damaged peritoneum has also been covered using various materials in the past, including amniotic membrane, gold beater's skin, oiled silk, calf peritoneum, free grafts of omentum and oxidized cellulose (75). Many of these failed, likely due to the simple tenet that barriers must carry no infection risk, and should not create an environment for fibroblast localization. As well, for a barrier to be clinically effective, it should be easy to use in both laparoscopic and open procedures, should be applicable over oozing surfaces in addition to adhering to the desired tissue long enough to prevent adhesions. How long is this period? It is difficult to determine with certainty, especially given the limited clinical data, however, many hold the view that critical events in adhesion formation occur by day 7 (82-85).

As mentioned previously, it is known that the mesothelial cell layer of the peritoneum is coated with a natural anti-stick coating, a thin film of surface-active phospholipid (29). Experimental application of exogenous phospholipids has been a promising area of development. Phosphatidylcholine, sphingolipid, and galactolipid (56) have decreased the areas of postoperative adhesion in animal studies. Phosphatidylcholine has been shown in several studies to have a

beneficial effect in the rat (86-89), but with the side effect of impaired healing of intestinal anastomoses at higher concentrations. SprayGel, a novel hydrophilic polyethylene glycol based adhesion barrier, is formed by spraying two liquid precursors onto surgical sites. It is reabsorbed and renally cleared by 5 days post application, and using a porcine model was found to significantly reduce the incidence of adhesions (90). Applying Poloxamer 407, a polymer of hydrophilic non-ionic surfactant, after adhesiotomy in an animal model has reduced the incidence of re-formation of adhesions. It has yet to be studied in humans (91).

Interceed barrier (Johnson and Johnson, New Brunswick, New Jersey) is a fabric made of oxidized-regenerated cellulose and was the first barrier used that demonstrated an improvement in postoperative adhesions in humans (92). However, further studies have suggested it is not suitable for use in the face of significant bleeding and may actually increase the incidence of adhesion formation in this setting (92-93). Expanded polytetrafluoroethylene (Gore-Tex Surgical Membrane) has also been studied in an animal model and compared to oxidized-regenerated cellulose. There was a localized injurious effect on the peritoneum resulting in de novo adhesions in the case of the cellulose, while Gore-Tex showed neither peritoneal injury nor adhesions (93).

Hyaluronic acid, also called hyaluronan or hyaluronate, has long been a subject of interest in antiadhesion research. It is a naturally occurring glycosaminoglycan and forms a highly viscous solution to coat serosal surfaces. There has been much work with this substance with varying outcomes. In a single intraperitoneal dose at time of operation, Treutner et al. found the mean area of adhesions reduced by 84% compared to control in their animal model (56). Other animal studies have shown a reduction of adhesion formation when the hyaluronic acid was applied before the trauma, but not a reduction in reformation after the division of existing adhesions (94-95). A cross-linkable hyaluronan hydrogel has been developed for use with or without nanoparticles (96-97) and initial animal studies have been promising.

A bioabsorbable membrane consisting of hyaluronic acid in combination with carboxymethoxycellulose is produced under the name Seprafilm (Genzyme Corporation, Cambridge, Mass.). There is also a viscous liquid form of the product, Sepracoat (Genzyme Corporation, Cambridge, Mass), which has shown favourable results in gynaecological procedures (98). Seprafilm is a thin membrane which is placed in the abdomen before closure at sites of surgical trauma for the purpose of adhesion prevention. Its hydrophilic properties cause it to swell and take on a gel-like consistency over the subsequent 24-48 hours. It is resorbed within the first 7 days of placement and is fully excreted by 28 days. Several animal

and human studies with Seprafilm have shown promising results; a systematic review and meta-analysis in 2007 suggested that Seprafilm can decrease abdominal adhesions after general surgery. However, it did increase the rate of abdominal abscesses and anastomotic leaks (99). Seprafilm is not recommended for use around fresh intestinal anastomoses.

While barrier methods attempt to abort adhesion formation through prevention of surface contact, their introduction into general clinical practice has been restricted by several factors. Though a few are commercially available, drawbacks include difficulties in preparation and application, the need for absolute hemostasis, insufficient pliability, intricate product fixation techniques, and incompatibility with laparoscopic surgical procedures (100). In addition, concern regarding possible adverse outcomes has been raised. For example, while preventing adhesion formation is a priority, any device would be of little benefit if the rate of anastomotic leak increased. Without clear understanding of how these agents might interfere with normal intra-abdominal wound healing, their use deserves caution.

Pharmacological methods

Many different agents have been used in the effort to arrest the adhesion pathway or to tip the balance in the favour of fibrinolysis. Drugs may be administered systemically, or ideally, locally at the site of adhesion formation with minimal systemic effect. Due to the physiology of the peritoneal cavity, it can serve as a site for uptake of large molecules; even particles the size of cells may pass through the lymphatic lacunae located in the submesothelial layer. There are obvious implications for biocompatibility and toxicity of any material used for adhesion prevention (31).

As most share the view that critical events in adhesion formation occur in the first days following tissue insult, the durations of drug release required for adhesion prevention may be quite short.

The inflammatory component of the pathogenesis of adhesion formation has been a target for pharmacotherapy, and a variety of steroidal and anti-inflammatory drugs have been studied, including aspirin, dexamethasone, methylprednisolone, estrogen, progesterone and budesonide (101-108). However, the effectiveness of these agents has not been consistent in animal models and clinical trials (109). Yeo et al. showed in an animal model that the co-delivery of budesonide with a hyaluronan hydrogel

prevented adhesions completely in the majority of animals (106), but has yet to be tested in humans. Additional insight into development of delivery vehicles has been gained through a model for sustained local delivery of dexamethasone using poly(lactic co-glycolic) acid(PLGA) microparticles. This method was more effective than a dexamethasone crystal suspension in preventing adhesions (102), however a small amount of microparticles actually worsened adhesions and blank PLGA microparticles caused adhesions (110).

As fibrin deposition and activation of the clotting cascade have been identified as major parts of adhesion formation, heparin has been suggested in an attempt to reduce the contribution of clotting to the process. There is some evidence from animal models that suggests local intraperitoneal administration of low-dose heparin throughout the immediate postoperative interval may result in fewer adhesions (111).

Other targets of the fibrin-fibrinolysis pathway include use of fibrinolytic drugs and plasminogen activating factor (82, 103, 112-114). These have been effective in a dose-dependent manner (115) but are noted to impair wound healing. Ancrod, an experimental defibrinogenating agent made from Malayan pit viper venom, has been used experimentally in combination with a hydrogel to reduce adhesions (116). Since all fibrinolytic drugs can potentially induce bleeding, their general acceptance

for routine use in the prevention of postoperative adhesions is not to be expected.

There may be a role for several medications approved for other indications, such as mitomycin C, paclitaxel and sirolimus. Mitomycin C is an antitumour antibiotic that is also known to inhibit fibroblast proliferation for several weeks in vitro. It has been combined with a crosslinked hyaluronan hydrogel for the purposes of adhesion reduction in an animal model (100). It has also been used locally in strabismus surgery to limit postoperative adhesion, a property which is attributed to its antifibrinolytic activity (117), and for prevention of recurrent intra-abdominal adhesions in rats (118). Antiproliferative agents such as paclitaxel and sirolimus may also have promising applications in adhesion reduction devices. Paclitaxel-loaded crosslinked hyaluronic acid films reduced adhesion incidence in a rat model (119). Sirolimus, a macrolide group antibiotic with immunosuppressive and antiproliferative properties, will be discussed later in this chapter.

Lastly, there are a number of novel compounds which have been used in an attempt to prevent adhesions. Tranilast, an anti-proliferative medication, leads to reduced collagen formation in vitro. It has been combined with a gel in an experimental animal model to inhibit development of adhesions (120) and has yet to be tested clinically for this purpose. There is evidence to suggest methylene blue dye may act as an

antioxidant in the postoperative peritoneum and serve to reduce the incidence of adhesion. In one study it was also found to enhance fibrinolytic activity in peritoneal fluid (121). Postoperative stimulation of motility using prokinetic drugs has also been found to reduce adhesions in a rat model, and conversely, administering atropine worsened them (122). Taurolidine is a drug with antimicrobial and anti-lipopolysaccharide properties, and has immune modulatory action via priming and activation of macrophages. It has been used in experimental animal models to reduce adhesions, with inconsistent results (56, 123-124).

A summary of selected pre-clinical and clinical trials of devices and drugs is provided in **Table 1-1**. Given the location of drug delivery and barrier placement, there are obvious implications for biocompatibility and toxicity of any material used for adhesion prevention (31). The future of adhesion prevention strategy likely has the most promise in a device which combines targeted pharmacology with a barrier method; the device developed in this thesis work follows this strategy.

Sirolimus in a Pharmacologic + Barrier Adhesion Prophylaxis

Sirolimus is the generic name for the natural product rapamycin, a lipophilic macrocyclic lactone, which was isolated from a strain of *Streptomyces hygroscopicus* found in soil on Easter Island (Rapa Nui) in

1975. It is a macrolide group antibiotic which was initially isolated as an antifungal agent, and discovery of its antitumor/antiproliferative and immunosuppressive properties followed. Sirolimus binds to an intracellular binding protein termed FKBP (FK binding protein) and this complex targets the pivotal regulator of cell growth and proliferation: the mammalian target of rapamycin (mTOR).

Blocking the mTOR has a very wide range of downstream effects, all of which may play a potential role in halting adhesion formation at a cellular and humoral level. Briefly, in possibly the most universal of its effects, blocking the mTOR impedes cell proliferation of many cell lines, including those of connective tissue origin, by arresting the cell in the G1 and S phases. Sirolimus is also a potent inhibitor of cell proliferation and protein synthesis mediated by growth factor (125). The blocking of mTOR by the sirolimus:FKBP complex also negatively affects CD28-mediated sustained upregulation of IL-2 transcription in T cells (126). In resting cells, a binding protein (4E-BP1), when phosphorylated by mTOR is responsible for translation of some specific mRNAs required for cell growth and differentiation (127). Sirolimus thus inhibits this process.

Sirolimus is known to inhibit the proliferation of tumor cell lines (recently reviewed by Alvarado et al.(128)), and it or related mTOR inhibitors have been approved for use in treating malignancies such as renal cell

carcinoma (129), mantle cell lymphoma (130), and subependymal giant cell astrocytoma (131). Its potent antiangiogenic effects are accomplished in part by inhibiting PDGF-stimulated proliferation of smooth muscle cells(132). As adhesions at all measures of maturity contain vessel structures and connective tissue elements (52), perhaps arresting their formation early in development would deprive the tissue of necessary promoting factors and be an effective detriment.

It has been reported that administration of systemic sirolimus in hamsters with an implanted surgical mesh resulted in inhibition of angiogenesis, reduction of collagen deposition, and an overall impairment of incorporation of the mesh when compared with cyclosporine-treated and control animals(133). Systemic sirolimus has been shown to contribute to the formation of incisional hernias after liver transplantation, a consequence of this impaired wound healing (134). Risk factors such as age >40 years, BMI >26, use of thymoglobulin for induction immunosuppression, and larger cumulative doses of sirolimus have been related to having wound complications (135).

Perhaps the most direct role of sirolimus in preventing adhesion formation might be its ability to block basic fibroblast growth factor (bFGF)-induced proliferation of endothelial cells and fibroblasts (136). It has become well-known for its use in decreasing the rate of restenosis in coronary artery

stents (137). Fibroblasts synthesize collagen, and dense adhesions have more total collagen and higher collagen type I/III ratios. Indeed, fibrosis of many organs and tissues has been reduced with administration of sirolimus in preclinical models, including hepatic fibrosis in rats (138), renal fibrosis in mice (139), subglottic stenosis in a rabbit model (140), pulmonary fibrosis in transgenic mice (141), and most recently urethral stricture in rabbits (142). Sirolimus has been used experimentally in an animal aortic PTFE vascular graft model to reduce retroperitoneal adhesions (143). Anecdotally, some surgeons suggest transplant patients receiving sirolimus have fewer adhesions on relaparotomy. The potential for one drug to intervene at multiple points along the known cellular and humoral pathways of adhesion formation makes a compelling argument for further investigation of the effect of sirolimus on adhesion formation. Dosing to achieve maximum therapeutic effect with a minimum of known immunosuppressive and wound healing side effects would be imperative. This may be accomplished by avoidance of systemic administration or absorption, and instead, locally delivering the sirolimus to the site of tissue trauma and inflammation.

A summary of *in vitro* cellular effects of sirolimus is found in **Table 1-2**.

Surgeon Factors

There are some technical points which deserve mention in any discussion of tissue trauma and adhesion formation. Careful and atraumatic handling of tissue is essential to reducing serosal damage. Grafting or suturing of peritoneal defects increases tissue trauma, ischemia, devascularisation and necrosis, predisposing the site to decreased fibrinolysis and adhesion formation (144). Choice of scalpel may also influence development of adhesions as several experimental studies have suggested that sharp mechanical tissue transection was followed by the least amount of reaction, and resulted in less necrosis than the CO_2 laser, electrocautery, or the ultrasonic scalpel (145-147).

The first reports of granuloma and adhesion formation from magnesium silicate dusting powder from sterilized surgical gloves appeared in 1937 (148), and the association of talc, starch, and silicates with adhesions has been established and reviewed extensively since (43, 149-150). Surgeons should therefore choose to use gloves which contribute little adhesiogenic contamination to the surgical field. Foreign bodies have been found on microscopy in a large percentage of adhesions (151). Commonly found items include wood particles from paper products, lint from cloth drapes and packs, and suture materials. With regard to suture material choice in particular, the type of suture material (including surgical staples) seems to

be less of an adhesiogenic factor than the amount used (knot configuration or suture gauge), and the finer the gauge, the less adhesiogenic the suture (152-153).

Reduction in the magnitude of the activation of coagulation cascade by careful attention to hemostasis can be supplemented by use of newer adjuncts. However, the choice of hemostatic agents has been shown to influence adhesion formation in a rat model (154). In a comparison of six materials (oxidized cellulose, polyethylene glycol polymer, activated starch microspheres, thrombin-coated microspheres, fibrin glue, and glutaraldehyde activated collagen), polyethylene glycol polymer and activated starch microspheres appeared to reduce the incidence of adhesions compared to controls at day 7 (154). Glutaraldehyde activated collagen had an adverse effect of tissue necrosis and adhesion formation. In terms of surgical technique, the majority of studies on the subject favour the idea that laparoscopic surgery is less adhesiogenic than open abdominal surgery (155-158). Recent work by Brokelman and Holmdahl has assessed the peritoneal fibrinolytic response to both types of surgery by measurement of serum tPA antigen and tPA activity, and PA-I during and at the conclusion of surgery. It was found that open surgery creates a worse hypofibrinolytic state than laparoscopic surgery earlier in the case, but that the levels of markers were no different at the end. Additionally, these parameters were measured during use of heated CO_2 versus room-

temperature CO_2 for insufflation, and showed a significantly higher level of PA-1 in the room-temperature patients (159), suggesting a change in peritoneal biology with this stress. The clinical outcome of adhesion formation from these serum measurements has yet to be observed and quantified. Other aspects of laparoscopic surgery, such as light intensity, intra-abdominal pressure, and choice of dissection device have not been shown to affect peritoneal activity during short-term laparoscopy (158).

Whether or not to perform peritoneal closure on conclusion of laparotomy has been a topic of debate. As previously outlined, the healing of peritoneal defects is accomplished at the same rate regardless of the size of the defect. Closure of the peritoneum subjects tissue to additional factors which are known to be adhesiogenic: suture material, tissue hypoxia and ischemia, activation of the coagulation cascade, and trauma due to tissue handling. Studies have also demonstrated the safety in general surgery and gynaecological patients of not closing this layer, prompting surgeons to reconsider this practice (160).

There are a number of operative techniques which surgeons can adopt in an attempt to reduce the burden of adhesion formation. It is unlikely that any of these will lead to a complete reduction in post-operative adhesions. However, employing multiple techniques is not only good surgical practice

but also possibly a means of reducing the future impact of any adhesions which do form.

IV. Conclusion

Postoperative adhesions represent a problem of considerable magnitude for surgeons, patients and the health care system worldwide. Morbidity resulting from adhesive tissue following an abdominal or pelvic surgical procedure is significant and is a burden the patient carries with them for the rest of their lives. The search for a solution to the problem has been extensive with many promising results in animal studies, but few of these developments have been extended to clinical trials. Human data is also difficult to acquire for the ethical reasons of planned relaparotomy. From our increased understanding of the multifactorial nature of adhesion pathogenesis, and our beginning knowledge of the effects of cellular and molecular mediators of the process, there is room for significant individual variation in adhesiogenesis.

Development of a successful adhesion reduction system will likely come from the interface of biophysics and pharmacology, and the use of an agent that exploits a facet of the cellular signaling or molecular pathways. A device that will release controlled amounts of this agent, over a prescribed period of time and only in the location and cellular milieu that it

is needed is a worthwhile endeavour. The reasons for failure of previous designs are varied, and rapid clearance of drugs from the peritoneum could be a cause of the limited effectiveness of intraperitoneally applied drugs. As well, our use of small animal models can be limiting, as they prevent the use of laparoscopic devices, have reduced organ volume, and it can be theorized that forces may be insufficient to challenge the ability of a barrier to remain attached to the desired site, as suggested by Ferland (90).

To date, there is no method or device in widespread use, and there has been a failure of intraperitoneal adjuncts to improve the outcomes (30, 33, 109). Despite much progress in elaborating the complexity of the problem, it remains a critical surgical challenge.

Table 1-1. Selected methods of adhesion prevention.

	Reference	Model
Barrier methods		
Aloe vera gel	Aysan et. al, 2009	Rat
Soybean oil	Aysan et. al, 2009	Rat
Octyl methoxycinnamate	Aysan et. al, 2009	Rat
Icodextrin	diZerega et.al, 2002, Menzies et. al, 2006	Clinical trial, Clinical trial
Phosphatidylcholine	Ar'Rajab et. al, 1991, Roszga et. al, 1990, Snoj et. al, 1992, 1993, Treutner et. al, 1995	Rat, Rat, Rat, Rabbit
SprayGel	Ferland et. al, 2001	Pig
Poloxamer 407	Steinleitner et. al, 1991	Rabbit
Interceed	DeLaco et. al, 1998, Haney and Doty, 1992	Rabbit, Mouse
Gore-Tex Surgical Membrane	Haney and Doty, 1992	Mouse
Oxidized-regenerated cellulose	Haney and Doty, 1992	Mouse
Hyaluronic acid/hyaluronan/hyaluronate, and Seprafilm	Numerous	Models have included mouse, rat, rabbit, as well as clinical trials
Pharmacological methods		
Aspirin	Golan et. al, 1995	Rat
Dexamethasone	Buckenmaier et. al, 1999, Gazzaniga et. al, 1975, Hockel et. al, 1987, Kucukozkan et. al, 2004	Rat, Rat, Rat, Rabbit
Methylprednisolone	Gazzaniga et. al, 1975	Rat
Estrogen	Bozkurt et. al, 2009	Rat
Progesterone	Maurer and Bonaventura, 2003	Guinea pig
Budesonide	Yeo et. al, 2003	Rabbit
Heparin	Bahadir et. al, 2007, Fukasawa et. al, 1991	Mouse, Rabbit
tissue plasminogen activator	Buckenmaier et. al, 1999 and others	Rat
Streptokinase	Buckenmaier et. al, 1999	Rat
Urokinase	Buckenmaier et. al, 1999	Rat
Ancrod	Chowdhury and Hubbell, 1996	Rat
Mitomycin C	Cubukcu et. al, 2001 and 2002, Liu et. al, 2005	Rat, Rat
Paclitaxel	Jackson et. al, 2002	Rat
Tranilast	Petrilli et. al, 2008	Rabbit
Methylene blue dye	Heydrick et. al, 2007	Rat
Taurolidine	Bahadir et. al, 2007, Tarhan et. al, 2008, Treutner et. al, 1995	Mouse, Rat, Rabbit

Table 1-2. Selected *In Vitro* Cellular Effects of Sirolimus. Modified from Sehgal, S.N. Sirolimus: Its Discovery, Biological Properties, and Mechanism of Action. 2003: *Transplantation Proceedings* 35 (Suppl 3A), 7s-14S.

Cell Type	Stimulus	Function	Response to Sirolimus
T lymphocytes	Mitogen, antigen	Proliferation	Inhibition
	IL-2, IL-4, IL-7, IL-12, IL-15	Proliferation	Inhibition
Mast cells	IL-4, IL-3	Proliferation	Inhibition
Smooth Muscle Cells	Growth factors	Proliferation	Inhibition
Fibroblasts	Growth factors	Proliferation	Inhibition

Figure 1-1. Artistic rendition of the evoked emotional response of the surgeon on encountering intra-abdominal adhesions.

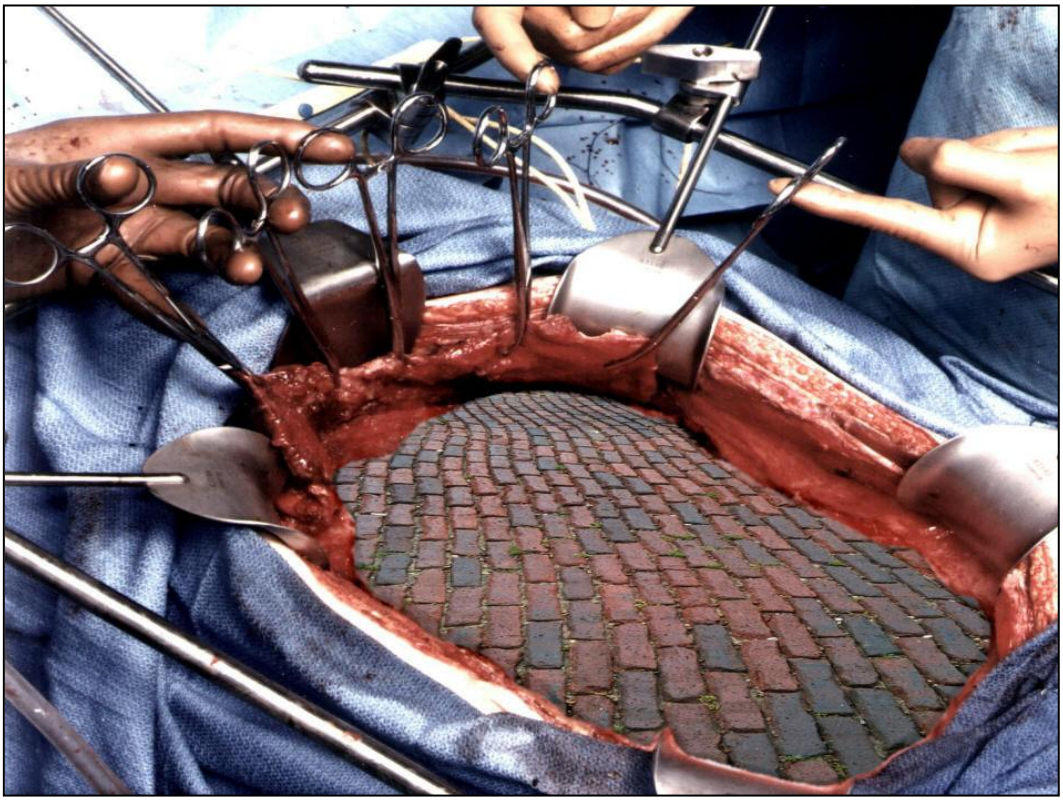
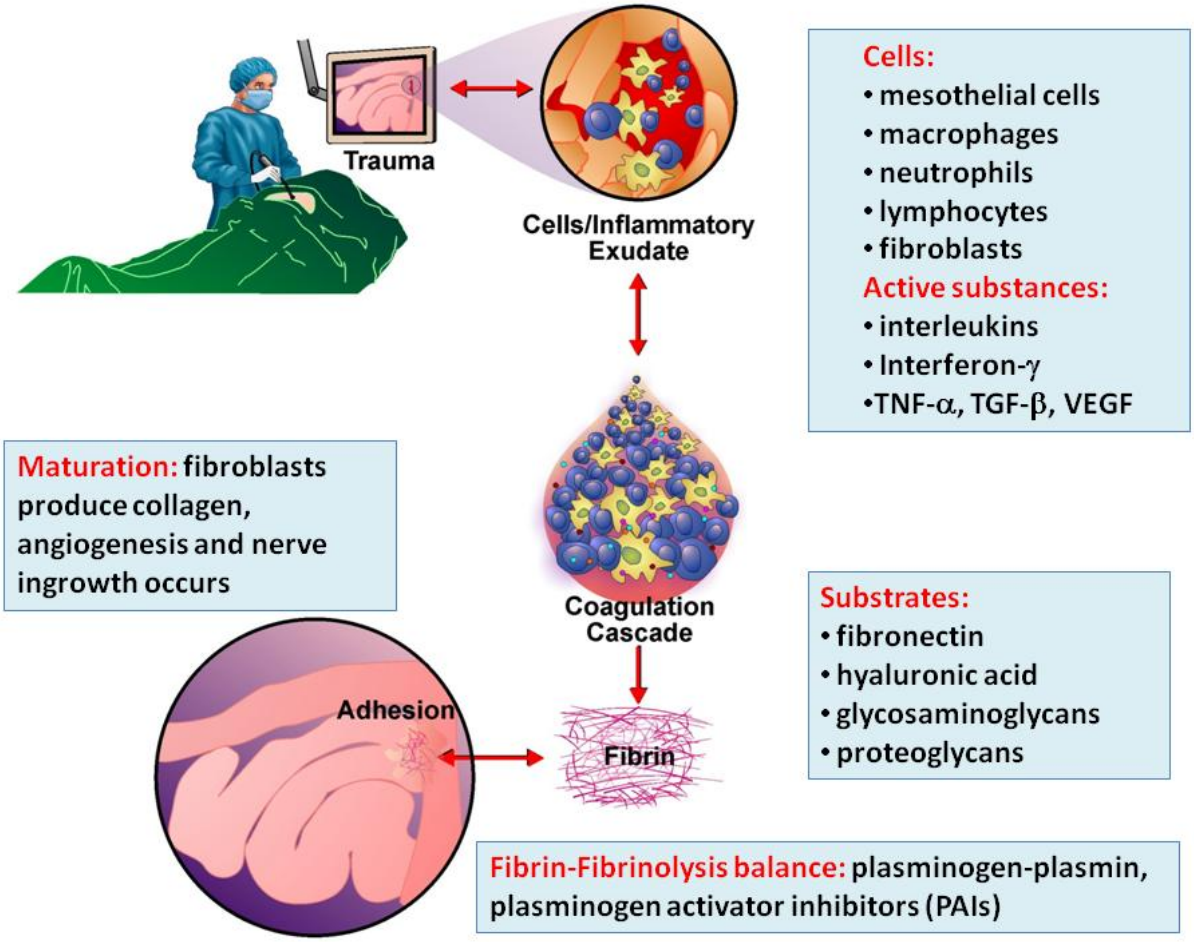


Figure 1-2. Summary of postsurgical adhesion formation. Trauma to peritoneal and other intra-abdominal tissue results in an exudate rich in proinflammatory cytokines and various cell types. These in turn serve to activate the coagulation cascade and a fibrin mesh forms, eventually resorbing or maturing into an adhesive connection between the surfaces.



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Chapter 2

Sirolimus drug-eluting, hydrogel-impregnated polypropylene mesh reduces intra-abdominal adhesion formation in a mouse model

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Abstract

Background: Prosthetic mesh is frequently employed in abdominal wall hernia reconstruction, but is prone to postoperative adhesion formation. Complications resulting from intra-abdominal adhesions represent a considerable clinical and cost burden. We herein investigate the anti-proliferative and antiadhesiogenic properties of sirolimus and hydrogel-impregnated drug-eluting mesh to reduce such complications in a mouse model of abdominal wall hernia repair.

Methods: A 1x1cm^2 polypropylene mesh from one of three groups (plain control, hydrogel (2% agarose), or hydrogel + 10 mcg sirolimus) was surgically implanted into the peritoneal cavity of BALB/c mice, and followed for up to 4 weeks, with adhesions scored by percent surface area of mesh (0-100%), severity (0-3) and tenacity (0-4). Representative samples were assessed by scanning electron microscopy.

Results: The combination of hydrogel and sirolimus-impregnated-mesh led to significant reduction in adhesion formation. The percent surface area of adhesional attachment to mesh was reduced from 100.0 $\pm$ 0% in the plain mesh control group versus 17.5 $\pm$ 8.4%, ($p<0.001$) in the combined impregnated mesh group. Similarly, adhesion severity scores were reduced from a score of 2.9 $\pm$ 0.1 (plain mesh) versus 1.4 $\pm$ 0.1 (sirolimus/hydrogel mesh) ($p<0.001$). Scores for tenacity were also markedly reduced from 3.5 $\pm$ 0.2 (plain mesh) versus 1.5 $\pm$ 0.1

(sirolimus/hydrogel impregnated mesh, ($p<0.001$).

Conclusions: Creation of a sirolimus drug-eluting and hydrogel impregnated polypropylene mesh resulted in marked reduction of adhesion formation in this mouse model, was well tolerated without side effects, and has potential for clinical application.

Introduction

Intra-abdominal adhesions are a challenging problem for surgeons and their patients, developing as sequelae of tissue trauma following surgery. They are despairingly common, and a near certainty on re-operation: in a prospective analysis of 210 patients undergoing a laparotomy with one or more previous abdominal operations, 93% had adhesions, compared with 10.4% in first-time laparotomy patients (1). In a survey of UK surgeons, adhesions and their sequelae represent a large portion of the general surgical workload, though treatment and prevention practice varies widely (2). Adhesions are known to cause a range of serious complications including small bowel obstruction, chronic pain, and female infertility, increasing the risk of enterotomy and bleeding on re-operation (3-7). This resulted in an estimated $1.3 billion/year additional cost burden attributed to adhesions in the U.S. alone (5).

The repair of abdominal wall hernias may be accomplished by a variety of options, including open primary suture repair, open prosthetic mesh repair, and laparoscopic repair with intraperitoneal placement of prosthetic mesh. Primary suture repair carries a high rate of recurrence (8), prompting most surgeons to consider and choose a repair using mesh. The most common prosthetic meshes are knitted (with spaces or pores), synthesized from polypropylene or polyethylene, and share the potential for causing

intestinal complications via bowel loops adhering to mesh, a consequence which carries risk of fistula formation. Smooth-surfaced and impermeable materials such as silicone are resistant to adhesion formation; however, these materials may lack sufficient fixation to adjacent tissues to provide strength and support to the repair.

Multiple approaches have been taken pre-clinically and clinically in an attempt to reduce the incidence and severity of adhesions, and have recently been the subject of two Cochrane reviews (9-10). There are three main approaches to preventing postoperative adhesion formation: 1) provide a barrier between peritoneum and viscera or two visceral surfaces, 2) deliver a pharmacological intervention along the hypothesized pathway to mature adhesions; or 3) combine both, as accomplished in our current approach - a drug-eluting mesh.

Hydrogels are natural or synthetic compounds made of hydrophilic polymer chains. Recently there has been interest in their potential use as a barrier to adhesion formation (11-12). Their high water content, flexibility and effectiveness as a sustained-release drug delivery system make them ideal for development as an adhesion prophylaxis device. There has been very limited evaluation combining hernia repair mesh with a hydrogel, but results have been promising in a small study in rats (13).

Sirolimus is a macrolide group antibiotic with immunosuppressive and antiproliferative properties (14). It has become known for its use in decreasing the rate of restenosis in coronary artery stents (15) and it has also been employed in novel treatment of conditions such as choroidal neovascularisation and renal cell carcinoma (16-17). The aim of this study was to investigate the impact of a SRL drug-eluting, hydrogel impregnated polypropylene mesh in reducing adhesions to surrounding intestines. Local delivery of sirolimus has been examined previously in the context of adhesions to abdominal prosthetic vascular grafts in a rat model (18), however, its use in conjunction with an adhesiogenic hernia repair mesh is novel. Hydrogels have been used previously for intraperitoneal delivery of microencapsulated insulin-producing cells as a physiological barrier in clinical studies (19, 20) and have also been found to be effective in form of hyaluronic acid (Seprafilm^{TM}) to reduce adhesions in clinical studies, but direct impregnation of polypropylene mesh with agarose hydrogel has not been reported previously. We herein explore the potential of combined sirolimus drug-eluting and hydrogel impregnated mesh as a means to reduce postoperative adhesion incidence, severity and tenacity.

Methods

Animals and Reagents

Male BALB/c mice were obtained from Jackson Laboratories (Bar Harbour, ME) and housed under conventional conditions (standard lab chow and water *ad libitum*, 12-hour light-dark cycle in a temperature- and humidity-controlled environment). All animals were cared for according to the guidelines of the Canadian Council on Animal Care, and ethical approval was obtained from the animal welfare committee at the University of Alberta. All reagents including sirolimus (SRL) were obtained from Sigma Aldrich (Oakville, Ontario, Canada) unless otherwise specified.

Mesh materials

Plain monofilament polypropylene mesh was obtained (ETHICON, Somerville, NJ), and three experimental groups were generated – i. plain mesh (control), ii. mesh + 2% agarose hydrogel (control for effect of hydrogel alone), and iii. mesh + SRL + hydrogel (SRL drug-eluting treatment group). In all groups, the mesh implants were cut into 1 cm^2 squares. SRL was solubilised in 95% alcohol (1mg SRL dissolved in 1cc), and diluted in phosphate buffered saline (PBS) to a concentration of 10

mcg of SRL per mesh (100μL of 2% agarose + 10mcg SRL). The dose of 10mcg was approximately twice the suggested daily intraperitoneal dose equivalent to a clinically relevant (immunosuppressive) exposure, 0.2mg/kg/d ip injection, as determined by previous work in our islet transplantation laboratory (19). The solution was injected in a warmed, viscous form onto a petri dish in 100μL aliquots and a mesh square was placed onto each droplet. The solution was then distributed gently to all edges of the mesh with forceps, fully impregnating the mesh in the gel which became solid when cooled to room temperature. Mesh were sealed in petri dishes and refrigerated >24h before use to facilitate solidification of the hydrogel. The light-sensitive nature of SRL was respected throughout.

Surgical Technique

The abdomen was shaved and the mouse was then anaesthetized and maintained in surgical plane using 1% isoflurane. Procedures were done under aseptic conditions. No prophylactic antibiotics were given. Mice were randomly assigned to each group.

A midline laparotomy was performed to expose the viscera. Surfaces of the abdominal contents were swabbed once with a cotton-tip to mimic conditions of routine surgical trauma and tissue handling. Pilot studies demonstrated that this enhanced the adhesiogenicity of the model. It was

also found that there was a propensity of the epididymal fat pads to prevent adhesion of intestinal loops to the mesh, and as such, all mice underwent lipectomy using a 2-0 vicryl suture (ETHICON, Somerville, NJ). The mesh square was placed directly over the abdominal contents in the midline, below both costal margins, and then each corner was sutured to the overlying parietal peritoneum and muscle using 4-0 PDS suture (ETHICON, Somerville, NJ). The peritoneum and muscle was then brought together over the mesh and reapproximated with a running 2-0 vicryl suture. No defect in the abdominal wall was created in this process, and thus our model investigates the adhesiogenic and lytic properties of treated polypropylene mesh, and does not specifically investigate the impact of mesh pretreatment upon strength of mesh incorporation and repair. The subcutaneous tissue and skin was clipped together using surgical clips.

Assessment of Systemic Sirolimus Levels

A separate cohort of mice (n=20) underwent implantation of SRL-containing hydrogel mesh, and were euthanized at 1, 2, 3 and 4 week time points. An intracardiac blood sample was withdrawn and analyzed for serum SRL levels to detect systemic absorption of SRL.

Assessment of Adhesions: Necropsy studies

The animals in the time course study (n=5 at each week) received plain polypropylene mesh and were evaluated at necropsy at 1, 2, 4, 8, 12, and 24 weeks; this established the relevant time point for assessment of the study groups. Hence, all animals in the study groups (plain n=5, agarose n=13, agarose + SRL n=12) were euthanized and underwent necropsy at the 4 week time point.

At necropsy, the abdomen was opened in a C-shaped window around the mesh prosthesis and gently elevated for visualization and scoring. Adhesions were assessed quantitatively and qualitatively by two blinded observers (M.M. and R.E.) who had not participated in the surgery, and were unaware of the assigned treatment group. Adhesions were scored using a scale modified from those previously published by Linsky(20) and the Surgical Membrane Study Group(21), according to the following parameters: percentage of mesh covered by adhesions, severity, and tenacity **(Table 2-1)**. Scores given by each observer were analyzed as independent observations.

Assessment of Adhesions: Scanning Electron Microscopy

Representative specimens (n=4 per group) were obtained by necropsy at 2 and 4 weeks. Adherent viscera were separated from the mesh with

blunt and sharp dissection. Specimens were prepared for scanning electron microscopy by fixation in 2.5% glutaraldehyde solution, and stored at 4°C until processing. The samples were post-fixed in 1 % osmium tetroxide in Milonig's buffer, pH 7.2 at room temperature for 1.5 hours. They were then dehydrated in ethanol. The samples were further dried by critical point drying at 31°C for 5 – 10 minutes, then mounted on a stub and were sputter coated with gold (Edwards model S150B Sputter Coater). Samples were examined using a Hitachi SEM S-2500.

Statistics

Statistical analysis was carried out using GraphPad Prism (Version 5.0b, GraphPad Software Inc., San Diego, CA). P values less than 0.05 were considered statistically significant. Graphical representation of data is represented as mean ± SEM, unless otherwise mentioned. Means of % surface area were compared using Bartlett's test, ANOVA and *post hoc* Newman-Keuls Multiple Comparison Tests. Means of ordinal data were compared using Kruskal-Wallis test, followed by *post hoc* Dunn's tests.

Results

All animals tolerated the surgical procedure well and gained weight appropriately following surgery according to growth curves for age. Of all mice that underwent surgical placement of mesh, including pilot study data, there were no wound dehiscences, no issues with local wound healing of the skin, and no significant differences in infection rate (necrosis or purulent discharge) noted on necropsy: incidence was 0% among animals with plain mesh (n=30), 5.7% in those with agarose mesh (n=35), and 8.0% in animals with agarose + SRL (n=50). Mortality from all causes was 6.7%, 8.5% and 8.0% in the plain, agarose and agarose+SRL groups respectively, with no significant differences among these experimental groups. At necropsy, all animals demonstrated incorporation and adhesion formation at the edges of the mesh. In one case we encountered a complete adhesive small bowel obstruction at necropsy (**Figure 2-1**). This edge value was not included in calculation of surface area coverage.

Serial Systemic Sirolimus Levels

All animals sacrificed for serum SRL levels at weeks 1, 2, 3 and 4 had undetectable levels at their respective time point (<1.0 micrograms/L). This signified that there was no detectable systemic absorption of SRL when applied locally to the mesh beyond the first week, and a lack of systemic toxicity.

Macroscopic Observation of Adhesions: Time Course

Representative macroscopic images of mesh samples are shown in **Figure 2-2A-C**. Percent surface area of plain polypropylene mesh covered by adhesions was marked and consistent early in the postoperative course (91.5 $\pm$ 4.8 % at week 1), and remained constant through time to the 6-month point (98.0 $\pm$ 2.0 % at week 24), with no significant difference among groups (**Figure 2-3A**). Mesh severity scores (**Figure 2-3B**) and tenacity scores (**Figure 2-3C**) were similarly high and consistent over the time points of interest. Severity of adhesions differed significantly between weeks 1 and 4, and between 1 and 24 ($p<0.05$ and $p<0.01$ respectively), and tenacity scores differed significantly only between weeks 1 and 4 ($p<0.05$). The 4-week scores were therefore chosen as the end point for subsequent studies.

Macroscopic Observation of Adhesions by Group: Plain, Hydrogel, Hydrogel + SRL

The combination of SRL drug-elution and hydrogel was found to be the most potent inhibitor of adhesion formation. Comparing measured surface area (**Figure 2-4A**), there was a significant difference between all groups: plain mesh had significantly more surface area covered with adhesions than mesh treated with hydrogel or SRL + hydrogel (100.0 $\pm$ 0% (plain mesh) vs. 47.2 $\pm$ 10.6% (hydrogel), $p<0.01$, vs. 17.5 $\pm$ 8.4% (SRL + hydrogel), $p<0.05$. Severity scores (**Figure 2-4B**) were also significantly lower in the treated groups (2.9 $\pm$ 0.1 (plain mesh) vs. 1.8 $\pm$ 0.2 (hydrogel), $p<0.01$ vs. 1.4 $\pm$ 0.1 (SRL + hydrogel), $p<0.001$. Tenacity scores (**Figure 2-4C**) were also substantially reduced in the treatment groups, 3.5 $\pm$ 0.2 (plain mesh) vs. 1.8 $\pm$ 0.2 (hydrogel), ($p<0.001$), vs. 1.5 $\pm$ 0.1 (SRL + hydrogel), $p<0.001$.

Scanning Electron Microscopy

Images of the plain mesh (**Figure 2-5A**) and mesh treated with hydrogel (**Figure 2-5B**) reveal the ultrastructural architecture. Of the representative sections of mice undergoing scanning EM evaluation, we found evidence of mesh incorporation in all cases (16 tissue blocks), with trans-growth of fibroblasts and collagen throughout. Macroscopically, the hydrogel coats the surface of and spaces between the polypropylene fibres, rendering it

smooth and filling the interstices; rough areas as seen in Figure 5B likely reflect artifact caused by the desiccation process. When implanted in the abdominal wall and examined at 2 and 4 week end points, all mesh from all groups were covered by proliferations of cells, with no exposed polypropylene. Fibroblasts were more numerous in the untreated mesh at 2 and 4 weeks (**Figures 2-6A and B**), with populations of fibroblasts and mesothelial cells seen in all groups.

Discussion

In the current study, we have demonstrated a potent protective effect of a sirolimus drug-eluting, hydrogel-impregnated polypropylene mesh in reducing adhesion formation to viscera, in a mouse model. The model was intensely adhesiogenic, with all animals receiving untreated mesh developing a demonstrable reaction to the abdominal wall implantation of the mesh within one week of surgery. These adhesions remained stable through a 6-month period, and it is likely that observations at this late time point represent permanent, mature adhesions. We found a significant decrease in adhesion surface area, severity and tenacity with the maximal impact observed when SRL was combined with hydrogel.

Prosthetic mesh used in hernia repair surgery must provide sufficient scaffolding for tissue ingrowth on the parietal side and edges. However, a casualty of this desired characteristic is the proximity of nearby visceral organs, as postoperative adhesions connect these tissues to the mesh surface, risking complications such as fistula formation. Relaparotomy in the setting of postsurgical adhesions puts patients at increased risk of complications such as enterotomy and hemorrhage (4).

Changing porosity of the mesh by coating with a hydrogel may have prevented fibroblast ingrowth and tissue integration. It has been

established that the structure and porosity of the mesh play a fundamental role in adhesion formation, consistency, and in the organization of the neoperitoneum formed between the prosthetic and the visceral peritoneum (22). It has been well-established that peritoneal mesothelial cells prevent adhesions (23-24), and it appears as though the hydrogel coating permits early and swift migration of these cells, covering the surface in sheets by 2 weeks' time.

Agarose hydrogel offers a barrier to adhesion formation and serves as an effective vehicle for SRL drug elution. It is an inert, bio-compatible hydrogel with significant potential for use as a barrier against adhesions especially immediately adjacent to bowel or an adhesiogenic substance/implant. We anticipate that unlike hyaluronic acid preparations (25), agarose hydrogel may be less likely to interfere with the process of wound healing. Hyaluronan is known to bind to cell surface receptors and participate in the signaling of a variety of physiologic processes (26). Pure agarose by comparison is commonly used for electrophoresis of proteins and nucleic acids, and does not interact with those substrates. The recent work by Engelsman *et al.* implanting different surgical meshes in mice showed that multifilament and hydrophobic meshes were associated with high infection rates (27). Coating a monofilament mesh with a hydrophilic hydrogel, as in our model, may potentially improve this outcome.

Recently there has been an increased appreciation for the role of immune cells in the process of normal peritoneal healing and the pathogenesis of adhesions (28-30). Early events in adhesiogenesis include recruitment and interactions of these cell types, and their signaling via cytokine/chemokine release may be an important place for intervention. SRL is a small, lipophilic molecule that forms a complex with the immunophilin FK506 binding protein-12, which subsequently inhibits the regulatory kinase mammalian target of rapamycin (mTOR) (31). This protein kinase regulates cell growth and metabolism in response to changes in the cell environment. SRL may work to reduce adhesions via several mechanisms as it has anti-inflammatory, antifibrotic, antiproliferative, and antiangiogenic properties. In their 2009 study, Laschke *et al.* (32) reported that administration of systemic SRL in hamsters with an implanted surgical mesh resulted in inhibition of angiogenesis, reduction of collagen deposition, and an overall impairment of incorporation of the mesh when compared with cyclosporine-treated and control animals. Systemic SRL has been shown to contribute to the formation of incisional hernias after liver transplantation, a consequence of this impaired wound healing (33). Interestingly, it is our current clinical practice to convert patients from sirolimus and replace with tacrolimus and cellcept for a period of time before and after incisional hernia repair in the setting of liver transplantation. We anticipate that the small, single, local dose of SRL used in the present study would have minimal impact on

wound healing, but we acknowledge that the present study does not formally evaluate wound and hernia repair strength.

Local delivery of SRL to reduce adhesion formation appears to be a worthwhile endeavour given the undesirable side effects of systemic treatment with mTOR inhibitors, such as severe infection, pneumonitis, and renal function deterioration (34). In our model, we found no significant difference in infection rates between the groups at the single SRL drug doses administered, which was approximately twice the suggested daily intraperitoneal dose equivalent to a clinically relevant (immunosuppressive) exposure, 0.2mg/kg/d ip injection, as determined by previous work (19). As the SRL was solubilised before incorporation into the hydrogel, we assume the drug is eluted locally over time, but this has not been measured directly in the present study. There is potential to increase the amount of drug applied to this mesh as we found no evidence of systemic sirolimus absorption as early as 1 week, and further study of elution pharmacokinetics is required to optimize drug delivery. Scanning electron microscopy images suggest mesh incorporation in all cases, with trans-growth of fibroblasts and collagen throughout. We believe this provides surrogate data to suggest that the presence of hydrogel or sirolimus elution does not completely prevent wound healing. If this approach is developed further for clinical study, it might be advantageous to treat only the peritoneal surface of the polypropylene mesh, and spare

the side that is in direct contact with parietal peritoneum, abdominal wall musculature and fascia, to further facilitate incorporation and healing.

Our model suggests that adhesions present and persistent by day 7 are likely to be durable over time, and this had been confirmed by others (35-38). It is possible that the agent used to prevent recruitment of fibroblasts and other cellular players of the initial adhesiogenic pathway, whether a pharmacologic or mechanical barrier, may be most important in this early window. A caveat is that we cannot determine in this mouse model whether the intervention with hydrogel and/or SRL will weaken the effectiveness of the hernia repair over time. This effect while important was not formally assessed in the current study.

In summary, the creation of a SRL drug-eluting hydrogel-impregnated polypropylene mesh substantially reduced intestinal adhesion formation to mesh in a mouse model of abdominal wall hernia repair. This combination of pharmacological and physical barrier was safe, effective and offers promise as an alternative approach to reduce postoperative adhesions. Local delivery of SRL was well tolerated, in the absence of systemic absorption or lethal immunosuppressive side effects, and warrants further consideration in future clinical trials.

Table 2-1. Adhesion score.

ADHESION PERCENTAGE	Percentage of mesh involvement in mm^2
ADHESION SEVERITY	0 = No adhesion 1 = Filmy and avascular 2 = Moderately filmy and vascular 3 = Dense and significantly vascular
ADHESION TENACITY	0 = no adhesion 1 = no resistance to separation, easy to separate 2 = moderate force required for separation, blunt dissection 3 = sharp dissection required for separation, cannot separate without damaging serosa 4 = adhesions in a conglomerate with the intraperitoneal organs, impossible to divide and count

Figure 2-1. Necropsy photo: intestinal obstruction with adhesive loop of intestine to edge of mesh in one case (hydrogel control group).

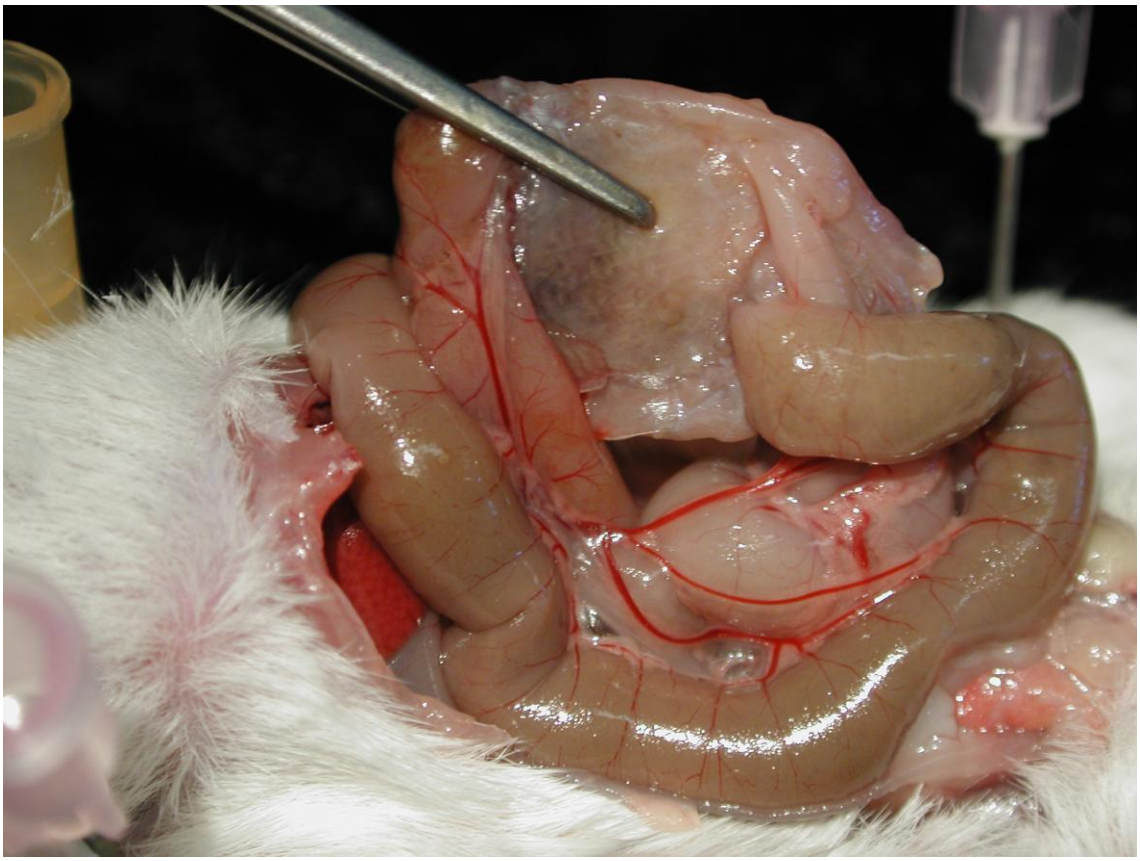


Figure 2- 2A. Macroscopic image of selected representative mesh specimen at 4 week necropsy. Plain mesh became completely covered with adhered abdominal viscera, obscuring mesh.

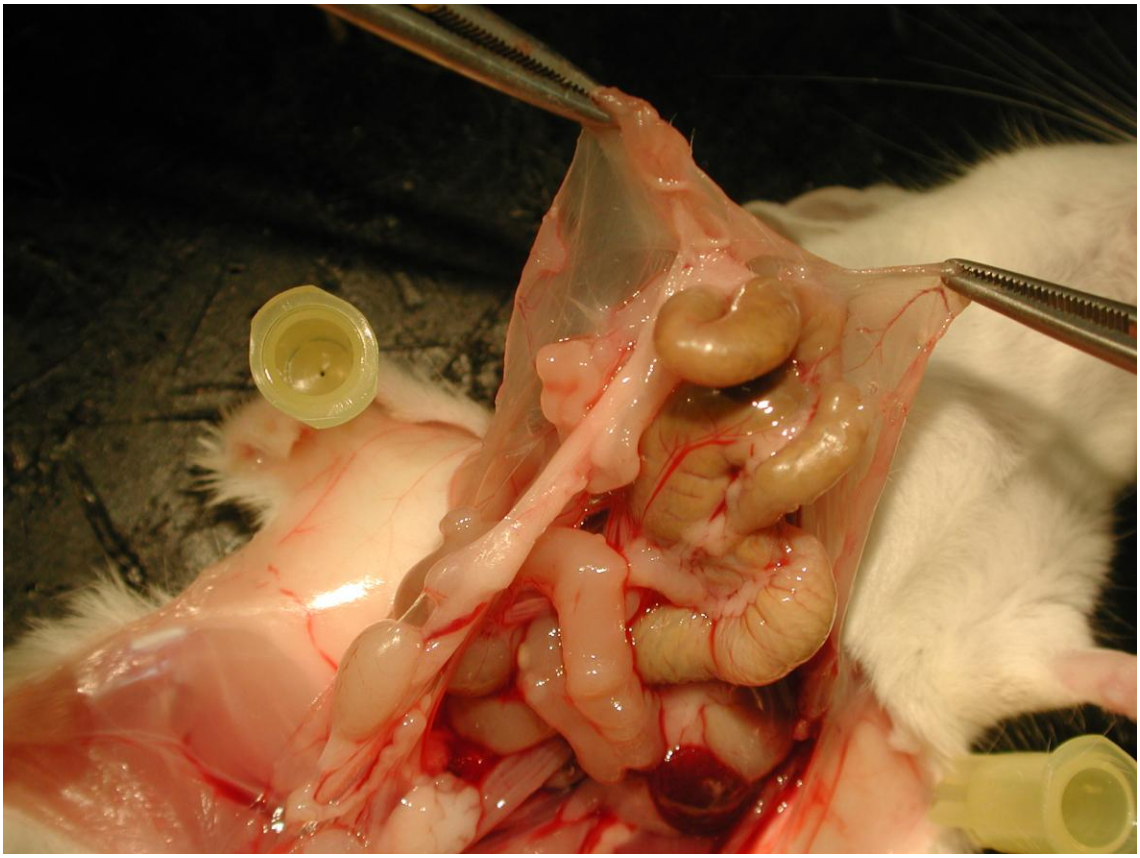


Figure 2-2B. Macroscopic image of selected representative mesh specimen at 4 week necropsy: notable central sparing of mesh in the hydrogel group.

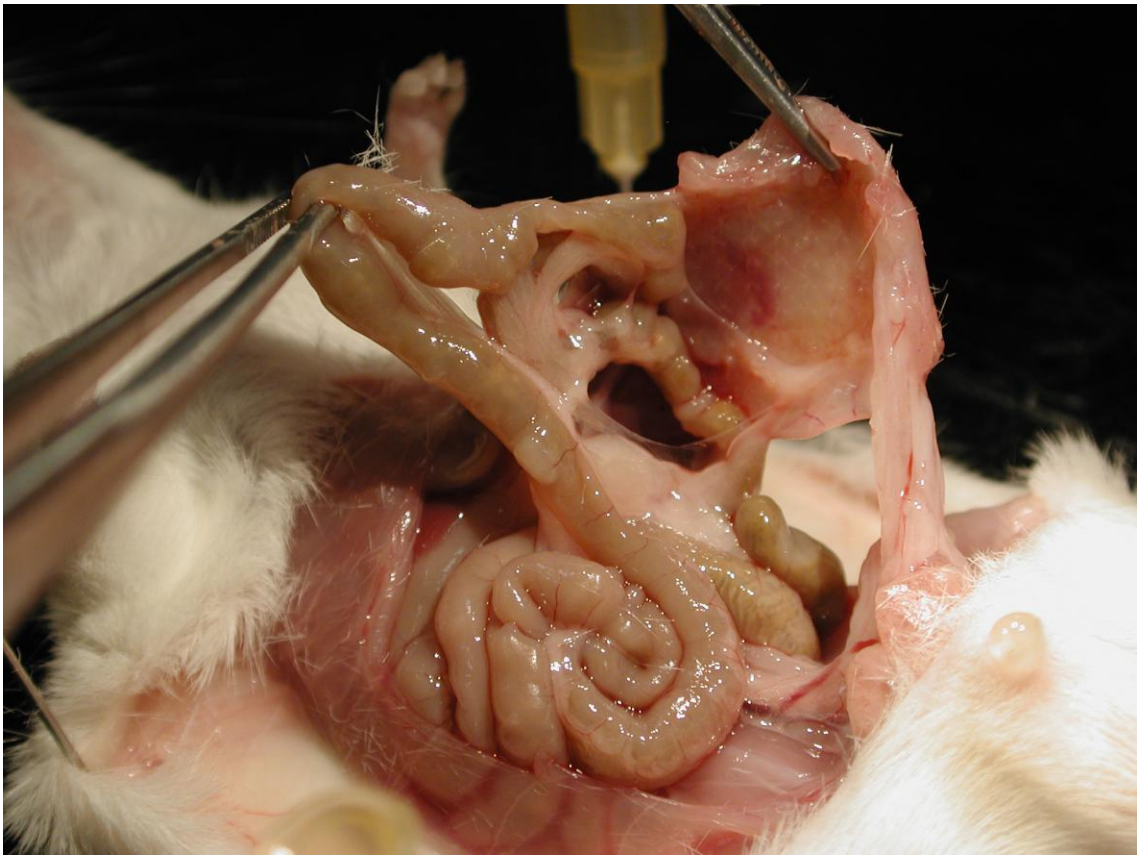


Figure 2-2C. Macroscopic image of selected representative mesh specimen at 4 week necropsy: notable central sparing of mesh in the hydrogel + SRL group, further reduction in adhesion incidence.

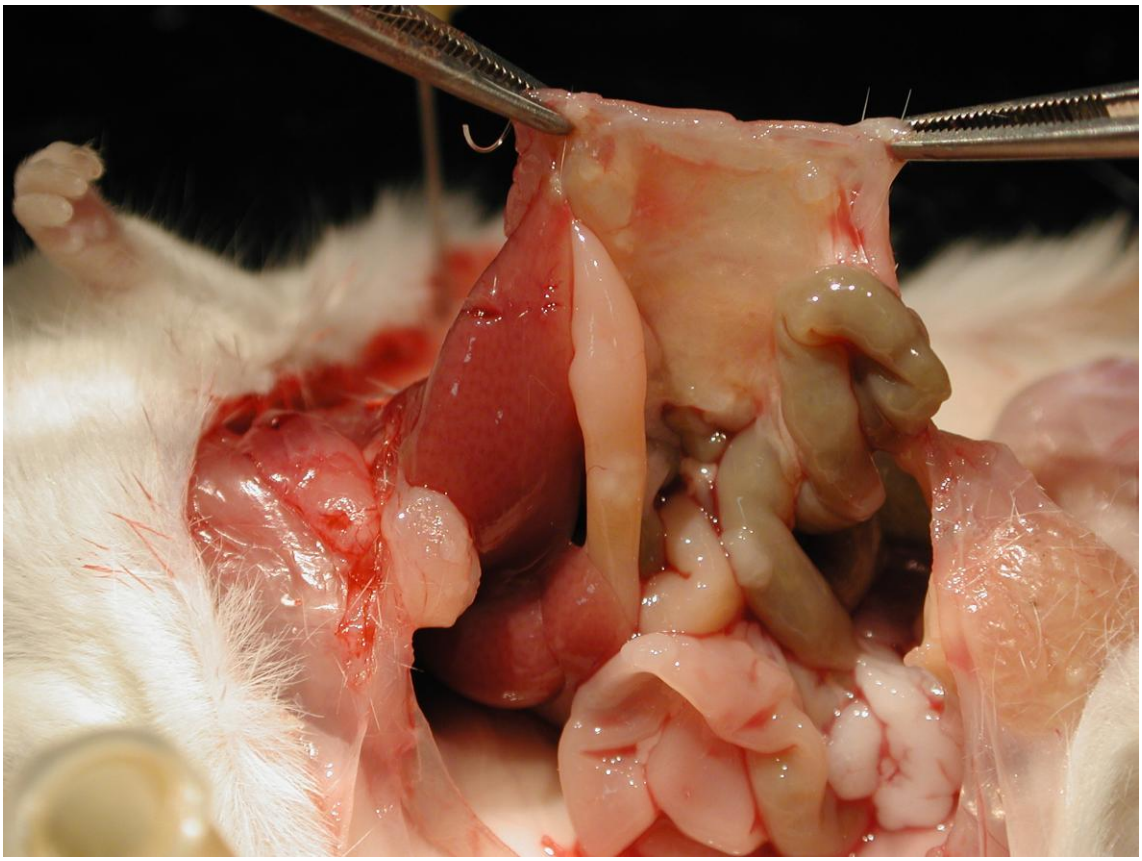


Figure 2-3A. Time-course study of the model using plain polypropylene mesh: Percentage of mesh surface area covered by adhesions, assessed at necropsy at sequential time points (n=5 weeks 2-12, n=4 weeks 1, 24).

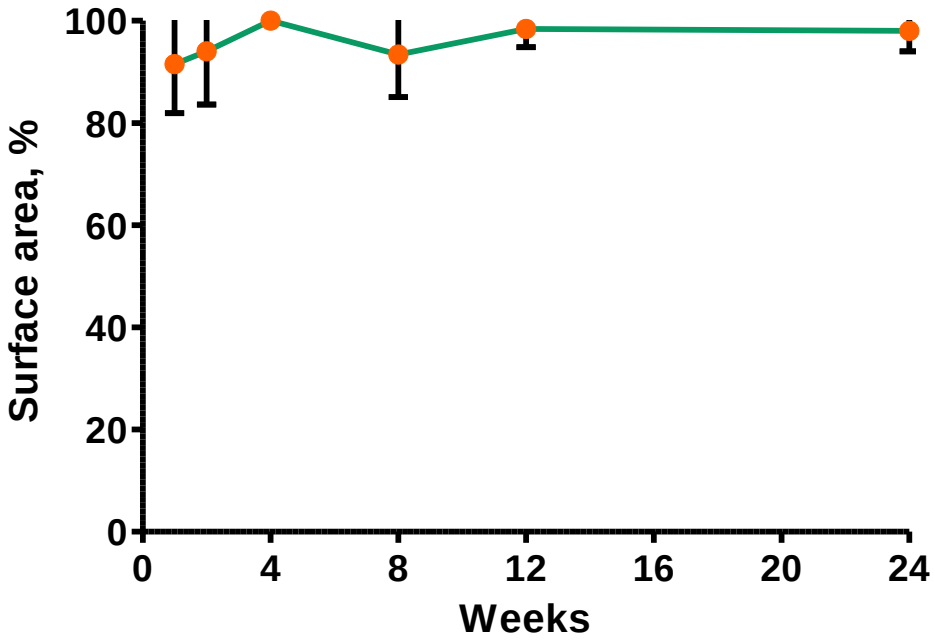


Figure 2-3B. Time-course study of the model using plain polypropylene mesh: Severity score of adhesions, assessed at necropsy at sequential time points (n=5 for all groups with exception of n=4, weeks 1 and 24). *p<0.05, **p<0.01.

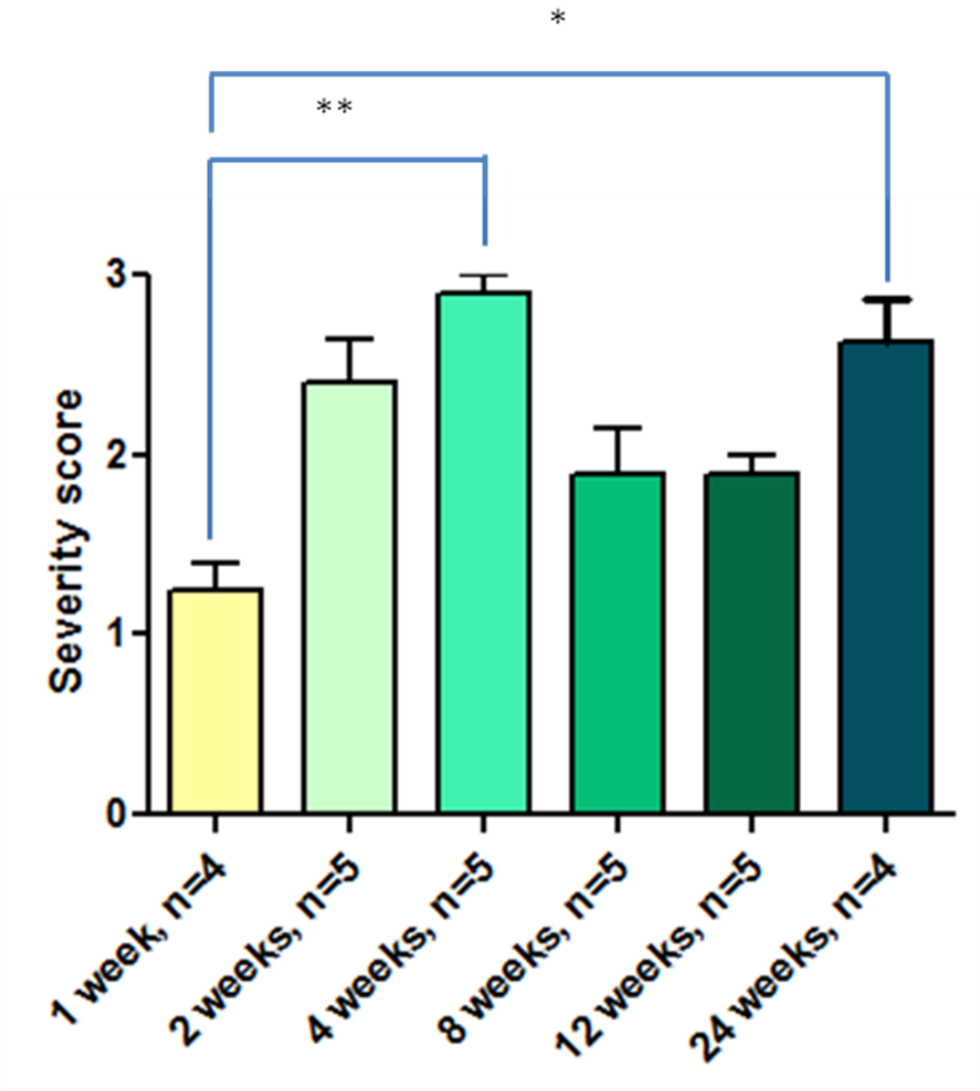


Figure 2-3C. Time-course study of the model using plain polypropylene mesh: Tenacity score of adhesions, assessed at necropsy at sequential time points (n=5 for all groups with exception of n=4, weeks 1 and 24). *p<0.05.

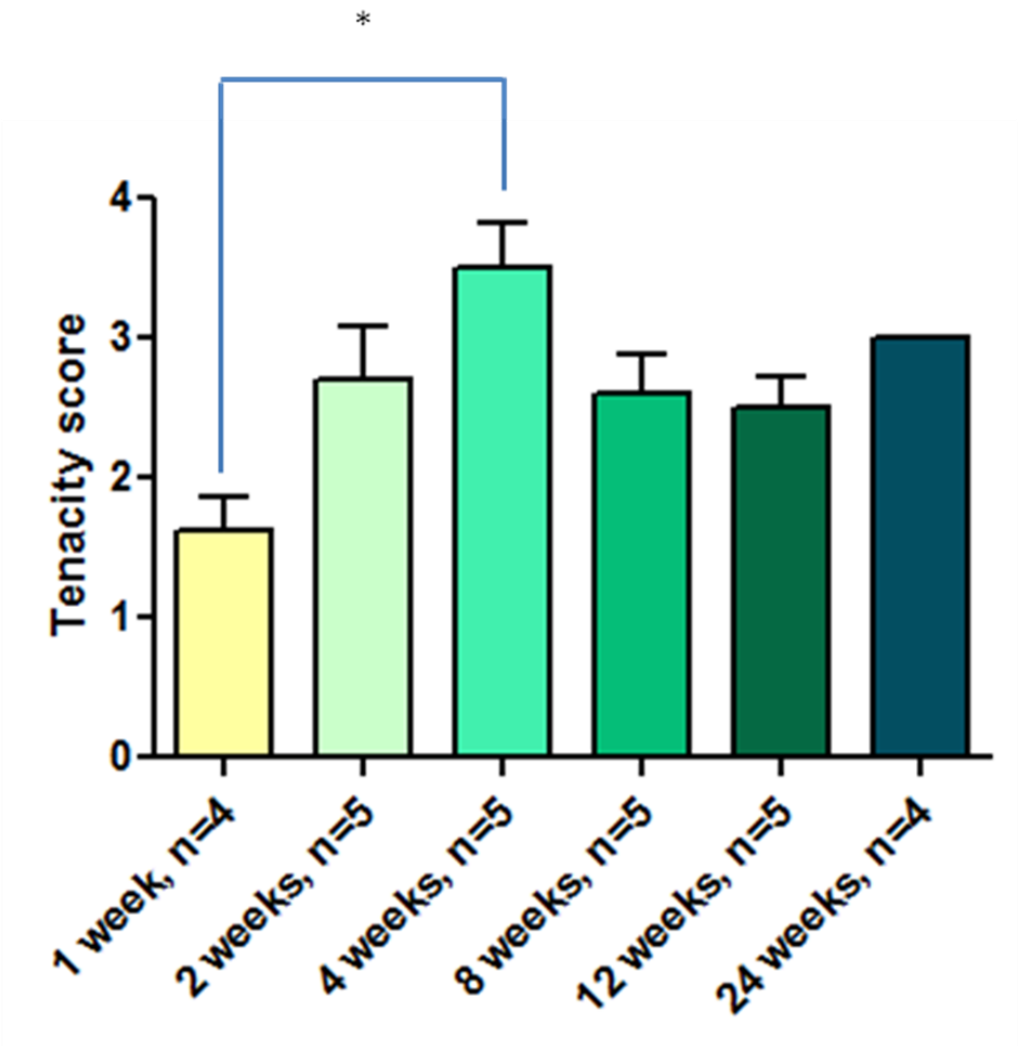


Figure 2-4A. Percentage of mesh surface area covered by adhesions, by group, at 4-week necropsy (n=number of observations). *p<0.05, **p<0.001, ***p<0.001.

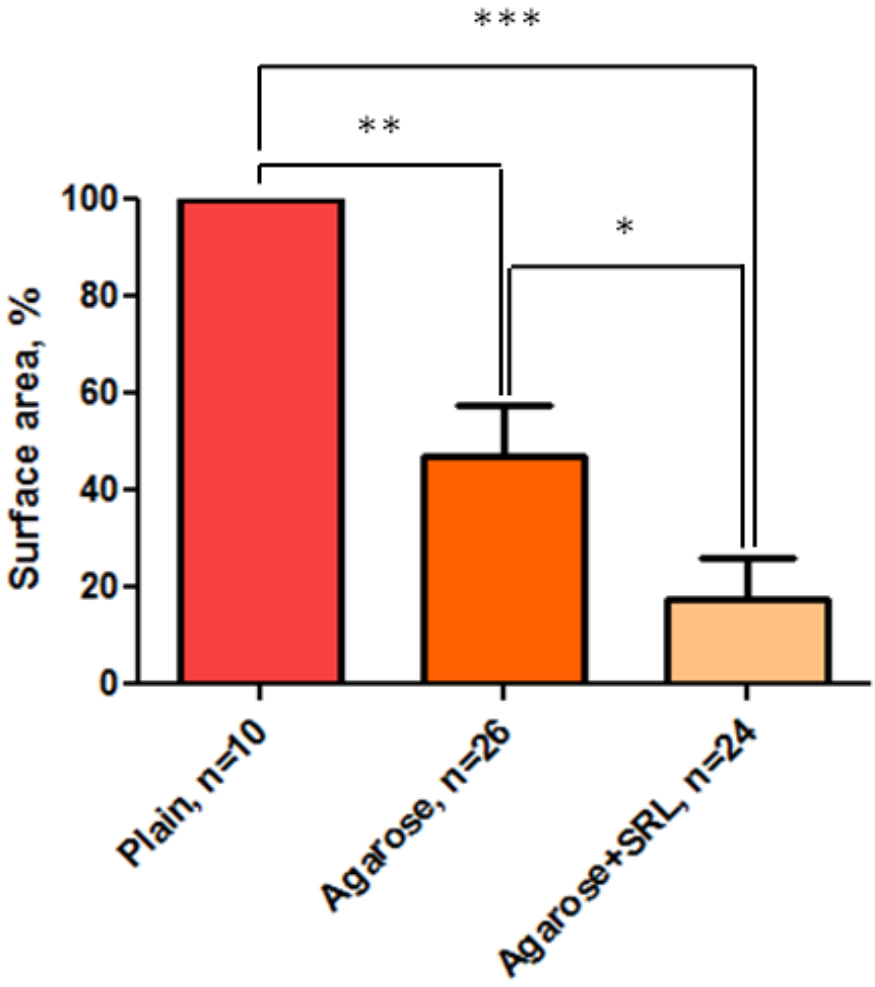


Figure 2-4B. Severity scores of adhesions, by group, at 4-week necropsy (n=number of observations). **p<0.01, ***p<0.001.

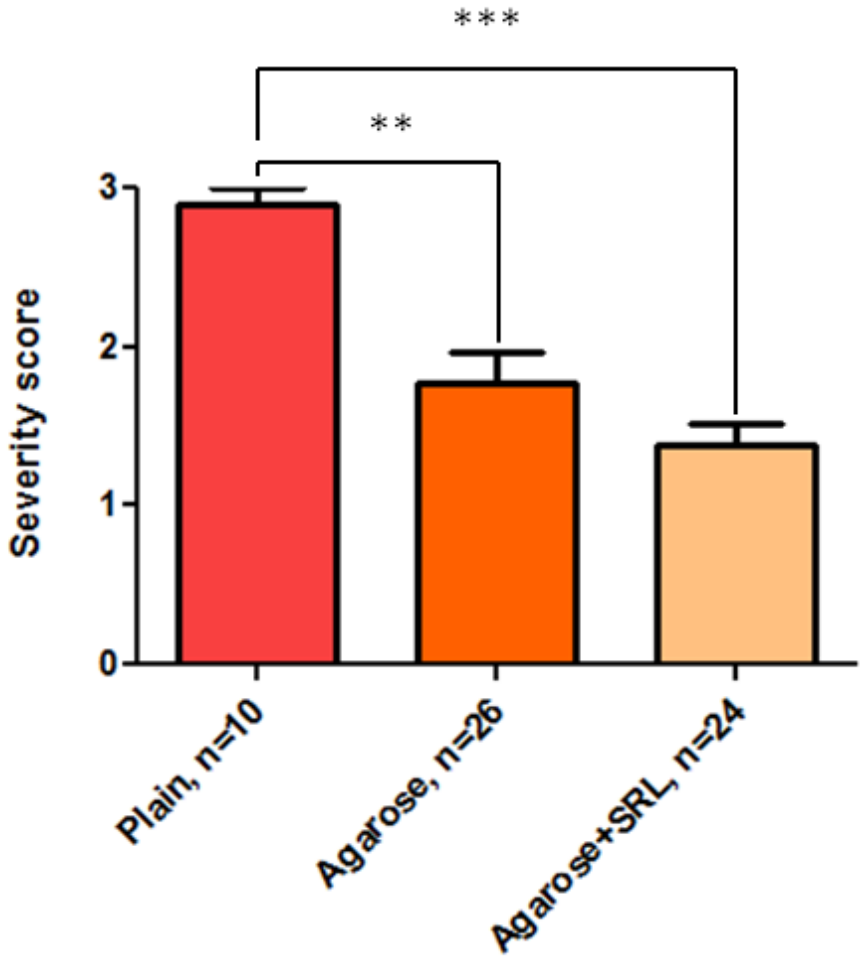


Figure 2-4C. Tenacity scores of adhesions, by group, at 4-week necropsy (n=number of observations). ***p<0.001.

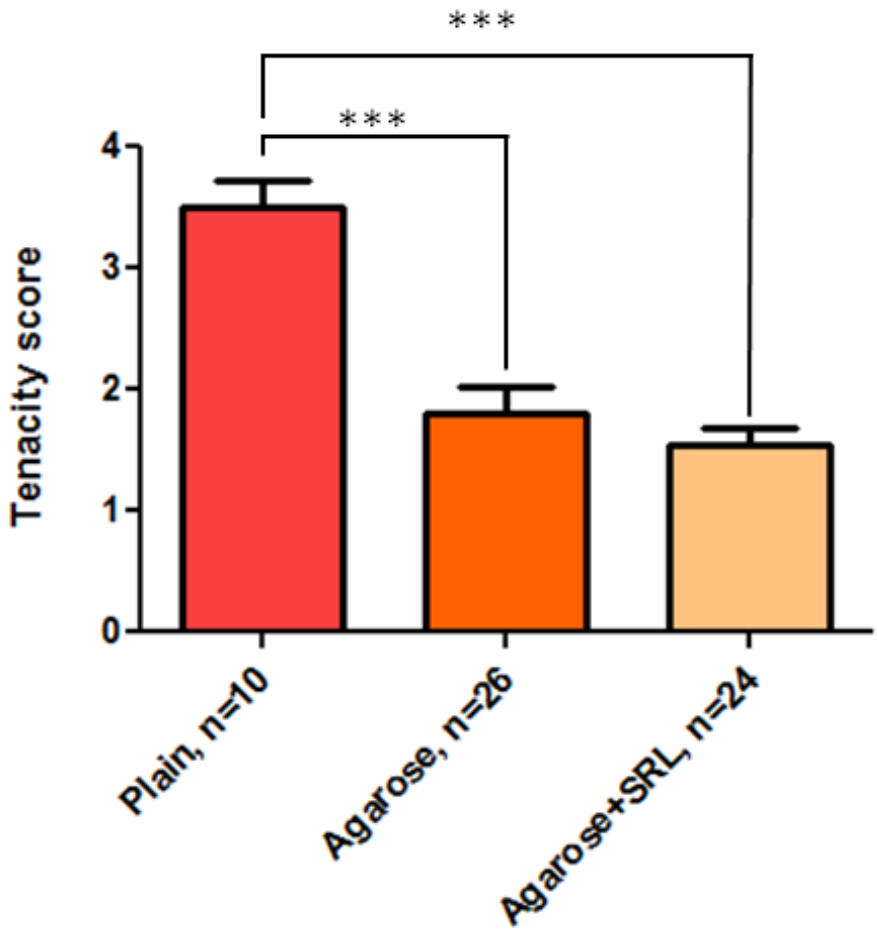


Figure 2-5. Scanning electron microscopy of mesh before implantation, plain polypropylene (A) and after treatment with agarose hydrogel (B). Porosity of the mesh is decreased (original magnification X 25).

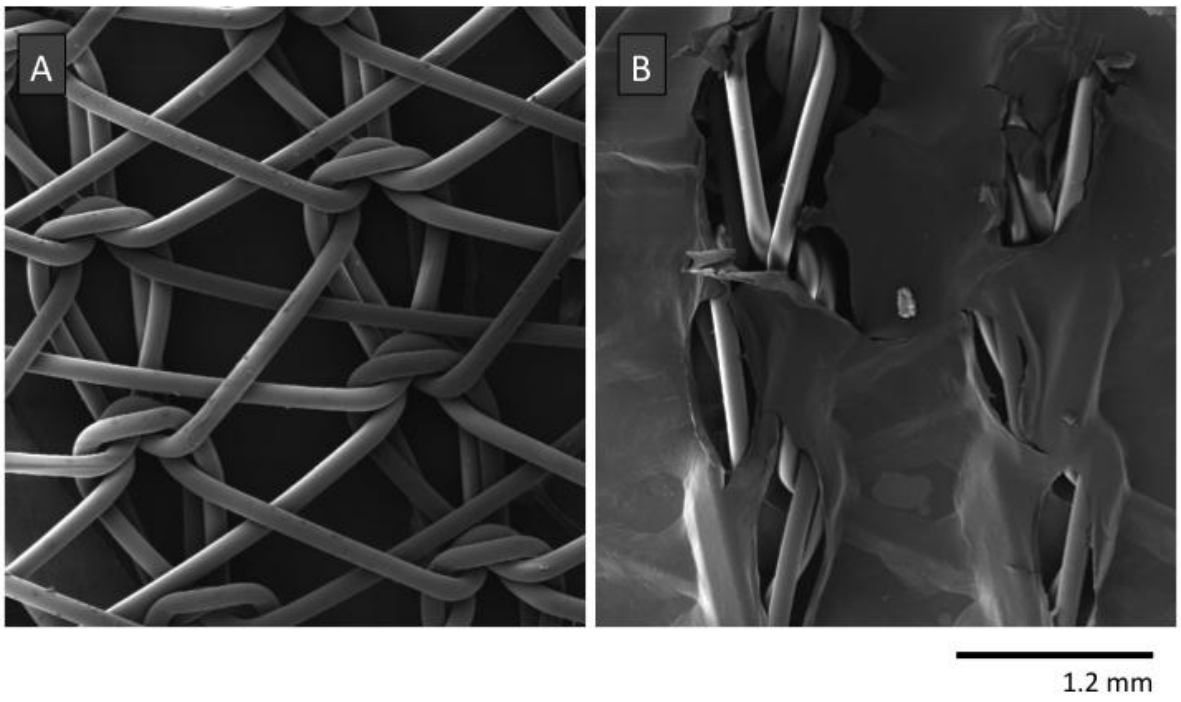
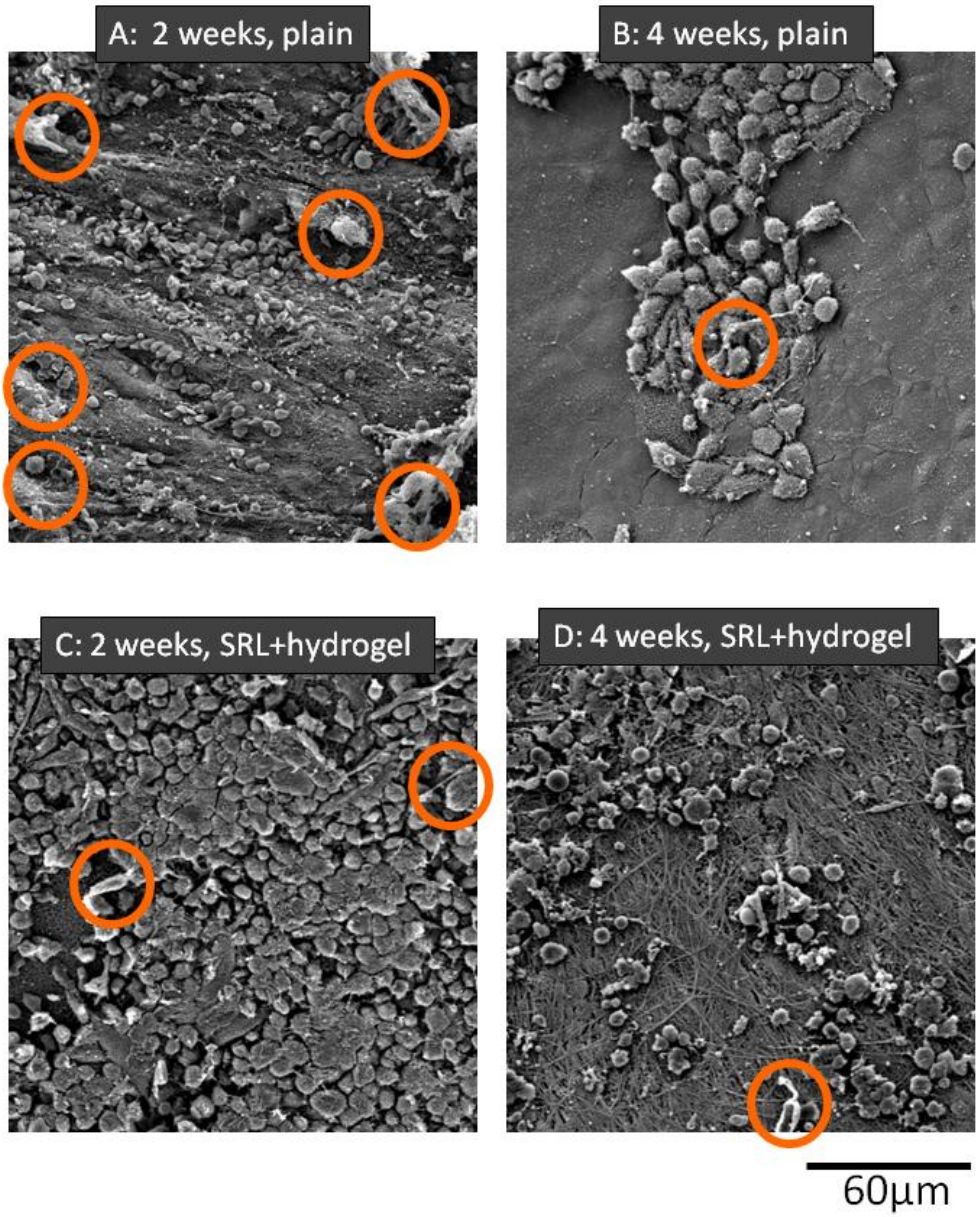


Figure 2-6(A-D). Scanning electron microscopy of viscera-facing surface of representative polypropylene mesh samples; shown are 2 weeks plain (A), 4 weeks plain (B), 2 weeks SRL+hydrogel (C), 4 weeks SRL+hydrogel (D) (original magnification X 500). Fibroblasts are circled; peritoneal mesothelial cells are round regular cells with microvilli. Fewer fibroblasts and more mesothelial cells are noted in the mesh treated with SRL-eluting hydrogel.



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Conclusions and Future Directions

Postoperative intraperitoneal adhesions remain a vexing clinical problem. While it can be debated whether their formation represents a disordered form of peritoneal wound healing or a normal physiologic response to injury, the complications arising from their occurrence pose an enormous clinical and cost burden to patients. As more of the world's population gains access to surgical therapies, an unprecedented incidence of small bowel obstructions, female infertility, hemorrhage and enterotomy on relaparotomy, and adhesion-related chronic pain may be expected if there are not effective therapies to reduce or prevent adhesion formation. Given that adhesions are almost a certainty on reoperation, one might argue it is unacceptable that there has been comparatively very little investigation to date into finding targeted therapies, and efforts to bring these into clinical use.

Our interest in using sirolimus as both a pharmacologic and barrier method to reduce adhesion formation was based on two principles: firstly, on the unpublished observation that transplant recipients receiving rapamycin for immune suppression seem to have fewer adhesions on relaparotomy, and the mounting evidence that this drug has potent antifibrotic, anti-inflammatory, antiproliferative, and anti-angiogenic properties for which there may be a wider range of applications than it is currently used for.

A mouse model was developed to create a stable, reproducible adhesion formation response that could be quantified using published scoring systems. Plain polypropylene mesh is used in the setting of abdominal hernia repair, and incites extensive adhesion formation when placed in the peritoneal cavity and a surgical trauma is applied to the viscera in contact with it. Plain polypropylene mesh was chosen for this model to produce a robust adhesiogenic response but also to create a platform that facilitated quantification of the reductions achieved by the experimental agents. Preliminary time-course studies of this model using plain mesh suggested an ideal time for necropsy and adhesion score is 4 weeks, at which point adhesion incidence, severity and tenacity scores are more likely to be stable. This time point was also consistent with many other adhesion studies in animal models in the literature.

By applying the anti-adhesion substance or drug of interest directly to the mesh, a sensitive index of propensity for adhesion formation is created. Using hydrogel alone significantly decreased the incidence, severity, and tenacity of adhesions to the mesh after 4 weeks in this model, and addition of sirolimus to the hydrogel offered further significant reduction in adhesion incidence. Mesh in all specimens was incorporated into the contacting peritoneum on the free edges, but the two treated mesh groups (hydrogel and hydrogel + sirolimus) had significantly more surface area of

the mesh free of adhered viscera. The mesh intervention itself was well tolerated by the animals, there was no increased risk of infection, and there was no detectable level of sirolimus in serum as early as 1 week post operation. There were no differences in mortality from all causes between the groups.

Impregnation with agarose transformed the mesh from a textured, hydrophobic, macro-porous material into a smooth-surfaced, hydrophilic, micro-porous one. It has previously been established that these qualities play a fundamental role in adhesion formation, consistency, and in the organization of the neoperitoneum formed between the prosthetic and the visceral peritoneum. We would speculate the agarose provides a matrix for the drug of interest to elute from, and as the peritoneal fluid bathes and hydrates the hydrogel, a microenvironment of higher drug concentration is created locally as the drug diffuses into this fluid. Sirolimus had a local effect of reducing adhesion incidence in this model, an observation which may be attributed to one or more of its mechanisms as an antiproliferative and antifibrotic agent.

The device developed is inexpensively made, using easily obtained materials. Approximate cost of raw materials for one 1 cm^2 mesh, using the latest pricing from Sigma Aldrich and Ethicon, is less than $10 (**Table 3-1**). Making the mesh also required use of laboratory equipment and

space (hot plate, petri dishes, syringes, pipets, blunt tip large gauge needles, parafilm, foil, refrigerator, etc.) as well as technician time and assistance, and this estimate also does not include the cost of commercialization. It represents a relatively low-cost solution to the very costly problem of complications due to adhesion formation.

Future work with this model can improve its viability as a clinical intervention, both in the setting of hernia repair, and as a surgical adjunct. Hernia mesh requires ingrowth of native tissue to ensure an effective repair, and ways to quantify this have been developed such as explant strength testing. A future student may continue this work by attaching a dynamometer to the mesh and fixing the abdominal wall with sutures; the force required to disrupt the mesh from the surrounding tissue represents the explant strength. This information would address concerns that the hydrogel or sirolimus may have detrimental effects on repair success. Data regarding would healing may be developed further to address this complication of sirolimus in immunosuppressant use.

Histological analysis of specimens was impeded by the technical challenge of cutting mesh, however, accomplishing this will provide valuable information regarding the presence of fibroblasts, collagen and fibrosis, and confirm the SEM observations of populations of peritoneal mesothelial cells. Future development of dose response curves may be

used to minimize the effective concentration of sirolimus required, as well as determine side effect profiles at higher doses. A sirolimus-containing hydrogel sheet may be made and trialed in other established models of adhesion formation, such as the rabbit uterine horn or rat cecal abrasion model, to evaluate the benefit in a different surgical challenge. While a small cohort of 4/5 animals receiving sirolimus-eluting, hydrogel-impregnated mesh survived to necropsy at 1 year, long term follow-up is necessary to document complications from the use of the device.

The vast majority of discoveries made in animal models in the area of adhesion research have not been employed clinically. The reasons for this are many-fold, and may include inaccessibility of the device/drug, limited or no long-term safety data, and the challenges inherent in using a device which may never be visually reassessed for the life of the patient. Follow-up laparoscopy to identify adhesion formation puts patients at unnecessary risk, and so confirmation of findings in animal models may be a remote possibility for many of the interventions that have been proposed. As well, it is difficult to estimate the clinical relevance and meaning of adhesion reduction, if the end-points do not include the complications of adhesion – some of which are symptomatic in the early postoperative period, and others may take years, if ever, to manifest in an individual patient. We can only theorize that reducing adhesion incidence relates to an associated decrease in complications, and accordingly,

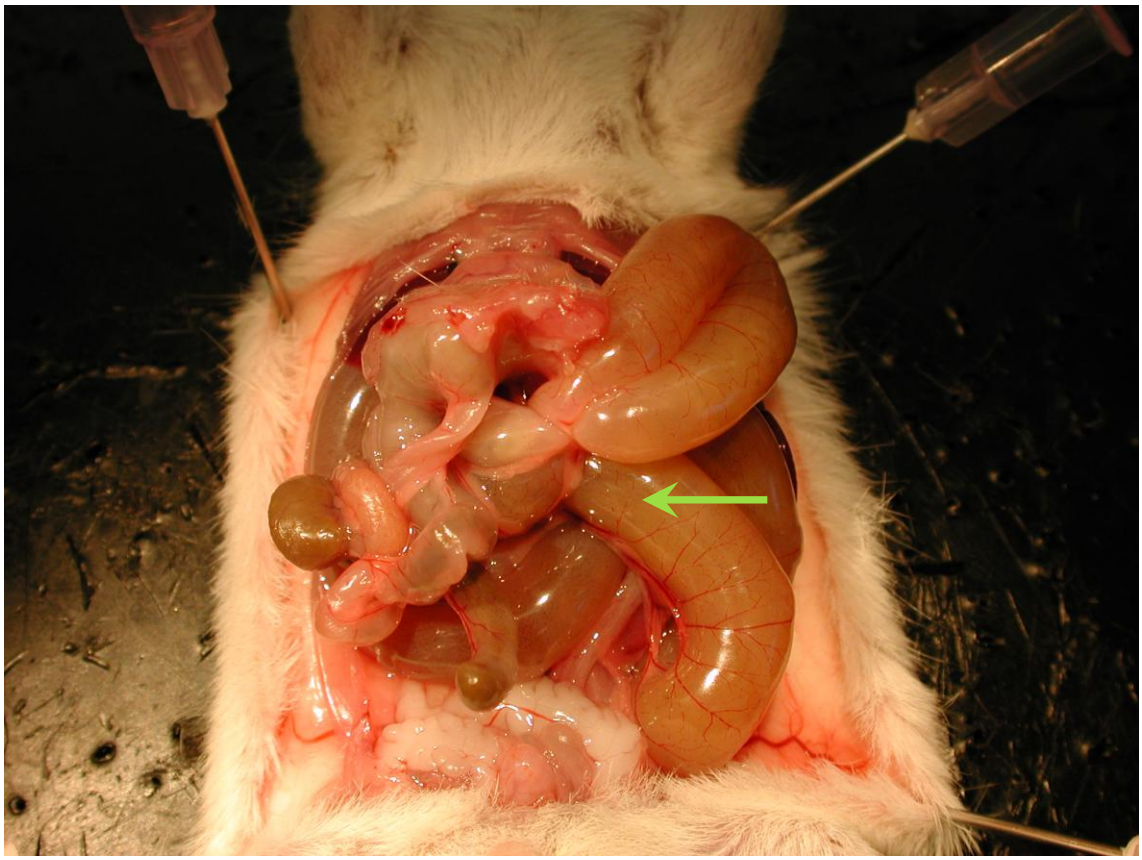
reducing adhesion incidence to zero may be the only true successful outcome to pursue (**Figure 3-1**).

The majority of what is known about adhesion formation has been discovered only within the last twenty years. Despite real difficulties in assessing the safety and clinical efficacy of anti-adhesion adjuncts, the sheer magnitude of adhesion-related complications and cost should compel surgical research to facilitate this leap: from a safe, successful model to a trusted, effective clinical intervention.

Table 3-1. Cost estimation of raw materials for 1cm^2 sirolimus-eluting, hydrogel-impregnated polypropylene mesh. Prices quoted are from suppliers Sigma Aldrich and Ethicon and are current at time of printing.

	Cost
Sirolimus, 10 mcg	$4.30
Polypropylene mesh, 1 cm^2	$4.97
Agarose, 0.002g	$0.005
PBS and EtOH to solubilize reagents	negligible
Total	**$9.28**

Figure 3-1. Necropsy photo from animal that required euthanasia before experimental end point. Mesh has been excised before image taken. A single adhesive band (arrow), at a location away from the implanted mesh, appears to be responsible for a volvulized segment of intestine and resulting obstruction. This image illustrates the potentially severe complications of any adhesion formation, regardless of extent.



APPENDIX

APPENDIX A: DEVELOPMENT OF THE MODEL

Several different materials and methods were trialed before arriving at the current model.

Selecting an Animal Model

There are several established animal models in the literature to study adhesions, including the rabbit uterine horn and the rat cecal abrasion model, as well as similar murine studies. Choosing a mouse model offers the benefits of a small scale, as well as the convenience of access to mouse equipment and housing in our lab facilities. Anecdotal reports suggest mice are more likely to form intestine-intestine adhesions than rats, which is a very clinically relevant finding.

Promoting Adhesiogenesis

It was found that implantation of the mesh alone was not a sufficient stimulus to adhesion formation in the majority of animals. Swabbing of the abdominal contents with a cotton-tip applicator was necessary: animals that underwent sham laparotomy (no rub, no mesh) were found to have no adhesions on necropsy.

Fixation of Mesh

In the first series of animals, the mesh was sutured to the parietal peritoneum at two points (superior right and inferior left corners). In several cases, abdominal organs (intestine) were then able to become positioned between the abdominal wall and the mesh and were found trapped in this space on necropsy. Securing the mesh with suture at all four corners minimized but did not entirely prevent this situation. Any abdominal viscera which had become incarcerated in this space were not scored as adhesions, as only the side of the mesh facing the peritoneal cavity was considered to be the relevant surface.

Lipectomy

In many of the first series of animals, the two epididymal fat pads had a propensity to migrate caudally and cover the mesh, preventing evaluation of the adhesiogenicity of the mesh. As these structures do not exist in humans, their removal is likely of no clinically relevant consequence. Studies in mice undergoing lipectomy in this fashion suggest the animal soon regains the lost body mass by gaining adipose tissue in other areas. To enhance the sensitivity and relevance of the model, all animals were lipectomized.

Intraperitoneal Delivery of Sirolimus

Sirolimus is commercially available for human pharmacologic use as an oral medication, in tablet or liquid form. Early trials were performed using the mouse mesh model and the crushed tablet form of the drug, however, a robust adhesion reaction was noted on necropsy: the confounding effect of additives (non-medicinal ingredients such as cornstarch) had not been taken into account.

A series of animals underwent mesh implantation and then daily intraperitoneal injections of sirolimus or PBS control for one month. These animals also showed significant adhesion formation on necropsy, and there was a presumed influence of additives to the oral liquid preparation on the adhesiogenicity of the model. These two pilot studies prompted the exclusive use of pure powdered sirolimus obtained by a chemical supplier.

Vehicle for Drug Delivery

Applying the drug to the mesh is a critical step in the process. In the first and perhaps most crude attempt, a sirolimus tablet was crushed and reconstituted into a paste using PBS. This paste was applied directly to the mesh square before implantation. It was then decided that a substance should be introduced to the model in order to keep the drug adherent to the mesh and permit elution of the drug over time.

Several industry contacts were approached, including Angiotech (involved in development of sirolimus-eluting and paclitaxel-eluting coronary stent)

and Wyeth (makers of Rapamune [sirolimus]) however proprietary issues prevented the release of specific and helpful suggestions regarding development of a drug-eluting mesh.

Two substances were trialed in the laboratory as potential vehicles for drug delivery and fixation to the mesh: the first was methylcellulose (10%), which did not solidify >24 hours after preparation and was therefore unsuitable for the purpose, and the second was agarose.

Agarose, in a 2% concentration (similar to that used in gel electrophoresis applications) was easily handled in a warmed, liquid form and quickly solidified to a gel when cooled to room temperature. By adding the sirolimus to the heated agarose while stirring, the drug could be evenly distributed and suspended in the mixture.

APPENDIX B: ELUTION STUDY

To determine the rate of elution of drug from the mesh, an elution study was devised based on consultation with Philip Toleikis at Sernova.

Method:

A 10 cm^2 piece of polypropylene mesh (2 x 5 cm) was prepared using the protocol as outlined in Chapter 2, with the additional condition of sterility imposed. A total of 100 micrograms of SRL in 1cc of 2% agarose was applied. The gel was allowed to harden for >24 hours before being placed in a 250cc bath of sterile fetal calf serum. The sterile container was sealed, placed in a 37C incubator, and agitated by mechanical shaker at approximately 2 cycles/second.

Aliquots of serum (1 cc) were removed at prescribed intervals and sent to the hospital lab for quantification of sirolimus levels.

The experiment was performed in triplicate and exposure to light was minimized.

Results:

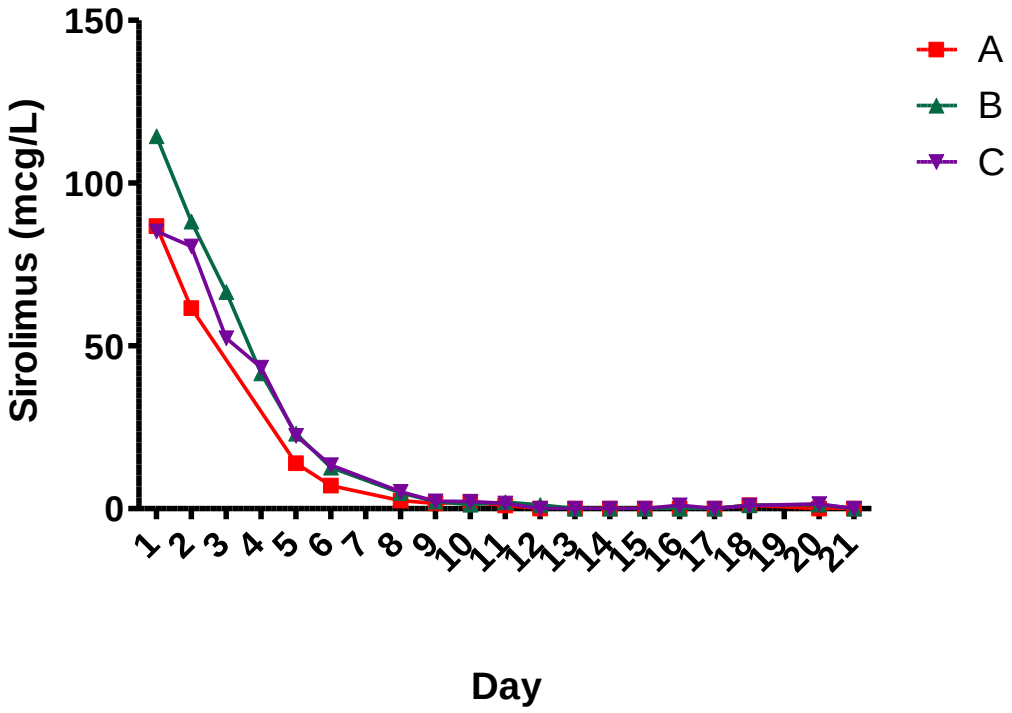
The elution curve produced is seen in Figure Appendix B – 1. The maximum amount of sirolimus that could be measured in the serum (assuming all drug leaves the mesh and becomes suspended in the serum) is calculated as 400 mcg/L. All three trials had a maximum level of sirolimus at first measurement (at t=6 hours) that declined precipitously over the first few days.

At the end of the experiment (day 21), the agarose was still present on the mesh and had not dissolved or degraded on gross inspection.

Discussion:

There may be several reasons to explain the lower than expected levels of sirolimus. Firstly, the laboratory testing is normally done on samples of whole blood, which has higher proteins and lipid content. Sirolimus binds to these substances and the assay may be exploiting this property to estimate the quantity. Actual levels of sirolimus in the serum may be therefore higher than reported, a caveat which we were warned of on submitting the samples for analysis. The assay should therefore be repeated using a different quantification method (eg. high performance liquid chromatography). It is also possible that a proportion of sirolimus bound to the polymer in the container or indeed remained bound to the mesh or agarose. Additionally, sirolimus is known to be light-sensitive and it is possible that the drug degraded with time.

Figure Appendix B-1. Elution of sirolimus into fetal calf serum over time. Experiment performed in triplicate (groups A, B, C).



APPENDIX C: HISTOLOGY

Randomly selected specimens of mesh from three experimental groups (plain control, hydrogel control and SRL + hydrogel) at the 4-week time point were excised and fixed in Z-fix solution. After preparation into slides using standard lab protocols, the specimens were stained to identify and characterize the tissue reaction that the implant incited. Opinion was provided by Dr. Aducio Thiesen of the Department of Laboratory Medicine and Pathology.

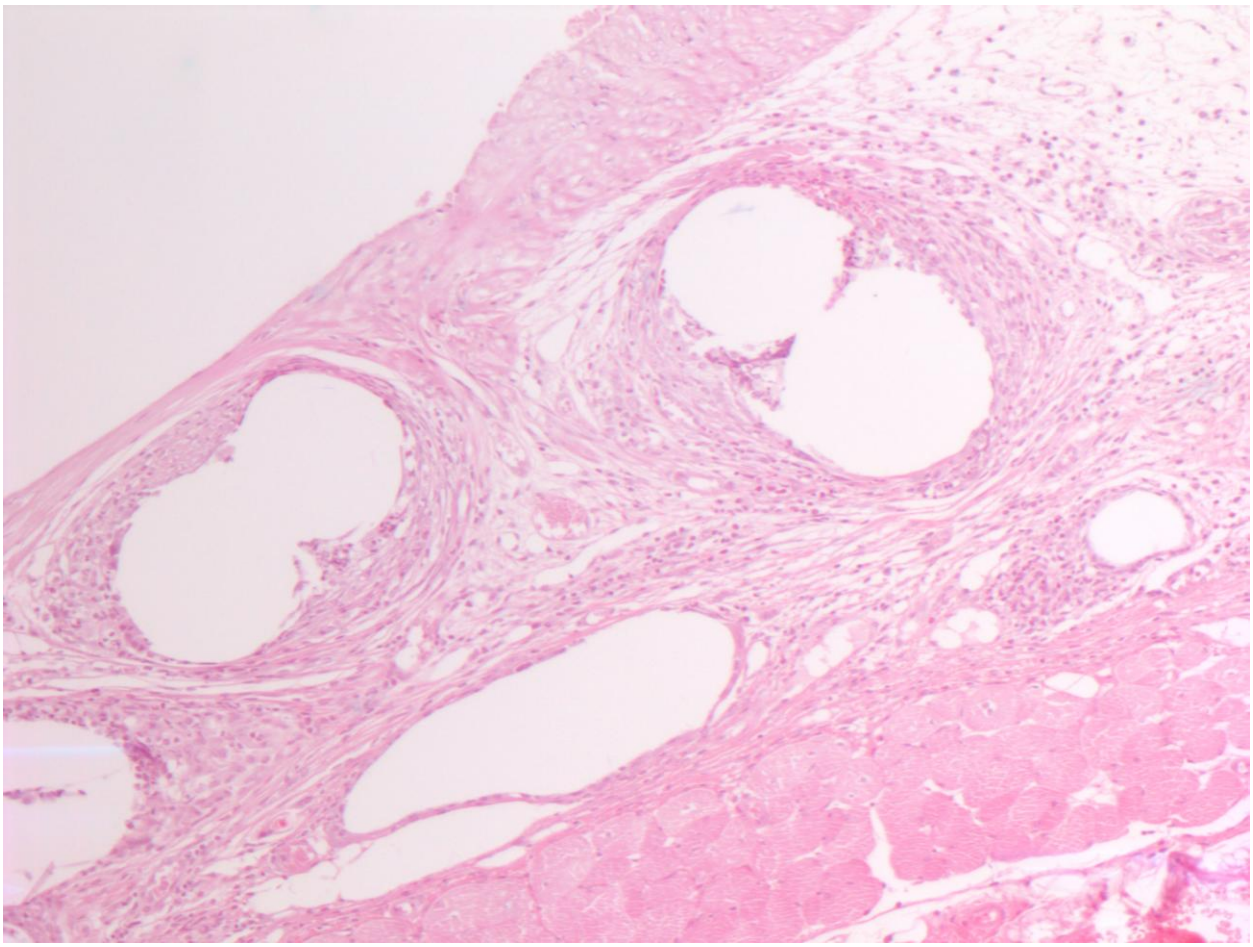
Hematoxylin and eosin stain (H&E) was first used to determine the cell populations and architecture present (Figures 1A-C). Masson's Trichrome stain was performed to identify areas of fibrosis, the presence of which is indicated by a bright blue staining (Figures 2A-C). Immunohistochemistry using D2-40 was also performed to mark the presence of peritoneal mesothelial cells (Figures 3A-C).

It was found on histologic examination that there was fibrosis and collagen around the mesh fibres in all specimens, but a more pronounced reaction was seen in the plain mesh samples. In mesh that had been treated with agarose hydrogel (with or without SRL), there was an increase in the population of mesothelial cells and their architectural arrangement was in keeping with an organized layer upon the agarose. It can be theorized that the fibrosis around the mesh filaments confers structural strength to

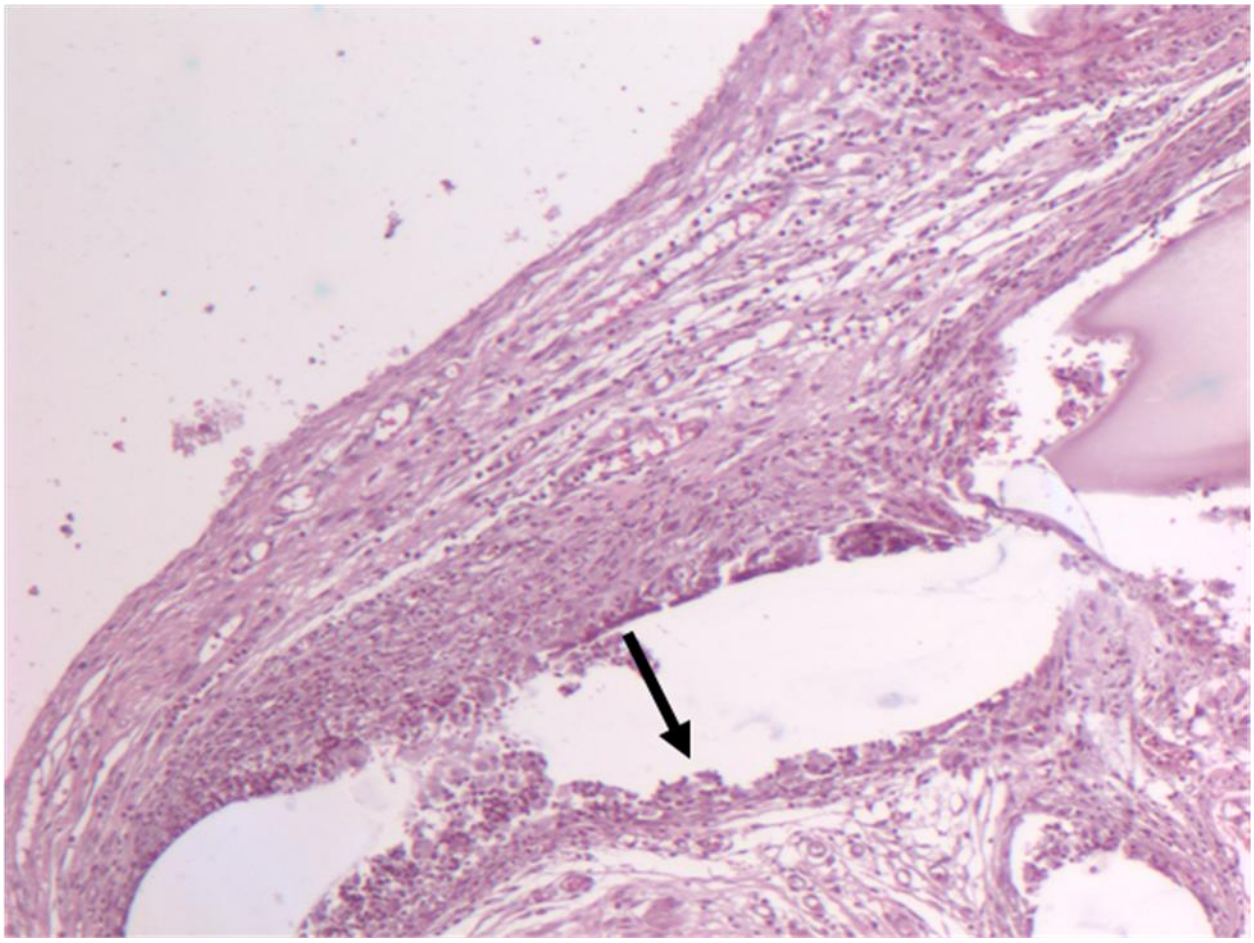
the hernia repair model, while the layer of “neoperitoneum” which forms over the agarose prevents adhesion formation.

Figure Appendix C-1 A-C: Hematoxylin and eosin staining of 4-week specimens from (A) plain mesh, (B) agarose hydrogel and (C) agarose hydrogel + SRL. Arrows denote the presence of peritoneal mesothelial cells.

(A)



(B)



(C)

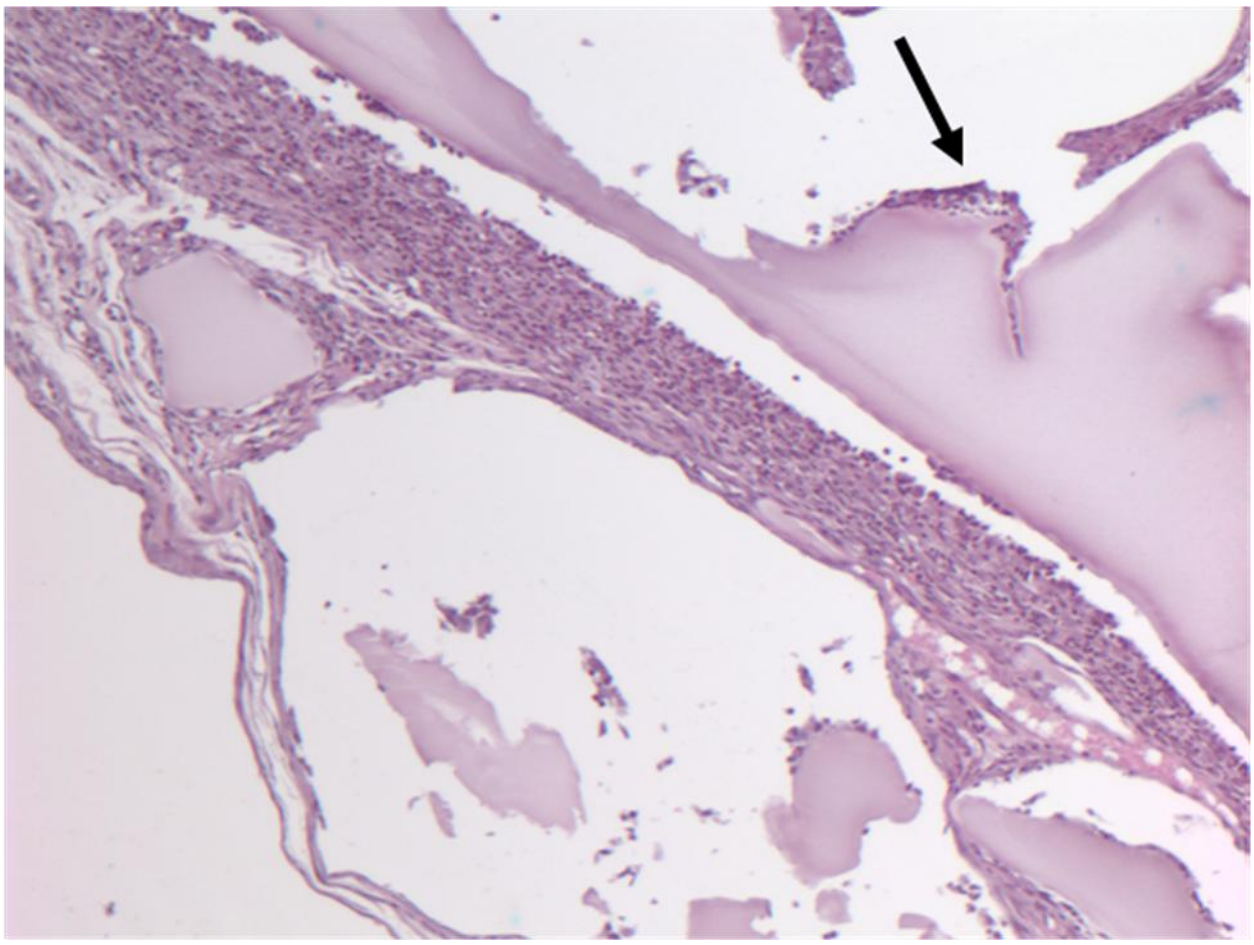
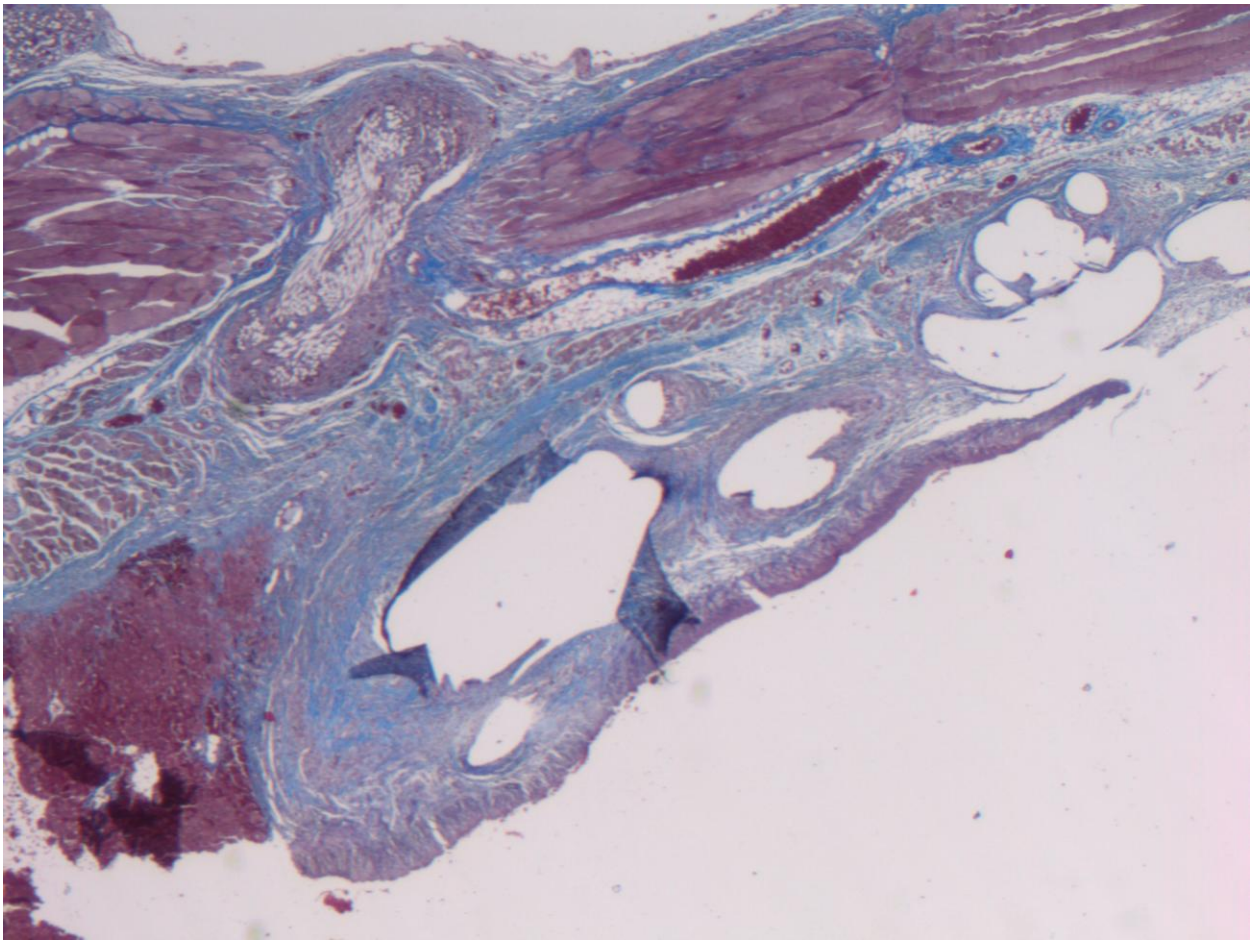
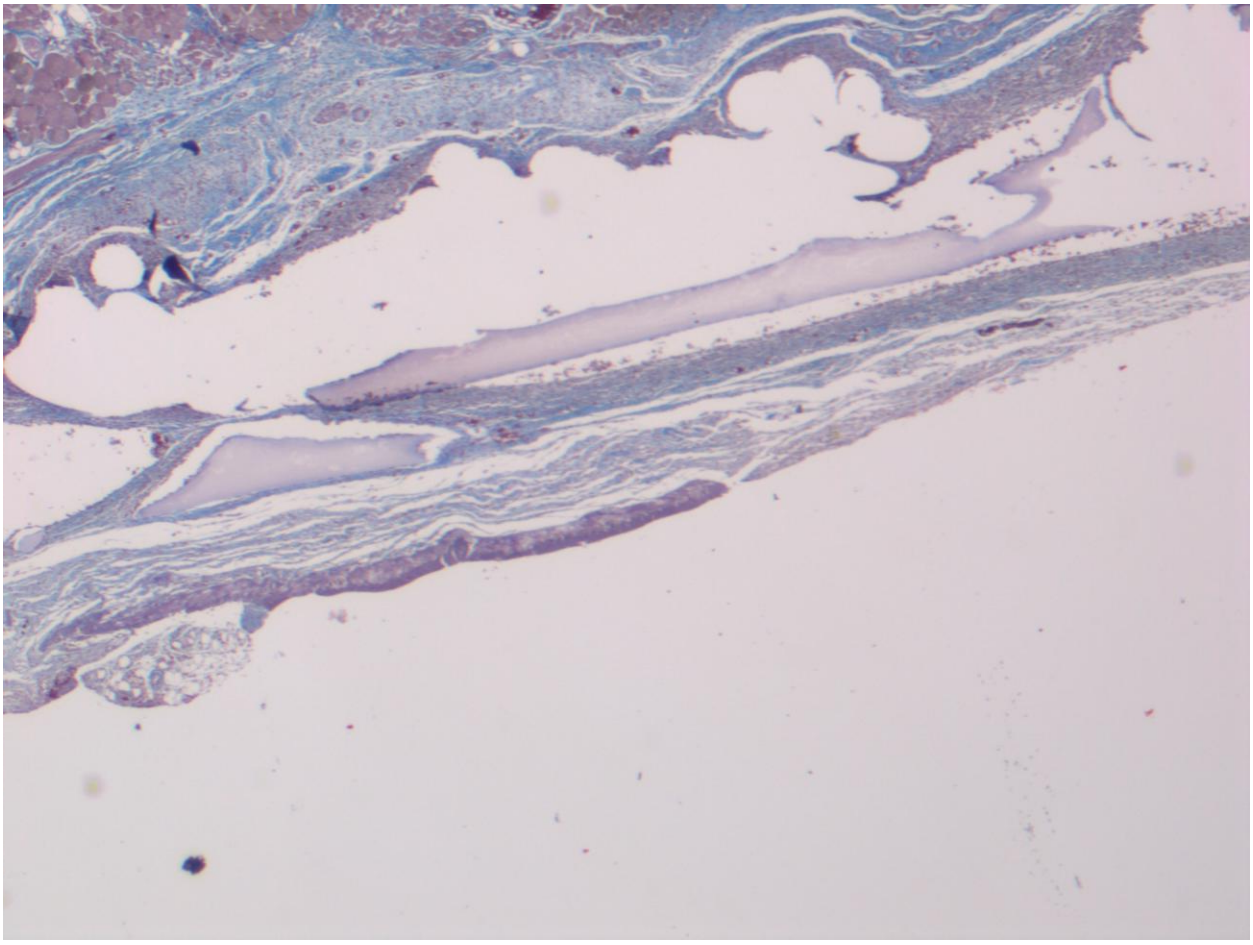


Figure Appendix C-2 A-C: Masson's Trichrome staining of 4-week specimens from (A) plain mesh, (B) agarose hydrogel and (C) agarose hydrogel + SRL. Fibrosis stains blue.

(A)



(B)



(C)

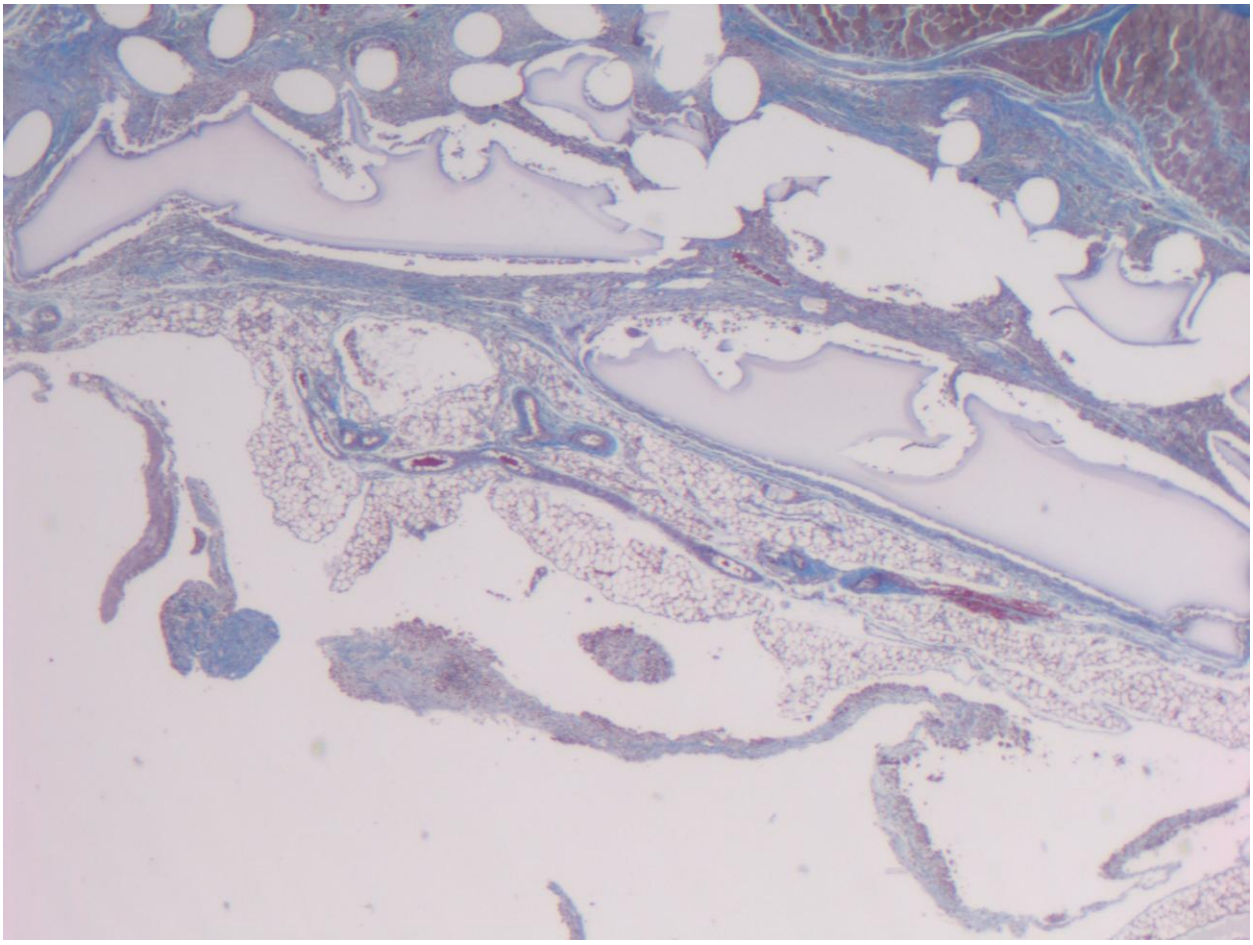
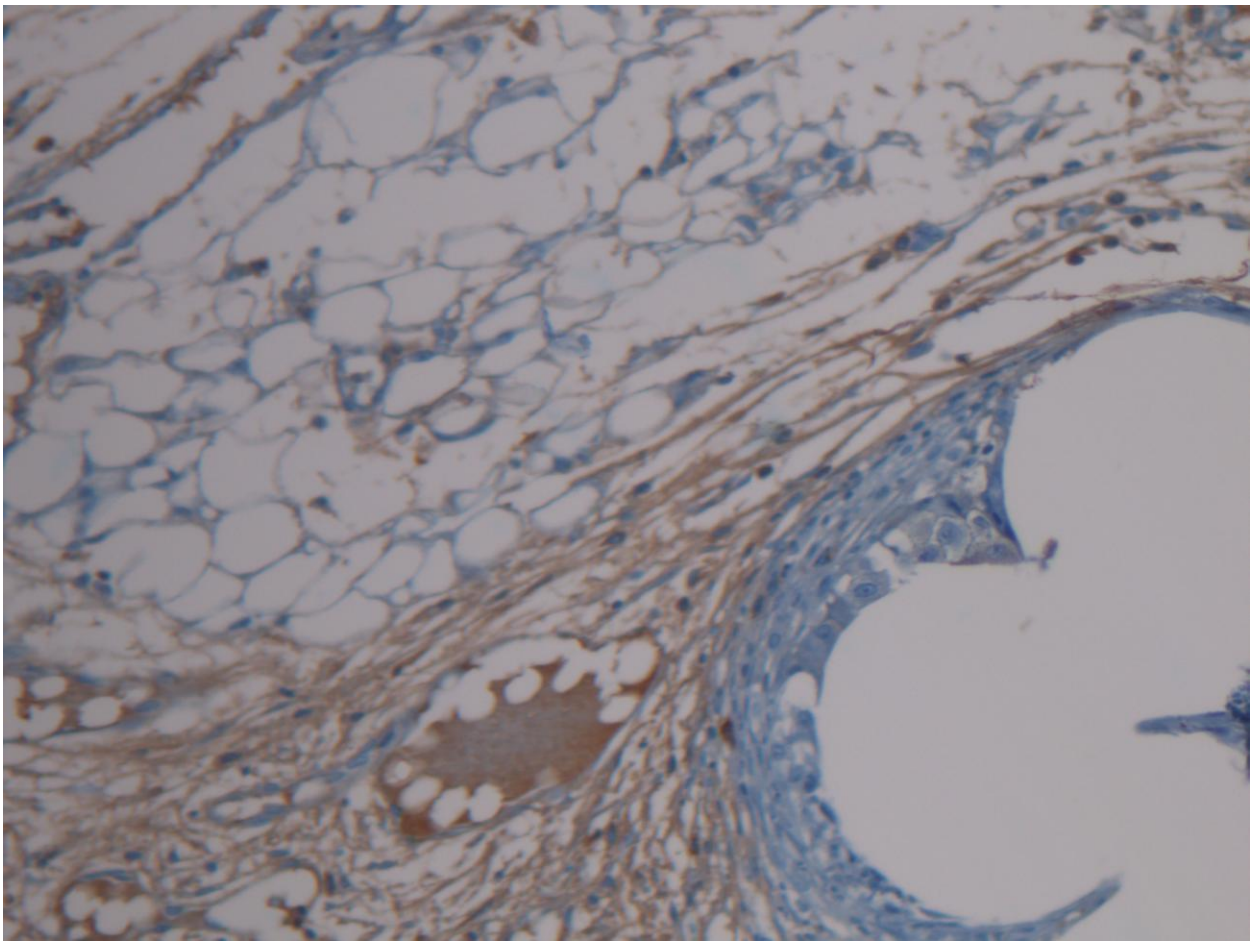
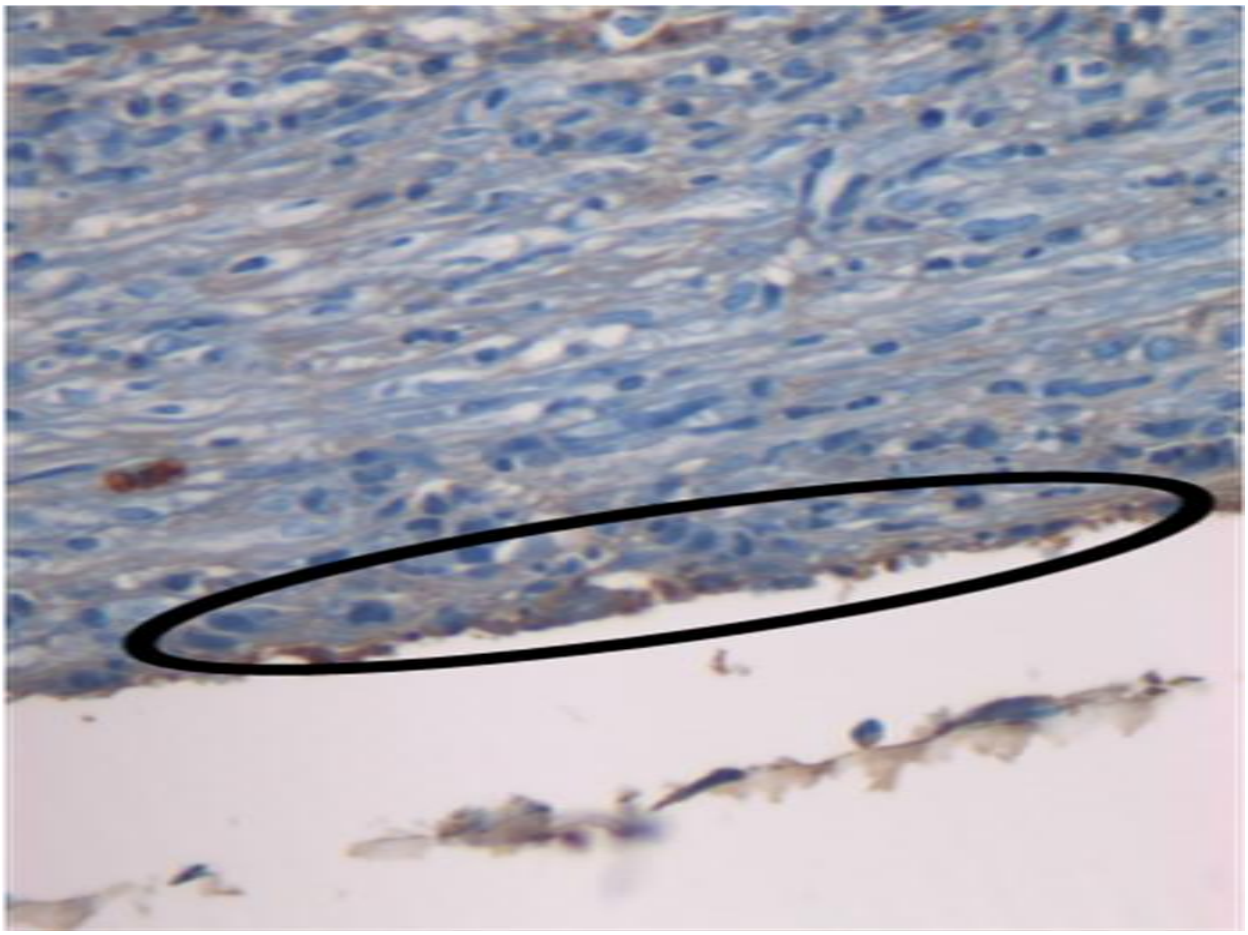


Figure Appendix C-3 A-C: Immunohistochemical staining for D2-40 in 4-week specimens from (A) plain mesh, (B) agarose hydrogel and (C) agarose hydrogel + SRL. This marker of peritoneal mesothelial cells appears brown. Lines of organized peritoneal mesothelial cells are indicated by black ellipses.

(A)



(B)



(C)