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Novel Therapeutic Approach to Post-Operative Adhesions: Enhancing Resident Repair Cells in the Abdomen

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Abstract

Background: This study proposed to identify the possible mechanism of action of novel treatment, alanyl-glutamine (AG) in adhesion prevention. The aim was to outline the natural history of adhesion formation in a randomized animal model and to confirm the effect of peritoneal infiltration of AG on adhesion formation in rats post-laparotomy. The study also challenged the role of macrophage chemotactic protein 1 (MCP-1) on adhesion formation.

Method: This study involved open abdominal surgery on 53 Wistar rats. They were assessed for AG's efficacy in preventing adhesion. Rats were randomly assigned to three groups: 1) Open laparotomy (no treatment), 2) Open laparotomy with saline, and 3) Open laparotomy with AG. Tissue explants were analyzed and assessed for fibrosis. Macrophage activity was also evaluated using ED1 and CD68 markers.

Results: 6 days after surgery, severe adhesion was evident in the saline and the non-treatment group. This persisted up to day 42, while the treatment group showed minimal evidence of adhesion, with 95%-100% adhesion prevention. MCP-1 did not have a clear role in controlling macrophage infiltration.

Conclusion: Our adhesion model showed a better outcome for adhesion prevention in all the rats instilled with AG after laparotomy. While MCP-1 is a marker of inflammation, it does not appear to play a role in preventing intraperitoneal adhesion, nor does it have a clear controlling role in macrophage infiltration.

Keywords: Post-operative adhesions, Alanyl-glutamine, Adhesion prevention, Animal study, Macrophages, Abdominal surgery

Introduction

Adhesions, or abnormal deposits of fibrous tissue that form within the peritoneal cavity, result in an estimated direct patient care financial impact of $1.3 billion in the USA and about $130 million in Canada.[1,2] Abdominal adhesions are the most common cause of small bowel obstruction and female infertility in developed countries.[3-5] Intraperitoneal adhesions are defined as either congenital or post-traumatic cicatricial adherences between two contiguous peritoneal surfaces that are normally unattached. Following surgical interventions that result in peritoneal trauma, abnormal scar tissue may form between peritoneal surfaces that are normally free, resulting in definitive adhesion formation.[6] Post-operative adhesions are a cause of considerable direct and indirect morbidity, and their prevention can be considered a major public health issue.[7]

Intraperitoneal adhesions develop between deperitonealized surfaces of abdominal organs, mesenteries, and the abdominal wall; the most common site of adhesion formation is between the greater omentum and the anterior abdominal wall.[8] After general surgical abdominal operations, the incidence of adhesions ranges from 67%-93% and up to 97% after open gynecologic pelvic procedures.[9] Clinical and autopsy studies show the incidence of intra-abdominal adhesions to be 70-90% in patients who had prior laparotomy.[10]

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Post-surgical adhesions are associated with the following factors: trauma, thermal injury, infection, ischemia and foreign bodies. Also associated with adhesion formation are tight suturing where tension within the sutured peritoneum produces ischemia, abrasion, and exposure to foreign bodies such as talc and powders from the gloves, and lint from abdominal packs or disposable paper items.[11-13] Neutropenia is associated with lower rates of adhesion and may play a role in modulating post-operative adhesion.[14]

The peritoneum

The peritoneum is composed of two mesothelial sheets that predominantly enclose adipocytes embedded in loose connective tissue, and also aggregates of mononuclear phagocytic cells. A fold in the peritoneum forms the omentum, an apron-like structure which extends from the stomach to the adjacent abdominal organs. This portion of the peritoneum has a rich vascular supply with numerous characteristic capillary convolutions, termed omental glomeruli, so named due to their similarity to renal glomeruli. These omental glomeruli are called milky spots and they measure 0.1-2mm in size. Under low magnification milky spots look like tufts of cotton wool[15-17] spots are characterized by a permanent glomus pattern of vascular structure, specific cellular population and a specialized mesothelial lining.

In humans, milky spots are comprised of macrophages (70%), B-lymphocytes (10%), T-lymphocytes (10%), mast cells, and stromal cells. Though they have a high functional potential and play a key role in antibacterial defense, we propose that they also play a significant role in adhesion prevention.

In contrast, macrophages and the macrophage chemotactic protein 1 (MCP-1) are thought to be significant players in adhesion formation. The macrophages in the mature omentum are essentially scavengers that appear to differentiate from monocytic precursors in the milky spots and are not dependent on precursors derived from the bone marrow.[18] When activated the macrophage precursors in the milky spots proliferate and migrate to the mesothelial surface. They transform into dendritic-shaped macrophages and have marked phagocytic abilities, such as being able to avidly phagocytose intraperitoneally injected carbon particles and bacteria. Patients with endometriosis have been shown to have statistically significant increases in levels of MCP-1 compared to patients without endometriosis.[18]

Following surgery and a resultant stimulation of the milky spots, there is increased microvascular permeability to fluid, neutrophils, monocytes and fibrin deposits within the connective tissue matrix of milky spots. This leads to a subsequent increased cellular migration across the mesothelial lining into the peritoneal cavity.[19]

MCP-1 is thought to play a role in the increase of the number of macrophages since these perform a different function from the resident macrophages. The new population of macrophages secretes variable substances, some of which include cyclooxygenase and lipoxygenase metabolites, plasminogen activator, and plasminogen activator inhibitor.

Most of the cells lining milky spots are dome-shaped, though some flattened cells are also observed. A small number of peritoneal cells (free peritoneal cells) are attached to the mesothelial surface. Many of the cells covering milky spots are separated at their lateral borders. This produces intercellular gaps or pores of various sizes between neighboring cells and limits the communication between the system circulation and the peritoneum.[19] Following surgery, inflammation or peritonitis the milky spots display higher numbers of round cells, creating gaps that allow the milky spots to serve as reservoirs of cells that can then migrate into the peritoneal cavity.[20,21] The purpose of change in cell architecture is to produce cellular replication that provides a source for replenishing cells for the omentum.

Alanyl-Glutamine

Alanyl-Glutamine (AG) is a dipeptide of glutamine which has been shown to be beneficial as a nutritional supplement in parenteral nutrition and has recently been shown to restore the cytoprotective stress proteome of mesothelial cells exposed to peritoneal dialysis fluids.[22-24]

A recent study by Fukuzawa et al concluded that glutamine enhances both phagocytosis and the production of Reactive Oxygen Intermediates by neutrophils in post-operative patients.[25] AG was chosen for this study because of its effect on macrophages and fibroblasts. The functions of glutamine have been previously outlined by Demling and Seigne[26] in Table 1.

Table 1: Key Functions of Glutamine

Function in Metabolism
Nitrogen shuttle: urea and ammonia clearance
Direct source of cell energy
Anabolism: Anti catabolism
Decreases protein breakdown
Rate-limiting factor for muscle growth
Stimulates release of human growth hormone
Effect on Wound Healing
Direct fuel for fibroblast and macrophages
Indirectly by preserving lean body mass
Preserves Gut Integrity
Primary fuel for gut enterocytes via glutathione antioxidant action
Immune Function
Improves neutrophil bacterial killing and is a lymphocyte fuel
Antioxidant
Substrate for the key cellular and plasma antioxidant glutathione

Source: RH Demling[26]

Our Pilot Study

10 Wistar rats (200g each) were assessed for the efficacy of alanyl-glutamine in preventing adhesion. The study was conducted after protocol and ethical approval from the Animal Care Committee. The experimental animals were randomly distributed into three groups 1) Open laparotomy (no treatment) 2) Open laparotomy with saline 3) Open laparotomy with AG.

The rats were anaesthetized with Halothane. Open surgery involved a midline sub-umbilical incision. The intestine was examined and 2.0 Maxon suture was inserted into the serosa of the caecum and a branch of the right colic artery. Alanyl-glutamine (varying dosage)/placebo (3.6ml) was instilled in the peritoneal cavity. The abdomen was closed in layers using the same suture and the skin was closed with surgical glue.

Postoperatively, the wound was infiltrated with local anesthetic and the animals received titrated doses of intramuscular buprenorphine for pain. The animals were sacrificed on day 10. The animals had sufficient food to eat (20g/day » 2mmol/kg/day). The gastrointestinal tract and omentum were harvested from the stomach to the sigmoid canal and fixed in formaldehyde solution. Paraffin embedded tissue blocks were used for microscopic analysis. The omentum and peritoneum were stained using hematoxylin and eosin, and Masson stains, and this enabled us to see evidence of fibrosis.

Results

Our preliminary study revealed some positive findings. On the 10th post-operative day, the histological appearance of the peritoneum in the glutamine treated rats displayed very minimal or no obvious adhesion when compared with the untreated or the saline treated rats and was almost comparable to the control rat peritoneum Plate 1.

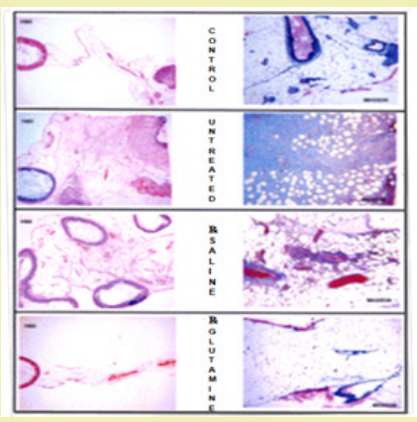

Plate 1: Hematoxylin & Eosin (H&E) and Masson-stained slides show absence of rat omentum adhesions 10 days after laparotomy in the animals treated with glutamine in comparison to the untreated and saline treated animals.

Thus, we developed a hypothesis based on the literature that adhesion following abdominal surgery develops as result of invasion of the peritoneum by circulating system cells that are able to enter the peritoneal cavity due to the gaps in the milky spots. We also felt that there might be some impact of MCP-1 in stimulating macrophages. We therefore focused on the role of milky spots and MCP-1 in the possible mechanism of adhesion. We suggest that this is based on the modulation of the normal peritoneal repair system as described by Cranshaw.[19]

The goal of the study was preventing adhesion in the post-laparotomy patient, by understanding the mechanism of adhesion formation. Our preliminary study in rats highlighted the role of glutamine in prevention of post-operative adhesion, a mechanism for adhesion prevention that had not previously been suggested or studied.

Main Study

Objectives

- To outline the natural history of adhesion formation in the animal model.
- To confirm the effect of peritoneal infiltration of AG on adhesion formation in rats post-laparotomy.
- To identify the possible mechanism of action of glutamine in adhesion prevention.

Method

53 Wistar rats (200g each) were assessed for the efficacy of AG in preventing adhesion. The study was conducted following protocol and ethical approval from the Animal Care Committee. The experimental animals were randomly distributed into three groups 1) Open laparotomy (no treatment) 2) Open laparotomy with saline 3) Open laparotomy with AG. They were anaesthetized with Halothane. Open surgery involved a midline sub-umbilical incision. The intestine was examined and a 2.0 Maxon suture was inserted into the serosa of the caecum and a branch of the right colic artery. AG (varying dosage)/placebo (3.6ml) was instilled in the peritoneal cavity. The abdomen was closed in layers using the same suture and the skin was closed with surgical glue. Postoperatively, the wound was infiltrated with local anesthetic and the animal received titrated doses of intramuscular buprenorphine for pain. The animals were sacrificed on days 1, 3, 6, 10, 21, and 42.

The animals had sufficient food to eat (20g/day » 2mmol/kg/day). The gastrointestinal tract and omentum were harvested from the stomach to the sigmoid canal and fixed in formaldehyde solution. Paraffin embedded tissue blocks were used for microscopic analysis. The omentum and peritoneum were stained using H&E and Masson stains. This enabled us to see evidence of fibrosis. In addition, we harvested the omentum and peritoneum from the negative controls and executed tissue culture as an explant to understand the peritoneum and omentum repair. Macrophage activity was evaluated using markers ED1[16] and CD68, both well-

known macrophage markers. Semi-quantitative analysis of positive stained cells was undertaken, scoring an average of 300 cells for evaluation of a percentage score using Northern Eclipse software. The results were analyzed using IBM SPSS Predictive Analytical Software, and ANOVA was used to compare the various groups.

Results

There was no development of adhesion in all groups in the first 3 post-operative days, however, by the 6th day there was evidence of severe adhesion as indicated by the Zuhlke score[27] that persisted in the saline and the non-treatment group up to the 42nd day. The study also revealed that AG prevented adhesion in all the rats that had an instillation of AG after laparotomy. These differences between groups are significant as evidenced in the ANOVA analysis and the corresponding bar graph in Figure 1.

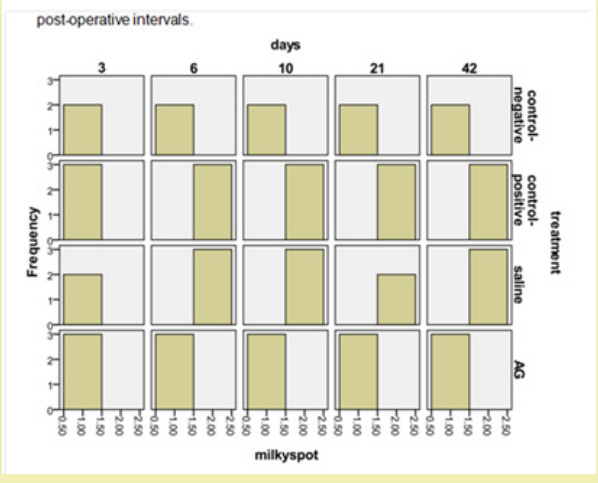

Figure 1: Frequency of milky spots within each treatment group at set

There was also a significant similarity between the negative control and the AG group, as well as no significant difference between the treated and the negative control in terms of milky spots as seen in Figure 2.

Experimental Groups	# of Milky Spots Per 7 HPF
Neg. Control no surgery	1
Positive Control Untreated non-saline	49
Rx-Saline	35
Rx-Evitar™	1

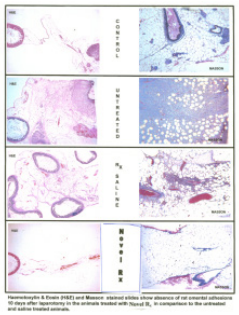

Figure 2: Comparison of milky spots per seven high power field in experimental groups

Our results also showed that AG enhanced the function of the mesothelial cell and peritoneal fibroblast, which were cultured from the peritoneal/omental explants as displayed in photographic Plate 2.

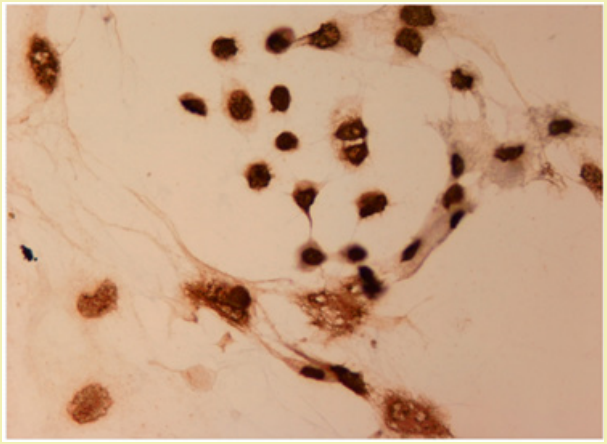

Plate 2: Tissue culture of peritoneal/omental explants.

Discussion

Our adhesion model with the Wistar rat showed a better outcome with 95%-100% adhesion prevention, than the previous animal study done by Genzyme.[28] It is also contrary to previous animal studies that implied that adhesion formation stopped at 7 days. More recent studies by Deerenberg[29] agree with our study, and Turza[30] showed adhesion 4 weeks after the surgery. Our study showed that adhesion formation started between the 3rd and 6th day and persisted until at least the 42nd day. It therefore implies that adhesion prevention is best started on the day of surgery.

The study showed that hemoperitoneum played a significant role in adhesion formation, which was not improved by a saline dilution as seen in Figure 2 when the number of milky spots marginally decreased from 7 per high power field to 5 per high power field. However, with the introduction of AG, the number of open milky spots stayed the same as seen in the virgin abdomen with only 1 per high power field.

MCP-1 is a significant marker of inflammation as seen in endometriosis and osteoarthritis. We had expected that MCP-1 would play a significant role in adhesion development; however, in our study the difference between the treated group and the non-treated/saline groups was not significant. It therefore appears that MCP-1 is a marker of inflammation and does not have a clear controlling role in macrophage infiltration.

We also wanted to evaluate the ability of the mesothelial and peritoneal fibroblasts to multiply in the presence of AG, and we did this by taking explant from the control, growing it in the culture media, and then adding AG to the group as seen in Plate 2. This independent group was enhanced by AG, which indicates that

AG played a role in maintaining the environment and therefore enhanced the repair without systemic help. This appears to be the mechanism post-hemodialysis.

Based on this study, we suggest that AG modulates the repair of the peritoneum and omentum by enhancing the function of mesothelial cells, macrophages and fibroblasts, keeping the milky spots closed and reducing the inflow of system monocytes and fibroblasts.

Conclusion

The role of macrophages has been the rate-limiting step in the repair of the peritoneum and has led to the development of the barrier methods to inhibit adhesion. However, our study revealed a fascinating process whereby mesothelial cells and peritoneal fibroblasts could multiply and function without macrophages, as was evident in the explant Plate 2.

Previous studies have focused on the role of AG in enteral and parenteral nutrition and its beneficial effects on patients undergoing chemotherapy. Our research indicates that intra-peritoneal administration of AG can prevent post-operative adhesion formation by limiting the number of milky spots that open after injury or surgery, and by enhancing wound repair via peritoneal fibroblasts, mesothelial cells, and macrophages. This ultimately limits the invasion of the peritoneum by systemic macrophages and fibroblasts. These findings underscore the potential of AG as a promising therapeutic agent in preventing post-operative adhesions, offering a novel approach that targets peritoneal cell dynamics and enhances wound healing processes.

This work laid the foundation for the recently reported human trial by Pierson et al, which proved the success of AG in the reduction of post myomectomy adhesions.[31] Further research is needed to fully understand the mechanisms involved and to optimize AG administration protocols for more clinical applications. This original study was conducted by Dr. Adebola Obayan and the patent was assigned to university of Saskatchewan and ADE Therapeutics.[32]

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Conflicts of Interest

Regarding the publication of this article, the authors declare that they have no conflicts of interest.

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