1 Platelet Rich Plasma Enhances Tissue Incorporation of Biologic Mesh

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19 **Keywords:** Platelet Rich Plasma, Biologic Mesh, Incorporation, Neovascularization,
20 Hernia, Strattice

21
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23 J.S.FM.: Animal surgeries, collection and assembly of data (Development of rat model,
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ABSTRACT

BACKGROUND: High recurrence rates due to poor tissue incorporation limit the use of acellular dermal matrices (ADM) in ventral hernia repair (VHR). Platelet rich plasma (PRP) is a growth factor-rich autologous blood product known to enhance tissue repair through cellular proliferation and neovascularization. We sought to study the effect of PRP on a porcine non-crosslinked ADM in an in vivo model of VHR. We hypothesized that PRP would enhance ADM-tissue incorporation in a rat model of VHR.

METHODS: Whole blood was extracted from Lewis rats followed by PRP isolation and characterization. Using a rat model of VHR a non-crosslinked ADM (Strattice) was implanted and activated PRP applied prior to closure. Rats were sacrificed at 2, 4, and 6 weeks. Immunohistochemical staining of CD 31 on endothelial cells was used to quantify neovascularization. Hematoxylin Eosin (H&E) stained tissues were measured to quantify tissue deposition.

RESULTS: Platelet concentration of PRP was standardized to 1×10^6 platelets/ μ l. Grossly, vessels were more evident in PRP treated rats. Immunohistochemical analysis demonstrated neovascularization was significantly greater in the PRP

48 treated ADMs at all time points. This increase in neovascularization correlated with
49 an increased thickness of tissue deposition at 4 and 6 weeks.

50 **CONCLUSIONS:** PRP enhanced neovascularization and incorporation in a rat model
51 of VHR. Enhanced neovascularization was associated with earlier and greater tissue
52 deposition on the ADM. This suggests that PRP could be used as an adjunct to VHR
53 in clinical scenarios where poor wound healing is anticipated and enhanced
54 neovascularization and early tissue deposition is desired.

55 **Keywords:** Platelet Rich Plasma, Acellular Dermal Matrix, Incorporation, Neovessel,
56 Hernia, Strattice

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59 **Introduction**

60 Over 250,000 ventral hernia repairs (VHR) are performed in the US annually,
61 making incisional hernias one of our most costly complications [1, 2]. Recently, the
62 Ventral Hernia Working Group published guidelines outlining the expansion of
63 indications for use of bio-prosthetic mesh over more traditional material in VHR[3].
64 Traditional repair materials comprise degradable and non-degradable synthetic
65 polymers that are associated with several complications such as infection, erosion,
66 adhesion formation, and contraction [4-6]. This has lead to interest in developing
67 more biological approaches to abdominal wall reconstruction. Acellular matrices
68 can be used as biomimetic scaffolds possessing the appropriate architecture for
69 cellular ingrowth and proliferation. Further, biologic materials have greater
70 biocompatibility and have been associated with fewer complications [7]. Bio-
71 prosthetic options include acellular extracellular matrices (ECM) derived from
72 human or animal sources such as dermis, intestinal submucosa, and pericardium [3,
73 8]. These prosthetics have proved useful in the repair of abdominal incisional
74 hernias but are themselves hindered by poor long-term mechanical strength and
75 high cost[9]. Following implantation, remodeling of the ECM by the body's natural
76 healing processes can weaken the mechanical strength of the mesh, leading to poor
77 integration and hernia recurrence or bulge when used to bridge unopposed
78 fascia[10]. The recurrence necessitates further reconstruction, limiting the use of
79 biologic meshes to act as a bridge to permanent synthetic repair [11, 12]. Further, if

neovascularization of the biologic matrix is delayed, infection, encapsulation and resorption are inevitable.

Platelet rich plasma (PRP), an autologous blood product rich in growth factors, is known to improve cellular migration, proliferation, ECM deposition, and neovascularization. PRP has been shown to contribute to enhanced neovascularization in tissue with poor perfusion [13]. The pro-regenerative effects of PRP may provide the necessary biologic instructions to speed the wound healing process in biologic mesh hernia repair, allowing neovascularization in a more rapid fashion.

We hypothesized that PRP could aid in successful biologic mesh incorporation through enhanced neovascularization and tissue deposition. We propose to investigate the effects of PRP on the non-crosslinked porcine ADM, Strattice. If PRP indeed speeds the rate of neovascularization of the biologic matrix, implications regarding mitigating infectious risk and speeding collagen crosslinking in VHR will be significant. This information could build a foundation of knowledge for further investigation regarding the use of both PRP in VHR with biologic materials.

Materials and Methods

Animals

Forty-two male Lewis rats weighing approximately 300-315g underwent ventral hernia repair with non-crosslinked porcine acellular dermal matrix

(Strattice, Life Cell). Because of their inbred nature and similar genetic composition, Lewis rats were chosen. Animals were randomly assigned to receive 200 µl PRP or 200 µl saline as placebo at the time of closure. All animals were purchased from Charles River (Wilmington, Mass.) and housed at Houston Methodist Hospital Research Institute animal facility. The study was approved by the Institutional Animal Care and Use Committee at Houston Methodist Hospital Research Institute and all investigators complied with the National Research Council's *Guide for the Care and Use of Laboratory Animals*.

Platelet Rich Plasma Isolation and Characterization

In order to attain sufficient amounts of blood, ten Lewis rats were used for the isolation of PRP. Their highly inbred nature allows for pooling of donor blood. Buprenorphine (0.03mg/kg) and Carprofen (5mg/kg) were administered for preoperative analgesia. Deep anesthesia was induced and maintained using a 2.5-3.0% Isoflurane/oxygen mixture through a non-rebreather mask. Using aseptic techniques, a sternotomy was made and the heart exposed. Using a 22 gauge needle a terminal cardiac blood draw was performed. The blood was then mixed with 10% Acid Citrate Dextrose (ACD) to prevent clotting, and centrifuged at 200g for 15min to remove the red cell fraction. The plasma was centrifuged at 1600g for 10min to pellet the platelets. The plasma is then removed leaving one milliliter total volume. In order to standardize the concentration of PRP, the number of platelets was quantified using a Multisizer Coulter Counter (Beckman Coulter, CA) in order to

have a final concentration of 1×10^6 platelets per microliter of plasma. The PRP was then stored at -80°C for further use.

Abdominal Wall Defect Creation

Rats were randomly allocated into treatment or placebo groups. Buprenorphine (0.03mg/kg) and Carprofen (5mg/kg) was administered for preoperative analgesia. Anesthesia was induced and maintained using a 2.5-3.0% Isoflurane/oxygen mixture through a non-rebreather mask. Aseptic techniques were used for the duration of the surgery. To create the abdominal wall defect all rats had a 3cm midline skin incision made and a 3 cm skin flap raised using sharp dissection (Fig. 1). A full thickness abdominal midline fascia/muscle/peritoneum defect measuring 2cm in length was then made. The skin was then approximated and stapled leaving the defect unrepaired. Fascial defects were allowed to mature over 28 days. The animals were monitored by physical exam and weight daily for 10 days and then weekly following that. Exclusion collars were applied for 5 days to ensure the animals did not disrupt the incision in the early postoperative period. After 14 days the staples were removed.

Abdominal Wall Defect Repair

At day 28 the animals underwent hernia repair. The protruding viscera were freed with sharp dissection and the fascial edges defined. A 2.5x1.5 cm piece of Strattice was customized to the shape of the fascial defect in order to allow for 5mm of overlap between the muscle and mesh. Then, an intraperitoneal underlay repair

was performed, where the mesh was placed between the contents of the abdominal cavity and the abdominal wall, using a 5mm overlap of each mesh with the respective fascial edge of the abdominal tissue. The mesh was fixed to the fascia with the use of eight transfascial 5-0 Prolene sutures spaced equally apart (Fig. 1). Following fixation of the mesh to the abdominal wall, 200 µl of PRP or 200 µl of saline was activated using 10% calcium solution and Thrombin (5000 units, Sigma-Aldrich) and then applied directly to the exposed anterior surface of the mesh. The skin was then closed using skin staples. Exclusion collars were then reapplied and the animals returned to their cages.

Buprenorphine (0.03mg/kg) and Carprofen (5mg/kg) were administered for postoperative analgesia. Postoperative daily physical exams assessing for signs of bleeding and infection as well as daily weight measurements were performed for the first week. Physical exam involved visual inspection and palpation of the abdominal wall for seroma formation and herniation of abdominal viscera. Animals who lost more than 10% weight were treated with saline injections and moist pellets. Rats were euthanized at 2, 4, and 6 weeks using 70% inhalant carbon dioxide method.

Euthanization and specimen harvest

At the predetermined times of 2, 4, and 6 weeks the animals were euthanized using 70% inhaled carbon dioxide and confirmed with thoracotomy. The samples were harvested with an equal amount of adjacent native abdominal wall tissue. The specimens were then fixed in either 10% buffered formalin or 4% paraformaldehyde for gross histology or immunohistochemical staining

respectively. Fixed tissues were then trimmed of excess tissue and embedded in paraffin.

Histopathology Evaluation

All paraffin embedded tissue was serially sectioned into 4µm sections. To assess neovessel formation and tissue deposition three non-consecutive sections were routinely stained with Haematoxylin and Eosin (H&E, Sigma-Aldrich). The thickness of tissue deposited on the mesh was then measured on three non-consecutive H&E slides at the rectus muscle-mesh interface and the middle of the mesh. The numbers of vessels per field of view at 20x magnification were counted. To reach statistical significance five fields of view were chosen per slide and three non-consecutive slides chosen were analyzed per each treatment. All slides were evaluated and scored by an independent blinded observer.

In order to further assess vessel formation and endothelial cell organization representative sections were prepared for immunohistochemical staining similarly by deparafinization with xylene and rehydration through a graded ethanol series. In order to reduce non-specific binding and expose antigens a heat-mediated antigen retrieval method was used. Samples were placed in a boiling 0.01M citrate buffer, pH 6.0 bath for 20 minutes. After allowing samples to cool, two separate 5 minute washes in Tris buffered saline, pH 7.4, were performed followed by one wash in PBS. Following antigen retrieval, sections were then incubated with anti-CD31 primary antibody (Abcam, Cambridge, MA). After washing, the sections were incubated in a peroxidase blocking solution for 10 minutes and then incubated with secondary

antibody room temperature for 30min. For detection, sections were incubated with horseradish peroxidase in PBS for 30 minutes at room temperature. Subsequently, the sections were subjected to DAB (substrate buffer+DAB chromogen [$\times 50$]) for 5 minutes. The slides were counterstained with haemotoxylin and then mounted. In order to properly quantify the number of vessels formed, the number of positively staining tubular structures per high-powered field was counted, averaged, and compared. To reach statistical significance five fields of view were chosen per slide and three non-consecutive slides chosen were analyzed per each treatment.

Statistical Evaluation

We compared the degree of tissue deposition and number of neovessels formed within non-crosslinked ADM groups implanted alone or with PRP treatment at 2, 4, and 6 weeks post-implantation using GraphPad Prism 5.02 software (San Diego, CA). Pooled raw data from the respective groups' was used and two-tailed, paired t-tests performed, with values reported as means with standard deviation. A 95% confidence interval (CI) was assumed and a p-value ≤ 0.05 was used to define a statistically significant difference between comparative groups.

Results

PRP Production

Concentrations of PRP were standardized to 1×10^6 platelets per microliter. An advantage to the study over other is the standardization of our PRP formulation. Having quantified growth factor release and cellular responses in vitro based on a

standardized platelet count we can now correlate those findings to an in vivo biological impact (Table 1).

Macroscopic appearance of mesh following PRP application

At the time of necropsy pictures were taken to document the extent of macroscopic neovascularization. The groups that received PRP had larger, more abundant, and macroscopically visible vessel growth. Extensive vessel ingrowth was observed in the groups that received PRP at all time points throughout the study and was most evident at 6 weeks (Fig 2).

PRP enhances neovascularization

The addition of PRP resulted in increased immunohistochemical staining of CD31 at all time points (Fig 3a). These findings correlated with an increased number of vessels seen per field of view ($p < 0.05$) as early as 2 weeks (Fig 3b). There was a heterogenous distribution of newly formed vessels with a higher density of vessels in the anterior surface of the mesh and vessels associated with adherent fat on the posterior surface. At 6 weeks this trend of increased numbers of vessels continued with a concomitant increase in their size (Fig 3a). Quantification of the numbers of vessels demonstrated a statistically significant increase in the neovascularization at 2, 4, and 6 weeks in the PRP treated group (Fig 3b). These data suggest that the biologic milieu of PRP provides is capable of accelerating granulation tissue formation and thus early neovascularization. This increased early neovascularization can translate to larger vessels at later time points.

Early neovascularization promotes greater tissue deposition

Tissue thickness above the mesh (Double sided arrows) was measured at 2, 4, and 6 weeks (Fig. 4a). A significant difference in the amount of new tissue coverage was seen between PRP treated and control groups at 4 and 6 weeks. PRP treated meshes demonstrated more dense, clearly defined, parallel collagen fibers oriented tangentially with a sparse network of small arteries oriented parallel to the collagen fibers when compared to control groups (Fig 4a). Muscle island formation was a unique observation only seen in the PRP treated groups (white arrows, inset). The mean thickness of PRP positive mesh was 1.5 fold greater when compared to the control groups (Fig. 4b). Overall Strattice with PRP demonstrated the greatest neovascularization and tissue deposition.

Discussion

Occurrence of incisional ventral hernia and acquired abdominal wall defects continue to be a significant complication of abdominal surgery[1, 14]. Patient's with multiple co-morbidities, previous abdominal infections or concomitant bowel surgery benefit from use of biologic matrix over synthetic due to infectious risk [15, 16]. The evolution of these biologic matrices has lead to varying degrees of chemical crosslinking in order to strengthen the device and limit the resorption. The crosslinking, however, inadvertently changes the nanostructured environment of the mesh making it more resistant to cellular migration and incorporation, factors necessary for proper mesh ingrowth [17-22]. Further, when primary fascial closure

is impossible even after attempts such as component separation, biologic matrix bridging unopposed fascial defects inevitably leads to some degree of bulge if not frank recurrence when the mesh is incorporated completely [23, 24]. The results of this study show that the use of PRP as a adjunct to bridging hernia repair with biologic mesh leads to both increased neovascularization and tissue deposition, factors that would ultimately lead to improved mechanical features and long term stability.

The use of supraphysiologic doses of platelets in the form of PRP has been studied for many decades [25-30]. Our group has previously reported that PRP is rich in angiogenic growth factors (VEGF), regenerative growth factors (TGFB, FGF, PDGF), and chemotactic factors (SDF1a and RANTES) [31]. While its use in oral maxillofacial, orthopedic, and plastic surgical applications has been widely accepted, the use of PRP has not been previously studied in the context of ventral hernia repair. Our findings correlate with previous findings that PRP contributes to increased cell migration, neovessel formation, and organized tissue deposition[32-35]. In order to have an effective regenerative process, the timing of growth factor release must mimic the natural environment. The biological activity of PRP is not limited to the activity of the growth factors but also to the chemoattractant factors it possesses and the natural scaffold it provides for cellular migration and organization. By forming a concentrated and organized pseudo clot at the site of repair, one can effectively accelerate the process of healing by “jump starting” the signaling cascade and cellular homing to the site of injury. This is demonstrated here by the early-enhanced neovascularization seen at weeks 2, 4, and 6. By providing

the ideal microenvironment and cellular signals, neovascularization was seen earlier in the healing process and with increased concentration of vessels. This finding translated to a thicker and more robust tissue deposited at 4 and 6 weeks. The maturity of the tissue was evidenced by the organized collagen formation and presence of muscle islands. Due to genetic similarities of Lewis rats, pooled donor PRP allows for a similar clinical similar scenario as autologous donation in the clinical human hernia model. An advantage of this study over other is the standardization of our PRP formulation. Studies reporting the use of PRP by volume may not be accounting for differences in concentration. Having quantified growth factor release and cellular responses in our previous studies we are now able to correlate those findings to an in vivo biological impact [31]. This has significant implications for the clinic where PRP studies are complicated by a lack of standardization of study protocols, platelet-separation techniques, platelet quantification and outcome measures [36, 37].

While a significant regenerative effect is seen up to 6 weeks, an inherent limitation to this study is the relatively short time frame investigated. To properly evaluate the effects of PRP on recurrence and mechanical strength studies must be taken out to 6 months or greater when recurrence begins to be evident. The scope of this investigation was not focused on mechanical properties but rather the biologic response. We appreciate the need for such quantification and mechanical studies are currently ongoing. As with many of the other studies, the mechanism of PRP's action has yet to be elucidated. While we believe that the biologic effect of PRP is the result of the cumulative effects of PRP's growth factors, chemoattractants, and

favorable biologic microenvironment; a more mechanistic approach to the study of PRP is needed in the literature. Standardization of the PRP concentrate is the first step to achieving that goal.

In summary, in this study we evaluated neovascularization and found that not only did the PRP increase neovessel formation at early time points, the increase in neovessel formation correlated with an increase in the tissue deposited at both 4 and 6 weeks on a non-crosslinked porcine ADM. The increased tissue deposited on the mesh may contribute to increased strength and ultimately limit recurrence rates. This study provides a proof of concept that PRP, an autologous and relatively cheap product, may be a useful tool for the surgeon using non-synthetic mesh in abdominal hernia repair. These results clearly show the enhancement of the biological process of granulation formation and tissue remodeling seen in secondary intention healing. By enhancing the early neovascularization of the mesh we have demonstrated that a greater amount of tissue deposition is possible and that this may contribute to downstream increases in mechanical integrity and overall hernia repair strength.

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Figures

Figure 1. Creation of chronic hernia. On day 1 the linea alba was incised and left unrepaired. By day 14 the edges of the hernia were palpable and a clinically evident was present. On day 28 rats underwent reduction of herniated abdominal contents and repair with either cross-linked or non-crosslinked porcine acellular dermal matrix. Prior to closure activated PRP was added to the ventral surface and the skin closed.

Figure 2. Gross Evaluation of Mesh at 6 weeks. Macroscopic appearance of mesh following implantation at 6 weeks demonstrated a pronounced neovascularization (arrows) in the PRP treated group when compared to the control group.

Figure 3. Immunohistochemical staining of porcine ADM. Staining for CD31 (brown) demonstrated more abundant positively staining structures (arrows) in PRP treated groups (A). Quantification of neovessel ingrowth shows increased vessel number in PRP treated groups at 2, 4, 6 weeks (B). 40x magnification, scale bar: 40 μ m. Values are mean $\pm$ SD (n=7). Asterisks depict highly significant; *(p<0.05), **(p<0.01) between groups.

Figure 4. Tissue deposition on porcine non-crosslinked ADM. Tissue thickness from tissue mesh interface (dashed line) to surface was measured (double headed arrow) (A). Organized heterogenous tissue with striated muscle islands were seen (insert,

white arrow). Addition of PRP resulted in a statistically significant increase in tissue deposition and thickness at both 4 and 6 weeks (B). 10x magnification, scale bar indicate 100µm. Asterisks depict highly significant; *(p<0.05) between groups.

Table 1. Characterization of PRP following centrifugation steps. Multiple centrifugations demonstrate erythrocyte and leukocyte reduction. Final platelet concentration in PRP was 1.002×10^6 .

Table 1

Whole Blood	Platelets (x10 ³ /μl)	415
	RBC (x10 ⁶ /μl)	5.56
	WBC (x10 ³ /μl)	6.08
Crude PRP (1 st centrifugation)	Platelets (x10 ³ /μl)	600
	RBC (x10 ⁶ /μl)	0.3
	WBC (x10 ³ /μl)	0.05
Final PRP	Platelets (x10 ³ /μl)	1002
	RBC (x10 ⁶ /μl)	0
	WBC (x10 ³ /μl)	0
Platelet Poor Plasma	Platelets (x10 ³ /μl)	2
	RBC (x10 ⁶ /μl)	0
	WBC (x10 ³ /μl)	0

Table 1.
Characterization of PRP following sequential centrifugation.

Figure 1

Figure 2

Control

PRP

Figure 3

Figure 4