Autologous Biological Glue and Aprotinin Prevent Ischemia in Latissimus Dorsi Muscle after Mobilization

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Abstract

The hemodynamic results of cardiomyoplasty, a promising form of surgical treatment for end-stage heart failure, do not support the subjective improvements seen clinically. We hypothesized that this disparity might be due to ischemia-reperfusion injury to the latissimus dorsi muscle (LDM) after mobilization. Having tested autologous biological glue (ABG) as a protective layer around traumatized muscle, as a means for facilitating revascularization, and as a drug depot to reduce local ischemia-reperfusion lesions, we wanted to determine if this protective and revascularization effect could be enhanced by adding aprotinin, a natural inhibitor of serine proteinases with the potential for preventing proteolytic degradation. To test for muscle damage and angiogenesis, we created pockets out of ischemic and nonischemic LDM. The control group had pockets without additives; the second group had pockets with ABG only; and the third had pockets with ABG and aprotinin. Light microscopy revealed that pockets treated with ABG, either alone or with aprotinin, had less leukocyte margination, fibrosis, calcified necrosis, and fibrous degeneration than in controls. In control pockets, after 56 days, capillaries occupied 4.1 ± 0.3% of the area in nonischemic LDM and 3.6 ± 0.7% in ischemic LDM (p > 0.05). In pockets treated with ABG only, capillaries occupied 5.5 ± 0.2% (p < 0.05) of the area in ischemic LDM; in pockets treated with ABG and aprotinin, 8.5 ± 1.1% (p < 0.05) area was occupied with capillaries. This data confirmed our hypothesis that aprotinin added to ABG prevents ischemia-reperfusion lesions after muscle mobilization, and enhances capillary ingrowth in both the ischemic muscle and the interlayer between ischemic and nonischemic muscle.

Key words: cardiomyoplasty, LDM ischemia, autologous biological glue, aprotinin.

The discovery that a training protocol stimulating skeletal muscle will increase its fatigue resistance has piqued interest in using latissimus dorsi muscle (LDM) in cardiac assistance [35-37]. There are four ways in which skeletal muscle could be used for cardiac assist: cardiomyoplasty, in which skeletal muscle is wrapped around the heart and electrically stimulated to contract synchronously with the heart [3, 24]; aortomyoplasty, in which skeletal muscle is wrapped around the descending aorta [6]; skeletal muscle ventricle, in which the skeletal muscle is connected to the circulation to assist in synchronous or counter pulsation [1]; and mechanical ventricular assist, in which skeletal muscle provides power for a device [41].

Of these four uses, only dynamic cardiomyoplasty is currently used as a surgical treatment for patients with congestive heart failure [7-8, 22, 29]. Potential benefits of cardiomyoplasty are attributable to the wrap itself, muscle flap stimulation, additional blood supply, or a combination of these factors.

Despite remarkable symptomatic improvement in patients, there is no concurrent objective hemodynamic improvement [11, 13]. A loss in hemodynamic benefits after cardiomyoplasty may be attributed to the damaged LDM's incomplete recovery after subtotal mobilization.

In this current study, we address the inability of the LDM to contract adequately after subtotal mobilization due to its ischemic state, in particular, muscle flap degeneration caused by reperfusion damage, which may impair LDM function. The endothelium has recently been shown to play a key role in the injury suffered after ischemia and sub-
sequent reperfusion [42]. We believe that enhancing the process of neovascularization (angiogenesis), and preventing ischemia-reperfusion injury immediately after muscle mobilization will preserve the LDM's angiogenic potential, and thus improve muscle performance for cardiomyoplasty and other types of skeletal muscle powered assistance.

In the past, we have shown that using ABG as an interlayer between the stimulated LDM and the mobile myocardium enhances adhesion between these tissues and improves cardiomyoplasty results [9]. We hypothesized that autologous biological glue (ABG) could also serve as a depot for drugs used to reduce local ischemia-reperfusion lesions. In this study we investigated the efficacy of adding aprotinin, a proteinase inhibitor, to the ABG.

Methods

Animal studies reported here conform to the Guiding Principles Regarding the Care and Use of Animals of the American Physiological Society and to all federal laws and regulations regarding animal use, and were approved by our institution's Animal Care Committee.

Operative technique

Animal preparation

Twelve adult sheep were operated on as described below. Amoxicillin (15 mg/kg IM) was administered to all animals 24 hours before surgery and continued for five days after surgery to guard against infection. Sterile technique was followed at all times to reduce the potential for infection. All surgical procedures and biopsies were conducted while the animals were under general anesthesia induced with diazepam (5 mg/kg IV) and thiopental sodium (20-25 mg/kg IV). The animals were intubated, placed on a Drager ventilator (North American Drager, Telford, PA), and maintained on halothane gas anesthesia (1-2% with 4.0 L/min Oz). Oxygen saturation levels and heart rate were monitored via a pulse oximeter placed on the animal's tongue.

Skeletal muscle pockets

After the animals were under general anesthesia, they were placed in a lateral position, and a longitudinal skin incision was made from the right axilla towards the costovertebral angle. A 6 x 16 cm flap of subcutaneous adipose tissue was dissected free, leaving the lateral portion connected. The anterior border of the LDM was completely mobilized. Several vessels originating from intercostal arteries that penetrated the muscle were ligated, but vessels entering the LDM from the spinal posterior, the profound posterior, and the superficial anterior areas were not disturbed.

Two distinct sections of the LDM were identified (defined by their blood supply): the posterior portion with its undisturbed vascular supply and the anterior portion that was still ischemic after partial removal of its blood supply. These sections were separated from each other, exposing the anterior flap to even greater ischemia (simulating the condition inflicted on the LDM after subtotal mobilization for cardiomyoplasty). We were left with three distinct tissue sections: nonischemic LDM in situ, ischemic LDM, and an adipose tissue flap.

The adipose tissue flap was placed on top of the in situ LDM, and the ischemic LDM section was placed on top of the adipose tissue, and, from this "sandwich", the three tissue layers were sutured together to form two double pockets (each 3 x 10 cm) (Fig. 1) in the medial and peripheral regions of the LDM. These regions of the LDM were chosen because the distal portion of the LDM flap undergoes the most dramatic change after muscle mobilization. In four sheep, pockets were left without ABG to serve as a control; in four sheep, pockets were filled with aprotinin.
ABG without any other additives; in another four sheep, pockets were filled with ABG plus 1000 U/ml of aprotinin (a serine proteinase inhibitor). Three hours after LDM mobilization (and before ABG application), biopsy samples were taken from the ischemic and nonischemic LDM. ABG was applied as needed, the wound was closed, and the animals allowed to recover. All animals recovered from the operation. Biopsy samples were taken again on days 14 and 56.

**Formation of fibrin interface**

*Preparation of autologous biological glue*

Autologous cryoprecipitate was prepared from each animal’s citrated blood (100 ml) under sterile conditions using a standard procedure ([AABB] Technical Manual, 10th Edition, 1990). Blood was centrifuged, and the decanted plasma was frozen for 48 hours at -80°C. The plasma was thawed at 4°C for four hours and centrifuged to collect a yellow-white precipitate; this was stored at -18°C. The resultant cryoprecipitate containing concentrated fibrinogen, factor XIII, fibronectin, and vitronectin was reconstituted in 40 ml of phosphate buffer solution (PBS) at a concentration of 10 mg/ml of coagulable protein. We used a FDA-approved thrombin bio-hybrid preparation (Johnson & Johnson Patient Care Inc., New Brunswick, NJ) to prepare ABG. This enzymatically active compound was dissolved in PBS (~40 ml) to yield an enzyme concentration of 250 U/ml.

*Application of biological glue*

Aprotinin (Pentapharm Ltd., Basel Switzerland) was added to the fibrinogen solution ex tempore at a concentration of 1000 U/ml. Since mixing fibrinogen and thrombin together immediately creates a meshwork of fibrin fibers (i.e., a fibrin clot), we used two separate syringes to apply the ABG to the tissue surface, one filled with thrombin and one filled with cryoprecipitate. An equal amount of each compound was applied at the same time for a total glue volume of 15-20 ml in each pocket.

**Histology**

*Biopsies*

Biopsies (3×4 mm) were taken from the LDM for light microscopy, immunohistochemistry, and transmission electron microscopy before mobilization, three hours after mobilization of the LDM, and on day 14. On Day 56 (the termination of the experiment), the LDM pockets were carefully excised and three random segments from all three layers of the pockets (nonischemic LDM, adipose tissue, and ischemic LDM) were removed for analysis. Samples (3 x 4 mm) for light microscopy and immunohistochemistry were placed in 10% formalin and taken to the hospital’s pathology department for embedding and sectioning. Samples for transmission electron microscopy (TEM) were placed in Karnovsky’s fixative.

*Indirect immunohistochemistry*

After fixation and proteolytic predigestion of formalin-fixed tissue, sections were incubated with von Willebrand factor (vWF) to serve as angiogenic markers. Vascular density was assessed by conventional indirect immunoperoxidase staining. Polyclonal rabbit antibodies to a-vWF (DAKO) were available as we had established in preliminary experiments that this rabbit antibody (originally obtained against human antigens) cross-reacted with antigen from sheep. By determining the percentage of area occupied by vessels (averaged from 10 different areas), we were able to calculate the degree of vascularization in the inter-layer between ischemic LDM and nonischemic LDM tissue.

**Results**

**Light microscopic data**

Three hours after LDM mobilization

In the proximal portion of the mobilized LDM flap, the specimen was relatively normal except for minimal peripheral eosinophilia. There were granulocytic pavements and, at the edges of the tissue, alternating areas of swollen and shrunken wavy fibers. In the medial portion, scattered fibers showed degenerative changes, primarily swelling, eosinophilia, or basophilic degeneration, and some fibers had apparently progressed to true necrosis of individual cells with marked margination of leukocytes. In the peripheral portion of the ischemic LDM, the degenerative process was pronounced with a high proportion of swollen degenerated cells and some progression to basophilic degeneration and necrosis. Visual inspection suggested significantly more leukocyte margination in the peripheral than in the medial portion of the LDM.

Days 14 and 56

Since previous biopsy extraction has a traumatic effect on the regeneration process biopsy samples on Days 14 and 56 were taken from LDM areas different from those previously biopsied.

a) Control pocket

Biopsies on Days 14 and 56 produced moderate blood oozing and showed no strong connections between the ischemic LDM flap and the adipose tissue. Leukocyte margination, present on Day 14, was absent on Day 56. In specimens obtained on both Days 14 and 56, various stages of necrosis were discernible; the muscle appeared damaged and edematous. By Day 56, some muscle fibers had developed a wrinkled appearance.

b) Pockets containing ABG only

Biopsies produced considerable bleeding. By Day 14, strong adhesions had grown through the adipose tissue connecting the ischemic and nonischemic layers of the LDM. On Day 14 and in some samples on Day 56, there was occasional fibrous degeneration, fibrosis, and calcified necrosis, but less than in control pockets.

c) Pockets with ABG plus aprotinin

On Days 14 and 56, biopsies produced intense bleeding and strong adhesions. Granulation tissue was observed invading the interface from the ischemic portion of the LDM, but leukocyte margination was not seen in capillar-
ies on either day. Fibrosis and occasional fiber degeneration were present in some samples, but no calcification or degenerative changes.

Immunohistochemistry

Before operation

Before LDM mobilization, in samples between the medial and peripheral portions of the LDM, capillaries occupied 3.99 ± 0.24% of the area. Days 14 and 56 after operation

a) Control pockets

On Day 14, capillaries occupied 3.0 ± 0.9% of the area in ischemic tissue (p > 0.05 vs. control) and 4.05 ± 0.36% in the nonischemic tissue (p > 0.05 vs. control). On Day 14, in pockets without ABG, there were few vessels between muscle fibers in the ischemic LDM. On Day 56, there was no statistically significant change in the fractional area occupied by capillaries between ischemic tissue (3.6 ± 0.7%, p > 0.05 vs. control) and nonischemic tissue (4.12 ± 0.29%, p > 0.05 vs. control). (Table 1). Diameter of the vessels was 44 ± 12 um (Fig. 2a).

b) Pockets containing ABG only

On Day 14, the area occupied by capillaries was 4.1 ± 0.4% in the ischemic tissue and 4.12 ± 0.3% in nonischemic tissue (p > 0.05 vs. control) and there were numerous small capillary structures. By Day 56, this had increased to 5.5 ± 0.2% in ischemic tissue (p < 0.05 vs. control) and 4.26 ± 0.2% in nonischemic tissue (p > 0.05 vs. control) (Table 1). Diameter of the vessels was 49 ± 14 um (p > 0.05 vs. Control) (Fig. 2b).

c) Pockets with ABG plus aprotinin

In these fortified pockets, new vascular structures (i.e., an increased number of capillaries) revealed vigorous neovascularization in the ischemic tissue (Fig. 2c). On Day 14, the area occupied by capillaries was 5.2 ± 2.1% in ischemic tissue (p < 0.05 vs. control), but only 4.3 ± 0.15% in nonischemic tissue (p > 0.05 vs. control). By Day 56, this had increased to 8.5 ± 1.1% in ischemic muscle (p < 0.05 vs. control and Day 14), but only 4.5 ± 0.4% in nonischemic muscle (p > 0.05 vs. control and Day 14) (Table 1). Many vessels had diameters larger than 50 um (75 ± 11 um) (p < 0.05 vs. control).

Transmission electron microscopy

Three hours after subtotal LDM mobilization

Within capillaries in the ischemic tissue, TEM revealed various stages of leukocyte-endothelium interaction: leukocytes binding to the endothelium (Fig. 3a); leukocyte destruction of endothelium (Fig. 3b); and leukocytes leaving capillaries through gaps in the endothelium (Fig 3c). In ischemic tissue, there were many leukocytes, macrophages, and mast cells with fibrous deterioration and swelling.

Table 1. Area of Muscle (%) Occupied by Capillaries.

<table>
<thead>
<tr>
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<th>Day 14</th>
<th>Day 56</th>
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</thead>
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<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic</td>
<td>3.0 ± 0.9%</td>
<td>3.6 ± 0.7%</td>
</tr>
<tr>
<td>Nonischemic</td>
<td>4.1 ± 0.4%</td>
<td>4.1 ± 0.3%</td>
</tr>
<tr>
<td><strong>ABG only</strong></td>
<td></td>
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</tr>
<tr>
<td>Ischemic</td>
<td>4.1 ± 0.4%</td>
<td>5.5 ± 0.2%*</td>
</tr>
<tr>
<td>Nonischemic</td>
<td>4.1 ± 0.3%</td>
<td>4.4 ± 0.2%</td>
</tr>
<tr>
<td><strong>ABG+Aprotinin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic</td>
<td>5.2 ± 2.1%*</td>
<td>8.5 ± 1.1%*</td>
</tr>
<tr>
<td>Nonischemic</td>
<td>4.3 ± 0.2%</td>
<td>4.5 ± 0.4%</td>
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* p < 0.05 vs. control
Day 56 after operation

a) Control pockets
   There was no new capillary growth, only a large amount of connective tissue between muscle fibers. In the interlayer between ischemic muscle and adipose tissue, there were newly formed collagen fibers, but no capillaries (Fig. 4a).

b) Pockets containing ABG only
   These samples looked healthier than controls, and had less connective tissue. In the adipose tissue interlayer, there were well-formed collagen fibers containing many newly formed small capillaries (Fig. 4b).

c) Pockets with ABG plus aprotinin
   This tissue looked healthy, and the adipose tissue interlayer contained well-organized collagen fibers with mature capillaries and arterioles (Fig. 4c).

Discussion

Skeletal muscle flap ischemia

As a promising surgical treatment for end-stage heart failure [4, 25, 31], cardiomyoplasty offers benefits from the girdling effect of the muscle wrap, from muscle flap...
stimulation (i.e., augmentation of systolic function), from indirect myocardial revascularization (i.e., augmentation of the blood supply), or from all of three factors. The marked symptomatic improvement in some patients notwithstanding, the full potential of this operation is still untapped [4, 30]. Enhancement of contractile performance of a weak cardiac muscle requires a healthy and strong LDM with an active angiogenic potential that can provide indirect myocardial vascularization.

Unfortunately, progression of heart failure is very evident in patients who have skeletal muscle flap ischemia in the immediate postoperative period [17,19], and there are reports of a correlation between lack of improvement in left ventricular ejection fraction and muscle flap ischemia after cardiomyoplasty [19]. Especially detrimental to long-term results is fibrosis and atrophy in the distal portion of the LDM damaged by the operation [21]. Probably due to ischemia and edema, the wrapped LDM was even occasionally unresponsive to electrical stimulation, negating the purpose of the cardiomyoplasty [18]. Postmortem histological analysis has frequently revealed pronounced fibrosis in the distal portion of the wrapped LDM [12], and evidence of inadequate revascularization and long-term tissue damage [21, 40]. Therefore, although the lack of a positive hemodynamic effect is probably multi-factorial [16], we believe the salient factor is muscle damage after cardiomyoplasty [19].

Collateral growth never occurs in cases of severe ischemic shock (i.e., when the angiogenic potential is greatly depressed). The distal LDM flap often in severe ischemic shock, has little impact on the LDM’s angiogenic potential. This sets up a vicious cycle in which the ischemic LDM flap, lacking angiogenic potential and adequate revascularization, only aggravates the damage, degeneration, atrophy, and necrosis of the rest of the LDM. However the cycle may be broken by local treatment with angiogenic growth factors (e.g., bFGF) to stimulate formation of extramyocardial collaterals to the heart and improve LDM function [42].

Tissue damage is also made worse when reperfusion occurs after periods of arrested blood flow (as little as 2-3 hours), because the cells that have remained viable can be injured by readmission of blood and oxygen. This sudden restoration of blood flow to previously ischemic tissue can lead to irreversible tissue injury, termed the “oxygen paradox” phenomenon [38]. Readmission of oxygen causes a burst of free-radical production that can quickly lead to cellular or tissue injury. Oxygen-derived free radicals are cytotoxic and damage endothelial and other cells [15]. Ischemia-reperfusion injury leads to an acute inflammatory response initiated by an overlapping cascade of inflammatory mediators, expressed both locally and systemically [20]. When ischemic tissue is reperfused with oxygenated blood, neutrophils accumulate rapidly in the infarcted tissue [23]. Thus, the endothelium, the source of future angiogenesis and capillary ingrowth, may be the first tissue injured during ischemic reperfusion [34].

The accumulation, and activation, of adhesion molecules on endothelial cells causes leukocyte-endothelial cell interactions and coagulation [14-15, 28]. In this study, we noted evidence for this in the margination of leukocytes both three hours after muscle mobilization and on Day 14. TEM revealed visible contact between leukocytes and endothelial cells, a clear sign of damage to the endothelium.

**Autologous biological glue application**

We believe that if the blood supply could be restored earlier, reperfusion damage would be minimized. Autologous biological glue may be a means to that end, since fibrin glue effectively stops bleeding by forming a layer around the traumatized LDM, thereby protecting it from severe damage. We noted on Day 14 after mobilization considerably less leukocyte margination, fibrosis, and calcified necrosis in LDM tissue treated with ABG than in nontreated ischemic LDM tissue.

Our study also showed that well perfused muscle apparently does not need additional capillarization, since is-
Aprotinin prevents latissimus dorsi ischemia

Chemic shock is enough to accelerate and mobilize angiogenesis presumably by enhancing the expression of hypoxia-sensitive growth factors (e.g., VEGF). ABG helped to initiate angiogenesis in the interlayer between the ischemic and nonischemic tissue. TEM showed newly formed capillaries by Day 56 in the adipose interlayer in ABG-treated pockets. It is important to note that these capillaries grew to the adipose interlayer only from the ischemic portion, not from the nonischemic portion of the LDM. In pockets without ABG, there was no capillary growth from either ischemic or nonischemic LDM. However, we cannot assert that capillaries will never grow from ischemic or nonischemic LDM into the interlayer, as 56 days may be too short a time for this process when angiogenic accelerants are not applied.

Proteinase inhibitor protecting against endothelial cell damage

Since a key event in ischemic muscle damage, proteinase release from leukocytes, may inhibit the active healing process (including angiogenesis), it seems logical that some undesirable leukocyte effects may be prevented by utilizing proteinase inhibitors. Aprotinin, a natural inhibitor of serine proteinase, may represent the ideal inhibitor in preventing proteolytic degradation [5, 32]. Topical application of aprotinin in the pericardial cavity has significantly reduced postoperative blood loss after cardiac surgery [5, 10]. The mechanism of action seems to be conservation of platelet function and inhibition of fibrinolysis [39].

In our investigation, light microscopy showed that muscle tissue looked healthier when aprotinin was applied to the mobilized LDM by means of ABG. Although some samples showed degenerative changes, these were considerably less than in ischemic samples from the control group.

The suggested mechanism leading to endothelial cell swelling and detachment, to increased vascular permeability, and to microvascular obstruction by detached cells and cellular debris is proteolytic digestion of endothelial basement membranes by migrating neutrophils. When aprotinin was added to the ABG, we noted considerably decreased damage of the endothelium and muscle tissue.

ABG plus aprotinin also invigorated the process of neovascularization, represented by an increased number of capillaries, and as reflected by the evident vascular structures (most capillaries were > 50 μm in diameter). TEM showed well formed capillaries in the interlayer between ischemic and nonischemic muscle. In the ischemic muscle, the area occupied by capillaries increased to 8.5 ± 1.1% - two times greater than in normal (in situ baseline) nonischemic muscle (p < 0.05).

Conclusion

Aprotinin added to ABG is effective for preventing ischemia-reperfusion lesions after muscle mobilization and can enhance capillary ingrowth in both ischemic muscle tissue and in the interlayer between ischemic and nonischemic tissue.

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