Methylene Blue Soldered Microvascular Anastomoses in vivo

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Objectives: solders containing chromophores and proteins enhance the strength of lasered anastomoses. Methylene blue (MB) solder anastomoses in vitro are strong but no in vivo work has been reported. We used an MB solder in vivo and studied the effects of two laser powers on patency and histological appearance.

Design, materials and methods: two groups of 15 rabbits had unilateral end-to-end carotid anastomoses (1.5–2.0 mm) formed using three stay sutures and MB solder. Group 1 anastomoses were formed at 5.7 Wcm⁻¹ and Group 2 at 2.8 Wcm⁻¹. The vessels were examined at various points by necropsy for patency and gross macroscopic appearance, with subsequent histological examination.

Results: group 2 showed patency of 93.3% v 0% (p<0.001) endothelialisation of 100% v 26.6% (p<0.001), giant cell formation 0% v 40.0% (p<0.01), but stenosis was not significantly different (0% v 13.3%; p=0.06). Group 2 showed a higher rate of intimal hyperplasia (IH) (66.6% v 20.0%; p<0.05) but neither group exhibited thermal injury or aneurysm formation.

Conclusions: laser soldered microvascular anastomoses were formed in vessels of 1.5–2.0 mm with a high degree of patency. A relationship appears to exist between laser power and anastomotic patency. Methylene blue fading has the potential to act as a switch against over exposure and a visual indicator of solder activation.

Key Words: Absorption; Anastomosis; Arteries; Laser Surgery; Methylene Blue; Microsurgery; Tissue adhesives.

Introduction

Laser activated tissue solders have been produced for the anastomosis of blood vessels,1–7 with a view to performing sutureless anastomoses. Solders were developed in response to the high rate of aneurysm formation caused by laser power alone.6,8 Jain,10 using an argon ion laser, showed that laser irradiation of a vein or muscle at the site of the join could reinforce the anastomosis. However it was not until later that chromophores, such as fuschin,4,11 fluorescein isothiocyanate5 or indocyanine green12 were used in an attempt to reduce the required incident laser power density by selectively absorbing the laser wavelength and converting the incident photon energy to heat. Further evolution in this field involved the addition of fibrinogen6 and later albumin13 as structural elements to enhance anastomotic strength and enable a reduction in laser power thus reducing damage to proteins in the tunica media and adventitia.

Methylene blue (MB) has been suggested as a chromophore in a laser activated solder.13 The same report describes no change in the absorbance profile of the methylene blue on addition of 50% human albumin. A recent report by this group has described the use of an MB based protein solder in vitro. This study, conducted on porcine splenic arteries, showed that the anastomoses were capable of withstanding high pressures (1188 ± 222 mmHg).5 However results from methylene blue soldered vascular anastomoses have not been reported in vivo.

The chromophore in a protein solder is considered to act primarily as a heat generator, absorbing the incident photon energy, becoming excited and generating thermal energy. This in turn causes protein polymerisation. The mechanism of this reaction may include crosslinking, disulphide bridge formation, or unraveling and tangling of the protein chains. However little is known of the precise nature of the cohesive and adhesive bonds formed with laser activated solder and the description of the process remains empirical.

Soldered vascular anastomoses have been reported using other chromophores but little is known of the optimal conditions for microvascular anastomosis to take place successfully. There are some indications of the optimal protein contents of the solder,14,15 but the effects of methylene blue concentration and of varying laser power density are not known.
With this in mind we set out to investigate the effect of two different power densities of laser light on MB based protein solder for microvascular anastomoses.

Materials and Methods

Solder preparation

The solder was prepared using reconstituted dried ingredients. Porcine albumin powder (Sigma Aldrich Chemicals, U.S.A.), and methylene blue powder (Sigma Aldrich Chemicals, U.S.A.) were mixed together and hydrated with “Water for Injection BP” (Phoenix Pharmaceuticals, U.K.). The resultant solution was mixed for 30 min and left to stand for 2 h. The final albumin concentration was kept constant at 41% w/w, while the MB concentration of the solder was 10 mg/ml. Solder was applied to the surface of the anastomosis using a micropipette set to 5 µl. This was the approximate volume delivered to each face, per layer.

Laser system

The system used was a CW laser diode (Laser Module – HPM250/3139, Laser 2000, Ringstead, Northants, U.K.) coupled to a silica optic fibre (50 µm core diameter) at a wavelength of 670 nm. The laser’s power was set to 5.7 W cm⁻¹ for Group 1 and 2.8 W cm⁻¹ for Group 2, with a focused spot diameter of 1 mm (± 100 µm) at 40 mm. The laser was applied by hand from approximately 40 mm directly onto the tissues. The operator used an operating microscope during the procedure and activated the laser by foot switch. Laser power was measured using a Coherent power meter (Model 210, Coherent, U.S.A.).

Surgery

Thirty male New Zealand white rabbits (2.5–3.5 kg) were divided into two groups, Group 1 – High Power (5.7 W cm⁻¹) and Group 2 – low power (2.8 W cm⁻¹). In each instance the animal received a premedication dose of 0.3 ml/kg of Hypnorm (Fentanyl citrate 0.315 mg/ml, Fluanisone 10 mg/ml) 0.3 mg/kg) IM 15–20 min prior to anaesthetic. The animal received a premedication dose of 0.3 ml/kg of Hypnorm (Fentanyl citrate 0.315 mg/ml, Fluanisone 10 mg/ml) 0.3 mg/kg) IM 15–20 min prior to anaesthetic. The animal was anasthetised using inhalational anaesthetic induction and maintenance (Halothane 5% reducing to 2% and 1.5% O₂) and monitored with an oxygen saturation probe.

The animal was placed supine, the skin shaved and prepared with aqueous chlorhexidine and povidone iodine, and draped for surgery. A midline incision was made and the left carotid (1.5–2.0 mm) was exposed and prepared for anastomosis with haemorrhage controlled by bipolar diathermy. Heparin was administered (1000–1500 iu, IV). The vessel was clamped using an Acland 3V clamp, transected and three stay sutures inserted (8/0 Polyamide) equidistant from each other (Fig. 1). Solder was applied to one face of the anastomosis at a time and the Laser (Laser2000) was applied at a power setting of either 2.8 W cm⁻¹ or 5.7 W cm⁻¹ for approximately 5 s per spot (Fig. 2). Typically 4–5 spots of irradiation are required for each face. The length of time required for laser exposure to each spot was judged by the operator according to the appearance of the solder and the degree of solder fade seen. Two layers of solder were applied to each surface in this manner. The clamps were then removed and Doppler flow measurement...
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Other histological data show the rate of endothelialisation was higher in Group 2 at 100% from day 7 compared to 26.6% of those in Group 1 (p<0.001). The incidence of intimal hyperplasia (IH) was higher in Group 2 at 66.6% as against 20.0% in Group 1 (p<0.05) (Fig. 4). No giant cells were evident in Group 2 but were present in up to 40.0% of the Group 1 (p<0.01) excluding those found adjacent to stay sutures. Stenosis was found in none of those performed at low power (Group 1) as opposed to 13.4% in the high power group (Group 2) (p=0.06) but was not statistically significant. Neither group showed evidence of thermal injury, aneurysm formation or medial disruption, while neutrophil infiltration into the solder was ubiquitous throughout all specimens.

Discussion

Despite the numerous reports of laser vascular anastomoses,16 little is recorded on the use of solders in vivo.3,6,12,17 Vascular anastomosis is a common surgical procedure and lasers offer the potential for fast, sutureless anastomoses, with reduced vessel trauma and good long-term patency. Initial work, while showing encouraging anastomotic times and patency, revealed a high rate of aneurysm formation and thermal damage.8 Since then efforts have concentrated on controlling laser power, to stay within the “therapeutic window” that allows both an adequate anastomosis to be formed and prevents thermal damage. Strategies have included the use of protein solders,18 chromophores3 and temperature controlled feedback systems.19

Chromophores previously described have included fuschin, eosin,11 indocyanine green (ICG)12 and fluorescein isothiocyanate (FITC)22 for applications such as urological reconstruction, choledochotomy repair and skin closure. These chromophores, while showing a good absorption match with laser emission, show no photobleaching, continuing to absorb long after solder polymerisation. ICG does show a change in colour23 but little in the way of a visual indication of complete solder activation. FITC has also been investigated as it undergoes a reduction in fluorescence with laser exposure. MB however displays photobleaching giving the surgeon a good visual indicator, as well as acting as an absorption “switch”, preventing over-exposure of tissue.

Soldered vascular anastomoses have been described in a canine model21 where a 50% albumin solder without chromophore was used. The anastomotic time was significantly reduced relative to sutured controls, but thermal damage was reported in 30% of the arterial...

Statistical analysis

This was performed using Fisher’s exact test calculated on Prism™ (Graphpad software Inc, U.K.) for non-parametric data.

Results

The results of the procedures are summarised in Tables 1 and 2.

There was a significant difference between the two groups in terms of patency and thrombosis. The low power group shows a patency rate of 93.4% as opposed to a rate of 0% for Group 1 (p<0.001). This is also reflected in the rate of thrombosis showing a 100% rate of thrombosis in Group 1 as opposed to 6.7% in Group 2 (p<0.001).
Table 1. Group 1 – High power (5.7 Wcm\(^{-1}\)).

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<thead>
<tr>
<th>Time period (days)</th>
<th>Patent</th>
<th>Thrombosis</th>
<th>Endothelium</th>
<th>Giant cells</th>
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\(+ = \text{Present}; − = \text{Absent})\).

Table 2. Group 2 – Low power (2.8 Wcm\(^{-1}\)).

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<th>Time period (days)</th>
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\(+ = \text{Present}; − = \text{Absent})\).

Lautos\(^{20}\) also compared Nd:YAG and laser diode to perform carotid anastomoses in the dog. These anastomoses were again formed without chromophore and are not, therefore directly comparable with the micro-anastomoses described here though the principles are similar. Lautos\(^{20}\) findings indicate that there was a greater rate of thrombosis in anastomoses formed using the Nd:YAG laser although patency was high. Thermal injury was also seen in these vessels although accurate measurements of the incident temperature cannot be made.

In this study we have described the use of a methylene blue based solder activated by a laser diode system at 670 nm. It has been found that this combination of chromophore and laser provides a good correlation between the absorption and emission (Fig. 2).

Fig. 4. 60 day anastomosis – Group 1 (5.7 Wcm\(^{-1}\)) (H&E, \(\times40\)).

Histological study of the anastomoses showed that there was a dramatic difference in patency between
the two groups and that the use of a power setting of 5.7 W cm\(^{-1}\) or above may result in thrombus formation at the site of the anastomosis. It is interesting to note that although thrombosis was seen to occur there was no evidence of thermal injury to elastic tissue. Although Lauto et al.\(^20\) considered that a high rate of thrombosis is associated with thermal injury it is possible that thrombosis may be due to mechanisms not involving thermal injury. Stenosis at the anastomotic site was observed but not quantified at the time of anastomosis. This subsequent observation correlated well with thrombosis at post-mortem. The operative constriction may occur as a result of cross-linking of the intrinsic proteins of the adventitia, or dehydration. Alternatively the effect may be an artifact, and simply the result of a restriction in expansion of the vessel from its unfilled to filled state. The current assay of thermal injury determines the amount of elastic tissue remaining at the site of the anastomosis, as well as the presence of charred tissue, collagen disruption and cell necrosis by examination with elastic Van Geison and H&E stains. In view of these findings, it may be possible to injure tissues in a way that cannot be detected by the methods employed in this study.

The inclusion of methylene blue in the formulation of the solder is intended to reduce any thermal effects in the tissue produced by the laser because of its ability to fade. This has a twofold function, which serves firstly to warn the surgeon that sufficient lasering has occurred and secondly as a switch to stop the absorption of laser energy. Thus a combination of surgical judgement and an in-built switch may serve to reduce and possibly eliminate thermal injury. Although the absorption switch mechanism has not been quantified, a reduction in thermal injury is apparent when compared to examples from the literature that have involved direct lasering.

Examination of the degree of endothelialisation shows that there is significantly more endothelium present in the low power group (Group 2) than in the high power group (Group 1) indicating that the regeneration of the endothelium may be inhibited by the overexposure of laser light. This may be caused by reduced regrowth or a more extensive area of endothelial damage as a result of high laser power. Either way the precise cause may not be deduced from the current study.

**Intimal hyperplasia**

An apparent paradox in the results indicates that the level of IH is greater in the low power group than in the high power group, which is perhaps a surprising finding. This effect has previously been reported by Chow\(^24\) who found similar results in the rat femoral model. This group postulated that it was possibly a repair process of the adjacent intimal cells attempting to cover over the raw area of the anastomosis. Quigley et al.\(^25\) compared the intimal hyperplasia in anastomoses formed in sutured and lasered micro-anastomoses in rat femoral arteries. A higher rate of Intimal hyperplasia was seen to occur in sutured controls at 2 weeks, but by 6 weeks there was no difference. This was thought to be due to the presence of medial damage in the laser formed anastomoses, inhibiting intimal proliferation. This inhibition was overcome by 6 weeks.

In the current study the presence of endothelium at the anastomotic site will be protective but this assumes that the endothelial cover is normal and functional. The exposure to laser may cause changes in endothelial reproduction or surface proteins rendering it dysfunctional. This in turn may result in exposure to stimuli producing IH.

The use of methylene blue, although a nitric oxide synthetase (NOS) inhibitor, appears to have little influence on the patency of the anastomoses. Nitric oxide, produced by the endothelium, is a vasodilator and prevents thrombosis. In a small vessel such as the rabbit carotid it is theoretically possible that NOS inhibition would result in thrombosis and vascular constriction. The fact that this is not seen is remarkable in itself but indicates that the dose of methylene blue used does not result in a significant vasoconstrictor or prothrombotic effect.

Previous reports have described the rate of patency in sutured micro-arterial anastomoses as being 90–100%.\(^24,26,27\) In these studies a number of experimental conditions were used, but a number a similar histological findings were seen. These included medial degeneration, intimal degeneration, and dehiscence of sutures in the short term with the later appearance of intimal hyperplasia.

The incidence of medial necrosis was as high as 70% in one series\(^28\) and 33% in another.\(^24\) Intimal damage was seen at some distance from the anastomosis, despite careful and non-traumatic handling of the tissues, upto 5–10 days with patchy regeneration thereafter.\(^28\) Reports of stenosis are rare in sutured anastomoses but were quantified by Acland\(^28\) as being 13% at 1 h and 2 days increasing to 16% and 19% at 5 and 10 days, but reducing again to normal after 21 days. Stenosis in this study was observed in the high power group but was not quantified. However as a 19% stenosis does not cause thrombosis it is unlikely that
the thrombosis seen in the high power group was caused by stenosis.

Further developmental work should be undertaken if the photofading properties of MB are to be used to their fullest potential as an absorption switch. This study has demonstrated that the solder works to the extent that thermal damage is not seen but the cause of the thrombosis associated with high power laser exposure is more complicated than previously thought. It is possible that a reduction in the concentration of MB will result in a more sensitive solder but may compromise anastomotic strength. Other alternatives are the inclusion of reducing agents to enhance MB fading and preventing re-oxidation. The use of multiple, thinner layers is also a strategy that may reduce the build up of temperature but would increase the time required to perform the anastomosis and require developments in solder application.

This study does however demonstrate that laser soldered anastomoses can be formed in an established microvascular model with a high rate of patency using a MB based protein solder. Relative to sutured controls described in the literature there is an equivalent patency rate, with a reduced incidence of medial and endothelial injury and a similar rate of intimal hyperplasia.

References


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