

Oxygen monitoring during 5-aminolaevulinic acid induced photodynamic therapy in normal rat colon - A comparison of continuous and fractionated light regimes.

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Summary

Currently, the clinical use of 5-aminolaevulinic acid (ALA) induced protoporphyrin IX (PPIX) for photodynamic therapy (PDT) is limited by the maximum tolerated oral ALA dose (60 mg/kg). Attempts have been made to enhance this treatment modality without increasing the administered dose of ALA. One way to do this is through light dose fractionation, where the irradiation is interrupted at a particular point for a short period of time. This can produce up to three times more necrosis than with the same light dose delivered without a break.

An oxygen microelectrode was employed to study the effect of continuous and fractionated light regimes on the level of oxygen in the colon of normal Wistar rats during ALA PDT. A rapid decline in pO_2 occurred close to the irradiation fibre as soon as the light dose commenced. With the fractionated regime, a partial recovery in pO_2 was observed during the dark interval which was reversed soon after the second light fraction commenced.

We have shown that the level of tissue oxygen at the treatment site is affected differently when the light dose is fractionated, than when continuous illumination is employed. This factor may at least partially explain the difference in outcome of these two treatment regimes. Further, oxygen measurements might prove to be a useful way of monitoring PDT treatments if they can predict whether tissue is likely to be viable following treatment.

Introduction

Photodynamic therapy (PDT) is a non-thermal technique which can be used to produce localised tissue necrosis. This requires activating a pre-administered photosensitiser with light of a specific wavelength to form a cytotoxic species from molecular oxygen. For a photodynamic reaction to occur, the photosensitising agent, activating light and oxygen must be present in sufficient amounts (1).

In this study we have administered 5-aminolaevulinic acid (ALA) intravenously to create a temporary build up of the photosensitiser protoporphyrin IX (PPIX) through manipulating the normal, mammalian, tetrapyrrole biosynthetic pathway. Because ALA, PPIX and all the haem intermediates produced are naturally occurring compounds they are rapidly cleared from normal tissues, limiting skin photosensitivity (the main side effect of PDT) to 1 or 2 days (2). The main drawback of this treatment however, is that preliminary clinical studies with the maximum tolerated oral dose of ALA (60 mg/kg) currently appear to produce only superficial necrosis (3).

It has been shown previously, that it is possible to increase the amount of necrosis produced by ALA induced PDT without increasing the administered dose of ALA or the applied light dose using light dose fractionation (4-7). This involves splitting the light dose into fractions by temporarily switching off the laser, at a particular point during illumination, for a specific length of time. It has been hypothesised that this results in a greater PDT effect as the break in illumination allows the treatment site to be reoxygenated. We have studied this technique in detail in normal rat colon (8,9) and this paper extends this work, comparing continuous illumination with the most beneficial fractionation regime.

It has been well established that PDT can produce vascular damage (in varying degrees with different photosensitisers) and this results in localised hypoxia (which limits the photodynamic effect) (10,11). Few studies however, have monitored the tissue level of oxygen at the treatment site in detail throughout PDT treatment, with more emphasis being placed on establishing above threshold light and drug doses. Reed *et al* (12) used Clark-style and Whalen type oxygen microelectrodes to measure tumour partial pressures before and after PDT with Photofrin II on a rat subcutaneous tumour model. They found that mean tumour pO_2 was significantly reduced 1 hour after treatment and this reduction persisted at 24 hours. Tromberg *et al* (13) also found that significant tissue hypoxia can be produced using Photofrin II PDT on a transplanted rabbit skin carcinoma (using a transcutaneous (non-invasive) Clark-style oxygen electrode to measure pO_2). More recently, Chen *et al* (14) and Sitnik *et al* (15) both used pO_2 microelectrode systems to monitor pO_2 in mice tumour models during Photofrin PDT. Chen *et al* found that the pre-PDT oxygenation level of the tumour had a profound effect on its post-PDT oxygenation and that a transient period of reoxygenation occurred following PDT. Sitnik *et al* also showed that a significant and rapid decrease in oxygen occurs shortly after the light dose commences (within one minute). It has therefore been shown that PDT using Photofrin and continuous illumination can induce a significant reduction in tumour pO_2 which may have a profound effect on the outcome of the treatment.

The degree of oxygenation in the treatment site and how it is affected by PDT is particularly crucial when investigating the mechanism of light dose fractionation. We have already shown in normal rat liver (using a non-invasive optical spectroscopic method of monitoring oxygen) that a significant and rapid decrease in oxygen can be induced by ALA PDT (16). The use of this technique to investigate fractionated PDT regimes is limited however, as it is not possible to

sample continuously throughout the treatment, as no measurements can be taken whilst the laser is in operation. In the present study we used an Eppendorf microelectrode (a polarographic measure of pO_2) to monitor the oxygen levels in the treatment area continuously before, during and after PDT, both with continuous and fractionated light dose regimes.

Experimental details

Chemicals

ALA powder (ALA.HCl, molecular weight 167.6, 99% purity, DUSA Pharmaceuticals, Inc., New York, USA) was dissolved in physiological strength, phosphate-buffered saline (PBS, pH 2.8) and administered intravenously at a concentration of 200 mg/ml.

Animal model

Normal, female, Wistar rats (120 - 200 g, University of Leeds, Leeds, UK) were used for all experiments. The animals were sedated for the ALA injections using inhaled halothane (ICI Pharmaceuticals, Cheshire, UK). Intraperitoneal injection of Hypnorm (Fentanyl and Fluanisone, Janssen Pharmaceutical Ltd, Oxford, UK) and Diazepam (Phoenix Pharmaceuticals Ltd, Gloucester, UK) was used as anaesthesia for the rest of the procedure, as halothane is known to affect the sensitivity of the Eppendorf system. Analgesia was administered subcutaneously following surgery (Buprenorphine hydrochloride, Reckitt & Colman Products Ltd, Hull, UK).

PDT studies

ALA was administered at a dose of 200 mg/kg i.v. and with a drug/light interval of 2 hours. PDT of the colon was conducted via laparotomy. The light source was a pulsed (12 kHz) copper vapour pumped dye laser (Oxford Lasers, Oxford, UK) tuned to 635 nm. A total energy of 25 J was delivered via a 200 µm plane cleaved optical fibre passed through the anti-mesenteric colon wall (approximately 1 cm distal to the caecum) so that it just touched the mucosa of the opposite side (area of contact = 0.03 mm²). The power output of the optical fibre was kept at 100 mW.

The rest of the abdominal viscera were shielded from forward light scatter by a piece of opaque paper positioned so that it did not touch the colon or affect its light distribution. This is a model that we have used many times in the past. The light fluence rate where the fibre touches the tissue is high (320 W/cm^2) but no thermal effect was observed macroscopically in the light only control group at three days. As the light fluence rate falls rapidly with increasing distance along the colon wall from the fibre tip, measuring the area of the zone of necrosis in the wall of the colon is a convenient way of comparing the efficacy of PDT with different treatment parameters.

Continuous illumination (25 J) was compared with a fractionation regime where the light dose was interrupted (after 5 J) by an interval of 150 seconds before the rest of the light was administered (20 J). This fractionation regime has been shown to produce more than three times the area of necrosis than that produced with continuous illumination (9). All animals were recovered following surgery and killed three days later, as mucosal damage is maximal at this time (17). The treated area of colon was excised and opened longitudinally so that the lesion could be measured macroscopically with a micrometer. The minimum (a) and maximum (b) perpendicular diameters of the lesion were recorded and the area (which was approximately elliptical) calculated using the formula $\pi ab/4$. Representative specimens were fixed in formalin, sectioned and stained with hematoxylin and eosin, so that conventional light microscopy could confirm the macroscopic findings. Laser only, drug only and complete blank (surgery only) control groups of animals were also included. There was a minimum of three animals in each group.

Measurement of pO_2

Colonic pO_2 was measured every 5 seconds, before, during and after PDT using the polarographic Eppendorf pO_2 histograph system (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). Before and after each treatment the instrument was calibrated in 0.9% saline bubbled alternatively with air and nitrogen. As the Eppendorf microelectrode is sensitive to temperature,

it requires a temperature value to operate. In this case, the temperature of the colon during the experiment was assumed to be 31°C (as previously measured by Barr *et al* (17) in similar conditions) and care was taken throughout to maintain the same ambient conditions. The 300 µm diameter polarographic needle probe was carefully inserted into the colonic wall (from the serosal side of the colon) and withdrawn slightly prior to measurements commencing, using the system's automated mechanism. It was not possible to accurately ascertain in which layer of the colon wall the tip of the probe was located whilst measurements were being conducted. The Eppendorf's automated probe is normally used in solid tissues to make a series of measurements, advancing and then retracting the probe a little each time to minimise the disruption to the local environment by this invasive technique. However, due to the thinness of the colon wall, this was not possible in this model. The microelectrode was positioned at varying distances along the colon wall (relative to the irradiation fibre) in separate experiments using different animals (one site per animal).

Results

At post mortem, the lesions seen in the colon were approximately elliptical, the longer diameter being found along the colon and the shorter being found across the colon. Lesion diameters ranged from 3 - 15 mm, with an average ratio of 1.5:1 between the maximum and minimum diameters (maximum ratio 3:1). The elliptical shape of the lesions was thought to be due to the geometry of applying light to a cylindrical organ and then measuring the effect when the organ had been opened and laid flat.

Control subjects

The blank control group of animals received neither ALA nor light. Surgery alone was performed and the partial pressure of oxygen in the colon was measured using the Eppendorf microelectrode. No significant increase or decrease of oxygen was observed in any of the animals in this group throughout the duration of the experiment. The tissue oxygen pressure (mmHg) of the normal rat colon of three separate animals is plotted as a function of time (seconds) in Figure 1a. The pO_2 of the untreated colon was relatively stable and no significant consumption of oxygen by the microelectrode was detected in these studies. The drug only control group (also three animals) received ALA and two hours later underwent surgery and oxygen monitoring but without laser irradiation (Figure 1b). Once again, the oxygen level remained stable throughout the experiment. No necrosis was observed at three days, in either the blank control or drug only control groups.

The tissue oxygen pressure of the continuous laser control groups can be seen in Figures 1c & d. These animals received 25 J of light without ALA administration. The oxygen level was

measured before, during and after light irradiation at 1 mm (Figure 1c) along the colon wall from the irradiation fibre and at 3 mm (Figure 1d). The period of illumination is denoted by the shaded area. Close to the irradiation fibre (1 mm away) the pO_2 increased sharply as soon as the irradiation started until it reached a plateau. Once the irradiation had finished the pO_2 level returned more slowly to the pre-laser ‘baseline’ level. Further away from the irradiation fibre (3 mm) the effect was less pronounced with only a slight increase being observed whilst the laser was switched on. Presumably these increases are the result of vasodilatation of the microcirculation and increased blood flow triggered by the heat of the irradiation. However, although the light fluence rate where the fibre touches the colon is high (320 W/cm^2) no thermal effect was observed macroscopically in either of the light only control groups at three days.

Continuous illumination

Figures 2a & b show the partial pressure of oxygen (mmHg) as a function of time (seconds) for animals receiving $200\text{ mg/kg ALA i.v.}$ two hours prior to continuous illumination with a total energy dose of 25 J . The partial pressure of oxygen was either measured 1 mm away from the irradiation fibre (Figure 2a) or 3 mm away (Figure 2b). The results from three animals in each group are presented. It can be seen that close to the irradiation fibre (1 mm) the pO_2 of the colon started to drop shortly after the start of the irradiation period, rapidly falling to zero. Further away from the irradiation fibre (3 mm away) no such dramatic effect was observed, and profiles more similar to the control groups were observed. The area of necrosis produced by the continuous PDT group was 27 mm^2 (average diameter = 6 mm). This is not significantly different from the results of our previous study using the same parameters (9). The first monitoring point was close to the irradiation fibre (1 mm) and well within this area of necrosis and the second point (3 mm away) was close to the edge of this zone of necrosis.

Fractionated illumination

Figures 2c & d show the partial pressure of oxygen (mmHg) as a function of time (seconds) for animals receiving 200 mg/kg ALA i.v. two hours prior to fractionated irradiation. These animals received the same total energy dose as the continuously irradiated animals (25 J) but fractionated by a 150 second interval after the first 5 J had been delivered. Once again the pO_2 of the colon was measured before, during and after irradiation both close to the irradiation fibre (1 mm) (Figure 2c) and further along the colon wall (3 mm) (Figure 2d). The periods of illumination are denoted by the shaded areas. The results from three animals in each group are presented. Close to the irradiation fibre the pO_2 of the colon fell to zero within the first fraction of light (5 J delivered over 50 seconds) in a similar manner to the continuous group (monitored at the same point). During the dark interval however, the pO_2 rose again although not as high as the 'baseline' pre-illumination levels. When illumination restarted at the end of the interval, the pO_2 fell back to zero. Further away from the irradiation fibre (3 mm), there was a much smaller fall in pO_2 following the first fraction of light and the fall in pO_2 to zero was delayed until after the second fraction of light had commenced. The pO_2 rose again, post-PDT, although never regained the pre-treatment level during the period of observation. No such decline in pO_2 was observed in the continuous PDT group also monitored at this distance. The area of necrosis produced by the fractionated PDT groups was 77 mm^2 (average diameter = 10 mm). This is not significantly different from the results of our previous study using the same parameters (9) and is approximately three times the area of necrosis produced by the same parameters using continuous irradiation. The greater area of necrosis produced in the fractionated PDT groups results in the second monitoring point (3 mm away from the irradiation fibre) falling well within the area of necrosis and may explain the different results observed between the continuous and fractionated groups monitored at this point.

Histological Findings

Histological analysis showed necrosis of the same extent as that measured macroscopically in all the sections examined. The maximum extent of necrosis was always seen on the mucosa, but in some instances there was full thickness necrosis in the centre of the lesion (where the light fluence and fluence rate were greatest), but only mucosal necrosis at the edges. Even though full thickness necrosis was observed in some sections and occasional lesions were circumferential, no animal showed evidence of colonic perforation or stenosis at post mortem.

Discussion

Many groups have found that light dose fractionation can produce beneficial results with a range of photosensitisers ((4) Haematoporphyrin Derivative; (5) Sulphonated Aluminium Pthalocyanine; (6) meta-tetrahydroxyphenylchlorin and (7) ALA induced PPIX) although the effect has been seen to be greatest with ALA. We have found it possible in the normal rat colon to increase the area of necrosis produced by ALA PDT by a factor of three by simply interrupting the light dose for 150 seconds, after 20% of the total energy had been delivered (9). The mechanism which enables such a short interruption in the light dose to produce such a large increase of effect is not yet clearly understood. Several hypotheses have been proposed, including relocalisation of the photosensitiser, reoxygenation of the treatment site during the dark interval and reperfusion injury. We used an Eppendorf pO_2 microelectrode to look at the oxygenation of the treatment site during PDT.

In the measurements taken just 1 mm from the irradiation fibre, a rapid decline in pO_2 was observed soon after irradiation commenced in both the continuous and fractionated groups. In the fractionated group, a small but definite recovery in pO_2 was observed during the dark interval. This recovery was reversed when the second fraction of irradiation commenced. This short period of reoxygenation during the dark interval was the only difference observed in the measurements taken 1 mm from the irradiation fibre when comparing fractionated and continuous light regimes.

This rapid fall in pO_2 close to the irradiation fibre is most likely due to the photochemical consumption of molecular oxygen outstripping the oxygen supply provided by the

microcirculation, which is itself impaired by PDT (10,11). Our results are consistent with the findings of other groups who have monitored oxygen during Photofrin PDT, particularly Sitnik *et al* (15) who observed a significant and rapid decline in oxygen shortly after commencing irradiation using a similar microelectrode system in a RIF tumour model.

The small rise in oxygen level observed during the dark was anticipated. By turning the light off, the photochemical consumption of molecular oxygen (to produce singlet oxygen) is temporarily paused and as long as the microcirculation remains viable the tissue should reperfuse (which appears to be the case with these particular fractionation parameters). When the light is restarted the photochemical oxygen consumption should restart and the pO_2 falls once more.

Differences in pO_2 monitored 3 mm away from the irradiation fibre were also observed between the two different PDT regimes. The monitoring position was chosen carefully so that it would fall on the edge of the zone of necrosis produced by the continuous regime. In the continuous treatment the pO_2 at this point was not affected significantly. With the fractionated regime, however, the pO_2 did decline to zero but this fall was delayed until after the second fraction of light had commenced. Further, the pO_2 recovered a little at the end of treatment (in some of the animals) and stayed above zero until sampling finished. These differences are important as the monitoring point was on the edge of the zone of necrosis in the continuous group and well within the area of necrosis in the fractionated group. It appears that in this model, necrosis occurred at all sites where a fall in pO_2 was documented during treatment (1 mm from irradiation fibre with both treatment regimes and 3 mm with fractionation). At the edge of the zone that was necrotic after treatment (3 mm, continuous), no change in pO_2 level was seen.

This result could be of importance for real time monitoring of PDT in clinical practice as it suggests that a documented fall in pO_2 at the time of treatment may be a useful indicator of later necrosis at that site. If such a change could be demonstrated at a series of strategically located sites around a tumour where it meets adjacent normal tissue, it could be a way judging whether sufficient light has been delivered at the time of treatment. However, many further studies will be required to establish whether our conclusion reached from experiments on normal rat colon will be valid when treating human tumours.

The fractionation parameters used in this study were found to be optimal in our previous work (9) simply by trial and error. The timing of the dark interval was found to be critical. If more than half the light dose was delivered before the interval, the fractionated regime was not significantly better than the same dose delivered in a single fraction. This may be due to the microcirculation not remaining viable past this point in the treatment (although, we have not studied the viability of the microcirculation directly in this model). The length of interval in this model has also been investigated (8) and longer intervals have not been found to be beneficial, indicating that 150 seconds is long enough for the tissue to reperfuse (in this situation). This is consistent with Foster *et al*'s calculations (18) which predicted that effective reoxygenation can take place in a hypoxic tissue in a period of about 45 seconds (depending on the degree of hypoxia caused by the previous light fraction and the maintenance of a viable microcirculation).

Multiple intervals have not been found to be any better than this optimal two fraction regime (9). This may be the result of irreversible damage occurring to the microcirculation when the second energy fraction commences resulting in subsequent intervals having little or no additional effect. The oxygen present when the irradiation restarts allows a greater PDT effect to occur when the

light dose is fractionated, although, it is unlikely that this is the sole factor which accounts for the difference in outcome between these two regimes. We have also observed a small increase in PPIX fluorescence during the dark interval using the same experimental parameters (9). This indicates that some PPIX relocalisation can occur in this period of time. The damage produced by the treatment will be increased if the amount of active photosensitiser available is increased, but once again it is unlikely that this small increase could account for the difference between the regimes. It is possible, that in addition to these effects, reperfusion injury may be playing a part. This would release free radicals and set off a cascade of tissue damage (19) which could explain the considerable difference between the continuous and fractionated regimes.

In conclusion, we have shown that there is significant consumption of pO_2 during ALA PDT in the normal rat colon and that there are marked differences in the pattern of pO_2 levels during PDT comparing continuous and fractionated light regimes. With further investigation we may be able to use the real-time monitoring of oxygen in patients to exploit these differences to monitor and improve the effectiveness of clinical PDT.

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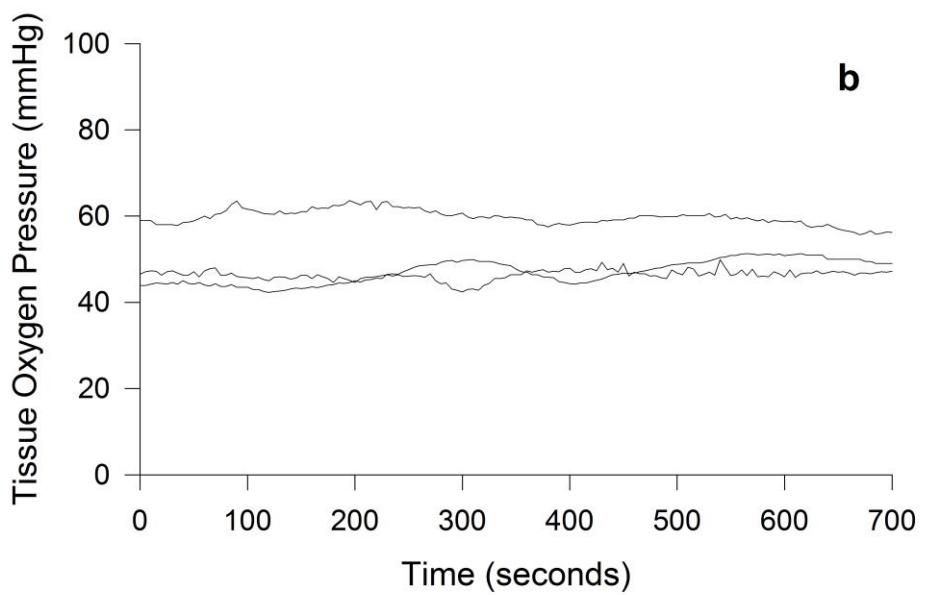
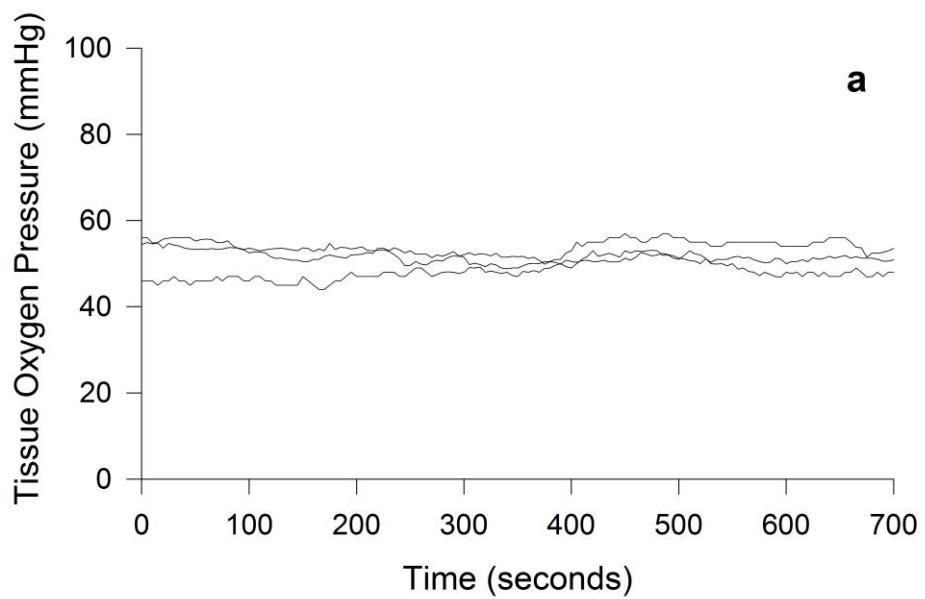
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Figure 1 The tissue oxygen pressure (mmHg) of the normal rat colon plotted as a function of time (seconds) for groups of three animals receiving a) neither ALA or light (surgery only blank controls); b) 200 mg/kg ALA i.v. 2 hours prior to surgery with no light administration (drug only controls) and c & d) 25 J of continuous light administration (635 nm, 100mW) without any ALA (light only controls) and the oxygen measured 1 mm from the irradiation fibre (c) or 3 mm (d). The period of illumination is denoted by the shaded area.



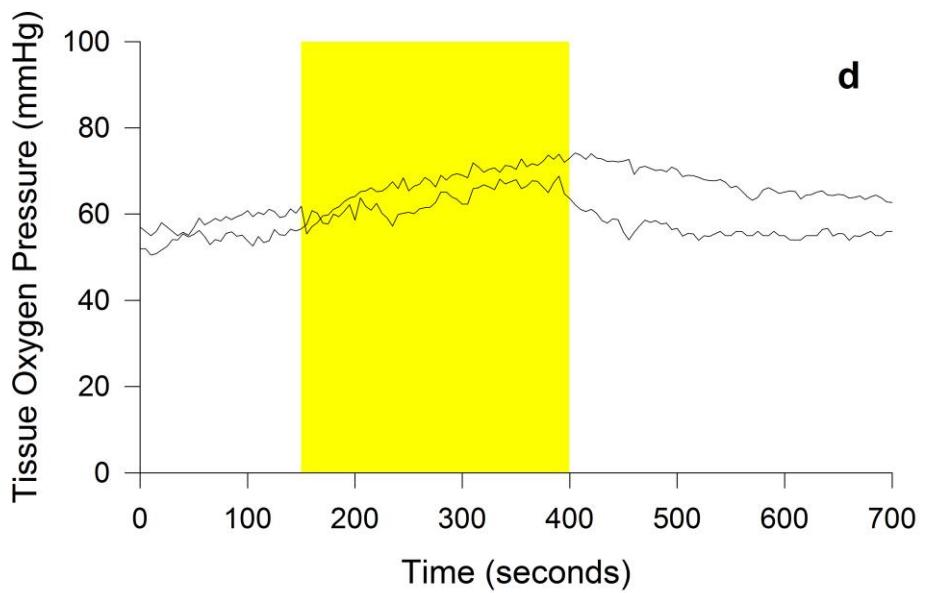
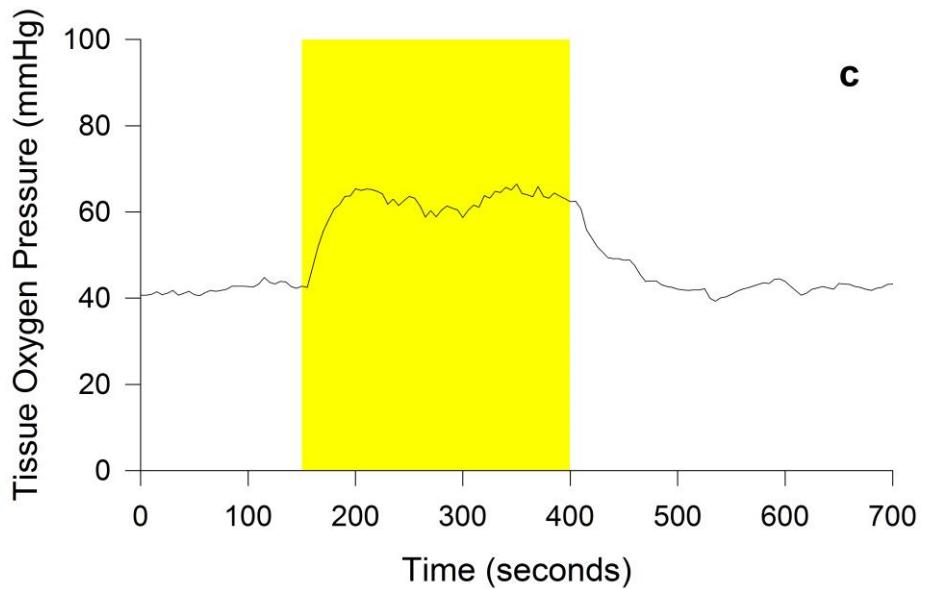


Figure 2 The tissue oxygen pressure (mmHg) of the normal rat colon plotted as a function of time (seconds) for groups of three animals receiving a & b) 200 mg/kg ALA i.v. two hours prior to continuous illumination with a total energy dose of 25 J (635 nm, 100 mW) with the oxygen measured at 1 mm (a) or 3 mm (b) away from the irradiation fibre and c & d) 200 mg/kg ALA i.v. two hours prior to fractionated illumination (50 seconds illumination (5 J), an interval of 150 seconds and then 200 seconds more illumination (20 J)) with a total energy dose of 25 J (635 nm, 100 mW) with the oxygen measured at 1 mm (c) or 3 mm (d) away from the irradiation fibre. The periods of illumination are denoted by the shaded areas.

