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Short Communication

Evaluation of toxicity of catfish skin toxin using diffuse reflectance methods

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Introduction

Fright induced epidermal secretions from Arabian Gulf catfish (*Arius bilineatus*, Valenciennes) are lethal when intravenously injected into rabbits at doses in excess of 2 mg of protein mixture per kg body weight [1]. Death is generally noted within 2 min. Sublethal, as well as lethal, doses induce major lesions in lung and liver tissues, as well as alterred levels of liver and plasma enzymes.

The toxic responses of rabbits to intravenous injections of the epidermal secretions (skin toxins, ST) can be largely blocked by pretreatment of test animals with indomethacin or hydrocortisone [2]. This had led to a conclusion that the toxic responses result largely from epidermal substances stimulating phospholipase A_2 activity in blood cells to yield arachidonic acid, which is metabolized to give toxic levels of prostaglandins [3]. Support for this conclusion was obtained by observations that an increase in prostaglandins was noted in plasma of injected animals [2], and that similarly prostaglandin release was noted following *in vitro* treatment of arterial tissue sections with the epidermal secretions [4]. Catfish epidermal secretions induced contraction of isolated smooth muscle tissues. The contraction was only partially blocked by indomethacin. This suggested that the secretions contain some neuroactive agents with acetylcholine-like muscarinic activation of cells, which could also play a rôle in toxic responses [4].

We have employed a novel diffuse reflectance spectroscopic method for monitoring responses to toxic substances. This method is capable of following some of the early events of toxicity which are induced by the epidermal secretion. The method demonstrated toxin-induced constriction and subsequent dilation of blood vessels, changes in ratios of oxy- to deoxyhaemoglobin and the formation of methaemoglobin. Blood flow rates were simultaneously monitored with a laser doppler (LD) flowmeter.

Experimental

Apparatus

Spectral measurements were recorded with a fibre optic spectrophotometer designed for non-invasive diffuse reflection studies of animals [8]. The quartz fibre optics (fibre bundle, dia 3 mm) was placed on the skin surface and the light was delivered through one branch of the bifurcated probe by a 150 W Xenon short arc lamp (Cermax Xenon illuminator, model LX 150 F, ILC Technology) through a UG3 filter (Schott Glass). The other branch of the probe carried light diffusely reflected by the skin, to a single monochromator (EG & G model 1229) with a 25-μm

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slit (bandwidth 0.5 nm), a 512 channel intensified photodiode detector (EG & G model 1454) and an optical multichannel analyzer (EG & G model 1451). Spectral scans were made over the range 378–648 nm). A single scan required 50 ms. The data were collected and averaged over 10 scans in 0.5 s to reduce noise. Each of the diffuse reflectance (DR) spectra was stored on a curve storage module (EG & G model 355).

Spectral data were collected prior to injection of the toxic material (control) and at various times after injection. The first nine DR spectra were recorded in quick succession at 10-s intervals and the remainder at 1-min intervals. Because of the hardware restrictions, a maximum of nine spectra were acquired in quick succession. Spectra were taken at 10-s intervals for the first 90 s after the injection, and then the system did number processing for the next 2 min.

The output of the LD instrument was recorded on a chart recorder for the duration of the diffuse reflectance measurements. The LD flowmeter was a Periflux (Pfld) and the potentiometric recorder was a Tacussel (EPL1). The recordings sometimes showed short term background oscillations with a period of 1.2–6 s. These have been ignored in our analysis as interpretations of these oscillations is uncertain at this point. All LD readings were considered as relative to the control values.

Materials

Catfish were caught on baited lines and their epidermal skin secretions were collected as described previously [5]. A soluble protein fraction was separated from the total epidermal secretion by homogenization in 0.15 M NaCl, 0.5 mM phosphate buffer, pH 7.5, and centrifuged for 15 min at 11,000*g* [6] and used subsequently for injection. Protein concentration in the aliquots was measured by the Lowry method [7].

Animals

All animals were anaesthetized by administration of sodium pentathal (30 mg kg^{-1}) through a 21-gauge butterfly cannula inserted into a marginal vein of the left ear. Soluble epidermal secretion in buffered saline was similarly injected into a marginal vein of the left ear. Rabbits were shaved to remove hair from the sites of injections and spectral measurements.

Measurements

Diffuse reflectance. Spectrophotometric measurements of animal cutaneous responses to ST were made at two separate locations on the rabbits. In the first case, readings were taken at the margin of the right ear distant from the site of the skin toxin injection. A black plate was placed on the side of the ear opposite the probe to prevent background reflectance. In the other tests the optical probe was placed at a site distant from the injection, on the upper portion of the left thigh.

Methods

Four locally available rabbits of mixed breed with an average weight of 1.2 kg, were injected with the soluble epidermal secretion (ST). ST was injected either in low dose of 0.5 mg kg^{-1} or as a high dose of 1 mg kg^{-1}. It was administered either in one injection or in successive doses of 0.1 mg kg^{-1}, reaching an accumulated dose of either 0.5 or 1 mg kg^{-1}. One rabbit was injected with the high dose (1 mg kg^{-1}), while the second was injected with the low dose (0.5 mg kg^{-1}). Diffuse reflectance (DR) measurements were taken on the right ear. The third and fourth animals were injected with a high and low dose, respectively; however, the DR measurements were taken on the thigh. Laser doppler (LD) measurements were made on the same location as the DR measurements.

Furthermore, three New Zealand white rabbits (average weight 5 kg) were injected with ST into the marginal vein of the left ear. The DR as well as the LD measurements were carried out on the thighs of the first two rabbits, while the same measurements were taken on the right ear of the third rabbit. The first and third animals were given a high dose of the epidermal secretion while the second animal was given two consecutive injections of the low dose material. As a control, two New Zealand white rabbits were injected with saline in the marginal vein of the left ear, and the DR and LD measurements were taken on the right ear for one animal and on the thigh of the second.

Results

A typical DR measurement taken at the thigh of an untreated rabbit is shown in Fig. 1. The absorption resonances of oxyhaemoglobin (HbO) are clearly observable at 415, 542 and

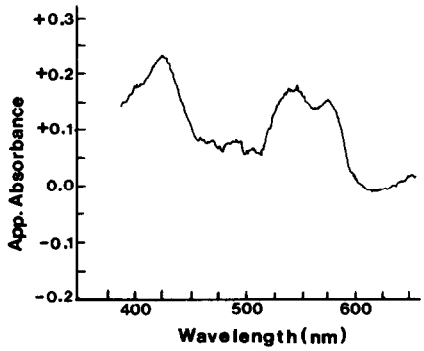

Figure 1
A diffuse reflectance spectrum (referenced to $BaSO_4$) from the thigh of one of the untreated rabbits tested. Units are apparent absorbance versus wavelength in nanometers. The relative intensities of peaks are altered due to scattering in the tissue. The absorption resonances correspond to those of HbO.

577 nm. The relative intensities of the absorption resonances are somewhat different from those of aqueous solutions of HbO. The 577 nm peak was used rather than the 415 nm, for determination of HbO, because it was minimally affected by Hb absorbance. The 430 nm peak was used for Hb determination as the 555 nm peak was difficult to measure due to the presence of the 542 and 577 nm HbO peaks. Methaemoglobin was followed at 404 nm as a shoulder to Hb peak at 430 nm.

The cutaneous response of a rabbit to an injection of 1 mg kg^{-1}, a relatively high, sublethal dose, of soluble epidermal protein into an ear vein, is shown in Figs 2(a) and 2(b). These spectra represent difference spectra prepared relative to control measurements recorded just prior to injection. Negative

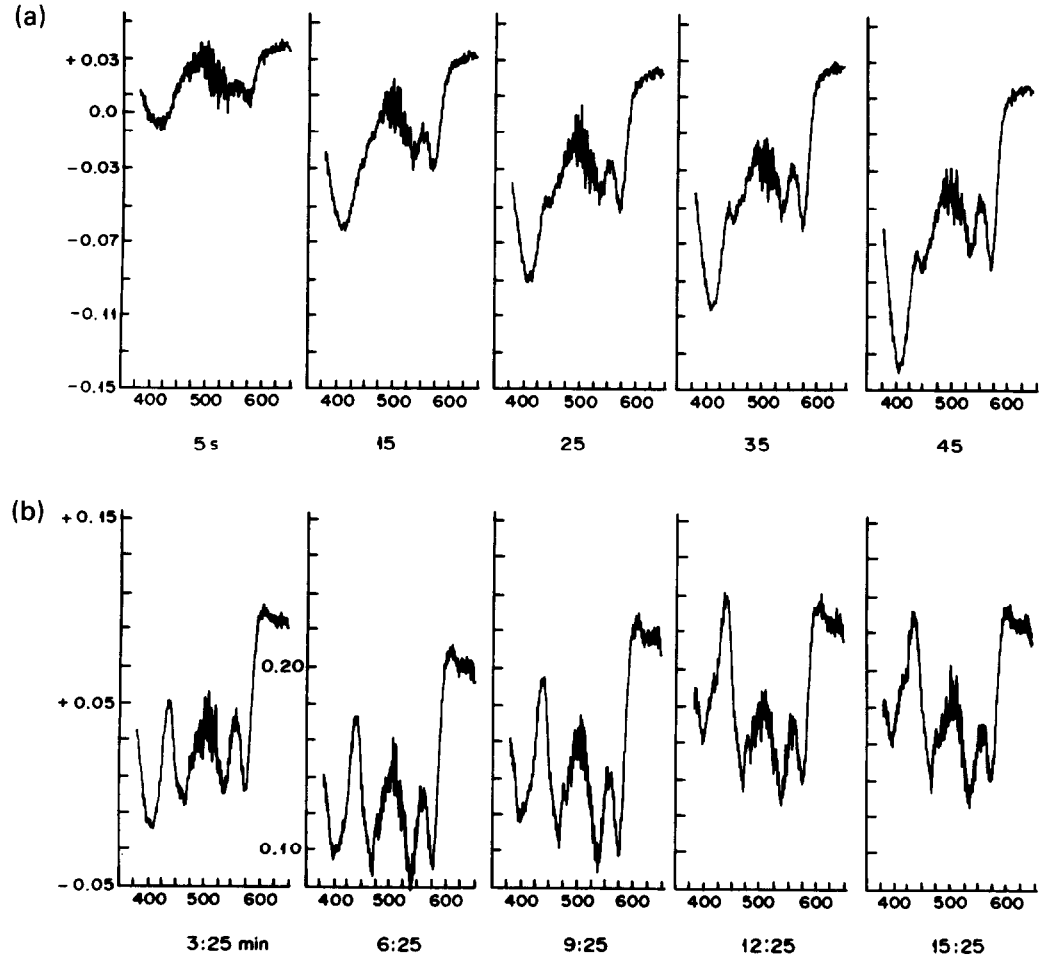

Figure 2
(a) Sequential difference DR spectra (referenced to normal skin before treatment) obtained from the thigh of a rabbit treated with epidermal catfish secretion (1 mg kg^{-1}) showing early responses. A significant decrease in the HbO absorption resonances can be seen. From 25 s onward, a positive absorption resonance due to Hb can be perceived. (b) Sequential DR spectra are presented at 3-min intervals as a continuation of the experiment of part (a). A prominent, increasing Hb absorption resonance was seen at 430 nm. Two negative resonances for HbO are also seen at 542 and 577.

values on these figures indicate a decrease, while positive values indicate an increase in that particular chromophore. In the first panel of Fig. 2 the spectra were collected at 10-s intervals, while in the second panel the spectra were collected at 3-min intervals.

In the first five spectra the absorption resonances of HbO were evident (415, 542 and 577 nm) and were all negative. Ratios of peak intensities remained essentially constant indicating simply a decrease in chromophore concentration. Absorption resonance for deoxyhaemoglobin (Hb) was evident at 430 nm and became prominent at 3 min. The very small absorbance at 25 s achieved significant dimensions. This change can be accounted for by the changes in the chromophores. The spectrum as a whole also shifted towards the negative side. The absorbance at 577 nm relative to that at 640 nm was used to indicate the change in concentration of HbO. The absorbance at 430 nm relative to the absorbance on the control curves which lacked this peak were used to estimate changes in the Hb concentration. Measurement of the changes at 577 nm and at 430 nm as functions of time yielded information on the changes in the concentration of the appropriate chromophore.

Figure 3 shows changes in absorbance and LD signals with time. A very rapid change in the LD signal preceded a still rapid change in the HbO. The LD signal plateaued within 0.2 min, while the HbO DR signal continued to decline during 1 min after injection. It should be noted that the LD signal is proportional to the number of scatterers and their velocity. Therefore, a change in both parameters enhances dramatically the change in the signal, but this does not alter the kinetic time course. The DR measurements depend only on the number of chromophores, assuming that scattering by the subcutaneous tissue does not change. After 0.4 min the Hb signal started to increase and continued to do so for more than 10 min, before it levelled off. While the results presented here were from the New Zealand white rabbits given the high dose, they are representative of all other experiments at similar dose.

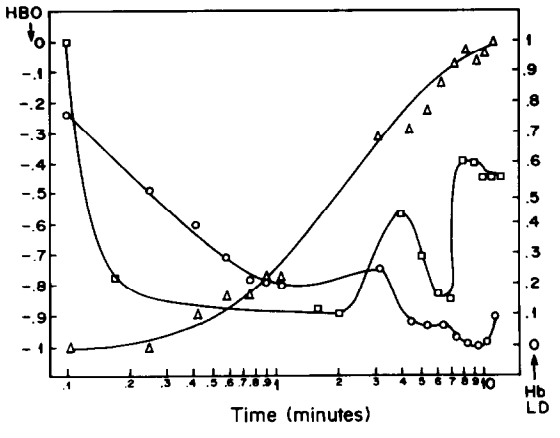

Figure 3
The changes in the DR values at 577 nm (HbO) and at 430 nm (Hb), and the change in the LD signal are shown following treatment (at time zero) with 1 mg kg^{-1} of epidermal gel. The change in the HbO (○) is negative, and is indicated on the left axis; while the change in the Hb (△) and the LD signal (□) are positive and are indicated on the right-hand axis, the units for Hb and HbO are absorbance, and those for LD are arbitarily normalized to 1 at time zero. The time axis is in logarithmic scale to show the rapid changes observed in the LD signal and the HBO absorption immediately following treatment.

Treatment of the animals with lower sublethal dose of ST (0.5 mg kg^{-1}) caused a small transient increase in the HbO absorption. Within 1 min, the increase changed to a strong decrease, reaching a maximum at 6.5 min postinjection. After that time, a slow recovery commenced.

The experiments that were conducted to examine the effects of saline on the animals showed no changes during 20 min of monitoring in both the DR and the LD readings.

Discussion

The DR spectral changes of animals injected with epidermal secretions can be divided into three phases. First, there was an initial decrease in total measured HbO levels. The rate of decrease in measured HbO was dose dependent. With low doses the rate of decrease was slow, starting within a few seconds but reaching a ½ maximum response in about 90 s.

As the dose was increased to 0.5 mg kg^{-1}, the rate of the initial response was roughly doubled, giving a half-time of about 36 s. Increasing the injected dose further to 1 mg kg^{-1} lowered response times to <5 s.

The second phase was a reversal of the initial decrease in HbO, back to a level near that of the control. Again the rate of this reversal was dose dependent and was much faster with higher toxin concentrations. At high dose levels the initial transient large decrease for HbO absorbance reached a maximum in <1 min.

The third phase occurred only where high doses of epidermal secretions [>1 mg kg^{-1}] were used. Following the transient decrease in absorbance of HbO, a slowly developing increase in absorbance due to the formation of Hb and methaemoglobin was observed as indicated by shifts in spectral maxima and band intensities.

High levels of toxin cause major tissue damage, including clotting in the lungs and leakage of blood into tissues [1]. As a result, lung function is impaired and shifts to higher levels of Hb are to be expected. Long term repeated low doses of toxin induce considerable lysis of red blood cells. This is consistent with the previously demonstrated presence of a haemolytic factor in the epidermal secretion [9].

The initial response of rabbits to injection of the skin toxin was too fast to be accounted for by circulation of materials through the vascular system. Circulation of blood in an average rabbit requires more than 2 min [10]. However, upon injection of toxin into an ear vein and subsequent spectral measurements at the other ear veins or on the thigh, showed responses within 5 s. Laser doppler studies showed significant response in <1 s. Since circulation from the site of injection to the heart and on to the test area requires longer times than 1–5 s, this initial response must have been mediated by some neural signal supplied in the toxin preparation or stimulated in the rabbit by toxin action. It is known that the epidermal secretions contain a molecule which may compete at acetylcholine receptor sites [4]. This may explain the observed neuro-responses.

Diffuse reflectance measurements provided simple, rapid measurements of rabbit responses to catfish toxin and helped define the mechanism of toxin actions by identifying key features of the animals' physiological responses. The information obtained supports and extends the conclusions reached through earlier toxicity and biochemical studies. The spectral detection of methaemoglobin and the increased Hb levels were consistent with enzymic and chemical analyses, and with histological studies of the effects of the toxin [11]. It was unexpected that initial responses to toxin would be so rapid that circulation of toxins through the vascular system could not account for the detected changes. These studies, therefore, extend our understanding of the toxic responses to the catfish secretions and at the same time open new directions for further study.

These studies also demonstrate a general applicability of diffuse spectral method. The method provides rapid non-invasive measurements of some important physiological parameters. It may be used to monitor various epidermal or subepidermal changes such as erythema or immune responses, in addition to the effects of treatment with drugs. Observation of changes in HbO–Hb ratios at different body sites following drug treatment or blockage of blood flow caused by injury or vascular damage resulting from strokes may be additional important applications.

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