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Research Paper

The CAM-LDPI method: a novel platform for the assessment of drug absorption

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Abstract

Objectives This study aimed to explore the use of the chicken chorioallantoic membrane (CAM) with laser doppler perfusion imaging (LDPI) as a platform to assess absorption of vasoactive drugs.

Methods The optimal age of the CAM to be employed in the test and the indicator of vasoactivity were first established. Test substances that included common solvents and vasoactive drugs were tested on the CAM surface to determine their irritancy and blood perfusion effects.

Key Findings Insignificant changes in blood perfusion were observed with deionized water, 0.9% w/v soldium chloride and 5% w/v glucose monohydrate, as well as theophylline and glucagon. Complex changes in blood perfusion were detected with ethanol, N-methyl-2-pyrrolidone, glycerin and propranolol. Both caffeine and glyceryl trinitrate resulted in a drop in blood perfusion.

Conclusions It was concluded that the LDPI offers a rapid and non-invasive method to measure blood perfusion in the CAM. The latter provides a potentially useful platform in formulation studies to evaluate the effects of additives on drug absorption using caffeine or glyceryl trinitrate as model drugs.

Introduction

In vivo drug absorption is one of the critical parameters used to determine the bioavailability of a drug. Drug absorption is a complex process that is affected by various drug properties, formulation factors and physiological variables. In formulation studies, there is ongoing investigation on the effects of additives on drug absorption. The traditional method, which involves the use of animals to assess drug absorption, is not only expensive but also time-consuming. In the pursuit of alternatives that employ living tissues but avoid the use of whole animals, it would be ideal to have an in-vivo method that is sensitive, inexpensive and capable of high throughput.

Chick chorioallantoic membrane

The chick chorioallantoic membrane (CAM) is a potential alternative to animal investigation of the effects of additives on drug absorption. The use of the CAM does not pose ethical issues and does not require ethics committee approval for use in countries such as the USA and Switzerland. Another attractive factor is its reasonable price (a few dollars). The CAM, also known as the chorioallantois, is derived from two extra-embryonic membranes: chorion and allantois. The chorion and allantois start to fuse together to form CAM at about 4 days after the egg is laid by a hen.[1] The incubation period of the egg is 21 days. The day that the egg is incubated, which may not coincide with the day that it is laid, is known as embryo age 0 day (EA 0). The following days are then known as embryo age 1 day (EA 1), embryo age 2 days (EA 2) so on and so forth. Histologically, the CAM consists of three layers: ectoderm, mesoderm and endoderm.[2] Besides being a respiratory and excretory organ, it provides support to the underlying extraembryonic blood vessels such as the vitelline vessels over the surface of the yolk. The CAM is also involved in the transport of sodium and chloride from the allantoic sac, and calcium from the eggshell to the vasculature. Through dilation of the associated blood vessels (known as chorioallantoic vessels), the embryo is able to avoid overheating for a relatively long time.[3]

Advantages and limitations of the CAM

The egg is disinfected and an opening is made in the shell to expose the CAM for investigation. The CAM can be viewed without disruption through the opening, which is sealed with transparent tape.[3] No restraint of the CAM is necessary in comparison with animal models. In the UK, fertilised eggs up to 10 days old can be used without the need for a licence for animal experimentation.[4] This is in line with the British

Animal Welfare Act (1986), which considers an embryo as an animal only when it has reached half its gestational age. In the case of the egg, this translates to the fact that eggs up to EA 10 can be used for experimentation without restrictions. The use of the CAM concurs with the initiative of the European Center for the Validation of Alternative Methods and the European Partnership for Alternatives to Animal Testing, which seek alternative approaches to animal testing. CAM also possesses advantages over non-animal models such as *caco-2 cells* as it represents an in-vivo system, unlike *caco-2 cells* which are ultimately in-vitro systems or at best, *in situ*.

The fertilised egg hatches in about 21 days. This relatively short incubation period of the egg, coupled with disturbances associated with the increasing embryo movements at older ages, decreases the period of time during which an egg can be employed for experimentation.[5] In angiogenesis assays, the naked eye is used to assess changes in vasculature in response to drug instilled. This method is subjective and not sensitive to minute changes in vasculature dimensions. The CAM is also not suitable for long-term studies as the partially deshelled egg is prone to contamination and the developing embryo is very fragile.[3]

The laser Doppler perfusion imager

The laser Doppler principle involves the use of a light beam directed onto a tissue sample with vasculature. The red blood cells in the vessels reflect light at an altered frequency, i.e. the Doppler effect. This frequency-shifted light mixes on the surface of a suitable photodetector with light scattered from static structures and the beat frequency generated is related to blood perfusion.[6] The laser Doppler perfusion imager (LDPI) measures a parameter termed as *perfusion*. Perfusion is related to speed times number concentration of red blood cells.[7]

The ease of use of the LDPI, as well as its non-invasive property, has resulted in its extensive use in various studies. Some examples include the characterisation of cerebral blood flow,[8] basal skin blood flow during the menstrual cycle,[9] retinal blood flow,[10] colonic blood flow,[11] microvascular blood flow in animal muscles,[12] the investigation of change in perfusion with epicondykitis[13] and rheumatic disease.[14] Treatments that induce change in blood perfusion have been investigated: capsaicin treatment,[15] corticosteroid therapy,[16]) penetration of methyl nicotinate[17] and permeation enhancer studies.[18]

LDPI imaging can be automated. It is simple to use and produces fast, objective and accurate results. A large test area can be monitored without compromising resolution. Measurement does not involve direct contact with the test site. This is advantageous as physical contact may elicit certain responses, such as a change in the blood flow rate of the tissue. Studies have shown that measurements are reproducible and the technique is selective in measuring the perfusion of the object in question.[19] Validated blood flow measurement methods such as hydrogen clearance, flow through glass capillaries and radioactive microsphere methods were conducted by Tanaka *et al*.[20] The results from these methods were found to be correlated with laser Doppler flowmeter (LDF) measurements, indicating that the LDF could provide quantitative determination of blood flow. A comparison of the LDPI with the LDF and dynamic thermographic imaging showed good correlation.[21,22] This indicates that LDPI measurements have good correlation with validated blood flow measurement methods. The LDPI method and laser speckle perfusion imaging have also been compared with the recently developed tissue viability imaging system. They were tested on different tissue compartments and the results demonstrated that each was more suited to different measurement sensitivities and could perhaps be used for complementary results.[23]

Assessment of drug absorption on the CAM

It was hypothesised that the CAM could be used to evaluate biological absorption of vasoactive drugs by measuring blood perfusion. The extent of change that vasoactive drugs exert on blood perfusion can thus be quantified and used as a marker to evaluate the influence of formulation factors on drug absorption.

The use of the LDPI to measure perfusion of the CAM has not been reported. Since the CAM is a potentially useful model for assessment of drug absorption, there is an impetus to develop the CAM-LDPI method as an alternative to animal studies. In this study, the ability of the LDPI method to quantify blood perfusion in the CAM was investigated. The influence of CAM maturity on blood perfusion was studied in optimising the CAM for use. The indicator for vasoactivity was also established. The changes in blood perfusion in response to different concentrations of solvents and drugs were further determined to explore the feasibility of the CAM–LDPI method to assess drug absorption through the CAM.

Materials and Methods

Freshly laid fertilised specific pathogen-free chicken eggs of the *White Leghorns* species were purchased from Agri-Food and Veterinary Authority of Singapore (AVA), Singapore. Test drugs used included propranolol (Zuellig Pharma, China), theophylline hydrate BP (Janson Chemicals, Singapore), caffeine BP (Luen Wah Medical Company, Singapore), glucagon (Novo Nordisk, USA) and glyceryl trinitrate. Two forms of glyceryl trinitrate, GTN 0.5 mg per tablet (Angised™, GlaxoSmithKline) and GTN 1 mg/ml (Merck, Germany), were used. The solvents used were deionised water, 0.09% w/v sodium chloride (Merck, Germany), 5% w/v glucose monohydrate (Merck, Germany), glycerin (BDH,

England), 70% v/v ethanol (Far East Distillers, Singapore) and N-methyl-2-pyrrolidone (NMP) (International Specialty Products, USA).

Preparation of the CAM

The entire exterior of the egg at embryo age 7 (EA 7) was swabbed with a disinfectant comprising 70% v/v ethanol and 10% w/v povidone iodine prior to manipulation. A hole of about 2 cm in diameter was made at the blunt end of the egg (Figure 1). The two outermost egg membranes were then removed to reveal the CAM for examination. This procedure was performed in a sterile environment maintained and provided for by the Cleansphere CA100 (Safetech Limited, USA). The hole was then covered with parafilm previously sprayed with 70% v/v ethanol. The egg was placed in the incubator and the condition of the CAM examined and its blood perfusion measured at specific embryo age.

Study of the relationship between CAM maturity and blood perfusion

The PERISCAN PIM II LDPI (Lisca, Sweden) uses a helium-neon laser light beam with a wavelength of 670 nm, with a maximum output power of 1 mW, beam diameter of 1 mm and a scan depth of approximately 0.5–1 mm depending on tissue properties. The LDPI was placed in an environment with a room temperature of about 22–23°C, allowing 15 min equilibration time before experimentation. A scan area of 2 mm by 10 mm was measured. Blood perfusion of the CAM (without any test substance) was measured daily at pre-determined operation settings of the LDPI (amplitude 1, threshold 6, measurement area 2 × 10 mm, 11 cm between Doppler head and CAM, low scan speed and very high resolution).

Study of the effects of test substances on blood perfusion in CAM

The test substances included common solvents and drugs (Table 1). Different concentrations of each of the drugs were prepared in 0.9% w/v sodium chloride solution. An amount of 30 µl of each sample was placed on the CAM and blood perfusion was measured at pre-determined operation settings of the LDPI (amplitude 1, threshold 6, measurement area 2 × 10 mm, 11 cm between Doppler head and CAM, low scan speed and very high resolution). The average perfusion of the 2 × 10 mm measurement area was obtained. The CAM and LDPI were not moved throughout the experiment to ensure that the same area on the CAM was measured. Each test substance was tested in quintuplicate, i.e. on five different CAMs, unless otherwise stated.

Statistical analysis

Statistical analysis of the changes in blood perfusion was performed using the paired samples *t*-test with significance level set at 0.05 (SPSS for Windows Version 11, Lead Technologies, USA). Statistical analysis of the changes in blood perfusion between treatment groups was performed using one-way ANOVA with significance level set at 0.05 (SPSS for Windows Version 11, Lead Technologies, USA).

Results

Influence of CAM maturity on blood perfusion

The influence of CAM maturity on the magnitude and reproducibility of the blood perfusion readings was determined. The blood perfusion values were relatively similar from EA 8 to EA 13 ($P > 0.05$), except for an increase in blood perfusion at EA 11. Beyond EA 13, the values were not similar ($P < 0.05$) as blood perfusion increased sharply and then decreased beyond EA 16 (Figure 2). Measurements were not taken at EA 7, which was the day de-shelling was performed, to avoid any

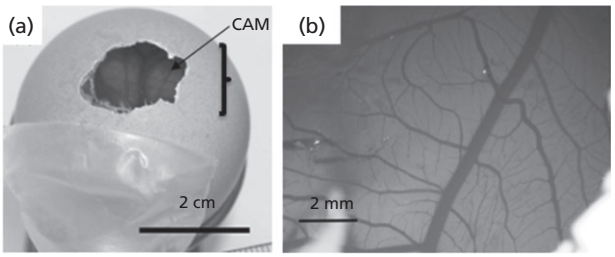

Figure 1 (a) Exposure of the CAM by partial deshelling method. (b) CAM surface.

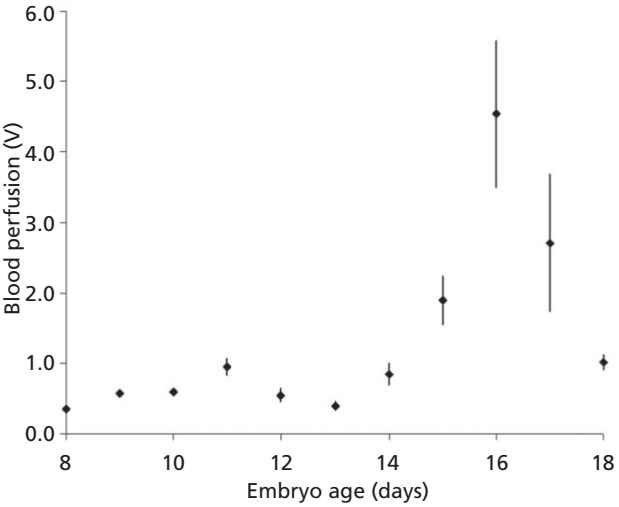

Figure 2 Baseline blood perfusion in CAM at different embryo ages. Values shown are expressed as mean ± SEM, $n = 5$.

Table 1 Perfusion ratio of test substances

	Test substance	Amount of test substance	Perfusion ratio	Standard error of mean	Coefficient of variance
Control (without test substance)	–	–	0.99	0.03	3.17
Solvents	Glucose monohydrate	5% w/v	0.98	0.04	3.30
	Deionised water	–	1.04	0.04	3.38
	Sodium chloride	0.9% w/v	0.96	0.02	2.44
	NMP	1% v/v	1.00	0.06	6.34
		10% v/v[a]	–	–	–
	Glycerin	100% v/v[b]	–	–	–
	Ethanol	70% v/v	0.99	0.05	5.36
Drugs	Caffeine	4 mg/ml	1.00	0.04	3.97
		8 mg/ml	0.95	0.02	2.44
		12 mg/ml	0.92	0.02	1.74
		16 mg/ml	0.99	0.03	2.67
		20 mg/ml	0.96	9.95	5.03
	GTN	0.016 mg/ml	0.76	0.03	3.42
		0.02 mg/ml	0.99	0.05	4.80
		0.04 mg/ml	0.84	0.03	3.71
		0.06 mg/ml	1.00	0.02	2.41
		0.1 mg/ml	0.94	0.02	2.59
		0.2 mg/ml	1.02	0.06	6.25
		0.3 mg/ml	0.93	0.04	4.81
		0.4 mg/ml	0.99	0.02	2.12
		1 mg/ml	0.95	0.03	3.60
	Propranolol	1 mg/kg	1.03	0.11	11.04
		2 mg/kg	1.12	0.14	13.73
		5 mg/kg	1.89	0.27	14.29
		10 mg/kg	1.40	0.33	23.93
		15 mg/kg	1.41	0.39	27.82
	Glucagon	0.1% w/v	0.99	0.06	6.61
	Theophylline	10 mcg/ml[a]	–	–	–
		20 mcg/ml[a]	–	–	–
		30 mcg/ml[a]	–	–	–
		40 mcg/ml[a]	–	–	–
		50 mcg/ml[a]	–	–	–
		60 mcg/ml[a]	–	–	–
		1.5 mg/kg[a]	–	–	–
		2.0 mg/kg[a]	–	–	–
		2.5 mg/kg[a]	–	–	–
		3.0 mg/kg[a]	–	–	–
		3.5 mg/kg[a]	–	–	–

[a]Denotes insignificant effect on blood perfusion of the CAM. [b]Denotes incidence of embryotoxicity. The amount of solvent was expressed in concentration while that of drug is in dose.

possible influences of the trauma of de-shelling on the perfusion measurements. The standard error of the readings generally increased with embryo age.

Indicator of vasoactivity

Variation in the baseline perfusion readings among the CAMs was significant because of biological variation. It is therefore inappropriate to compare the vasoactive effects of the test substance based on the absolute perfusion readings obtained. Hence, a ratio was calculated which provided a more meaningful comparison. The perfusion ratio was obtained by dividing the blood perfusion value of the test substance with the baseline blood perfusion value. The perfusion readings were taken at time intervals of 1 min. In general, significant changes in blood perfusion occurred 10–25 min after the application of the test substance and lasted for 10–20 min. Due to fluctuations in readings, the mean of the baseline perfusion values taken over 5 min prior to the application of the test substance was used to compute the perfusion ratio. Theoretically, a perfusion ratio greater than 1 would indicate an increase in blood perfusion whilst a ratio lower than 1 would

indicate a decrease in blood perfusion. The perfusion ratios of the controls were all very close to 1. However, blood perfusion in any biological system will be prone to fluctuations and biological variation, which explains some slight deviations. Taking biological variation into account, the SE of the control was used to calculate a range of blood perfusion ratio values, i.e. the mean of perfusion ratios for control ± 2SE. Hence, any perfusion ratio that fell within the range of 0.9 to 1.1 was not considered as significantly different. The perfusion ratios obtained for the various test substances are shown in Table 1.

Control and some common solvents

Blood perfusion studies were first conducted on the CAM without the application of any substance, drug or solvent to assess the baseline blood perfusion pattern of the CAM. The blood perfusion profile showed a very slight sinusoidal pattern, with small fluctuations in perfusion over time, giving perfusion ratios close to 1 (Figure 3a). The same was observed for deionized water, 0.9% w/v sodium chloride and 5% w/v glucose monohydrate, respectively. The above observation for 0.9% w/v sodium chloride concurs with the absence of irritation to the CAM by 0.9% w/v sodium chloride, as reported by other researchers.[24]

Glycerin

A slight drop in blood perfusion ratio immediately after the addition of glycerin was observed. This was followed by fluctuation generally within the baseline blood perfusion range before a significant drop after about 12 min (Figure 3b). Glycerin required an incubation period of at least 12 min for its vasoactive property to manifest. The depression in blood perfusion lasted for about 10 min after the onset of change in blood perfusion, without recovery to baseline level within the duration of the study.

Ethanol

Ethanol has been reported to be capable of causing both vasoconstriction and vasodilation.[25,26] Its effect on the blood perfusion of the CAM has not been reported. Ethanol can be used as a solvent, as well as a disinfectant. It should be pointed out that 70% v/v ethanol was used to disinfect the egg surface in this study. The egg shell possesses pores which allow the loss of water as well as gaseous exchange.[27] Hence, the disinfection process might potentially result in absorption of ethanol through the shell. It is important to know if this would affect the perfusion study of drug substances. The investigation on the effect of 70% v/v ethanol served two purposes in this study: to assess the influence of 70% v/v ethanol on the vasculature when it is used as a solvent for drugs or as a disinfectant. When 70% v/v ethanol was instilled on the surface of the CAM, an interesting blood perfusion profile

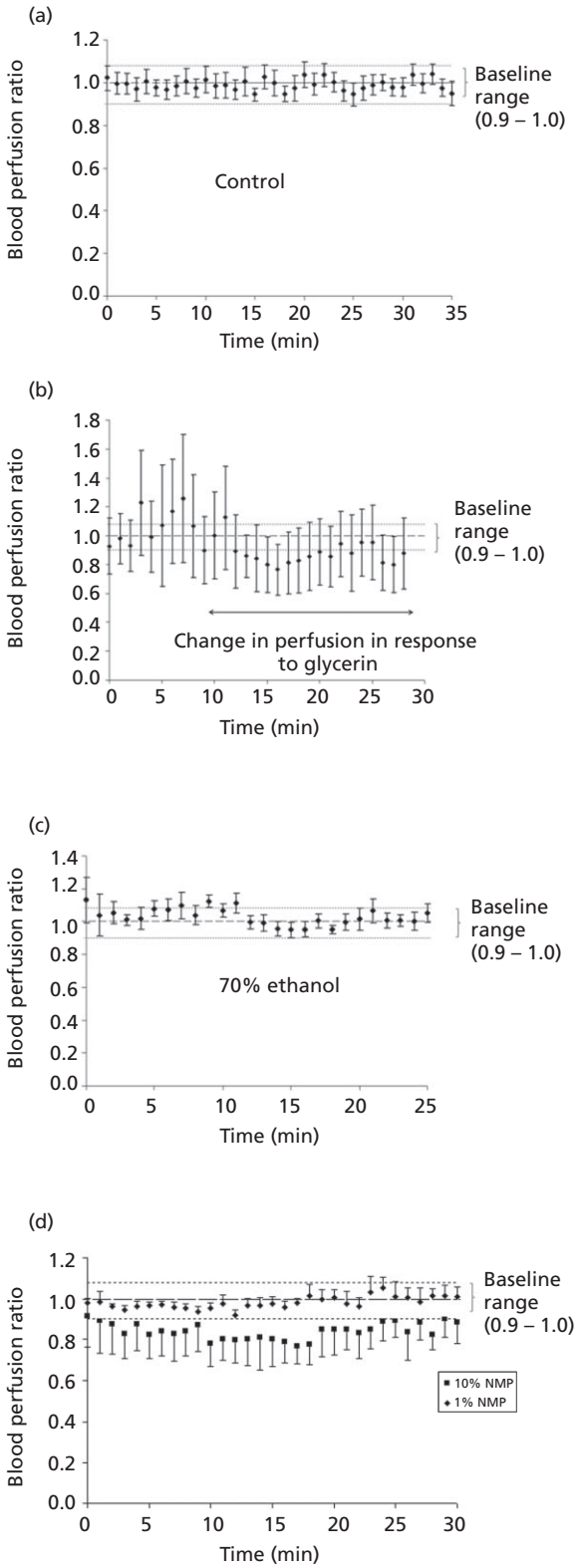

Figure 3 Blood perfusion ratio over time for (a) control, (b) glycerin, (c) 70% v/v ethanol and (d) NMP. Values shown are expressed as mean ± SEM, $n = 5$.

was observed (Figure 3c). Immediately after the application of 70% v/v ethanol, the blood perfusion ratio increased significantly. Fluctuation in blood perfusion was observed but it remained above the baseline. After 12 min, blood perfusion decreased to a level below the baseline. About 20 min after the application of 70% v/v alcohol, the blood perfusion returned to its baseline value. Overall, the blood perfusion ratios generally lie within the baseline range, indicating that 70% v/v ethanol exerted little and short-lived effect on the blood perfusion. The use of 70% v/v ethanol to disinfect the egg surface was therefore unlikely to affect the perfusion studies.

N-methyl-2-pyrrolidone

The effect of NMP was also evaluated as it has been demonstrated to be a good permeation enhancer.[28,29] NMP is used in numerous applications because of its good solvency properties. In addition to its use in textile or plastics, it is also used in the pharmaceutical industry in the formulation of drugs delivered through the oral and transdermal routes. Two concentrations of NMP (1% w/v and 10% w/v) were tested. A very significant drop in blood perfusion was seen about 1 min after application of 10% v/v NMP (Figure 3d). Blood perfusion continued to decrease gradually over time and reached the minima around 10 min after application of NMP. Overall, the depression in blood perfusion lasted for about 20 min, with almost full recovery to the baseline blood perfusion. In contrast, 1% v/v NMP did not exert any significant effect on blood perfusion.

Propranolol

Propranolol is a β-blocker that affects both the β_1 and β_2 adrenoceptors. The resultant decrease in blood pressure in human subjects has been attributed to the reduction in cardiac output and decreased peripheral vascular resistance brought about by the action of propranolol. It was also found to act on the peripheral presynaptic adrenoceptors to decrease sympathetic vasoconstrictor nerve activity.[30] It is not known if β_1 and β_2 adrenoceptors are found in the chick embryo and whether propranolol would affect the function of the CAM. Experiments were therefore conducted to determine the effects of different concentrations of propranolol on the blood perfusion of the CAM. Three different doses of propranolol, 7.5, 15 and 30 mg/kg, were used in the first set of experiments. The highest dose is close to the LD50 in rats whilst the lowest dose is close to the high doses used in pediatrics.[31]

A slight increase in blood perfusion was seen when propranolol was administered, followed by a decrease in blood perfusion. The CAM tissues were also found to undergo hemorrhage and the embryo died about an hour after drug application.

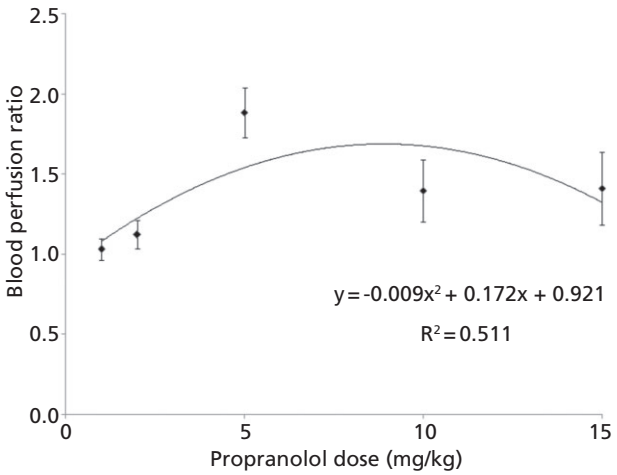

Figure 4 Relationship between blood perfusion ratio and propranolol dose. Values shown are expressed as mean ± SEM, $n = 5$.

The total blood volume of the egg was reported to be about 0.5 ml.[32] Further experiments were carried out with lower doses of propranolol, ranging from 50 to 100 ng in 1 ml of blood in the egg. These drug concentrations corresponded to the therapeutic concentrations in human blood for hypertension management. Perfusion measurement intervals were increased to 2 min over the duration of an hour after the application of propranolol. A transient drop in blood perfusion was seen after these lower doses of propranolol were applied to the CAM, followed by a slight increase. Higher doses of propranolol of 150, 200 and 250 ng/ml were further tested and found to have insignificant effect on the blood perfusion. The response of the CAM blood perfusion to propranolol appeared complex.

In subsequent experiments, the test doses of propranolol were readjusted based on egg weight, covering a wide range that included 1, 2, 5, 10 and 15 mg/kg. With the dose of 1 mg/kg, there was a slight increase in blood perfusion ratio. The latter increased with increasing dose of propranolol up to 10 mg/kg, beyond which blood perfusion ratio decreased (Figure 4). The higher doses of 10 and 15 mg/kg caused haemorrhage and the embryos died about an hour after application of the drug. This confirmed the earlier observations for similar propranolol doses and it probably accounted for the decrease in blood perfusion observed.

A plot of the blood perfusion ratio versus the concentration of propranolol used is shown in Figure 4. All of the propranolol doses caused an increase in blood perfusion. The extent of this increase reaches a maximum at the dose of 5 mg/kg, after which the magnitude of increase in blood perfusion drops. However, at these high concentrations, the change seen in blood perfusion might be complicated by the harmful effects of propranolol on the CAM.

Theophylline

Theophylline, which is a methylxanthine, produces bronchodilation in human subjects through the inhibition of phosphodiesterase in the smooth muscle of the airway. The methylxanthines also induce peripheral vasodilation. Theophylline has been reported to dilate coronary blood vessels.[33] In addition, it stimulates the central nervous system by antagonising adenosine and caused cerebral vasoconstriction, leading to a reduction in cerebral blood perfusion.[34] It has been employed in the treatment of ischemic stroke to produce cerebral vasoconstriction and increase blood perfusion to non-ischemic areas in the brain whilst increasing collateral blood perfusion to the ischemic region. The effect of theophylline on the CAM has not been reported. Doses in the range of 10, 20, 30, 40, 50 and 60 mcg/ml were tested. Blood perfusion readings were taken every minute for 25 min to capture any instantaneous change in blood perfusion. Readings were also taken at the first, second, third and fourth hour since theophylline concentration was previously found to reach a peak 1–2 hours after oral ingestion in humans.[31] However, insignificant effect on blood perfusion over time was observed with the theophylline doses used.

Further studies were carried out to investigate the influence of higher theophylline doses. The doses tested were equivalent to those recommended for pediatrics: 1.5, 2, 2.5, 3 and 3.5 mg/kg.[31] No significant changes in blood perfusion were observed. Theophylline undergoes metabolic transformation to result in the formation of metabolites such as caffeine before exerting a vasoactive methylxanthine effect.[33] The possible absence of this transformation could account for the lack of significant changes in blood perfusion. Hence, a further study was undertaken to establish if the CAM would respond to caffeine.

Caffeine

Vasodilation has been purported to be effected by increased concentration of cyclic adenosine monophosphate, inhibition of Ca^{2+} influx and inhibition of phosphodiesterase in humans. The binding of adenosine to adenosine receptors was found to cause vasodilation.[35] Caffeine is a methylxanthine. It has been reported to exert competitive inhibition of adenosine from binding to adenosine receptors, thereby causing vasoconstriction.[36] The effect of caffeine on the blood vessels of the CAM has not been reported. Vasoconstriction is expected to result in a decrease in blood perfusion. Hence, a decrease in blood perfusion with caffeine would suggest that caffeine causes constriction of blood vessels in the CAM.

The caffeine doses used in this study were 2, 4, 6, 8 and 10 mg/kg. These doses were chosen as they encompass the doses given to adults and children to treat various medical conditions. The choice of test dose also took into account the solubility of caffeine in 0.9% w/v sodium chloride. Blood perfusion was found to decrease almost immediately after the administration of caffeine and gradually returned to the baseline after 30–35 min.

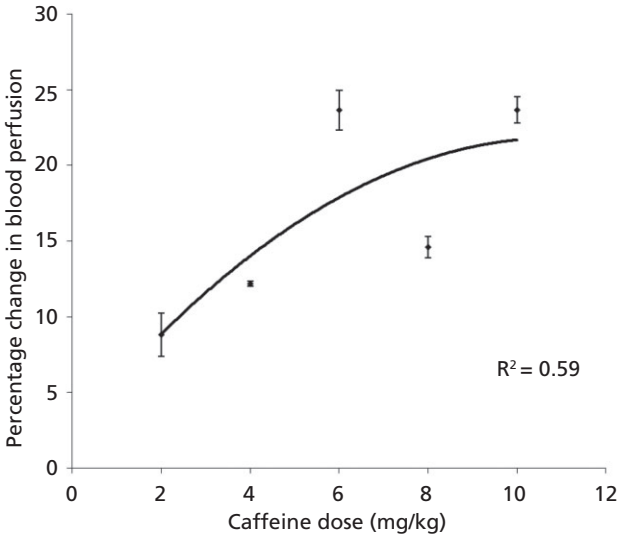

Figure 5 Relationship between percentage change in blood perfusion and caffeine dose. Values shown are expressed as mean ± SEM, $n = 5$.

The degree of change in blood perfusion was higher than that seen with theophylline. These results were also comparable to the change in blood flow seen in humans in LDPI studies conducted with vasoconstrictive topical corticosteroids.[37] The blood perfusion ratio was plotted against caffeine dose, where perfusion decreased slightly with increasing caffeine concentration. Further analysis of the data was conducted, whereby percentage change in blood perfusion was plotted against the caffeine dose to eliminate the variability in baseline blood perfusion of the different eggs used (Figure 5). The relationship found was not straightforward. According to the Pearson's correlation test, it is non-linear ($P > 0.05$) and best described by a curve that initially appears linear before gradually attaining a plateau.

Glyceryl trinitrate

GTN is part of the nitrate family, and releases nitric oxide in human vascular smooth muscle. Nitric oxide is a powerful vasodilator which elicits a rapid response from large arteries and veins. Venodilation, arterial-arteriolar dilation as well as dilation of vessels in the heart occur in the presence of GTN.[38] Veins are more sensitive than arteries to nitric oxide whilst arterioles are the least sensitive.[30] Owing to import restrictions, pure GTN was not available for this study. Hence, two different types of GTN were used to investigate if the results were affected by the additives in GTN tablets as opposed to GTN in injection form.

Tablet dosage form

GTN tablets were dissolved and diluted in 0.9% w/v sodium chloride to prepare the test solutions. Low doses of GTN were initially employed to determine the ability of GTN to elicit any vasoactive effects on the CAM model. Doses tested were 0.01, 0.0125, 0.025, 0.0375, 0.05, 0.075, 0.1, 0.12, 0.15, 0.2 and 0.25 mg/kg of the egg. These doses are close to the clinically used sublingual doses.[39] Blood perfusion was found to decrease immediately after the application of GTN. The perfusion values before and after application of GTN were compared. The depression of blood perfusion in the CAM was seen to last for at least 15 min before the blood perfusion level returned to the baseline. Blood perfusion was expected to increase as GTN is known to be a vasodilator. However, the opposite effect was observed. A study using optical Doppler tomography to monitor blood perfusion in the CAM showed an increase in artery blood perfusion and a decrease in vein blood perfusion following application of GTN.[40] GTN decreased the blood flow in arteries but increased the blood flow in veins. The mechanism behind this differing response to GTN is unknown. Arteries and veins can be distinguished by observing the direction of blood flow in the vessels with a stereo dissecting microscope.[2,41] In the present study, the vessels measured were found to be brighter red in colour, suggesting that they were veins. CAM veins are equivalent to the pulmonary veins in humans. They bring oxygenated blood back to the embryo and would hence deliver blood that is brighter than those carried by the CAM arteries. Coupled with the decrease in blood perfusion, this observation suggested that majority of the vessels measured were probably veins. The findings highlighted the significance of identifying whether the vessel measured was a vein or an artery. It also showed that the CAM studied had more predominant veins than arteries.

In this study, blood perfusion ratio decreased with all the GTN doses employed. The relationship was, however, not straightforward and it was of interest to determine a simple mathematical model that best describes the relationship. Hence, the data was further analysed by plotting the GTN doses with the various derivatives of perfusion, i.e. perfusion change, percentage or log perfusion change and square root of perfusion change. Except for the plots of GTN doses versus the square or square root of perfusion change, sine wave relationships were noted. Sine waves were also seen in the plot of the change in vessel diameter against GTN dose (not published). This suggested that the manner by which GTN interacted with the relevant receptors was complex and/or compensatory mechanisms existed in response to GTN. The plot of the square root of perfusion change versus GTN dose in the range 0.01–0.05 mg/kg showed a linear relationship with an R^2 value of 0.99 (Figure 6a). The presence of various excipients in GTN tablets could have served as a confounding factor in the above study. Hence, the investigation was extended to include GTN injections to address this.

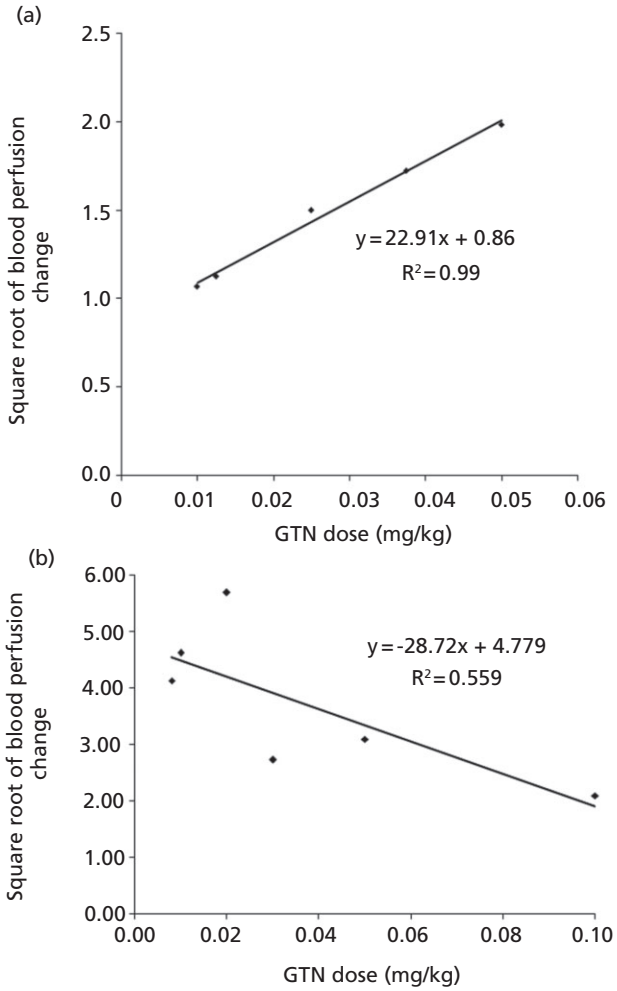

Figure 6 Relationship between square root blood perfusion change and GTN dose prepared from (a) tablets (n = 5) and (b) injections (n = 5).

Injection dosage form

GTN was diluted from a stock vial of GTN injection that consisted of GTN at a concentration of 1 mg/ml. This was carried out to determine if the presence of the excipients in the GTN sublingual tablet had any significant effect on blood perfusion. The doses tested were 0.01, 0.03, 0.05, 0.1, 0.15, 0.2 and 0.5 mg/kg of the egg weight, to correspond with the doses prepared from the GTN tablets. The trend obtained using the GTN injection was different (Figure 6b).

Blood perfusion in CAM veins and CAM arteries

Attempts were made to differentiate the veins from the arteries by their appearance. The blood perfusion in the respective

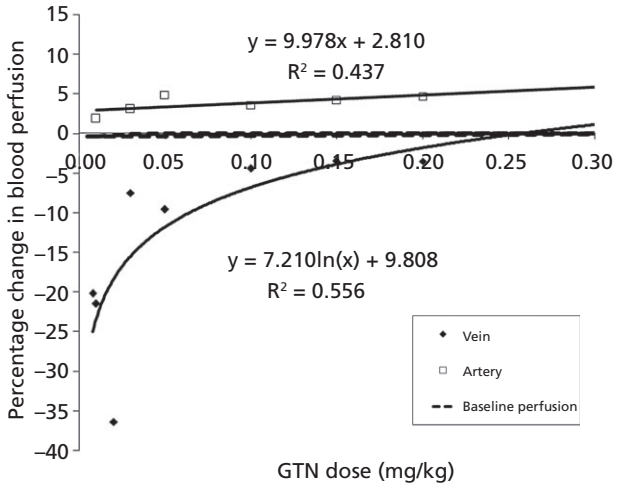

Figure 7 Relationship between percentage change in blood perfusion and GTN dose for veins and arteries ($n = 5$).

veins and arteries in response to the different GTN doses was then determined. The CAM arteries displayed vasodilation in response to GTN, as indicated by their greater blood perfusion with respect to the baseline. The reverse was exhibited by the CAM veins, indicating vasoconstriction. As most of the vessels measured previously were veins, an overall reduction in blood perfusion was obtained. Amongst the various derivatives of blood perfusion change, the percentage change in blood perfusion showed an interesting trend (Figure 7). The change in blood perfusion in the arteries was slightly increased by increasing GTN dose. Interestingly, the change in blood perfusion in the veins was decreased by increasing GTN and the effect was markedly greater.

Glucagon

The effect of 0.1% w/v glucagon on the blood perfusion of the CAM was evaluated. This concentration is commonly used in the regulation of high blood glucose levels. It did not have any significant effect on blood perfusion in the CAM. A slight sinusoidal blood perfusion pattern, similar to that of the baseline blood perfusion, was seen.

Discussion

Influence of CAM maturity on blood perfusion

EA 14–18 is classified as the late stage of embryo development during which the microvasculature of the CAM is fully matured.[27] This could have accounted for the sharp increase in blood perfusion of the CAM after EA 14. The subsequent decline was probably due to the degradation of the mesoderm of the CAM.[27] The blood perfusion readings from EA 9 to EA 11 demonstrated low standard errors. Since the fertilised egg at EA below 10 is not considered to be an animal and thus is exempted from ethical considerations, EA 9 was deemed as the optimal embryo age to perform the perfusion experiments.

Indicator of vasoactivity

The range encompassed by 2SE shows the 95% confidence interval. The baseline mean ratio ± 2SE values are included with all the relevant graphs. The former is indicated by a continuous line and the latter by two parallel dotted lines. Any test substance with a blood perfusion ratio that occurred outside this range was deemed to be able to cause a change in blood perfusion. Using this approach, the influence of normal biological fluctuation on blood perfusion changes was eliminated. No distinct relationship between the perfusion ratio was observed for some test substances. Most of the perfusion ratio values obtained were close to 1. This could be due to autoregulation mechanisms in response to the action of the test substance, thus not resulting in any large changes in blood perfusion.

Solvents

Solvents such as deionised water, 0.9% w/v sodium chloride and 5% w/v glucose monohydrate did not cause any significant change to the blood perfusion. The effects, if any, shown by the drug solutions prepared with these solvents were largely attributed to the drug. On the other hand, glycerin exerted a prolonged vasoactive effect on the CAM vasculature and the CAM was unable to re-establish the baseline blood perfusion level within 30 min. High concentrations of glycerin were reported to cause a reduction in blood flow of the CAM.[42] Use of glycerin as a solvent would confound the effects of the test substance.

Ethanol produced a distinct cyclic profile, in which blood perfusion increased and decreased alternately, indicating the existence of an autoregulation mechanism (Figure 3c). Compensation for the initial increase in blood perfusion resulted in a decrease in blood perfusion before achieving the baseline blood perfusion level once again. This study demonstrated that ethanol exerted an immediate effect on blood perfusion in the CAM and the effect was curbed by an autoregulation mechanism. Such mechanisms, which may involve a host of biological factors, have been reported in humans, where they play important roles in maintaining a stable system known as homeostasis.[43]

It was also possible that ethanol exhibited dual vasoconstrictive and vasodilative effects in the CAM. Ethanol first exerted a vasodilative effect on the CAM vessels, resulting in an increase in the blood perfusion rate. High concentrations of ethanol were found in another study to cause vasoconstriction, potentially through a redox sensitive and cyclooxygenase dependent pathways that affected the production

of reactive oxygen species and Ca^{2+} signalling.[26] Hence, as more ethanol reached the CAM vessels over time, the receptors that caused vasoconstriction were possibly overactivated, resulting in the decrease in blood perfusion henceforth. When used as a solvent, ethanol has the potential to trigger the autoregulation mechanism or cause vasodilation and vasoconstriction. Thus, it is not an ideal solvent to be used in the perfusion study of drug substances.

N-methyl-2-pyrrolidone

NMP at 1% v/v showed insignificant effect on blood perfusion. However, 10% v/v NMP resulted in a decrease in blood perfusion. Hence, the results illustrate that the effect of NMP on the blood perfusion of the CAM was dependent on concentration.

Propranolol

Propranolol has been reported to decrease peripheral vascular resistance in humans,[44] but it had little effect on blood perfusion in the CAM. It could therefore be inferred that the drug receptors found in the CAM are significantly different and less sensitive to propranolol although the CAM is relatively similar to the human buccal mucosa.[45] Propranolol was also found to be toxic to the CAM, resulting in haemorrhage and death of the embryo. Therefore, the use of propranolol as a marker drug for the assessment of blood perfusion is not recommended. A marker drug should not be toxic to the embryo and should preferably display reproducible change in blood perfusion that can be used to provide an accurate estimate of the influence of other variables on the absorption of the vasoactive marker drug.

Theophylline and caffeine

Theophylline had no significant effect on the blood perfusion of the CAM. The relationship of percentage change in blood perfusion with caffeine dose resembles the typical threshold model where the change in response reaches a maxima before levelling out.[31] In the case of caffeine, the maxima was reached with the 10 mg/kg dose.

The results seen with caffeine indicate the existence of autoregulation of blood perfusion by the CAM. Autoregulation involves an extremely complex array of mechanotransduction pathways.[46,47] Blood perfusion has often been regarded as a crucial modulator of vascular tone. Combined effects of myogenic and metabolic regulation in response to the changes in blood flow have been reported.[48] In human skin, perfusion changes are dealt with by the action of reflex neural control. An increase in skin blood perfusion would result in the activation of sympathetic vasoconstrictor nerves and a decrease in skin blood perfusion would result in the activation of vasodilator nerves.[49] It has been postulated that this results from a process known as mechanoreception whereby the mechanosensors located in the endothelium would be triggered by various factors.[50,51] Some proposed mechanotransducers include ion channels, integrins and heterotrimeric G proteins, but their mechanisms of action remain elusive.[52] Myogenic autoregulation involves the vasoconstriction and vasodilation of the vessel to bring about an increase and decrease in resistance, respectively. Metabolic regulation would result in the release of signal molecules to cause vasodilation and vasoconstriction accordingly. This would cause blood flow to change in accordance with the situation. It has been reported that the arteries in the CAM in the early stages of embryonic development adapt to the size of their lumen in response to the blood that flows through in order to maintain mean shear stress at a constant level.[53] Thus, in the CAM, changes in blood perfusion are also met with autoregulation responses.

Glyceryl trinitrate

A linear relationship between square root blood perfusion change and GTN dose prepared from tablets was observed (Figure 6a). This would imply that any change in GTN dose within the linear range would be manifested by a proportional change in the blood perfusion of the CAM. Hence, the effect of a formulation factor that result in a decrease or increase in GTN absorption within this range can be quantified. An estimation of the effect of the formulation factor can be deduced from the change in blood perfusion. Although a wider concentration range is desirable, the fivefold difference in concentration obtained for GTN (0.01–0.05 mg/kg) is useful for evaluating the influence of formulation factors on drug absorption.

However, the above study repeated with GTN doses prepared from injections showed a different trend. The latter suggested that the excipients either in the GTN tablet or injection affected the vasoactive effects of GTN on the CAM. GTN injections consist of GTN in 5% glucose monohydrate whilst GTN tablets consist of GTN with additives that are not disclosed. It is therefore most likely that the excipients of the tablets also exerted significant effect on the blood perfusion.

GTN has been reported to produce vasodilation.[35,38] This effect may arise through the formation of nitric oxide prior to a series of complex pathways that begins with the activation of the intracellular enzyme guanylyl cylase (sGC). This is followed by a cascade pathway that involves cyclic guanosine-3′,5′-monophosphate (cGMP), cGMP-dependent protein kinase and protein phosphorylation, leading to the activation of Ca^{2+}.

The above mechanism may not be the only way that GTN causes vasodilation to occur. Differing responses to GTN have also been reported.[54,55] In some studies, GTN was found to result in a decrease in systolic, diastolic, mean arterial and central aortic blood pressure.[56,57] A decrease in pressure

would ultimately result in a decrease in blood perfusion. GTN was also reported to cause a decrease in venous return, which in turn caused blood pressure and cardiac output to decrease.[58] This particular chain of events may have occurred and resulted in the decrease of blood perfusion in the CAM.

Interestingly, in this study GTN was found to increase blood perfusion in the arteries and decrease blood perfusion in the veins. Overall, the blood perfusion measured was found to decrease in response to increasing dose of GTN. This could be attributed to the measurement of predominantly veins which were clearly seen in the CAM.

Glucagon

Glucagon has been reported to exert a vasodilative effect and increase blood flow.[59,60] However, no effect on CAM blood perfusion was seen, possibly indicating the absence of the receptors associated with glucagon. Information on the glucagon receptors responsible for vasodilation is not readily available, but it has been postulated that the glucagon receptors affect cAMP levels to induce vasodilation.[60] Another possible reason for the limited effect of glucagon may be that an insufficient amount of glucagon was available to act on the CAM.

Conclusion

The CAM has been shown to be a potentially useful model for the assessment of drug absorption through biological membranes by the measurement of blood perfusion using the LDPI method. With an appropriate drug marker, such as GTN, it is able to provide an assessment of changes in blood perfusion and can be used to estimate the effects of pharmaceutical excipients on drug absorption. LDPI offers a potentially rapid and non-invasive way to measure blood perfusion. Hence, the development of CAM assays is timely as alternative 'living animal' models to reduce, refine and, if possible, replace testing in animals.

Declarations

Ethics approval

Approval from the Institutional Animal Care and Use Committee (National University of Singapore) was obtained for CAM older than EA 10.

Conflicts of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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