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Method Optimization on the Process of Iontophoresis with Laser Doppler Fluximetry in the Assessment of Microvascular Endothelial Function

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ABSTRACT

Introduction: Iontophoresis of vasoactive substances such as acetylcholine (ACh) and sodium nitroprusside (SNP) combined with Laser Doppler fluximetry (LDF) is a non-invasive tool used to determine microvascular endothelial function. This study aims to test the effect of sodium chloride on non-specific vasodilatation when used as a vehicle in the process of iontophoresis. This study also aims to define the number of current pulses needed to get the maximum effect during iontophoresis with ACh and SNP using low current strength. **Methods:** The experiment was conducted in five healthy females. Baseline skin perfusion was taken before administration of seven current pulses. Current strength of 0.007 mA and current density of 0.01 mA/cm^2 were used. Acetylcholine was used to assess endothelial dependent vasodilatation, while SNP was used to assess endothelial independent vasodilatation. The mean skin perfusion (AU) responses to the iontophoresis of ACh at the anodal and SNP at the cathode leads were recorded. Sodium chloride (0.9%) was used as a vehicle to obtain concentration of 1% for both ACh and SNP. Iontophoresis of pure vehicle (NaCl) was conducted on a separate day to observe the effect of vehicle only on the iontophoresis process at both anode and cathode. **Results:** Iontophoresis of NaCl showed no significant increase in perfusion compared to baseline at both anode and cathode. Significant increases in skin perfusion were observed with SNP and ACh; a plateau of ACh was reached from the 3rd pulse onwards; while the plateau of SNP was reached from the 4th pulse onwards. **Conclusion:** NaCl could be used as a vehicle for Ach and SNP during iontophoresis as it did not cause non-specific vasodilatation. Using five current pulses are adequate for iontophoresis of ACh and SNP to assess microvascular endothelial function.

Keywords: Microvascular endothelial function; iontophoresis; Laser Doppler Fluximetry; non-specific vasodilatation

INTRODUCTION

Endothelial dysfunction is considered to be the earliest sign of atherosclerosis [1, 2] leading to the development of cardiovascular diseases such as myocardial infarction and angina [3]. Thus, assessment of endothelial function can be used as a tool to detect early vascular changes that may occur due to medical conditions and diseases, or to monitor response to pharmacological interventions.

Skin can provide a good model to measure microcirculation as in diabetes; for example, measurable changes in the skin have been found to pre-date the symptoms of microvascular disease in other organs by many years [4]. Different methods to assess cutaneous endothelial function have been developed; non-invasive method using laser Doppler fluximetry (LDF) combined with the iontophoresis technique is used in the present study. LDF is a device that permits real-time continuous measurement of microvascular blood perfusion [5, 6]. Iontophoresis is a non-invasive method of introducing charged substances across the skin by means of a small electric current. When iontophoresis is combined with LDF, this method enables the detection of alterations in skin perfusion in response to time-controlled delivery of the vasoactive drug. Iontophoresis of acetylcholine (ACh) is often used to test endothelial dependent vasodilatation, while iontophoresis of sodium nitroprusside (SNP) is used to test 'endothelium-independent' vasodilatation [7].

A major disadvantage with drug administration via iontophoresis is the possibility of non-specific vasodilatation due to the influence of electric current on the blood flow. This is called galvanic response and is believed to be related to the voltage required to establish the iontophoretic current that stimulates the local sensory nerves. The resistance of

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the drug in its diluting solution might therefore be an important factor, and investigators have attempted to limit this by using sodium chloride instead of water as the vehicle [8].

Another extremely important issue is the charge density used for iontophoresis; the higher the charge density, the higher the chance to get non-specific vasodilatation. Use of larger chamber sizes and lower currents has been reported to lower the charge density and prevent current induced non-specific vasodilatation [9]. Recently, Droog *et al* (2004) suggested that it is possible to prevent nonspecific effects during iontophoresis of ACh and SNP, by limiting the current density (≈0.01 mA/cm^2) and charge density (<7.8 mC/cm^2). The study also suggested adjusting the concentrations of iontophoresis drugs to 1% and using sodium chloride 0.9% as a vehicle [10]. However, Droog *et al* (2004) employed the laser Doppler imager (LDI).

Therefore, this study firstly aims to test 0.9% NaCl on non specific vasodilatation when used as a diluent / vehicle to prepare acetylcholine and sodium nitroprusside solutions for iontophoresis. Secondly, this study aims to determine the number of current pulses needed for ACh and SNP iontophoresis to get the maximum effect using LDF and low current density.

METHODS

Subjects

For part 1 of the study, five healthy females with mean age of 23.8 ± 0.58 years and BMI of 20 ± 0.74 kg/m^2 were included. All were non-smokers; none had received any medications or supplements for at least 7 days before the experiments. They were fasted overnight and refrained from drinking coffee and high salted food for at least 12 hours before the experiments. All measurements were conducted between the 2nd and 5th days of their menstrual cycle. All subjects had normal hemoglobin (mean 12.40 ± 0.62 g/l), and hematocrit levels (mean 38.76 ± 1.17 %) and were normocholesterolemic (mean 5.36 ± 0.19 mmol/l). The protocol of the study was fully explained to the subjects; all subjects signed an informed consent. The study protocol was approved by the Ethical Committee of Universiti Sains Malaysia.

Equipment

Dual-channel DRT4 laser Doppler fluximetry (Moor Instruments, Axminster, United Kingdom) was used to measure skin perfusion during iontophoresis and a battery-powered iontophoresis controller (Moor Instruments, Axminster, United Kingdom) was used to deliver constant direct current pulses to the skin.

Principles and technique of laser Doppler fluximetry

Dual-channel DRT4 laser Doppler fluximetry is a non-invasive device that permits real-time continuous measurement of microvascular perfusion. In the present study, this instrument was used together with DP1T-V2 skin laser probe (Moor Instrument), which was held stable by using a PH1-V2 probe holder (Moor Instrument). LDF generates a low-intensity beam of infrared monochromatic coherent 780-nm light. This light was delivered to the site of measurement by a flexible fiber optic probe. The laser light usually penetrates to a depth of 1-2 mm of skin [11], and the measurement is therefore predominantly a reflection of perfusion in arterioles, capillaries, postcapillary venules and venules of the superficial dermal plexus [12].

Laser Doppler fluximetry uses laser Doppler shift principles to measure perfusion of blood cells, mainly erythrocytes, in the skin. Photons of laser light scattered in moving blood cells produce a Doppler shift on the reflected light. This reflected light is detected by a photo-detector, and the signal is processed to determine the amount of the frequency shift. Theoretically, the blood perfusion measured by Laser Doppler fluximetry is determined by the product of blood flow velocity and the number of moving red cell corpuscles within the surface micro-vessels of skin. The blood perfusion recorded is generally termed as "flux" and is expressed in perfusion using arbitrary units (AU); the DP1T-V2 probe used also monitors the skin temperature at measurement sites.

Iontophoresis operation principles

A battery-powered iontophoresis controller (Moor Instruments, Axminster, United Kingdom) was used to deliver constant direct current pulses to the skin. The technique of iontophresis is based on the principle that an electric potential difference will cause ions in solution to migrate according to their electrical charges. To transfer a drug into the skin the polarity of the active electrode has to have the same charge as the active ions of the drug, for example; if ACh is to be investigated, anodal currents are used to transfer the cation (ACh^+) during iontophoresis. The quantity of a drug delivered is directly proportional to the total charge that migrates through the skin. The charge measure in millicoulombs (mC) unit is equal to the current measured in milliamps (mA) multiplied by the duration of current flow in seconds.

Drugs

Acetylcholine chloride (ACh) powder (Fluka Chemie Gmbh, Japan) and sodium nitroprusside dehydrate (SNP) powder (Riedel-de Haen, C.O.O. Switzerland) were used to assess endothelial dependent and endothelial independent vasodilatation. Sodium chloride (NaCl) 0.9% at a physiological strength of 0.154M (Excel Pharmaceutical, Selangor, Malaysia) was used to prepare 1% ACh and 1% SNP (Droog, 2004). It was also used alone for iontophoresis to test the effect of pure vehicle on non-specific vasodilatation. Drug solutions were freshly prepared daily before each experiment.

Iontophoresis protocols

The experiments were conducted in a temperature – controlled room (22-23ºC). All measurements were performed in the morning. During a 10-minutes acclimatization period, the participants lie supine comfortably with the right forearm uncovered and supported by a hand supporter to reduce involuntary movement during measurements. The flexor surface of the right forearm was gently cleaned with alcohol to remove dead keratinocytes. Two iontophoresis chambers with 9.5 mm inner diameter for ACh and SNP were used simultaneously. The ACh chamber was attached to the anodal lead while the SNP chamber was attached to the cathodal lead. The two chambers were attached to the skin by special double adhesive discs. Skin sites with superficial veins, hair follicles and broken skin were avoided. The SNP chamber was applied 5 cm from the wrist creases while the ACh chamber was applied 5 cm proximal to the SNP chambers. The laser probes were fixed into the chambers. Each chamber was filled with 0.4ml of drug solution. Iontophoresis of sodium chloride was performed on day 1 while ACh and SNP iontophoresis was performed on day 2. The chambers were carefully washed in running water after each use.

Two minutes of baseline perfusion were recorded before each series of current pulses. Each series of current pulse consists of 7 pulses of 2 minutes each separated by 1 minute interval of current free interval. Current strengths of 0.007 mA (current density of 0.01 mA/cm^2 with chamber surface of 0.7 cm^2) and charge density [current density (mA/ cm^2) x duration of current (sec)] of 8.4 mC/ cm^2 were used. Current density is a measure of electric current per cross sectional area. The skin perfusion responses to iontophoresis of ACh and SNP at anode and SNP and sodium chloride at cathode were recorded after each current pulse (AU).

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Science (SPSS) Software version 12.0 (SPSS, Chicago, IL, USA). Data were presented as mean ± SEM. Statistical significance was sited at $P<0.05$. Repeated measures ANOVA was used for analysis of perfusion response and paired t test was used to determine the maximum effect of ACh and SNP. Multiple paired t tests with Bonferroni correction were performed to test for any elevation from baseline.

RESULTS

Anodal iontophoresis

Figure 1 showed microvascular perfusion with iontophoresis of 1% ACh (upper line) and NaCl (lower line) at the anode. There was a strong and significant increase in mean perfusion compared to baseline from the first pulse with iontophoresis of 1% ACh ($p = 0.003$). The plateau was reached from the third pulse onwards; no significant difference in perfusion was observed between the third pulse and subsequent pulses ($P = 0.267$).

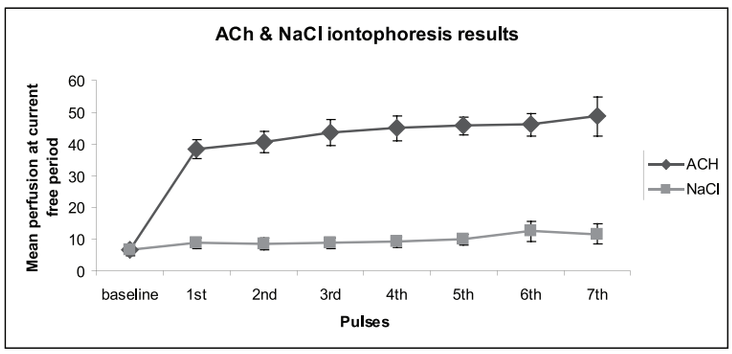

Figure 1. Microvascular perfusion with iontophoresis of 1% acetylcholine solution (ACh) and 0.9% sodium chloride solution (NaCl) at anode (n=5)

For iontophoresis with sodium chloride solution only, no significant increase in perfusion over baseline was observed (p = 0.396).

Cathodal iontophoresis

Figure 2 showed microvascular perfusion with iontophoresis of 1% SNP (upper line) and NaCl (lower line) at the cathode. There was a gradual and significant increase in mean perfusion compared to baseline from the first pulse with iontophoresis of 1% SNP (p = 0.029). The plateau was reached from the fourth pulse onwards; no significant difference in perfusion was observed between the fourth and subsequent pulses (P = 0.377).

For iontophoresis with sodium chloride 0.9% solution, although there appears to be a small increase in perfusion compared to NcCl iontophoresis at anode, the increase was not significant compared to baseline (p = 0.08).

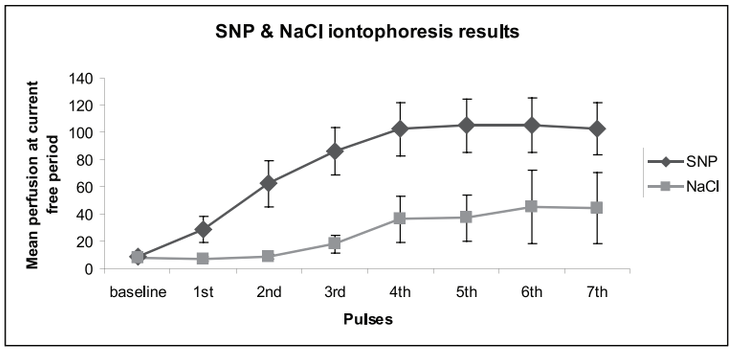

Figure 2. Microvascular skin perfusion with iontophoresis of 1% sodium nitroprusside (SNP) and 0.9% sodium chloride solution (NaCl) at cathode (n=5)

DISCUSSION

Our study used low current strength and density as the study by Droog *et al.* (2004) [10], however we used the laser Doppler fluximetry as we do not have the laser Doppler imager. LDI has the advantage over LDF as it is able to measure perfusion over a larger area compared to LDF. LDF, on the other hand gives a constant measure of perfusion, whereas LDI gives only a snapshot of the perfusion. Our results showed that with the current strength of 0.007 mA and current density of 0.01 mA/cm^2, the maximum response of 1% ACh diluted in 0.9% sodium chloride at the anode can be reached from a charge density of 3.6 mC/cm^2 without showing non-specific vasodilatation. At the cathode, the maximum response to 1% SNP diluted in 0.9% sodium chloride can be reached from a charge density of 4.8 mC/cm^2 without significant increase in perfusion due to iontophoresis of pure vehicle. Iontophoresis of SNP measures the maximum vasodilatation of the microvasculature, independent of endothelial function.

Non-specific vasodilatation during iontophoresis is considered a major disadvantage to the process of iontophoresis and different approaches have been used to suppress it. One approach was to subtract the response of NaCl from the response of the drug [6, 13]. However, this method could introduce erroneous results due to variability in perfusion response between different sites and difficulty in assessing the two responses independently. The other approach is to apply local anaesthesia to inhibit the stimulation of local sensory nerves [14, 15, 16]. Although this method successfully suppress the nonspecific response, local anaesthetic agents has vasoactive properties in itself [17]. Another approach is to use NaCl instead of water to minimize the non-specific vasodilatation as demonstrated by previous studies [8, 9, 10, 18] that conceded with our result as iontophoresis of NaCl showed no significant increase in perfusion compared to baseline. But this finding is in contrast to a study by Noon *et al.* (1998) which reported that saline (0.9%) and tap water delivered by iontophoresis alone produced vasodilator response [19]. This can be explained by the current strength that was used in that study which ranged between 0.1 to 0.2 mA and reached a higher charge density of 16 mC/cm^2.

Our findings suggest that the important factor inducing non-specific vasodilatation was not the vehicle in itself but the current threshold reached through current strength, density and charge density. Thus, an effective approach is to limit the current density and total charge during iontophoresis. This was applied successfully by several studies [4, 9, 20]. However, applying this method may lead to incomplete response as maximum drug response may not be reached. A study by Droog *et al.* (2004) had limited the current density and total charge while reaching the maximum response using the laser Doppler imager [10]. In our study also, the maximum change in perfusion response reached were approximately eight to nine fold increase over baseline perfusion for both ACh and SNP; this is consistent with previous studies (Kubli *et al.*, 2000). While ACh response in Figure 1 showed a lower value as it represented

the mean skin perfusion at current free period which was affected by the maximum and minimum value at that period. The response to ACh was not stable throughout the current free period and rapidly decline due to the effect of acetylcholine-cholinesterase; in contrast, SNP is more stable [21].

Larger chamber size is another suggested approach by Ferrell *et al* (2002) to reduce non-specific vasodilatation as it results in lower current density. However, in our study the current strength was calculated according to the inner diameter of our chambers and this limits the effect of chamber size to non-specific vasodilatation.

As the microvascular responses depend on the current strength and duration of current applied, constant current approach has been chosen by our study rather than a gradual increase in current over time approach. This is because the aim of this study is to obtain maximum microvascular responses at lower charge density to avoid non-specific vasodilatation.

In conclusion, our study showed that iontophoresis protocol using five current pulses with current strengths of 0.007 mA and current density of 0.01 mA/cm^2 was sufficient to get maximum effects for both ACh and SNP iontophoresis. 0.9% sodium chloride can be used as a vehicle for iontophoresis of both ACh and SNP, as iontophoresis of NaCl alone did not produce significant non specific vasodilatation at both anode and cathode.

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REFERENCES

[1] Ross R. Atherosclerosis: an inflammatory disease. N. Engl. J. Med. 1999, 340: 115-126

[2] Shimokawa H. Primary endothelial dysfunction: atherosclerosis. Journal of Molecular and Cellular Cardiology 1999, 31: 23-27.

[3] Manson JE, Stampfer MJ, Colditz GA *et al.* A prospective study of obesity and risk of coronary heart disease in women. N Engl J Med. 1990; 322: 882-889.

[4] Khan F, Elhadd TA, Greene SA *et al.* Impaired skin microvascular function in children, adolescents and young adults with type 1 diabetes. Diabetes Care 2000, 23: 215-220.

[5] Anderson TJ. Assessment and treatment of endothelial dysfunction in humans. Journal of the American College of Cardiologists 1999, 34: 631-638.

[6] Morris SJ, Shore AC, Tooke JE. Responses of the skin microcirculation to acetylcholine and sodium nitroprusside in patients with NIDDM. Diabetologia 1995, 38: 1337-1344.

[7] Moncada S, Palmer RMJ, Higgs E A. Nitric oxide: Physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 1991, 43: 109-142.

[8] Abou-Elenin K, Xydakis A, Hamdy O *et al.* The effects of aspirin and various iontophoresis solution vehicles on skin microvascular reactivity. Microvasc. Res. 2002, 63: 91-95.

[9] Ferrell WR, Ramsay JE, Brooks N *et al.* Elimination of electrically induced iontophoretic artefacts: implications for non-invasive assessment of peripheral microvascular function. J. Vasc. Res. 2002, 39: 447-455.

[10] Droog EJ, Henricson J, Persson K *et al.* A protocol for iontophoresis of acetylcholine and sodium nitroprusside that minimises nonspecific vasodilatory effects. Microvasc. Res. 2004, 67(2): 197-202.

[11] Anderson RR, Parrish JA. Optical properties of human skin. In R. Marks, & P. A. Payne (Eds.), Bioengineering and the skin. Lancaster MTP Press.1981, 147-194.

[12] Ryan TJ. Cutaneous circulation. In L. A. Goldsmith (Ed.), Biochemistry, physiology and molecular biology of the skin (2nd ed.). London Oxford University Press. 1992.

[13] Morris SJ, Shore AC. Skin blood flow responses to the iontophoresis of acetylcholine and sodium nitroprusside in man: possible mechanisms. J. Physiol. 1996, 496: 531-542.

[14] Hanneman MM, Lidell WG, Shore AC *et al.* Vascular function in women with previous gestational diabetes mellitus. J. Vasc. Res. 2002, 39: 311-319.

[15] Kubli S, Waeber B, Dalle-Ave A *et al.* Reproducability of laser Doppler imaging of skin blood flow as a tool to assess endothelial function. J. Cardiovasc. Pharmacol. 2000, 36: 640-648.

[16] Pellaton C, Kubli S, Feihl F *et al.* Blunted vasodilatory responses in the cutaneous microcirculation of cigarette smokers. Am. Heart J. 2002, 144: 269-274.

[17] Bjerring P, Andersen PH, Arendt-Nielsen L. Vascular response of human skin after analgesia with EMLA cream. Br. J. Anaesth. 1989, 63: 655-660.

[18] Asberg A, Holm T, Vassbotn T *et al.* Non-specific microvascular vasodilation during iontophoresis is attenuated by application of hyperosmolar saline. Microvasc. Res. 1999, 58: 41-58.

[19] Noon JP, Walker BR, Hand MF *et al.* Studies with iontophoretic administration of drugs to human dermal vessels in vivo: cholinergic vasodilatation is mediated by dilator prostanoids rather than nitric oxide. Br J Clin Pharmacol 1998, 45: 545-550.

[20] Hamdy O, Abou-Elenin K, Logerfo FW *et al.* Contribution of nerve-axon reflex-related vasodilation in the total skin vasodilation in diabetic patients with and without neuropathy. Diabetes Care 2001, 24: 344–349.

[21] Kvandal P, Landsverk SA, Bernjak A, Stefanovska A, Kvernmo HD, Knut Kirkebøen KA. Low-frequency oscillations of the laser Doppler perfusion signal in human skin. Microvascular Research (2006), 72: 120-127.