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Highlights

Methyl nicotinate at a concentration of 20 mmol/L results in a reproducible increase in skin perfusion, as measured with LSCI.

The perfusion increase has a stable plateau phase 5 min after application, which lasts for at least another 20 min.

The absolute perfusion response to topical methyl nicotinate differs among body sites, but is reproducible from day to day.
The microvascular response in the skin to topical application of methyl nicotinate: effect of concentration and variation between skin sites

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Abstract

Background

Methyl nicotinate (MN) induces a local cutaneous erythema in the skin and may be used as a local provocation in the assessment of microcirculation and skin viability. The aims were to measure the effects of increasing doses of MN, to find the concentration that yields the most reproducible effect from day to day and between sites, and to study the variation between skin sites.

Methods

Microvascular responses to topically applied MN at different concentrations were measured in 12 subjects on separate days and on contralateral sides, using laser speckle contrast imaging (LSCI). MN effects were measured in four different body sites.

Results

At 20 mmol/L, the response to MN was most reproducible day-to-day and site-to-site, and resulted in a plateau response between 5 and 20 minutes after application. The skin region of the lower back had a lower perfusion value compared to the epigastric region (p = 0.007). When responses were compared to nearby, unprovoked areas, a significantly larger increase in perfusion was seen in the forearm, compared to all other anatomical sites (p<0.03).

Conclusion

A concentration of 20mmol/L MN generated the most reproducible microvascular response in the skin. The response varies between different body sites.
**Introduction**

Several methods can be used to analyze the microcirculation of human skin and to measure its perfusion. Perfusion, which is defined as a function of the concentration of red blood cells and their velocity, can be used as a helpful variable in several clinical situations. In reconstructive surgery it has been used to predict wound healing, to assess tissue morbidity in complex wounds, in scald injuries and after flap reconstructions. The microcirculation of the human skin is often studied using non-invasive optical methods such as intra vital microscopy techniques. Various spectroscopy techniques are also used, such as white light spectroscopy and polarized light spectroscopy (TiVi)(1). Many of the more popularized non-invasive methods are based on illumination of the tissue by laser light, including laser Doppler flowmetry/imaging and laser speckle contrast imaging (LSCI).

The human skin has a large reserve of capillary capacity. A simple method to increase the basal perfusion is therefore needed for meaningful assessment of microvascular function or tissue viability. Methyl nicotinate (MN) is a nicotinic acid, which induces a local cutaneous erythema when topically applied to the skin. The physiologically active compound is the methyl ester of nicotinate, commonly known as the B vitamin niacin (2). Its rubefacient effect is related to a transient increase in the microcirculatory perfusion, which is hypothesized to be mediated through the prostaglandin D2 (PGD2) pathway(2). The mechanisms of action are however not presently completely elucidated, and like with most compounds that cause vasodilation, the effect is likely to be of multifactorial origin, including release of nitric oxide (NO) from the endothelium, and neural effects. MN reaches its receptor by epidermal diffusion and a possibly by other mechanisms involving transport by the blood flowing in the dermis, due to its rapid rate of spread (3). Clinically, Niacin has been used to study percutaneous absorption, sensitive skin, niacin mediated flush and its connection to schizophrenia. It can be found in different skin care products. Due to the harmless transient
flushing it generates it has been used in microvascular research. Topically applied MN is fast, and it has been speculated that its effect is reproducible. This makes MN a potentially valuable substance for non-invasive assessment of skin vasodilation as a measure of microvascular function.

There are several other methods to increase basal perfusion with the aim to study capillary capacity in the skin. Post-occlusive reactive hyperemia (PORH) and heat provocation are two reliable ways that both have been shown to have great reproducibility with LSCI (4). For PORH the main disadvantage is that it can only be used on limbs as it requires circular compression. The main disadvantage with heat is that the effect is somewhat hard to control if a non-contact delivery such as heat fan is used. Other methods, such as heating glasses and heat pads have the disadvantage that they are in the way of the LSCI beams. A topically applied agent thus has several advantages. Vasodilators, such as Nitroglycerine and Nitroprusside have been delivered through both iontophoresis and microdialysis (4, 5) however topical administration has only limited effect, with these compounds.

In previous studies on MN’s effect on cutaneous blood flow, several different concentrations and doses have been used (6). It has however been hard to establish the optimal dose and concentration interval of MN. The effect of MN varies between individuals, but so far, no correlation to sex, ethnicity or, age has been verified (7). Most studies have used a concentration of 100 mmol/L to ensure maximal effect, but lower concentrations have also been used to alter skin perfusion (8). It has been reported that a concentration of 100 mmol/L MN could generate transient pain, discomfort or a local inflammatory response in some patients. This makes it important to avoid unnecessarily high concentrations.

Despite previous reports on MN, it is unknown at which concentration of MN the perfusion response will be saturated and how the variation in response from day to day and
from site to site depends on the given concentration. The perfusion increase caused by the MN seems to reach a steady state (8) however the length and stability of the time window of this plateau phase remains unclear. We have previously studied the effect of MN on the microcirculation of the skin with LSCI. However, day-to-day and site-to-site variability was not investigated, and only 10 mmol/L was included (9). All these aspects are important to study, particularly if MN provocation is to be used in a clinical setting to evaluate its effect on the capillary capacity or tissue viability.

The aims of this study were therefore to (1) examine the relation between the concentration of MN applied to the skin and the microvascular response, and the reproducibility of the microvascular responses, and (2) to study the variation in microvascular responses to MN in different sites on the human body.
Method

Subjects

Healthy non-smoking subjects where recruited and gave their informed consent. None of the subjects used regular medication, except for oral contraceptives. Female subjects were included regardless of their menstrual phase. All subjects were asked to abstain from caffeine and strenuous exercise for at least 24 h before the measurement, which was recorded with the subject in a supine position. They were acclimatized for 10 mins before the start of the measurements. Room temperature was kept at 21.0 ± 1.0 °C. All provocations and measurements were done after the skin had been gently cleaned with chlorhexidine ethanol (5 mg/ml, Fresenius AB, Uppsala, Sweden). All experiments were done by the two first authors of the study. The study was carried out according to the Declaration of Helsinki and was approved by the Regional Ethics Committee at Linköping University Hospital, Dnr 2014/299-31.

Equipment

A Laser Speckle Contrast Imager (PeriCam PSI System, Perimed AB, Järfälla, Sweden) was used to measure the perfusion of the skin. The measurement principle of LSCI has previously been described in detail (9). This system uses a divergent class 1 laser with a wavelength of 785 nm to illuminate the skin. The system was calibrated at regular intervals as recommended by the manufacturer. The same acquisition parameters were used for all perfusion recordings. The measurement distance was between 20 and 30 cm. The sampling rate was set to 21 images per second, with an average of 5 images, with an effective frame rate of 4.2 images per second. Averaging of the images was done to reduce signal to noise ratio, to improve image quality, and to reduce data size.
Experimental procedure

Experiment 1: different concentrations and reproducibility

The first experiment was designed to investigate the microvascular response to different concentrations of MN in the skin and to assess the reproducibility of the responses. Twelve healthy, non-smoking subjects (5 male), with a mean age of 28.4 (range 25.9–30.9) years, participated. Two series of concentrations were tested, and both used the same volume of 50 μl diluted MN. The first series consisted of the following concentrations: 0, 0.01, 0.1, 1 and 2.5 mmol/L. The second series consisted of 0, 1, 2.5, 5, 10, 20 and 40 mmol/L. Eighty mmol/L was also tested in a pilot study and showed a similar perfusion response profile compared to 40 mmol/L; we therefore decided to not include 80 mmol/L in the investigated range of concentrations.

To achieve the different concentrations, the MN was diluted in 70% ethanol, which was also used as the negative control. A foam dressing, Mepilex® XT (Mölnlycke Health Care AB, Sweden, was used to create wells, each with a diameter of 2 cm, resulting in three wells per dressing. Two of these dressings were then applied to the volar side of the forearm and a baseline measurement was acquired (see Fig 1). Each dose was applied in its assigned well with a pipette and then gently rubbed into the skin of the subject by hand while wearing latex gloves, using different fingers for each concentration. After application of all MN concentrations, perfusion data was recorded with LSCI for 20 minutes. Skin temperature was measured in the wells before application of MN and after 20 minutes. All region of interest (ROI) were marked in the central part of the wells. Blood pressure, saturation, pulse and room temperature was also measured. Tissue edema was repeatedly evaluated with visual assessment of edema formation.

After initial analysis of the data, the following concentrations were selected for further testing the day-to-day and site-to-site reproducibility: 0, 1, 2.5, 5, 10, 20 and, 40 mmol/L.
This was achieved by repeating the first measurement of the same forearm as in the initial trial in addition to the contralateral arm. At least 48 hours passed between two consecutive measurements.

**Experiment 2 – variation between different anatomical sites**

To investigate the variation in the microvascular response to MN between different anatomical sites, MN was applied to four different body sites: the lower back, the epigastric region, the volar side of the forearm and the dorsal side of the hand (see Fig 1). Twelve healthy, non-smoking subjects (5 male), with a mean age of 27.2 years (range 22 to 36 years) were recruited. A foam dressing, Mepilex XT (Mölnlycke Health Care AB, Sweden), with a well in the middle with a diameter of 5 cm, was attached to the skin. In all anatomical regions described above, 50 μl of diluted MN at a concentration of 20 mmol/L was rubbed on the skin of the subject by hand, according to the same protocol as in Experiment 1. Perfusion images were acquired 15 minutes after application of MN of all four sites.

**Data-analysis**

All data in the text are given as mean (SD). Images were analyzed using the LSCI system’s software (PimSoft 1.5, Perimed AB, Järfalla, Sweden). Regions of interest (ROI) were selected manually in the first image of each series. Then, the locations of ROI in subsequent images were verified and corrections were made as needed. The day-to-day and the site-to-site variability was analyzed using correlation analysis and with Bland-Altman plots. Normal distribution of the data was tested using D'Agostino & Pearson omnibus normality test. As the perfusion data obtained with different concentrations of MN was not normally distributed, differences between concentrations were analyzed with a Dunn's multiple comparisons test. The data from different anatomical sites were normally distributed and were analyzed with a
one-way ANOVA with multiple comparisons using the Tukey correction method. A two-tailed paired Student’s t-test was used to compare the difference in perfusion between the control and MN area. Statistical calculations were performed using GraphPad Prism version 6 for Windows (Graphpad Software, San Diego, CA, USA). A probability of less than 0.05 was accepted as significant.
Results

Different concentrations and reproducibility

Descriptive data on the study participants are shown in table 1. Dunn's multiple comparisons test revealed a significant difference between concentrations 10, 20, and 40 mmol/L vs 1 mmol/L (p<0.0008) and 20, and 40 mmol/L vs 2.5 mmol/L (p<0.0007).

The MN concentrations of 5, 10 and, 20 mmol/L proved the most reliable in day-to-day and site-to-site reproducibility. Concentrations of MN lower than 5 mmol/L were found to be less reproducible day-to-day and site-to-site and varied more between subjects (Figure 2). The mean perfusion during baseline was 42.2 (8.4) PU. The mean perfusion with 5 mmol/L MN was 152.8 (32.8) PU at 5 mins, and 158.5 (31.3) PU at 20 mins; with 10 mmol/L it was 156.4 (29.1) PU at 5 mins, and 162.4 (27.5) PU at 20 mins; with 20 mmol/L, the mean perfusion was 159.7 (36.1) PU at 5 mins, and 171.2 (29.9) PU at 20 mins. Perfusion with 0.1, 1, 2.5, 5, 10, 20 mmol/L of MN are shown in Figures 2 and 3.

The day-to-day reproducibility of the response to 5, 10 and, 20 mmol/L of MN were excellent, with Pearson’s r-values of 0.94 (0.05), 0.95 (0.04) and 0.90 (0.18), respectively. Site-to-site reproducibility for the MN concentrations 5, 10 and, 20 mmol/L resulted in an r-value of 0.90 (0.15), 0.92 (0.10) and, 0.94 (0.05), respectively. Bland-Altman analysis of the day-to-day and site-to-site data showed excellent reproducibility of MN concentrations 5-20 mmol/L. Bland-Altman plots are shown in Figure 4. Data on all other concentrations are presented in Supplemental material Figure 7.

For concentrations below 40 mmol/L no study participant complained of tissue edema.

Variation between different anatomical sites

Perfusion increased significantly in all four areas after application of MN with an average increase of 85.7 PU (209 %, p<0.0001). A one-way ANOVA showed a significant difference
in absolute perfusion units 15 minutes after application of MN (p<0.01, Figure 5). Differences in perfusion are presented in Figure 5. Multiple comparisons showed the skin region of the lower back to have a lower perfusion compared to the epigastric region (p<0.007). Differences in perfusion among the other areas were not significant.

When changes in perfusion relative to a nearby control area were analyzed there was a significant difference among anatomical sites (p<0.001). These relative responses at different anatomical sites are shown in Figure 6. The forearm site had a significantly larger relative increase in perfusion compared to all other anatomical sites (p<0.03).
Discussion

To evaluate cutaneous microvascular reactivity at rest is difficult. Blood flow is a self-regulating physiological parameter, and that a wide range of the blood flow can therefore be observed in the normal resting condition of the cutaneous tissue. By stimulating a vasodilatory response in a standardized way, evaluation of tissue microcirculation can be done. Previous studies on topically applied methyl nicotinate have proven it a candidate for standardized assessment of skin microcirculation in a resting state.

The absorption rate and duration of action for different solutions of MN was studied for the first time 1969 by Fountain et al. (6). Since then, the drug has been used to stimulate local vasodilatory response in various ways, although there is no consensus regarding the optimal concentration or measurement protocol to use.

We define the optimal concentration of topically applied MN as the concentration that provides a quick vasodilatory response, that has an as long plateau phase (to allow for a prolonged, stable perfusion measurement) as possible, and that causes minimal tissue edema. In this study we have found that, using these criteria, the optimal concentration range for MN was between 5 mmol/L and 20 mmol/L, with a slight preference for the concentration of 20 mmol/L.

When using low concentrations of MN, <5 mmol/L, we observed large inter-individual differences in perfusion response, a non-reliable plateau phase, and poor reproducibility. For concentrations of MN higher than 20 mmol/L there was no significant difference in response or in the duration of the plateau phase as compared to 10 and 20 mmol/L. On the other hand, concentrations above 20 mmol/L occasionally caused tissue edema and, in some subjects, even slight discomfort. In previous studies, it has been discussed whether the presence of tissue edema might even be counterproductive to the perfusion increasing effect of MN due to the mass effect compressing capillaries and making it difficult for MN to reach its receptors.
Intermediate concentrations of MN, 10 mmol/L, produced equal or better responses than 20 mmol/L with respect to day-to-day and site-to-site reproducibility. As observed in an earlier study, however, the lower concentrations gave higher inter-individual variations (10). Specifically, two subjects in this study had a substantially lower perfusion response to 10 mmol/L MN compared to the mean response among all participants for this concentration, and could therefore be regarded as “non-responders” (Table 2). When a concentration of 20 mmol/L was applied, this issue did not persist in these individuals.

Based on the above findings, we consider the lower concentrations (5 and 10 mmol/L) to be unreliable as some individuals may not respond with a stable plateau phase at these concentrations. It therefore seems like the concentration of 20 mmol/L MN is best choice to successfully achieve a stable perfusion plateau in most individuals. This concentration also caused a prolonged plateau phase during which perfusion was increased. This plateau appeared 5 min after application of MN and lasted for at least 15 min. Therefore, we suggest that the response is measured 15 minutes after application of MN. Preferably, skin perfusion is measured during 1 minute to allow for the selection of a measurement period free from motion artefacts (9).

To measure the effects of MN on skin microcirculation, a measurement method is needed that should ideally be non-invasive and without causing any patient discomfort. We used LSCI, which fulfills these criteria and is validated against other laser-based perfusion measurement methods such as laser Doppler flowmetry. When measuring perfusion, using LSCI, both the concentration and velocity of red blood cells is taken into account. This enables LSCI to detect changes in both erythema and microvascular blood velocity. The effects of MN on skin erythema and skin blood flow have been investigated in a number of studies. Oestmann et al found that changes in skin blood flow are measurable before any
erythema is visible (11). This contrasts with Guy and Bugatto, who found a good correlation between erythema onset and blood flow changes (12).

When topical application of MN is used to assess microvascular reactivity, care should be taken to compare responses between different anatomical sites of the body. In this study we investigated the perfusion response in the epigastric area, the lower part of the back, the volar side of the forearm, and the dorsal side of the hand to a dose of 50 µl of 20 mmol/L MN. The absolute perfusion values were significantly higher in the epigastric region compared to the lower back. However, when the increase in perfusion was expressed relative to the perfusion in a nearby control area, the forearm had a higher response compared to all other regions. The observed differences between these different body sites can be related to microvascular density or differences in peripheral vasoconstriction. There were no significant differences between the other regions.

To evaluate tissue microcirculation in a setting of wound healing, a reproducible provocation method is needed to determine the tissue viability over a large area, in different body sites. This must be combined with a perfusion measurement method to generate objective measurements, which can be converted to clinical cut-off values. Our study has further characterized methyl nicotinate as a substance for stimulating skin microcirculation. We found that MN is easy to apply, and Ross et al showed that it is both chemical and biological stable, when stored at 4 °C (13). At a concentration of 20 mmol/L, MN gives a reliable and reproducible response after 5 min with minimal tissue edema and discomfort. The responses vary between measurement sites on different body parts, depending on whether the measured values are expressed as absolute or relative to a nearby control area. These findings show that MN is a promising agent for the safe and reliable evaluation of tissue viability and microvascular reactivity.
This study has a number of limitations. Only young healthy individuals participated, while in a clinical setting MN would be used for a wider range of ages. The effects of caffeine, nicotine, or strenuous exercise have not been considered. Arterial blood pressure was not monitored during the experiments and is not correlated to our response. All these factors might have an impact on the perfusion response caused by MN and could potentially raise issues in a clinical setting. Therefore, it would be advantageous to relate clinical measurements of microvascular reactivity to a control site in the same individual. For instance, when assessing microvascular reactivity in a skin flap after reconstructive surgery, a nearby control site could be assessed in the same patient.

Although the same volume of 50 µl was applied to each site and for each concentration of MN, we did not objectively investigate the actual levels of MN present in the microcirculation of the study participants for the different concentrations. This would otherwise have provided interesting insights into the local concentration of MN compared to the observable perfusion plateau phase.

**Perspectives**

To conclude, our findings suggest that topical application of 50 µl methyl nicotinate at a concentration of 20 mmol/L results in a reproducible increase in skin perfusion. The perfusion increase occurs from the first few minutes from application and reaches a stable plateau 5 min after application, which lasts for at least another 15 min. The absolute perfusion response differs among body sites. Methyl nicotinate seems to be a safe and easy to use method for the evaluation of the reactivity of the microcirculation in the skin.
References

Figure 1. Overview of experiment 1 and 2. The left part of the image describes the well placement on the volar side of the forearm in the 1st experiment (the contralateral forearm was used for testing site-to-site reproducibility). The right part of the image shows the four different anatomical sites in the 2nd experiment, where topical methyl nicotinate provocations were done: a = the volar part of the forearm, b = dorsal side of the hand, c = the epigastric region, d = the lower back.
Figure 2. Mean (SD) perfusion response in the skin after topical application of different concentrations of methyl nicotinate (MN). Note the similarities between the concentrations 5-20 mmol/L and the increased standard deviation in the lower concentrations in combination with the lack of response from the lowest concentration 0.1 mmol/L.
Figure 3. Perfusion in the skin after topical application of increasing concentrations of methyl nicotinate. Different graphs show the response at different time points (see heading). Boxes and whiskers are shown according to the Tukey method.
Figure 4. Bland-Altman plots of the concentrations 1, 5, and, 20 mmol/L showing day-to-day and site-to-site reproducibility of the response to methyl nicotinate in the skin. Each blue dot represents a comparison between two measurements in the same individual and time from application of methyl nicotinate. The blue dotted line shows the average bias between the two different measurement point. The red dotted lines indicate ±1.96 SD.
Figure 5. Perfusion in the skin 15 min after topical application of methyl nicotinate (MN) in different anatomical sites. The perfusion was statistically higher in the epigastric region compared to the lower back ($p=0.007$). Boxes and whiskers are shown according to the Tukey method.
Figure 5. Perfusion in the skin 15 min after topical application of methyl nicotinate (MN) in different anatomical sites. The perfusion was statistically higher in the epigastric region compared to the lower back ($p=0.007$). Boxes and whiskers are shown according to the Tukey method.
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