



RESEARCH ARTICLE

Changes in labial capillary density on ascent to and descent from high altitude [version 1; referees: 2 approved]

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Abstract

Present knowledge of how the microcirculation is altered by prolonged exposure to hypoxia at high altitude is incomplete and modification of existing analytical techniques may improve our knowledge considerably. We set out to use a novel simplified method of measuring *in vivo* capillary density during an expedition to high altitude using a CytoCam incident dark field imaging video-microscope.

The simplified method of data capture involved recording one-second images of the mucosal surface of the inner lip to reveal data about microvasculature density in ten individuals. This was done on ascent to, and descent from, high altitude. Analysis was conducted offline by two independent investigators blinded to the participant identity, testing conditions and the imaging site. Additionally we monitored haemoglobin concentration and haematocrit data to see if we could support or refute mechanisms of altered density relating to vessel recruitment. Repeated sets of paired values were compared using Kruskal Wallis Analysis of Variance tests, whilst comparisons of values between sites was by related samples Wilcoxon Signed Rank Test. Correlation between different variables was performed using Spearman's rank correlation coefficient, and concordance between analysing investigators using intra-class correlation coefficient.

There was a significant increase in capillary density from London on ascent to high altitude; median capillaries per field of view area increased from 22.8 to 25.3 ($p=0.021$). There was a further increase in vessel density during the six weeks spent at altitude (25.3 to 32.5, $p=0.017$). Moreover, vessel density remained high on descent to Kathmandu (31.0 capillaries per field of view area), despite a significant decrease in haemoglobin concentration and haematocrit.

Using a simplified technique, we have demonstrated an increase in capillary density on early and sustained exposure to hypobaric hypoxia at thigh altitude, and that this remains elevated on descent to normoxia. The technique is simple, reliable and reproducible.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: Braedius Medical, a company owned by a relative of Can Ince, has developed and designed a hand held microscope called CytoCam-IDF imaging. Can Ince has no financial relation with Braedius Medical of any sort; he never owned shares, or received consultancy or speaker fees from Braedius Medical.

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List of Abbreviations

EBC	Everest Base Camp
FOV	Field of view area
[Hb]	Haemoglobin concentration
Hct	Haematocrit
IDF	Incident Dark Field
KTM	Kathmandu
LON	London
SpO ₂	Peripheral oxygen saturation

Introduction

The physiological processes involved in acclimatisation to high altitude attempt to maintain adequate oxygen delivery as the partial pressure of oxygen decreases. Traditionally, research has concentrated on global haemodynamics and the macrocirculation, variables such as cardiac output¹, oxygen saturations² and haemoglobin concentration [Hb]³. Far fewer studies have focused on the microcirculation, which regulates blood flow to match micro-regional oxygen demand. Disruption of microvascular blood flow could explain a failure of acclimatisation in some individuals as well as the well-documented exercise limitation that occurs at altitude despite normalisation of systemic oxygen delivery⁴. The precise role of the microcirculation in acclimatisation to hypoxia, however, remains unclear.

Teleological reasoning would suggest that increasing capillary density could provide a means to augment oxygen flux and tissue oxygenation through a reduction in the inter-capillary distance⁵. Whilst plausible, data on this theory remains contradictory, though this may in part relate to the dissimilar tissues observed. In human skeletal muscle biopsy samples previously exposed to hypobaric hypoxia, no evidence of neovascularisation has been demonstrated⁶⁻⁹. Interestingly, in each instance whereby the capillary density was initially thought to increase, no change in the capillary-to-fibre ratio was observed. The perceived rise in capillary density were therefore interpreted as being secondary occurrences in response to a reduction in skeletal muscle mass. Conversely, an increase in the density of sublingual microcirculatory vessels to >25 μm was demonstrated on ascent to high altitude^{10,11}, a response that was further amplified after prolonged exposure to hypoxia¹⁰. In this instance, what remains to be determined is whether the observed changes in vessel density are due to microvascular recruitment secondary to increased blood viscosity (and thus quickly reversible), or neovascularization (which is likely to be sustained). Moreover, the question of what happens to vessel density following re-exposure to normoxia remains to be elucidated.

We therefore piloted a novel modification of a previously described technique for calculating changes in capillary density^{12,13} on ten individuals, to see if we could firstly support or refute previous findings on ascent to high altitude, and secondly see if the changes

observed persist on descent. Additionally we monitored haemoglobin concentration and haematocrit data to see if we could support or refute mechanisms of altered density relating to vessel recruitment.

Methods

The study was undertaken as part of the Xtreme Everest 2 research expedition (XE2)¹⁴. The study design, risk management plan and protocol were approved (in accordance with the declaration of Helsinki) both by the University College London Committee and the Ethics of Non-National Health Service Human Research, and the Nepal Health Research Council (Reg no. 139/2012). Written consent was obtained from all participants. Baseline images of the labial capillaries were initially obtained from ten individuals in London (LON) (35m) in December 2012 and January 2013. Sequential images were taken after an 11 day ascent to Everest Base Camp (EBC-early) (5300m), then after 6 weeks residence at Everest Base Camp (EBC-late), and finally on descent, over 5 days, to Kathmandu (KTM) (1300m) in May 2013.

Images were obtained using a CytoCam-IDF video microscope (Braedius, Medical BV, Netherlands). This new device is based on the principle of Incident Dark Field (IDF) imaging, which uses polarized green light (wavelength 548nm) produced from LEDs to visualize, in real time, the sublingual microvasculature. Its high resolution imaging sensor (14 Mpixel) allows for a 50% increase in optical resolution (300 lines/mm) compared to previous Sidestream Dark Field imaging devices, and it generates a far larger field of view. With the participant lying in the supine position having rested for a minimum of 10 minutes, the CytoCam-IDF device's probe was introduced into their mouth and placed on the mucosal surface of the inner lip. Once a suitable image was visualised on the screen of the CytoCam-IDF monitor, (Figure 1), 1 second of digital video footage was recorded.

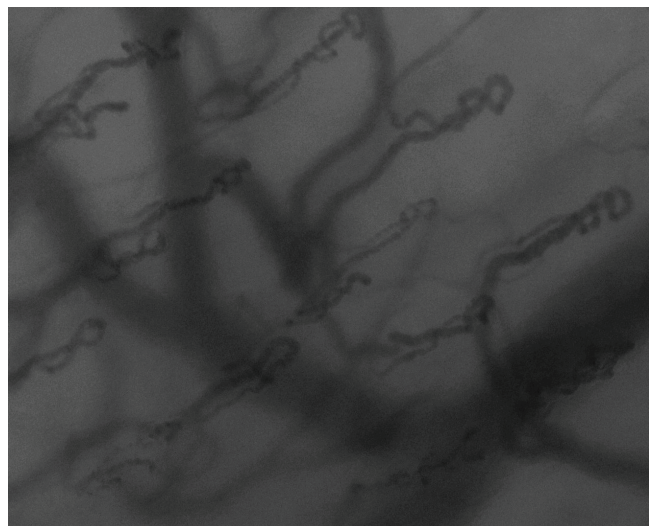


Figure 1. An example of a labial capillary density image.

This process was conducted on all four lip quadrants (right upper lip, left upper lip, right lower lip, left lower lip), and at each quadrant four separate videos were acquired. Two trained investigators (EGK, PH) obtained all the data.

To determine capillary density, analysis was conducted offline by two independent investigators blinded to the participant identity, the testing conditions and the imaging site (EGK, JC). Using the company's own video software (CytoCamTools V1, Braedius, Netherlands), a single still frame was projected on the computer screen and the number of capillary loops per image frame was counted manually. Partly visualised capillaries were included if the observer was assured that the vessel was a capillary due to its morphology. Subsequently, the mean capillary density was calculated from the four images obtained in each lip quadrant, and from these four results, the mean total lip density obtained. Capillary density was defined as the number of capillaries counted per field of view area (FOV), which equates to 1.79 mm². The haemoglobin concentration ([Hb]) (Hemocue AB, Hemocue, Sweden) and haematocrit (Hct) (Sigma 1–14 microcentrifuge, Sigma, Germany) were obtained from whole blood samples, and peripheral arterial oxygen saturation (SpO₂) measured (Nonin Onyx 9500, Nonin Medical Inc, Minnesota, USA) on the same days as microcirculatory imaging was performed.

Statistical analysis

As data were not normally distributed, they were described by median and interquartile range. Repeated sets of paired values were compared using Kruskal Wallis ANOVA, whilst comparisons of values between LON baseline and other sites was by Wilcoxon Signed Rank Test. Correlation between different variables was performed using Spearman's rank correlation coefficient, and concordance between analysing investigators using intra-class correlation coefficient. All statistical analysis was undertaken on SPSS version 21 (SPSS Inc., Chicago, IL, USA), and a P value of <0.05 was taken to indicate statistical significance.

Results

Dataset 1. IDF values

<http://dx.doi.org/10.5256/f1000research.7649.d134021>

The number of loop capillaries seen per field of view

Dataset 2. Physiological values

<http://dx.doi.org/10.5256/f1000research.7649.d134022>

The haemoglobin, haematocrit and peripheral oxygen saturations at each laboratory.

CytoCam-IDF imaging was conducted on all ten individuals on the first two occasions, however only eight individuals had data captured on descent. No problems were encountered with the device or image acquisition. Mean laboratory barometric pressure and mean temperature for each location is shown in [Table 1](#).

Table 1. Mean (standard deviation) atmospheric pressure and temperature at each laboratory.

Site	Atmospheric Pressure (kPa)	Temperature (°C)	Humidity (%)
London	100.6 (0.2)	16.9 (1.8)	35.4 (6.5)
Everest Base Camp	53.0 (0.2)	12.9 (8.2)	37.8 (17.5)
Kathmandu	86.8 (0.4)	23.8 (3.4)	47.4 (15.7)

Changes in labial capillary density are shown in [Figure 2](#). Compared with LON (median 22.8 capillaries per field of view area (20.7–26.8)), capillary density was significantly increased at EBC-early 25.3 (24.5–30.6; p=0.021), EBC-late 32.5, (28.4–36.63; p=0.012), and on descent in KTM 31.0 (24.0–35.13; P=0.017). Between EBC-early and EBC-late, capillary density increased significantly (p=0.017), however there was no significant decline in density between EBC-late and KTM (p = 0.069).

Changes in [Hb], Hct and SpO₂ at each site are shown in [Table 2](#). There was a significant increase in [Hb] between LON and EBC-early (p=0.007), and EBC-early and EBC-late (p=0.011), and a decrease between EBC-late and KTM (p=0.008). There was also a significant increase in Hct between LON and EBC-early (p=0.007), and EBC-late and KTM (p=0.012), but no significant change between EBC-early and EBC-late (p=0.191). Between the sites on ascent, the increase in vessel density demonstrated an inverse relationship with the SpO₂, however at each altitude there was no correlation between vessel density and [Hb], Hct or SpO₂.

To assess whether an image capture time of 1 second was indicative of that captured over longer periods of time, we obtained 30 seconds of footage from four individuals at two different locations. From this we randomly selected one frame per five seconds of footage, and counted the number of capillaries per field of view area. The values of these may be seen in [Table 3](#), as too can the mean and standard deviations for each set of frames, the latter of which demonstrates a highest value of only 0.52 capillaries per field of view area.

Discussion

This study demonstrates for the first time, persistence of *in vivo* sublingual microvascular density increase on re-exposure to normoxia after a prolonged period of hypobaric hypoxia at high altitude. We utilised an infrequently used imaging and analysis technique that we had purposefully adapted to suit our needs, and found our data aligned with previously published work on blood vessel density at altitude¹⁰.

Using the data obtained from corresponding blood samples, it is possible to speculate on the adaptive processes occurring at each measurement point. As previously described, we observed a significant rise in [Hb] (14.5 g/dl to 16.4g/dl; p=0.007) and Hct (44%

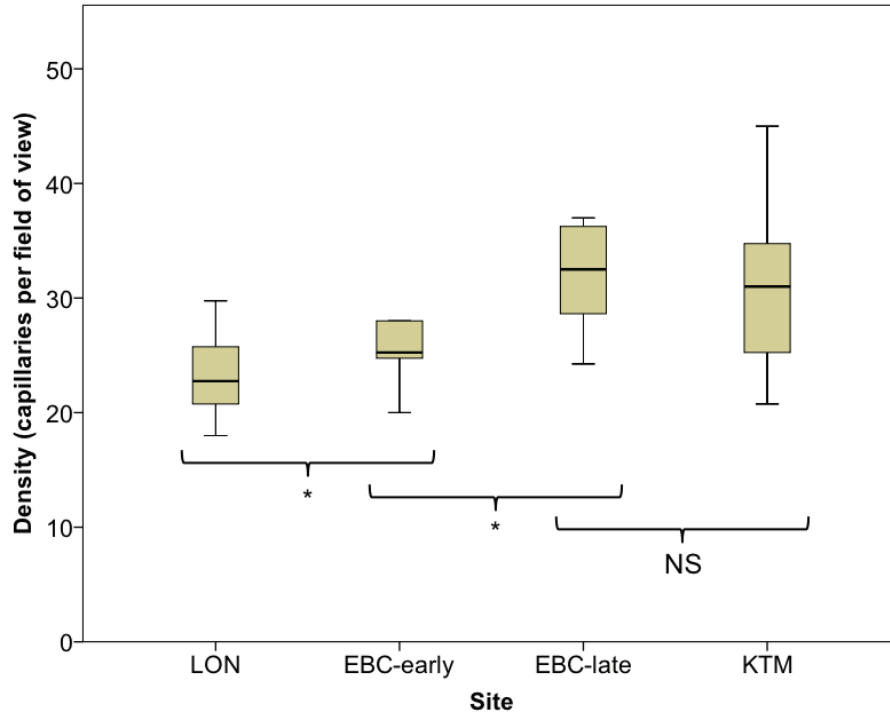


Figure 2. Box-whisker plots of labial capillary density on ascent from London to Everest Base Camp, after six weeks at Everest Base Camp, and on descent to Kathmandu.

Table 2. Median (interquartile range) haemoglobin concentration, haematocrit and peripheral oxygen saturations at each measurement point.

Site	Hemoglobin concentration (g/dl)	Hematocrit (%)	Oxygen saturation (%)
London	14.5 (13.5–15.3)	44.0 (41.5–47.5)	99 (98–100)
Everest Base Camp - early	16.4 (15.7–17.2)	52.0 (51.5–56.3)	81 (79–86)
Everest Base Camp - late	18.3 (17.1–19.7)	56.0 (51.0–61.5)	88 (85–91)
Kathmandu	16.0 (14.0–17.0)	50.0 (46.5–55.0)	99 (99–100)

Table 3. Number of capillaries counted per field of view area from one randomly selected frame per five seconds of footage, with mean and standard deviation demonstrated.

Code	Site	Frame 1 (0–5 s)	Frame 2 (5–10 s)	Frame 3 (10–15 s)	Frame 4 (15–20 s)	Frame 5 (20–25 s)	Frame 6 (25–30 s)	Mean	SD
1	LON	30	30	30	30	30	30	30.0	0.00
2	LON	24	25	24	24	24	25	24.3	0.52
3	KTM	27	27	27	27	26	27	26.8	0.41
4	KTM	22	23	23	23	23	22	22.7	0.52

s = seconds; SD = standard deviation

to 52%; $p=007$) on ascent to altitude. Whilst this polycythaemia increases arterial oxygen content, blood viscosity also rises, altering its rheology. Under normal physiological conditions, a considerable proportion of the microcirculation is thought to be 'unrecruited', acting as a reservoir for times of increased metabolic needs¹⁵. As Hct rises, these reserve vessels are recruited, and microvascular density increases, along with functional capillary density¹⁵⁻¹⁸. Thus a secondary benefit to increased Hct is achieved; a reduction in the diffusion distance from capillaries to mitochondria. Importantly however, it should be noted that in normal capillary Hct is generally 50% less than systemic Hct owing to the streamlined blood flow in narrow capillaries¹⁹. The effect of hypoxia on this association is unknown. Whether or not neovascularisation had occurred on arrival at high altitude is difficult to say, although due to the short time between measurement points the chances of this being the case are low²⁰.

After 6 weeks spent at altitude, a further, and far greater, increase in vessel density was apparent; EBC-early 25.3 capillaries per field of view area, EBC-late 32.5 ($p=0.017$). Over the same time period, [Hb] had significantly risen, whilst Hct had not. As Hct is a more reliable indicator of viscosity between the two variables²¹, it seems unlikely that further recruitment of the microvasculature had occurred, yet it is plausible that neovascularisation had. Increased levels of vascular endothelial growth factor (VEGF) have been detected in subjects ascending to high altitude²²; its role in angiogenesis perhaps explaining the observed rise in microvascular density¹⁰. Such adaptations lead to improved tissue oxygenation by a reduction of the inter-capillary distance, whilst maintaining a sufficiently low, and thus fluid Hct to permit flow of red blood cells in the microvasculature.

On descent to a lower altitude (KTM) there was no significant fall in microvascular density when compared to EBC-late ($p = 0.069$), however, a much greater number of vessels (36% increase) was evident when compared with baseline testing in LON. Whilst vessel density was thus unaltered on descent, over the same time point [Hb] and Hct values significantly declined ($p=0.012$). When compared to the original LON values, Hct on descent to KTM was significantly higher (44.0% and 50.0% respectively ($p=0.011$)), however, [Hb] was not (14.5 and 16.0g/dl ($p=0.052$)). Teasing apart the relative contributions of vessel recruitment and neovascularisation to the observed changes in sublingual microcirculatory density is challenging. Whilst the failure of vessel density to return to baseline after descent suggests some neovascularisation, neither [Hb] nor Hct had normalised at the time of the final readings so a raised blood viscosity could perhaps be maintaining a heightened level of capillary recruitment. A combination of the two processes would make sense as continually increasing [Hb] to improve oxygen delivery would eventually be counter productive. Indeed in Tibetans, who have been exposed to environmental hypoxia for many generations, there is a clear reduction in [Hb] compared to populations who have been exposed to these conditions for less time²³⁻²⁶. This suggests Tibetans utilize alternative long-term strategies for chronic adaptation to hypobaric hypoxia, ones that do not rely on maintaining a high [Hb]. It is plausible that one such means would be to increase their capillary density.

Technique and analysis

The use of the described methodology was also novel. A similar technique has been used twice previously; once in the assessment of coronary artery disease in diabetes¹³ and the other in a study investigating hypertension and rarefaction during treatment with Telatinib¹². In these instances, data capture involved recording sublingual images for 1 minute¹³ or 30 seconds¹² per quadrant, however we altered this time period by using an extremely short capture phase for data acquisition (< 1 second). Crucially, this allowed us to readily obtain snap shot images to reveal data about microvasculature density, whilst avoiding concerns surrounding probe and patient movement, in addition to issues relating to pressure artefact. Analysis was rapid, simple and reproducible; in this study it had an observer mean intra-class correlation coefficient of 0.91 (95% CI 0.84 – 0.96). Previously no difference in capillary density was observed in ten individuals between lip quadrants, and the reproducibility of the technique to determine capillary density was moderate to high with a coefficient of variation of 4.6%¹². Of note, the technique does not allow assessment of microvascular flow, nor does it yield information on heterogeneity of microvascular blood flow, however, we propose it to be a robust method for the assessment of labial vessel density that could be conducted after only a short user training period.

Study limitations

The small number of participants used in this study could be considered a study limitation. As we were both employing a newly-adapted data acquisition technique, and using a novel device at altitude, no power calculation was performed. This therefore increases the risk of a type 2 error. Other limiting factors include the environmental considerations associated with high altitude research in a remote field environment. These include fluctuations in laboratory temperature, humidity (Table 1) and participant hydration status, all factors that may alter microvascular blood flow and density. Attempts were made to limit these potential confounding factors by performing CytoCam-IDF imaging at the same time of day in heated purpose-built laboratories, and encouraging participants to maintain a good state of hydration. Previous studies at altitude have also raised concerns over the development of tissue oedema^{10,11} that can occur on ascent to altitude²⁷. Whilst this could potentially reduce image quality and lead to false measurements of flow and density, our IDF camera provided us with a depth of focus reading, thus allowing us to confirm that we were recording at the same depth under the tongue on each time point. Finally, we have discussed alterations in capillary or vessel density. It is important however to clarify this nomenclature. IDF imaging cannot image blood vessels directly but rather uses the fact that polarized green light is optimally absorbed by red blood cells within the microvasculature regardless of oxygenation status. Absorption of light by haemoglobin, but not by surrounding tissues, therefore creates a distinct contrast of dark and light colour respectively, and red blood cells moving through the mucosal microcirculation thus appear as dark globules moving along the axis of flow. All vessels visualized are therefore only seen if they contain erythrocytes. The variables measured by IDF imaging (and its precursor SDF imaging) include a measure of total vessel density (TVD) and perfused vessel density (PVD)²⁸.

A distinction is made between the two depending on the speed of red blood cell flow within the observed vessels. TVD includes vessels which contain erythrocytes flowing at any velocity (or even at standstill), whilst PVD only includes vessels with continuously moving erythrocytes. As we cannot measure erythrocyte velocity with this simplified method, our observations therefore describe the TVD.

Conclusions

This study demonstrated an increase in sublingual microvascular vessel density on early and sustained exposure to hypobaric hypoxia; and, for the first time, that no significant change in vessel density occurred on immediate descent. The technique used to capture the images provided a rapid and reliable means for assessing changes in vessel density, and could be applied in future studies of microcirculatory vessel density. Further research in this area may allow a more complete comprehension of the multidimensional response to sustained hypoxia that occurs during pathophysiological situations.

Data availability

F1000Research: Dataset 1. IDF values, [10.5256/f1000research.7649.d134021](https://doi.org/10.5256/f1000research.7649.d134021)²⁹

F1000Research: Dataset 2. Physiological values, [10.5256/f1000research.7649.d134022](https://doi.org/10.5256/f1000research.7649.d134022)³⁰

Author contributions

E G-K: design of study, collection of data, analysis of data, writing manuscript

JC: collection of data, analysis of data, writing manuscript

PH: analysis of data, writing manuscript

MG: design of study, writing manuscript

CI: design of study, writing manuscript

DM: design of study, analysis of data, writing manuscript

All authors have seen and agreed to the final content of the manuscript

Competing interests

Braedius Medical, a company owned by a relative of Can Ince, has developed and designed a hand held microscope called CytoCam-IDF imaging. Can Ince has no financial relation with Braedius Medical of any sort; he never owned shares, or received consultancy or speaker fees from Braedius Medical.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Open Peer Review

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Referee Report 28 September 2016

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The authors report on the use of a relatively novel method to determine capillary density during acute and chronic exposure to hypobaric hypoxia. Previously reported results of an increase in capillary density during acclimatization are confirmed, but it is for the first time demonstrated that upon immediate descent some changes persist.

Overall, this is a well-conducted study that adds to the current knowledge base of microcirculatory changes during adaptation to hypobaric hypoxia, and provides an innovative approach to vessel density measurement.

Specific comments:

Introduction

In the introduction the absence of neovascularization in muscle samples during adaptation to high altitude is discussed. It is however also known that mitochondrial density increases during this process (PMID 26339730). The authors may want to include this in the discussion about capillary-to-fibre ratio, the mitochondria arguably representing the circulation's endpoint.

Methods

The KTM time point seems difficult to me for use as a “post-hypoxia” measurement, since it is not comparable to the LON baseline. This is not a severe limitation since “immediate descent” was one of the study endpoints rather than a comparison to the initial (LON) baseline. I still suggest mentioning this in the limitations.

Microcirculation capture time is very short (1 sec). I am not sure if the analysis in four subjects given in table 3 is sufficient support for the use of much shorter capture intervals than recommended by current consensus guidelines. This could be discussed in more detail in the limitations.

Hb and Hematocrit values, as well as SpO₂ were determined “on the same day as microcirculatory imaging was performed”. Given the influence of hydration status changes throughout a day, especially in a mountain setting, either specify the time frame more clearly or mention this time relation in the limitations.

Results

Figure 2: It is unclear to me what the results of the Kruskal-Wallis ANOVA described in the methods section were for vessel density measurements, consider mentioning these in the results section. Further, are the P values given in the text references to Wilcoxon Signed Rank tests as mentioned in the methods section? Consider describing these tests as post-hoc analysis for the non-parametric ANOVA. And if so, was a correction for multiple testing applied (Bonferroni, or one of the newer algorithms)? Please describe this more clearly.

It is mentioned that at each altitude there was no correlation between vessel density and Hb, Hct or SpO₂. In the methods section it is stated that Spearman's rank correlation coefficient was calculated. Consider adding correlation coefficients and P values to the results section to provide the readers some insight into the data.

In addition to Hb, Hct and SpO₂ given in Table 2, it would be of interest to know more about the subjects' physiologic parameters, such as blood pressure, cardiac output and even RV/RA – including a discussion about the changes in macrocirculation in relation to the changes in microcirculation.

Discussion

Given borderline significance ($p=0.069$) for a decrease in vessel density from EBC-late to KTM and the lack of a power calculation, as well as the lack of real comparability to the initial (LON) baseline it is somewhat difficult to argue that there really was no change between EBC-late and KTM. This is discussed in the discussion to some extent, but it should be stated more clearly that a verification of these results is needed in the future.

The method for vessel density estimation used in this study differs from the effective calculation of total vessel density (TVD), being the total length of small vessels divided by the surface area covered in the recording, that is recommended by current consensus guidelines. Please discuss the relationship between vessel density as measured in this study with vessel density as assessed by TVD, and potential confounders.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 14 September 2016

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Sam Hutchings

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Edward Gilbert-Kawai and colleagues involved in the Xtreme Everest 2 study report further work on the effects of altitude and hypoxaemia on the microcirculation. They describe a technique for rapidly measuring microcirculatory capillary density, using a method of counting the number of capillary loops in the buccal mucosa of healthy volunteers at sea level, progressive altitude acclimatisation and descent.

They also report systemic haemoglobin and haematocrit values in an attempt to elucidate whether changes in microcirculatory parameters are paralleled by changes in blood viscosity. Their results show, in line with previous work, that microcirculatory vessel density increases after prolonged exposure to hypoxic conditions. However, they also show that there is some persistence of this effect on partial descent. The authors pose a question as to whether this increased vessel density is due to neo-vascularisation or recruitment of existing capillaries secondary to an increase in haematocrit.

This is generally a scientifically sound paper and a well written manuscript. I have several comments for the authors:

Major Comments

- It strikes me that the authors are trying to achieve two things in this paper. Firstly, to confirm that there is microcirculatory adaptation to altitude and hypoxaemia and to provide more information regarding the mechanisms surrounding this and secondly to describe a method of rapidly assessing vessel density. Both subjects are not quite covered in enough detail to do either justice.
- With regard to the assessment method, this has been described previously in both buccal mucosa but also in other areas such as the nail bed. It has the advantage of being quick to perform but only provides information on one variable – number of capillary loops. The authors state and assume that this measure equates to Total Vessel Density (TVD) but it would be very useful to confirm this by actually measuring capillary TVD using sublingual IDF video capture and analysis. More importantly, the described method provides no measure of microcirculatory flow. This is crucial when discussing the impact of capillary haematocrit and viscosity on microcirculatory performance. Personally I think the paper would have been improved had the investigators used sublingual images and analyzed these to produce data on a fuller range of flow and density parameters.
- Although the authors give two possible mechanisms for the observed increase in capillary density, I would be interested to know if these changes could possibly be accounted for by an increase in systemic haemodynamic parameters, such as cardiac output. Unless there is a loss of haemodynamic coherence one would expect that micro would follow macro. Measurement of these values using a non invasive technique such as trans thoracic echocardiography would have been interesting and added value to the paper.
- The authors discuss that systemic haematocrit and haemoglobin do not necessarily equate with capillary haemoglobin. This is a crucial point and one often observes that “shocked” capillaries have a visibly lower number of erythrocytes even in the presence of a reasonably preserved systemic Hb level, especially post resuscitation. Being able to quantify this difference would allow us to answer crucial questions but is not possible with current analysis methods using this technology.
- I would be very interested to know how long this microcirculatory adaptation lasts. Did the authors consider repeating the measures after return to sea level and after a more prolonged period – e.g. one month?

Minor Comments

- Southampton is spelt incorrectly in several places within the list of authors
- Citing a validation paper of the IDF camera (e.g. Hutchings S, Watts S, Kirkman E. The Cytocam video microscope. A new method for visualising the microcirculation using Incident Dark Field

technology. Clin Hemorheol Microcirc. IOS Press; 2015 Oct 16;62(3):261–71) would be useful as the authors have stated that it is a new device.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Discuss this Article

Version 1

Reader Comment 07 Oct 2016

Dan Milstein, Department of Oral & Maxillofacial Surgery, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands

Some important elements regarding design and textual accuracy were completely missed by the authors and the reviewers.

The authors state that the CytoCam operates by emitting polarized light, this is incorrect.

Assessment of capillary density has been validated before and there are numerous reports available in humans and animals that show the applications of capillary density measurements (e.g. by Lindeboom *et al.* 2005 and onwards). The type of data presented in the manuscript may be infrequently used for investigating the topics of the current report on hypobaric hypoxia, but not in other fields and was drawn entirely from oral medicine and dentistry. The authors unfortunately did not refer to the source publications correctly referencing reproducibility and validation of capillary density measurements. The two citations that were used do not report validations of the analysis methodology. Why was intra-class correlation data not presented in the Results section of the report?

The microcirculation data was not divided by 1.79 to present a standardized unit of capillaries per mm^2 , which is identified in several papers as cpl/mm^2 for capillary density (CD) or functional capillary density (FCD), a completely different unit of analysis. As the data stands, it is not clear what the units are and it appears that the density data was interpreted as a number per 1.79 mm^2 and not per mm^2 . Others might argue, but the only reason that dividing by 1.79 for capillary density counts is permissible for labial mucosa capillary density is because of counting of repeating (identical) vascular loops, a population count essentially, dividing by 1.79 would not work for branching vascular networks as in TVD or PVD.

Standardizing the data serves also another interesting role because the data from the CytoCam can then also be used to compare all previously obtained data with SDF imaging that also presents data on capillary density per mm^2 in the visual field, this yields progress of the science and not always starting anew. Was normalization of the data thought of? One cannot assume that everybody has the same number of capillaries in their lips, some have more and others have less, that is why standardizing the data would allow clustered analysis and ease in trend assessments, especially when a repeated measurement is executed to trace the effects of a condition, in this case hypobaric hypoxia from ascending or return to normobaric normoxia from descent back to sea level.

The observations do not describe the consensus reports regarding TVD nor PVD, as detailed in the manuscript. TVD is reserved for network type (e.g. sublingual) analysis. This is why there should be different nomenclature for different anatomic compartments. Was there angioarchitecture classification performed, as presented by Weber *et al.* 2015? This classification provides a way of sorting the angioarchitecture and matching it with analysis methodology. For example, an array of capillary loops is a Class 1 angioarchitecture and fits with a field of view counting type analysis, a Class 2 is a hybrid between capillary loops and network vasculature and fits either a counting or AVA type analysis depending on the research question, and finally a Class 3 is exclusively network microcirculation fitting AVA and CCTools type analysis with TVD as mm/mm^2 . The data should be in total capillary density (TCD) or actually functional capillary density (FCD). The data of capillary density is not the same as TVD. Depending on the scientific or clinical questions the investigators are trying to answer, the design of the study should take into consideration whether the study objectives are to investigate mucosal state implicitly or translate measurements of the reticular layer of the oral mucosa as a model representing central or systemic microhemodynamics.

In the first line of the Discussion, the first line in the Conclusions and in two more instances in between the Discussion and Conclusions, the authors refer to measurements of sublingual microcirculation obtained in their report. The report describes lip (labial) mucosa microvascular density measurements and not sublingual microvascular measurements.

There are more publications that report on validation of the CytoCam instrument before the report by Hutchings S *et al.* 2015. Other topics that were mentioned such as neovascularization was not properly addressed.

Finally, to understand the oral measurements, some knowledge of oral physiology is recommended. For example, since the oral mucosa of both the upper and lower lips are peppered with minor salivary ducts, their salivary secretions and associated microcirculation are controlled by shifts in sympathetic and parasympathetic mechanisms that may not lineup with identifying changes in capillary density in for example dry mouth from breathing, cold temperatures, high altitude hypoxia and compensatory autonomic activity in response to low atmospheric pressures and/or hypoxia.

Addressing these points would strengthen the scientific integrity of this report and improve research design for future studies.

Competing Interests: No competing interests were disclosed.
