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RESEARCH ARTICLE

REVISED Do fluorocarbons substantially increase transdermal oxygen delivery? A proof-of-principle study in mice

[version 2; peer review: 1 approved, 1 approved with reservations, 2 not approved]

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Abstract

Background: Coronavirus disease 2019 (COVID-19) patients who need intensive medical care often require oxygen ventilation, but the number of ventilation machines is limited, and in some parts of the world, they are not available at all. In addition to patients for whom there is no access to ventilation machines there is also a considerable population of patients for whom ventilation is not sufficient for them to survive a critical state.
Methods: Here, we propose and test an alternative oxygen supply through accelerated transdermal oxygen delivery. Covering the entire body with liquid fluorocarbons, which can dissolve 20 times more oxygen than water, we hypothesized to increase the contribution of transcutaneous respiration by a sustained amount.
Results: Experiments applying pure medical grade perfluorodecalin on nude mice did not change their oxygenation in the blood under induced hypoxic conditions compared to control mice. However, increases in blood oxygenation below 2% could not be detected with the applied method.
Conclusions: We could not establish a proof-of-principle for a substantial increase in oxygen supply by transdermal oxygen delivery in mammals.

Keywords

transcutaneous respiration, transdermal oxygen supply, perfluorodecalin, pulse-oximeter, nude mice

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REVISED **Amendments from Version 1**

A small paragraph was added to the 'Introduction' referring to the expectable effect of fluorocarbons.

The design and the motivation of the applied protocols are now better explained in the 'Methods' section.

The paragraph 'Conceptual considerations' was moved from the 'Results' section to 'Discussion and Conclusions'.

Any further responses from the reviewers can be found at the end of the article

Plain language summary

There are life-threatening situations of patients due to insufficient oxygen supply. Here we propose and test a method to increase oxygen uptake by the skin, similar as reptiles do. The trick is to wet the skin, but instead of water using a special substance, best described as 'liquid Teflon', that can take up 20 times more oxygen than water can do.

This idea was tested on nude mice that were ventilated with a low amount of oxygen (comparable to the oxygen concentration at the highest mountains of the Alps) and at the same time brushed with the 'liquid Teflon'. Then the oxygen saturation in the blood was measured with a special mouse oximeter. We could not find differences with or without the 'liquid Teflon' treatment.

There might be small improvements but if they are below 2% of oxygen saturation, we could not detect it with the method we applied, because there is a limited accuracy of the oximeter and also a considerable variation from mouse to mouse. However, a relevant improvement for the patients would require an increase in the range of 5%.

Introduction

The major cause of death in coronavirus disease 2019 (COVID-19) patients is severe atypical pneumonia and respiratory distress (Hui *et al.*, 2020). Severe acute respiratory distress syndrome in these patients is treated mainly with mechanical ventilation, which supports O_2 delivery through the lungs, and with anti-inflammatory and anti-viral medication or even targeting the receptor used by the virus to invade the lungs and the epithelium, angiotensin-converting enzyme 2 (Zhang *et al.*, 2020). Unfortunately, the increasing number of patients and the finite number of healthy and operationally trained medical care personnel and machines limits the choice of therapeutic strategies that are available for all that require assistance. The efficacy of the oxygen supply of blood provided by the ventilation of the lungs varies depending on the amount of fluid in the lungs, as the Krogh diffusion coefficient of oxygen in water is 2×10^5-fold lower than that in the gas phase (Dejours, 1989). Therefore, approaches used for the treatment of high-altitude pulmonary edema were recently suggested for patients with COVID-19 infection associated with severe pneumonia (Solaimanzadeh, 2020). Other strategies use complementary O_2 delivery options that bypass the lungs, such as extracorporeal membrane oxygenation (ECMO) (Matthay *et al.*, 2020). However, ECMO is associated with thromboembolic and hemolytic complications and, when performed, requires constant attention of skilled medical care experts (Ramanathan *et al.*, 2020).

Here, we propose and test a simple method to increase the systemic oxygen supply by bypassing the lungs and boosting transdermal oxygen delivery. Evolutionary vertebrates that were equally efficient in aquatic and terrestrial habitats used lungs, skin and gills for oxygen uptake (Dejours, 1989; Jorgensen, 2000). In frogs, transcutaneous respiration was shown to be responsible for approximately 10–20% of oxygen uptake, the amount sufficient to support animal survival during dives (Vitalis, 1990).

In humans, transcutaneous respiration mediates oxygen uptake of 0.53 ml $O_2/(m^2$ min), contributing 0.4% to the total oxygen supply under normoxic conditions (Stucker *et al.*, 2002). A decrease in oxygen uptake through the lungs (e.g., under hypoxic conditions) results in a compensatory increase in transcutaneous oxygen flow that may be boosted up to 25-fold, reaching 10% of total oxygen uptake under certain conditions. This oxygen uptake pathway could support patients with severe respiratory distress on or off mechanical ventilation, allowing them to avoid barotrauma to the lungs (Ioannidis *et al.*, 2015).

Studies on healthy humans revealed that transdermal oxygen travels 400 µm, reaching well into the dermis and thus to blood capillaries (Stucker *et al.*, 2002). Apart from the oxygen gradient between the skin surface and dermisa that supports diffusion, physical parameters in favor of transdermal respiration are temperature (Jaszczak, 1988) and skin humidity (Vaupel, 1976).

However, the crucial intervention will be the increase in available oxygen by replacing a putative aquatic phase with a liquid that could provide much more oxygen. Such compounds are fluorocarbons, which can dissolve 20-fold the amount of oxygen compared to aqueous solutions (Riess, 2005).

If humidity favors oxygen uptake by the skin, i.e. wet skin has a better oxygen uptake than skin in a gas phase (air), a liquid dissolving 20-fold the oxygen of water could result in an increased oxygen uptake by the skin. However, the entire process is multifactorial. In addition to the diffusion step at the skin surface, there is the diffusion deeper into the tissue, the putative uptake of the oxygen by the red blood cells, the role of vasodilation, possible differences at different parts of the body, just to name a few obstacles. Therefore, it seems impossible to estimate an expectable effect size.

Fluorocarbons are not new in circulation and respiration research as well as in dermatological and ophthalmological applications. Three major applications were suggested and have partly been tested decades ago. (i) Perfluorocarbons have already been proposed and tested as blood substituents in the 1980s (Waxman, 1986). This approach never made it into clinical

practice mainly because of emulsion issues. In the future, the situation may improve by combining oxygen carrier compounds with a metal-organic framework (Gao *et al.*, 2018). (ii) Fluorocarbons were tested in liquid ventilation either as total liquid ventilation or as partial liquid ventilation (Kolla *et al.*, 1997). Although initial tests on humans showed beneficial effects in patients with acute respiratory distress syndrome (Hirschl *et al.*, 1996) and acute hypoxemic respiratory failure (Kolla *et al.*, 1997), this treatment was never approved for routine clinical treatment. (iii) Suggested for human use are hydrogels containing fluorinated methacrylamide. Chitosan has been proposed to support wound healing (Patil *et al.*, 2016). Furthermore, perfluorodecalin is used to support wound healing (Jalani *et al.*, 2017) (and as an ingredient in cosmetics) and is applied in ophthalmic surgery (Dk-line®, Bausch+Lomb). Perfluoro-n-octane (e.g., Auro octane®, Aurolab; Okta-line®, Bausch+Lomb) is approved for application in the treatment of traumatic ocular trauma and retinal detachment or in ophthalmic surgery. However, topical whole-body skin application has to our best knowledge not been tested. Therefore, the goal of this work was to test if oxygen supply through the skin enforced with applications of oxygen-concentrating fluorocarbons may result in better oxygenation of blood under conditions of poor absorption of oxygen through the lungs. This aim was tackled as a proof-of-principle studies in mice.

Methods

Animal experiments

Experiments with mice were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal experiment protocol was approved by the State Office for Health and Consumer Protection (permit number: 08/2020). All efforts were made to ameliorate harm to the animals. This was achieved by performing the entire experimental procedure with the mice in anesthesia and by providing an additional pain therapy (details are provided below).

For this study, we used sex mixed population of CD1nu/nu mice (Charles River, Wilmington, MA, USA) at an age of 50.3±8.5 weeks with a bodyweight of 29.9±3.7 g, which were housed under a 12h/12h day-night rhythm in the animal husbandry of the Institute for Clinical & Experimental Surgery (Homburg, Germany). The animals were kept in individually ventilated cages (IVCs) in groups of 5 animals per cage. They had free access to drinking water and standard pellet food (Altromin, Lage, Germany). No additional inclusion criteria were set. Animals were randomly distributed in two groups (control group and treated group). Randomization was done by choosing lots and experiments were performed by alternating measurements of control and treated mice with a cage of animals per (experimental) day. The number of animals per group could not be precalculated because we established this kind of measurements and had no reliable information about the variances of the measured parameters. Therefore, we performed a statistical analysis on a daily base and as such decided after we reached a group size of five animals to stop the experiments of protocol 1 and after reaching four mice for protocol 2.

During the experiment, the mice were anesthetized with isoflurane (5%: initiation, 1%: maintenance of anesthesia). Immediately after anesthesia initiation, the mice received 5 mg/kg carprofen (Rimadyl; Zoetis, Berlin, Germany) i.p. for intraoperative pain therapy. Afterwards, they were fixed on a heating pad in supine position and tracheotomized for the insertion of a catheter for ventilation. Breathing rate and stroke volume could be set on the mouse ventilator (Minivent; Harvard Apparatus, March-Hugstetten, Germany) and was adapted according to the body weight of the mice in the range of 200–270 per minute and 120–140 µL, respectively. The body temperature was kept constant in the range between 37–38°C, which was monitored with an anal probe (GTH1170, Greisinger Messtechnik, Regenstauf, Germany). The heart rate was documented with the help of a pulse oximeter (MouseSTAT Jr. with Paw sensor, Kent Scientific, Torrington, CT, USA). The control group contained 5 animals at an age of 47.4±2.4 weeks with a body weight of 28.8±3.7 g and the test group contained the same number of animals (n=5, age: 49.3±3.3 weeks, body weight: 29.4±4.9 g; no significant difference to control group).

Experimental protocol

All mice in all groups and all protocols were given an initial acclimatization phase as outlined in Figure 1B (-10 to 0 min). Mice of control and treatment groups experienced the same hypoxic protocol. Control mice received no additional treatment whereas mice of the treatment group were brushed with prewarmed (30°C) perfluorodecalin (medical grade, Pharmpur, Königsbrunn, Germany). This included a full-body application with the exception of the area of the head and the paw to which the sensor of the pulse oximeter was attached. The procedure was continuously renewed in the course of the experiment to compensate the perfluorodecalin evaporation. The oxygenation of the blood was monitored and documented (in addition to the heart rate) with the pulse oximeter. After the oxygen saturation had reached a stationary level, the oxygen content of the ventilation was reduced according to the test protocol (see below). For this purpose, a gas mixer (KM10–2 FLEX, Witt-Gastechnik, Witten, Germany) was used, which mixed oxygen and nitrogen in an adjustable ratio. In addition, the setting was monitored with an oxygen meter (GOX100, Greisinger Messtechnik, Regenstauf, Germany). The mouse group used for protocol 2 consisted of 4 mice at an age of 55±15 weeks with a body weight of 31.9±1.1 g.

The initial idea (represented as protocol 1A in Figure 1B) was to reduce the available oxygen in steps from 20% - 15% - 10% - 9% - 8% -7%, each step lasting for 5 min. While oxygen supply, heart rate and oxygen saturation were recorded, the rationale was to detect differences in the survival time between perfluorodecalin treated mice and control mice. However, it turned out that it was impossible to properly determine the time point of death of the mice. The pulse-oximeter was thought to provide the information of the termination of the heart beat but it turned out that the pulse-oximeter stopped working at a weak pulse before the death of the animal (alternating periods of no signals and measured signals). Since the animals were ventilated stop of breath was neither a criterion. A mouse

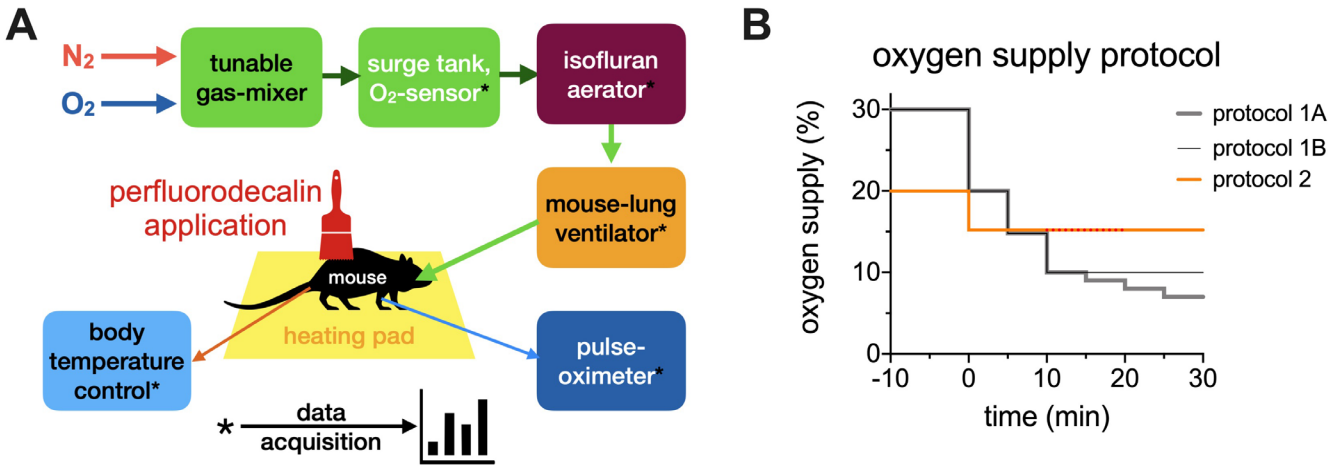


Figure 1. Experimental design. (**A**) Experimental setup: A tunable gas mixer was connected to oxygen and nitrogen gas bottles. The percentage of oxygen could be adjusted. This gas mixture was collected in a surge tank, where the oxygen content was continuously measured. The required amount of isoflurane was added in an appropriate aerator and finally connected to the mouse-lung ventilator. The mouse was monitored for body temperature, heart rate and oxygenation level using a pulse-oximeter. (**B**) Time line of the oxygen supply in the different protocols applied. Protocols 1A and 1B are performed in the presence and absence of topical application of 100% pure perfluorodecalin. The red dots in protocol 2 indicate the period of perfluorodecalin application.

electrocardiogram was not available. Therefore, after the measurement of 6 animals, the protocol was modified to protocol 1B in Figure 1B, i.e. the available oxygen was reduced in 2 steps of 5 min from 20% to 10% and then kept constant at 10%, still with the intention to determine the survival time expecting a better reliability of the pulse oximeter at higher percentage of oxygen supply. Because this was not the case, we stopped to apply protocol 1B after further 4 animals and changed the concept of measurements. Instead of recording the survival time at decreasing oxygen availability, we measured the blood oxygen saturation (as well as the heart rate) under hypoxic conditions (15% oxygen supply) before, during and after termination of the perfluorodecalin application, each period lasting for 10 min.

Statistical analysis

All data were initially tested for normal distribution using the Shapiro-Wilk test. Figure 1 does not contain any statistical data. When the normality test was passed, an unpaired Student's t-test (Figure 2) or a paired one way-ANOVA (Figure 3) was performed to determine the p-value. P-values below 0.05 we regard as significant. When data are presented as box-plots, whiskers depict the minimal and maximal value (Figure 2). Single points show medians with error bars representing the standard error of mean (Figure 3). Age and weight of mouse groups are given as mean value ± standard deviation. All statistical tests were performed in Prism 9 (GraphPad Software, San Diego, CA, USA).

Results

Comparison of perfluorodecalin-treated mice with untreated controls

The results of the measured heart rate and the oxygen saturation as a function of oxygen supply using the pulse oximeter in the presence and absence of topically applied perfluorodecalin are depicted in Figure 2A and 2B, respectively, following the timing protocols 1a and 1b (Figure 1B) (Kaestner, 2021). It turned out that the heart rate and oxygenation readouts were unstable at the lowest oxygen concentrations in the gas phase used for ventilation. Therefore, protocol 1A was adapted and instead of determining the lowest tolerable gaseous oxygen concentration, we measured and compared the survival time at 10% oxygen supply (protocol 1B in Figure 1B).

Figure 2A and 2B reveal a high variability between the mice (heart rate between 490 and 750 per minute and an oxygen saturation between 58% and 75% at a value of 15% oxygen supply). No obvious differences was observed between perfluorodecalin-treated and untreated mice. Figure 2C shows the lowest oxygenation measured independent of the particular amount of oxygen supply. Despite a huge variability between the mice, there is a lack of a significant difference between the 2 groups. Such a difference was not expected and therefore this parameter can be seen as an internal quality control. Panels 2D and 2E show the envisaged final read-out parameters of protocol 1A and 1B, respectively. However, their value is limited due to the low number of animals (n = 4 and 6, respectively) and the huge variation between the mice. Based on these results (Figure 2), experiments following protocol 1 were stopped, because it was judged that the variability of the results did not justify continuing measurements.

Comparison of hypoxic mice before, during and after treatment with perfluorodecalin

A new protocol was designed, where the oxygen supply was set to 15% and then the oxygenation (and heart rate) was measured before, during and after a 10 min period of perfluorodecalin treatment, as outlined in Figure 1B (protocol 2).

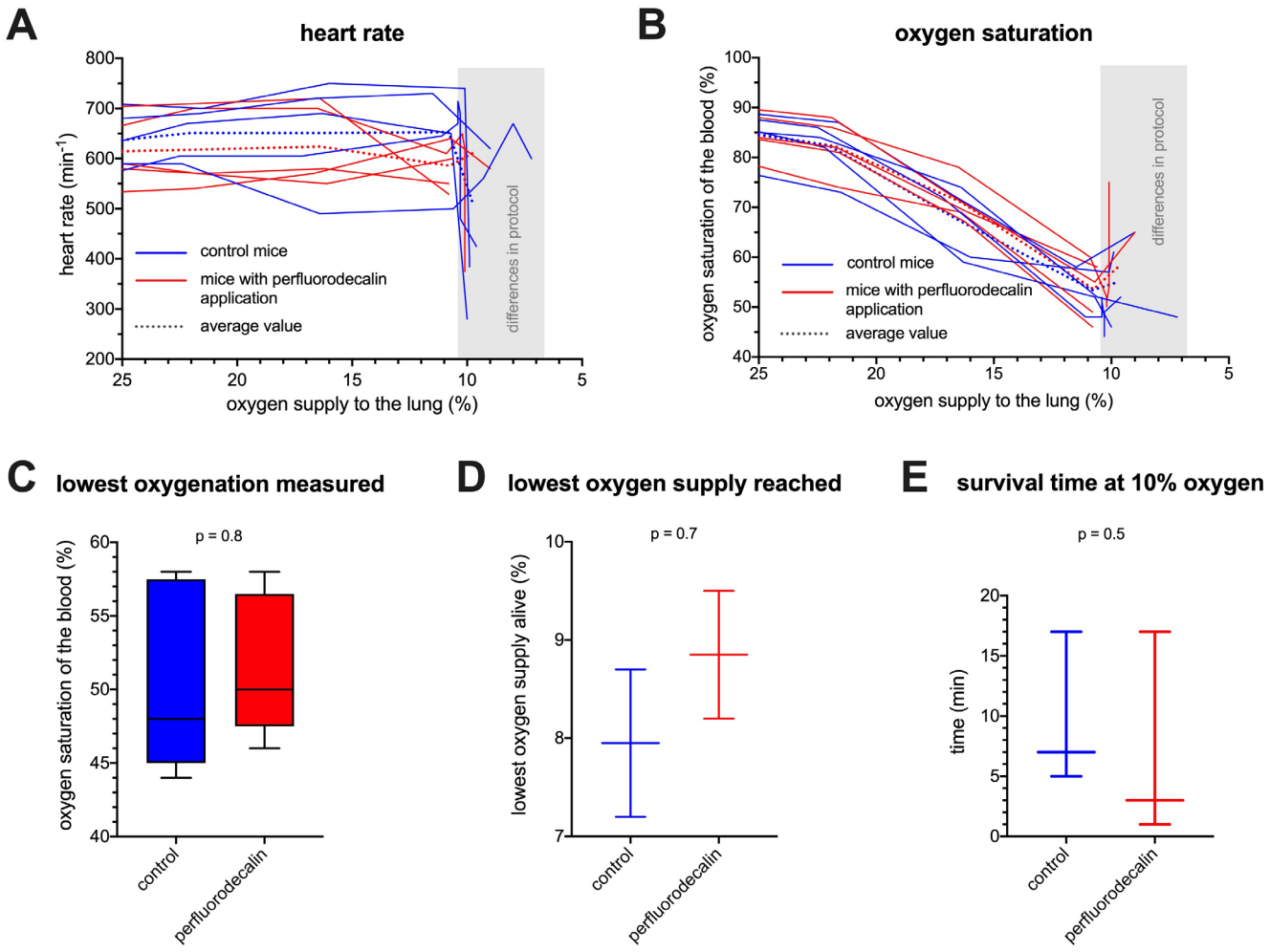


Figure 2. Results of a comparison of perfluorodecalin-treated mice with untreated controls. (**A**) Heart-rate plotted against the percentage of oxygen supply for 5 control mice and 5 perfluorodecalin-treated mice. When an oxygen supply of 10% is reached, mice follow two different protocols (compare Figure 1B). (**B**) Oxygen saturation of the blood plotted against the percentage of oxygen supply for 5 control mice and 5 perfluorodecalin-treated mice. When an oxygen supply of 10% is reached, mice follow two different protocols (compare Figure 1B). (**C**) Lowest blood oxygenation measured during the experiment for each mouse. (**D**) Lowest oxygen supply reached before the mice died (protocol 1A). (**E**) Survival time of the mice at 10% oxygen supply (protocol 1B).

This approach has the advantage that presence and absence of perfluorodecalin can be analyzed on a paired basis within the same animal. Figure 3A and 3B depict the heart rate and the oxygenation, respectively, at 15% oxygen supply before, during and after perfluorodecalin application. These measurements reveal a high variability even within the defined experimental intervals of a particular mouse, i.e., the measurement itself shows a high 'noise' level. To approach this effect the median of each experimental period was determined for every mouse and then the mean of the four measured mice for the three experimental intervals were plotted in Figure 3C. A paired one-way ANOVA test showed no significant differences. However, a linear fit of the oxygenation over time revealed a goodness of fit R^2 of 0.997 with a significant non-zero slope ($p = 0.035$). This shows that (i) indeed a considerable portion of the variation is likely to be measurement noise and (ii) in our experimental approach, we could not improve the blood oxygenation level. Instead, a decline of the oxygen saturation along the experimental periods was detected, which is most likely a temporal effect of the low oxygen supply duration.

Discussion and conclusions

Conceptual considerations

An experimental setting had to be developed to assess a possible transdermal oxygen delivery. Although measurements on human volunteers would be attractive, a pilot animal study was regarded as more appropriate. For the animal experiments, we considered young house-pigs and nude mice, because in both cases a fur-less skin is available. While porcine skin is anatomically and physiologically very similar to human skin (Avci *et al.*, 2013), it is thicker and less vascularized. Murine skin, with an average body epidermal thickness of approximately

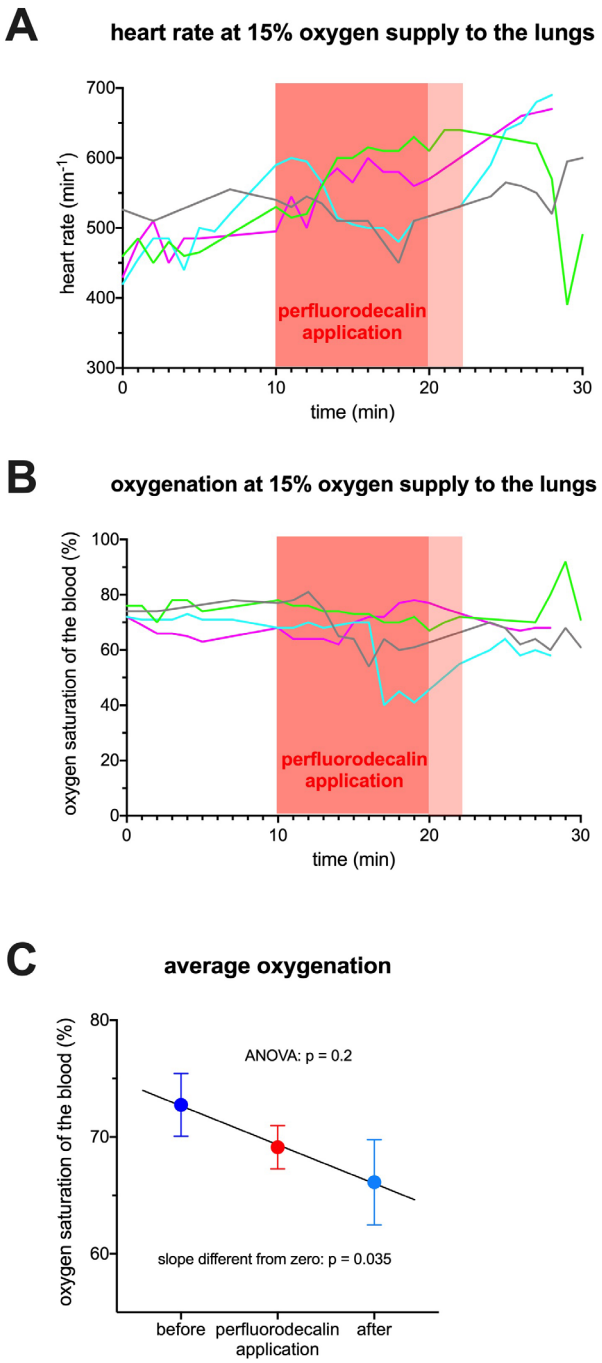


Figure 3. Results of the comparison of hypoxic mice before, during and after treatment with perfluorodecalin. (**A**) Heart-rate plotted against the time at 15% oxygen supply for 4 mice. The region highlighted with a red background indicates the perfluorodecalin application. The light red region indicates the interval, when active perfluorodecalin application was stopped but still not the entire substance evaporated and remains were present on the skin. (**B**) Blood oxygenation plotted against the time at 15% oxygen supply for 4 mice. The region highlighted with a red background indicates the perfluorodecalin application. The light red region indicates the interval, when active perfluorodecalin application was stopped but still not the entire substance evaporated and remains were present on the skin. (**C**) Statistical analysis of the blood oxygenation before, during and after perfluorodecalin application.

8.5 µm and a cutaneous blood flow of 17.8 mL/min/100 g (Monteiro-Riviere *et al.*, 1990), is an ideal candidate. Accordingly, we used nude mice (CD1nu/nu, Charles River, Wilmington, MA, USA) in the present proof-of-principle study.

Next, a fluorocarbon had to be selected. The physico-chemical property to dissolve about 20 times more oxygen than water (Riess, 2005) applies to more or less all fluorocarbons. However, it has previously been shown (ECETOC, 2008) that adverse effects of fluorocarbons when topically applied are mainly caused by impurities of the preparations. Therefore, it was compulsory to use a fluorocarbon with medical purity. Pharmpur (Königsbrunn, Germany), a manufacturer of liquid medical products, has the fluorocarbon perfluorodecalin (also known as perflunafen) in its portfolio and kindly provided it for this study. It has a boiling point of approximately 142°C, which is less than other long-chain fluorocarbons depict and leads to a considerable evaporation on the 37°C skin surface of mice. However, this problem can be handled by repetitive/continuous brushing of the mouse skin with perfluorodecalin.

Our approach was that transdermal oxygen is only relevant, if a difference can be detected on the systemic level. As a major read-out device, a dedicated rodent-optimized pulse oximeter (MouseSTAT Jr. with Paw sensor, Kent Scientific, Torrington, CT, USA) was chosen. Preliminary experiments revealed that the maintenance of the body temperature at 37°C or slightly above is vital to obtain reliable read-outs of the pulse oximeter. Therefore, body temperature was continuously monitored and subsequently adjusted with help of a heating pad (Figure 1A).

To mimic a hypoxic condition that can also be found in the COVID-19 disease situation, an artificial reduction of the oxygen supplied to the lungs was envisaged, while the skin of the body was exposed to the normal atmospheric oxygen concentration of above 19%. This was realized by the combination of a tunable gas-mixer and a dedicated mouse lung-ventilator as detailed in Figure 1A. The initial concept was to subsequently reduce the oxygen supply and compare the survival time of mice at a reduced oxygen supply in dependence of dermal whole-body perfluorodecalin application, as outlined in Figure 1B (protocol 1A).

Transdermal oxygen delivery to increase systemic oxygen

Using CD1nu/nu mice and medical grade perfluorodecalin we were not able to measure a significant increase in the oxygenation of the blood. We have to admit that the resolution of our blood oxygenation measurements was not very high. The display resolution is 1% but we have the impression that the hypoxic conditions, the mice are exposed to, are not in favor of pulse oximeter reliability (jumping values, i.e. noise). Measurement variations even on the same mouse over time require a certain averaging. Therefore, we conclude that increases in blood oxygenation below 2% cannot be detected with the method applied and the number of animals investigated. However, since our goal was a proof of principle, which may have helped to improve the situation of COVID-19 patients, a dermal oxygen supply of 5% of the total would be a minimal requirement

for a practical application. Accordingly, we falsified the hypothesis that perfluorodecalin improves the transdermal oxygen supply to be relevant for a clinical application. On the other hand, we like to stress that this does not generally disprove an increase in transdermal oxygen delivery by topical application of fluorocarbons.

Methodological inferences

The manipulative part of the setup introduced in the upper part of Figure 1A seems to be a reliable approach for a (hypoxic) stress model for anesthetized animals with full manual access to the animal, i.e. without the use of hypoxia cages or boxes.

For the detection part of the system we experienced shortcomings and for future studies we propose to complement pulse-oximetric measurements with a simple ECG for a more reliable detection of the vital function. Furthermore, a more sophisticated measurement of tissue oxygenation, e.g., by applying fibre-optic micro-sensor technology, could be a valuable extension of the measured parameters.

Data availability

Underlying data

Dryad: Test of perfluorodecalin to increase transdermal oxygen delivery in mice. https://doi.org/10.5061/dryad.931zcrjjp (Kaestner, 2021).

This project contains the following underlying data:

- Heart_rate_protocol_1.csv (data provide the heart rate of the mice (per minute) in dependence of the oxygen supplied (%) according to protocol 1, described in the methods)
- Oxygenation_protocol_1.csv (data provide the oxygenation of the mice (%) in dependence of the oxygen supplied (%) according to protocol 1, described in the methods)
- Heart_rate_protocol_2.csv (data provide the heart rate of the mice (per minute) in dependence of the experimental time (min) according to protocol 2, described in the methods)
- Oxygenation_protocol_2.csv (data provide the oxygenation of the mice (%) in dependence of the experimental time (min) according to protocol 2, described in the methods)

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

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Open Peer Review

Current Peer Review Status: ✔ ✘ ✘ ?

Version 2

Reviewer Report 14 August 2024

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?

Michele Samaja
University of Milan, Milan, Italy

This article reports a proof-of-concept approach to the problem of bypassing lung oxygenation in hypoxic subjects. The lack of positive results in this study should not discourage the Authors from pursuing their goal, which may be of tremendous relevance for many hypoxic patients. Regarding the submitted article, there may be several ways to improve transdermal oxygenation that should be mentioned in the article or tested experimentally.

First, it is unclear if perfluorodecalin was equilibrated with air (How? Shaking?) or pure O2. Perhaps, equilibrating the perfluorodecalin emulsion with 100% O2 increases by five times the amount of O2 that can diffuse through the skin.

Second, more details on how the animals were “brushed” with perfluorodecalin should be given. I suspect that the thickness of the layer of perfluorodecalin in contact with the skin plays a key role. If it is too narrow, O2 may escape back in the direction of the environment and not towards the skin. It would be important to measure the PO2 in the perfluorodecalin liquid, if possible.

Third, the Authors may have neglected the role of vasoconstriction. It appears from the text that perfluorodecalin was prewarmed at 30°C, but why not 37°C, which may have induced greater vasodilation? This, with the simultaneous systemic administration of a pharmacological vasodilator as a NO-donor or a phosphodiesterase inhibitor, may have induced persistent vasodilation with greater transdermal O2 diffusion.
Fourth, the great mice-to-mice data dispersion may be managed by statistical treatments based on paired data comparisons.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Hypoxia, cardioprotection, hypoxic conditioning, altitude, oxygen carriers

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 25 May 2024

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Amin Shavandi
Université Libre de Bruxelles (ULB), Brussels, Belgium

This article proposes a method to increase oxygen uptake through the skin using a liquid perfluorocarbon.

One missing aspect in my opinion is the hyperbaric oxygen therapy which hasn't been mentioned in the state of the art, among other techniques of oxygen delivery that are mentioned.
One important aspect is also the potential safety concerns associated with topical application of perfluorocarbons. While the goal is for PFCs to deliver oxygen locally through the skin, there's a chance they might be absorbed into the bloodstream. Maybe the authors could measure perfluorocarbon levels in animal blood after topical application to assess potential systemic effects. There is also a need to mention or discuss the skin physiology of the animals and the need to use animals with skin physiology closer to humans.

Again in line with PFC safety, the article focuses on short-term application. However, the long-term effects of repeated or continuous exposure to PFCs are unknown. Maybe it could be applied to animals for extended periods and monitoring for any changes in skin health. These safety

concerns related to PFC could be also mentioned in the introduction to show the state of the art in that regard.

The second point is related to the hypothesis of the article concerning oxygen diffusion through the skin. If the oxygen in the environment dissolves in the PFC it may not necessarily diffuse through the skin layers. Due to the concentration difference, oxygen molecules in the environment will tend to move from the environment and dissolve into the perfluorocarbon layer on the skin. There's also a chance for oxygen molecules to back-diffuse from the PFC layer out towards the environment. This back diffusion would reduce the overall efficiency of delivering oxygen into the skin. We know that the key driver is the concentration difference between oxygen in the PFC layer, the skin layers, and the surrounding environment. Oxygen will naturally move from areas of high concentration, PFC, to lower concentration areas (Skin). In my opinion, the article likely simplifies the process without considering the potential competition between the two pathways.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biomaterials and tissue engineering

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 07 October 2021

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Alessandro Santini
Department of Anesthesia and Intensive Care Medicine, Humanitas Clinical and Research Center-IRCCS, Rozzano, Italy

Dear Lars,

I appreciate your responses and the effort you made to perform these experiments, and I completely agree with you that negative results are worth to be considered for publication as well as positive results.

However, it seems to me that this report, at this time, is more suitable for a lab meeting than for a publication.

What I mean is that there is still a lot of troubleshooting to be made in order to be sure - to be really sure - that the results are negative.

Mice of the protocol 1A should not be presented at all, regardless of the total number of mice remaining (you can perform more experiments if you wish to increase your numerosity). As you pointed out, your monitoring was not adequate to detect the time of death. I suggest to change your monitor. One again, you can easily cannulate the carotid artery in mice during general anesthesia and connect a pressure probe (the same used in humans) to measure blood pressure beat-by-beat on a monitor.
Regarding blood withdrawal, I am aware that you cannot measure 6 timepoints of PaO2 in the same mouse, however you can use more mice and withdraw blood at different timepoints (e.g. 4 mice per group, first group you withdraw blood at 10 mins, second group at 20 min, third group at 30 mins and so on). Also, but this is more costly, you can cannulate the carotid artery with a PtiO2 probe (the one used for brain tissue oxygenation measurements) and have a continuous measure of PaO2 without withdrawing blood.
Lastly, even if putting the mouse inside a cage with a fixed fraction of oxygen is not feasible for the reasons you explained, you might "ventilate" the body of the mouse with a continuous source of oxygen (e.g. a simple oxygen mask over the body of the animal) - clearly the fraction of oxygen will not be "fixed" and not even measurable, but will surely be higher than 21% and I think it will help you to prove or disprove your hypothesis.

Once again, these are only suggestions to increase (in my opinion) the quality and reliability of your research. As already pointed out, I think the research question is of importance and interest, however I don't think these results can be indexed without further experiments.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Acute respiratory failure; Ventilator-induced lung injury

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 06 Oct 2023

Lars Kaestner

As a general statement: We agree that the experimental procedures could be improved by using more sophisticated techniques. Also increasing the number of experiments would improve the statistics. However, all of these measures would increase the number of animals included in this study. We provided strong evidence for negative results in the dimension of a potential application for patients and we are convinced that a further killing of mice is neither justified nor timely.

Competing Interests: No competing interests were disclosed.

Version 1

Reviewer Report 31 August 2021

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Alessandro Santini
[1] Department of Anesthesia and Intensive Care Medicine, Humanitas Clinical and Research Center-IRCCS, Rozzano, Italy
[2] Department of Anesthesia and Intensive Care Medicine, Humanitas Clinical and Research Center-IRCCS, Rozzano, Italy

Kaestner and colleagues performed a preclinical study to assess the feasibility of delivering oxygen to the systemic circulation via the skin using perfluorocarbons in situations of low oxygen supply (e.g. respiratory failure).

They were not able to find a clinically meaningful increase in oxygen saturation in mice with skin covered with a specific perfluorocarbon during hypoxic ventilation (FiO2 10%-15% depending on the protocol).

The topic is very interesting and actual, given the ongoing COVID-19 pandemic.

However, their track into this topic seems to me to be well ahead to be completed, and overall the results are not ready to be published in this form.

My major concerns and suggestions are the following:

- The three protocols used should be better explained to the reader in the Methods section. In the present form, the reader starts to understand what happened to each mouse at the end of the Results section.

- Protocol 1A should probably not even be shown, it seems to be very explorative and not reproducible.

- I agree with the Authors that the SpO2 probe is probably not very sensitive – as such it should not be used for the purposes of a study which aims to find. differences in oxygenation as its main outcome. SpO2 values at the beginning of the protocols, with FiO2 around 25%, should be well above 90% (see Figure 2B); since mice are anesthetized and tracheotomized it is very easy to withdraw blood from the carotid artery for blood gas analyses.

- I suggest the Authors, in order to maximize a signal if there is one, to check for increases in oxygen saturation (or PaO2, see above) using perfluorocarbon coated skin and 100% O2 around the body of the animal (using a cage with tunable fraction of oxygen). The only good reason for using mice in these kinds of experiments is to have reliable proof of principle studies, which then need to be adapted to what is feasible in a human being. Trying to match proof of principle demonstration and clinical feasibility in a single study on mice will never give any answer.

- “Conceptual Considerations” paragraph should be moved to the Discussion section.

I thank the Authors for their effort and out of the box idea, and invite them to go on in their research endeavors, using different experimental settings and more reliable measurements, in

order to give perflouorocarbons their best chance to show a possible clinical role, if there is any.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
No

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Acute respiratory failure; Ventilator-induced lung injury

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 24 Sep 2021

Lars Kaestner

Dear Alessandro, thank you very much for your report and the associated effort. We like to respond to your points of criticism:

- We agree that it makes a lot of sense to better explain and motivate the use of the different protocols. Therefore we added the following paragraph in the Methods section: "The initial idea (represented as protocol 1A in figure 1B) was to reduce the available oxygen in steps from 20% - 15% - 10% - 9% - 8% -7%, each step lasting for 5 min. While oxygen supply, heart rate and oxygen saturation were recorded, the rationale was to detect differences in the survival time between perfluorodecalin treated mice and control mice. However, it turned out that it was impossible to properly determine the time point of death of the mice. The pulse-oximeter was thought to provide the information of the termination of the heart beat but it turned out that the pulse-oximeter stopped working at a weak pulse before the death of the animal (alternating periods of no signals and measured signals). Since the animals were ventilated stop of breath was neither a criterion. A mouse electrocardiogram was not available. Therefore, after the measurement of 6 animals, the protocol was

modified to protocol 1B in figure 1B, i.e. the available oxygen was reduced in 2 steps of 5 min from 20% to 10% and then kept constant at 10%, still with the intention to determine the survival time expecting a better reliability of the pulse oximeter at higher percentage of oxygen supply. Because this was not the case, we stopped to apply protocol 1B after further 4 animals and changed the concept of measurements. Instead of recording the survival time at decreasing oxygen availability, we measured the blood oxygen saturation (as well as the heart rate) under hypoxic conditions (15% oxygen supply) before, during and after termination of the perfluorodecalin application, each period lasting for 10 min."

- To remove the results of Protocol 1A is not a real option, because it would reduce the number of animals presented in Figure 2 to 2 per group, which would not make sense at all.

- When planning the experiments we indeed considered the blood gas analysis to monitor the oxygen saturation of the blood. The blood gas analyser we have available requires sample volumes of 60 µL. This would be in the range of 3 to 3.5% of the total blood volume of the mouse. Apart from the fact that we don't know the temporal information of the endpoint in advance, we would need to record a time-course of at lest 6 points, which corresponds to approximately 20% of the blood volume of the mouse, which would screw up the entire experimental setting.

- We indeed also considered to place the mice in a cage with 100% oxygen and we agree with the reviewer that this is a good idea for a proof-of-principle study. The reason for not doing it was of practical reasons. The only fluorocarbon we could get hold of at the required purity was perfluorodecalin. Although perfluorodecalin has a higher boiling temperature than water (142^{o}C), it has a lower evaporation enthalpy 41.5 kJ/mol (vs. approx. 44 kJ/mol for water). This volatility required the continuous application of perfluorodecalin to the mouse skin (as described in the methods section) and therefore a continuous accessibility to the mouse skin, which prevented us from using a closed cage filled with 100% oxygen.

  Furthermore, we completely agree, that a putative demonstration in mouse doesn't tell anything about the clinical feasibility. The way round if there is no effect in mice (with the high skin surface / body volume ratio, the comparably thin skin and high cutaneous blood flow), an effect in humans is unlikely. Thus, the paper presents a negative result, but we believe the negative results are also worthwhile to report. However, the results achieved so far, do not justify further animal experiments to possibly show small effect sizes below 2%.

- The paragraph "Conceptual Considerations" was moved as proposed to the Discussion section.

I sincerely hope these explanations allow you to approve our report. Best regards, Lars

Competing Interests: No competing interests were disclosed.

Reviewer Report 20 April 2021

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Christian Bollensdorff
[1] Precision Medicine, Research Branch, Sidra Medicine, Doha, Qatar
[2] Precision Medicine, Research Branch, Sidra Medicine, Doha, Qatar

It is a very valuable point, which has been picked up by the authors, that oxygenation via the skin plays a crucial role, especially in situations where every little additional supply is of importance. Substances like fluorocarbons could eventually be used in support during situation of low oxygen supply. That could also be expanded to other situations where blood supply is limited and therefore parts of a body are in need of alternative routes for a period of time. Unfortunately the authors could not show that the used substance is improving the oxygen supply significantly, which does not mean that a similar approach with higher levels of oxygen could not be successful. Overall the publication might benefit form some minor additions. The authors may expand on the idea why a fluid which carries less oxygen content than the surrounding air would have a better transmission of oxygen through the skin as a result. A more elaborate introduction into experiments using oxygen supersaturated fluids, or gases exposed to skin and its effects would be of benefit. More background should be given about the expected effect and effect size, and when it will become impactful. Although some animals are known to benefit from oxygen absorption through skin, the special anatomical preconditions playing a role as well – that was only partially discussed. The influence of vasodilation can be discussed, and how would that interfere with a severe condition. Some information about the nature of fluorocarbons in terms of oxygen content retention, i.e. how good of an oxygen donor is it, would also inform the reader more about the suitability of such substance.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cardiac research.

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

Author Response 24 Sep 2021

Lars Kaestner

Dear Christian, thanks for your report. We added a new paragraph in the introduction of the new version. Best regards, Lars.

Competing Interests: No competing interests were disclosed.