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Short-Duration Hypothermia Induction in Rats Using Models for Studies Examining Clinical Relevance and Mechanisms

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TITLE:

Short-Duration Hypothermia Induction in Rats Using Models for Studies Examining Clinical Relevance and Mechanisms

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KEYWORDS

hypothermia, animal model, short-duration hypothermia, translational, rats, neuroscience

SUMMARY

This article describes two methods of whole-body short-duration hypothermia induction in rats. The first, rapid induction method, employs active cooling using fans and ethanol spray for a rapid decrease in temperature. The second method allows for a slower time to target and does not rely on external cooling devices.

ABSTRACT

Therapeutic hypothermia (TH) is a powerful neuroprotective strategy that has provided robust evidence for neuroprotection in preclinical studies of neurological disorders. Despite strong pre-clinical evidence, TH has not shown efficacy in clinical trials of most neurological disorders. The only successful trials employing therapeutic hypothermia were related to cardiac arrest in adults and hypoxic ischemic injury in neonates. Further investigations into the parameters of its use, and study design comparisons between pre-clinical and clinical studies, are warranted. This article demonstrates two methods of short-duration hypothermia induction. The first method allows for rapid hypothermia induction in rats using ethanol spray and fans. This method works by cooling the skin, which has been less commonly used in clinical trials and may have different

physiological effects. Cooling is much more rapid with this technique than is achievable in human patients due to differences in surface area to volume ratio. Along with this, a second method is also presented, which allows for a clinically achievable cooling rate for short-duration hypothermia. This method is easy to implement, reproducible and does not require active skin cooling.

INTRODUCTION

TH is the practice of cooling body or brain temperature in order to preserve the viability and function of the organ/system[1,2]. The role of hypothermia in neuroprotection has been investigated and has shown benefits in a range of preclinical models of neurological diseases such as stroke[3], subarachnoid haemorrhage[4], and traumatic brain injury[5]. In terms of clinical applications, TH has shown efficacy in patients post-cardiac arrest and in neonatal hypoxic-ischemic injury[6].

TH induction is achieved using either surface or endovascular cooling methods. The majority of pre-clinical hypothermia studies perform surface cooling by applying water or ethanol to the animal's fur, or by using a cooling blanket to achieve target temperature[1]. In humans, systemic surface cooling is achieved by using ice packs and cooling blankets[7,8]. More rapid cooling has been shown in patients using endovascular methods, which couple an induction infusion of cold saline through an intravenous or intra-arterial catheter, with the placement of an endovascular cooling device within the inferior vena cava[9,10]. For example, a moderate target temperature of 33 °C can be reached in 1.5 h with endovascular cooling compared to 3-4 h with surface cooling in patients[11]. The endovascular approach has also become more popular in recent years because it has been reported to reduce some of the side effects seen in systemic surface cooling, such as shivering[12,13]. The European multicenter, randomized phase III clinical trial of hypothermia for ischemic stroke (EUROHYP-1) used mostly surface cooling[14]. Results recently published from this trial showed that shivering was a major complication and might have limited the ability to achieve the target temperature[10]. The shivering response is known to be primarily driven by skin temperature. Some efforts have been made to develop a rodent endovascular cooling method[15], but the highly invasive nature of the technique compared to that used in humans, risks confounding any results obtained from that model.

Temperature is the key modulator of biological processes in the body and is tightly regulated by homeostasis. Therefore, any manipulation of the body temperature can have associated risks. Cooling duration is a factor that may have limited the success of hypothermia clinical trials. These trials use a long-duration cooling method, with many maintaining hypothermia from 24-72 h[11]. This extended duration poses a risk for infection during the cooling protocol. Pneumonia is the most common complication from hypothermia, affecting between 40-50% of patients who undergo the procedure[13]. This is in contrast to what is normally seen in animal studies of hypothermia where a short-duration paradigm is used (>6 h)[3]. The success of these pre-clinical animal studies will likely result in the adaptation of short-duration hypothermia for the use in clinical trials. As a result, it is necessary to have an animal model of short-duration hypothermia that resembles the cooling rates of future clinical trials. Further details pertaining to other temperature parameters and the validity of short-duration hypothermia have been discussed in

several review articles[1,16-18].

Demonstrated here is a gradual model of cooling that is more clinically achievable than current experimental hypothermia models. This novel method has a much slower rate of cooling and therefore, the time to target temperature is closer to the range of those seen in clinical trials of hypothermia[11]. It also avoids direct surface cooling, which has specific physiological effects, and may, therefore, be more comparable to endovascular cooling, which has been the most commonly used cooling method in clinical trials[9,12]. This model allows animals to be cooled gradually over 2 h followed by a short period of maintenance at target temperature. Additionally, the rapid cooling short-duration hypothermia method[19] is also demonstrated. The fast-cooling method allows target temperature to be achieved rapidly after hypothermia onset. While this approach is not as clinically relevant as the gradual cooling method, it is useful for studies that aim to explore the mechanisms of hypothermia neuroprotection to potentially mimic its powerful neuroprotective effects pharmacologically. This method also has potential applications outside of neuroscience and could be adapted to any number of pre-clinical studies. Another advantage of both methods compared to other approaches is that they are inexpensive and do not require specialist equipment. Finally, this protocol also demonstrates implantation of temperature dataloggers, since post-operative warming and monitoring thereof are important to prevent inadvertent post-operative hypothermia, with its potential to confound study results[20].

PROTOCOL

All experimental procedures were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Care and Ethics Committee of the University of Newcastle (A-2013-343 and A-2020-003). In addition to the hypothermia induction methods described below, the following protocols are routinely done in conjunction with hypothermia: femoral line cannulation to monitor blood pressure and heart rate[21], and experimental stroke[22].

1. Datalogger implantation

NOTE: The datalogger device used in this protocol was not capable of providing real time read-out of body temperature. Read out is possible once the datalogger has been removed from the animal and connected back to the computer. As a result, the rectal temperature probe is used to provide real time information during the cooling and rewarming process. Additionally, the rectal probe is also vital to this method because the surgical heat mat on which the animal is placed during the procedure is regulated by the rectal probe system. The datalogger also serves a valuable purpose of providing temperature data in freely moving, awake rats and is important for ensuring that normal body temperature is maintained after rewarming. Therefore, both temperature-monitoring devices are important for this protocol.

1.1. Anesthetize 10-12-week-old male outbred Wistar rat with isoflurane (5% for induction and 2-2.5% for maintenance) in a 50% N_2 and 50% O_2 mixture.

1.2. Following induction, place the rat in the prone position on a surgical heat mat.

1.3. Position the rat so that the nose is sitting in the nose cone. Secure the nose with surgical tape to ensure no gases are escaping.

1.4. Shave the fur from the lower right abdomen and inject the site subcutaneously with a local anesthetic, Bupivacaine 0.2 mL, 0.05%.

1.5. Apply antiseptic solution to the freshly shaved region.

1.6. Using sterilized surgical tools, make a 2 cm longitudinal incision along the right abdominal region, proximal to the right thigh. Make the incision deep enough to expose the space at the ventral thigh crease.

1.7. Use hemostats and forceps to create a 'pocket' under the skin that is large enough to hold the device.

1.8. Insert the temperature monitoring datalogger device into the pocket and close the muscle and skin using 5-0 silk sutures. The subcutaneous method described here is preferred to the intra-peritoneal method as it is less invasive and allows for the better recovery after the procedure.

1.9. Ensure datalogger and rectal probe are cross calibrated for temperature monitoring (see Discussion).

1.10. Ensure the datalogger is not resting against the animal's heat mat after insertion, as this will influence temperature readings.

[Place Figure 1 here]

2. Induction of active (fast) hypothermia for mechanistic studies

2.1. Set up for hypothermia (see **Figure 2**). Place two retort stands with clamps at either side of the rat's body.

2.2. Attach a 60 mm 12 v/130 mA fan to each retort stand ensuring that the fans are aimed towards the lower back of the rat. The distance between the clamp and the rat is approximately 20 cm. The fan use must have a speed of 4,000 rpm.

2.3. Have an animal heat lamp ready either at the side or an on third retort stand.

2.4. Commence hypothermia by adjusting the animal heat mat to the desired target temperature. In this example, 32.5 °C is the target temperature (3.75 on the temperature control unit).

2.5. Turn both fans on and apply three to four sprays of 70% ethanol (standard plastic spray

bottle) on the lower back of the rat. Ruffle animal fur when spraying for faster cooling induction.

NOTE: Ethanol is used as a preferred solution over water because it has a faster rate of evaporation and therefore results in more rapid hypothermia induction.

2.6. Take care not to oversaturate the fur, since this can contribute to overshooting the target temperature.

NOTE: The fans will accelerate ethanol evaporation and the cooling process.

2.7. Allow short intervals between ethanol applications while keeping a close eye on the rectal temperature of the rat.

2.8. Cease any further application of ethanol once rectal temperature reaches within 1 °C of target temperature.

2.9. Turn off both fans once the temperature has reached within 0.5 °C of target temperature (33 °C in this case).

NOTE: Turning off fans before the target temperature is reached helps prevent the rat from overcooling beyond required temperature.

2.10. Allow the temperature to drop to 32.5 °C.

2.10.1. If overcooling does occur, use animal heat lamp to mildly warm the animal back up to target. The assistance of one fan may be used to prevent a rewarming overshoot.

2.11. Once target temperature is reached and has stabilized, continue to monitor temperature. Temperature will usually remain very stable for the remainder of the hypothermia period without the need for spraying, use of fans or use of heat lamp.

2.12. To rewarm the animal at the end of hypothermia, adjust the heat mat temperature back to 37 °C (6 on the temperature control unit used in this example) and allow the animal to thermoregulate over a 30 min period.

NOTE: Temperature settings on temperature control units may vary and it may, therefore, be necessary to determine settings for target hypothermia and normothermia on individual devices.

3. Induction of clinically achievable gradual onset hypothermia without active skin cooling

3.1. Achieve hypothermia by reducing the temperature of the core temperature regulated homeothermic heat mat in small increments to the required target temperature. In the illustrated example (**Figure 3B**), a 1 °C increment every 30 min was used.

3.2. Cool the animal to target temperature over the desired period (2 h in the example described). Once cooled, maintain at target for the desired interval. Usually, no further intervention is required if they are maintained on the homeothermic heat mat set for the desired target temperature.

3.3. Do not provide any external cooling is necessary with this protocol as anesthesia prevents the normal regulation of core body temperature.

NOTE: Isoflurane requirements decline with hypothermia. In most animals, a starting isoflurane concentration of 2% can be reduced in 0.1% increments every 20-30 min to 1.5% to maintain stable respiratory rate (>50 breaths/min), heart rate and blood pressure, and preserve suppression of reflex responses.

3.4. To rewarm the animal after hypothermia, adjust the heat mat to allow the animal to rewarm over the desired interval. In the example, rewarming with a single adjustment to 37 °C (6 on the FHC temperature control unit used in the example) was achieved over a 30 min period.

3.4.1. For longer-term studies that require animal recovery, keep animals in a cage that is placed half over a heat mat to allow the animal to thermoregulate and to avoid inadvertent post-operative hypothermia.

REPRESENTATIVE RESULTS

Figure 3A is a representation of how a Wistar rat responds to hypothermia using the rapid cooling approach. Hypothermia induction is achieved by the use of fans and 70% ethanol spray. Hypothermia to a target of 32.5 °C is achieved in 15 min. Care must be taken to ensure a delicate interplay between the use of the fans/ heat lamp and ethanol spray to maintain target temperature. As can be seen from **Figure 3A**, a slight temperature overshoot is observed, which may occur if cooling is not ceased from about 0.5 °C above target temperature. Target is maintained and stabilized at the 30 min mark and rewarming is initiated at 1.5 h.

Figure 3B shows the gradual protocol in which target temperature to 33 °C is reached at 2 h and maintained for 30 min before rewarming at 2.5 h. Here, the temperature is adjusted in increments which prolongs the duration necessary to reach target temperature. Vertical dotted lines in both graphs represent the duration of cooling.

Figure 3A and **Figure 3B** are obtained from the datalogger device. At the start of the experiment, the datalogger is programmed to initiate recording prior to implantation. At the end of the experiment, the datalogger is removed from the animal and connected to the provided temperature reader via USB port. The software (e.g., eTemperature) reads and generates the data, which can then be exported to a spreadsheet software.

FIGURE LEGENDS

Figure 1: Implantation of datalogger device. (**A**) Panels left to right show an incision of approximately 2 cm being made on the right side of the lower abdomen of the rat. (**B**)

Temperature monitoring datalogger was inserted subcutaneously into the pocket incision. (**C**) The incision was closed with nylon sutures.

Figure 2: Set up of rapid cooling protocol. (**A**) Two fans (black arrow) were situated over the lower back region of the rat. At hypothermia initiation, both fans were turned on and ethanol spray was applied to the lower back. The combination of ethanol and the fan facilitates and accelerates hypothermia to rapidly achieve target temperature. (**B**) A heat lamp (white arrow) was used to prevent hypothermia overshoot. Once target temperature was reached, the heat lamp was used to prevent the rat core temperature from dropping any lower. Once target had stabilized, the heat lamp and/or remaining fan was turned off.

Figure 3: Hypothermia induction using active (A) and gradual (B) methods. (**A**) Target temperature was reached in 15 min using the active cooling process and was maintained for 60 min in the above example before the animal was rewarmed. (**B**) Target temperature was reached in 2 h using the gradual cooling method and was maintained for 30 min before the animal was rewarmed. Shaded regions in both graphs represent time points in which target temperature was maintained. Dotted perpendicular lines in both graphs refer to the overall cooling duration.

DISCUSSION

The procedures described here are easily implemented, non-invasive, and provide reliable and reproducible decreases in core body temperature to a desired target temperature.

There are several critical steps in the rapid cooling method include following. Do not oversaturate ethanol spray - care must be taken not to soak the animal in ethanol, as this will interfere with results. Monitor the animal during hypothermia induction- care must be taken to closely monitor animal responses to rapid hypothermia induction. A close watch of rectal temperature is important to ensure that temperature does not go below the desired target- if this happens, turn off the fans and allow the heat lamp to gently rewarm the animal back up to the required target.

In both methods, physiological monitoring is important to ensure appropriate adjustment of anesthetic dose. For prolonged cooling, inadequate anesthetic dose may prolong the duration of cooling. In this case, isoflurane concentration can be increased until an adequate cooling rate is achieved. Another critical step is the cross-calibration of temperature devices. When using a temperature probe regulated heat mat and a datalogger in the same experiment, it is best practice to cross-calibrate the datalogger with the rectal probe, in vivo, since there may be minor variations in the recorded temperature of the two devices.

These methods are suitable for studies that wish to explore the use of hypothermia as a potential treatment for neurological disorders. The specific aim of the study should dictate which method is used. Both methods can be classed as systemic surface cooling, however the second method does not require any active cooling. The gradual cooling model described above has important potential applications for the use of hypothermia in ischemic stroke treatment. Long-duration hypothermia and their resulting complications pose a challenge to elderly stroke patients. Moreover, the shivering response makes it difficult to achieve target temperature in some

patients[10]. While anti-shivering medication can help to reduce the shivering response, short-duration gradual cooling could more effectively ameliorate the issue. Having a shorter cooling period is also likely to reduce the incidence of pneumonia often reported in trials. Another potential benefit of this short-duration method is that the speed of rewarming might not be as important when compared to long-duration cooling. Very early clinical studies of long-duration cooling in stroke patients with large infarcts found that fast rewarming led to large elevations in intracranial pressure (ICP), which worsened outcome and was often fatal. This led to the development of gradual rewarming paradigms, which further extended the overall duration of cooling. Short duration cooling only maintains target temperature for a short period and may less likely result in rebound ICP. Previous work which has investigated hypothermia treatment for ICP elevation, using a similar rapid cooling and rewarming protocol as the ones described here, have shown no rebound ICP elevation after rewarming[23,24].

Clinical trials of hypothermia for ischemic stroke treatment have not been able to translate the benefits of hypothermia that are reported in experimental studies. The mismatch in cooling rates and duration between experimental models and patients, are important variables that may account for this discrepancy. Having an experimental model of hypothermia that better resembles the clinical rate of cooling will allow for a more informed investigation into the benefits of hypothermia as a treatment measure for stroke patients.

ACKNOWLEDGMENTS

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DISCLOSURES

The authors have nothing to disclose.

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Figure 1: Datalogger insertion

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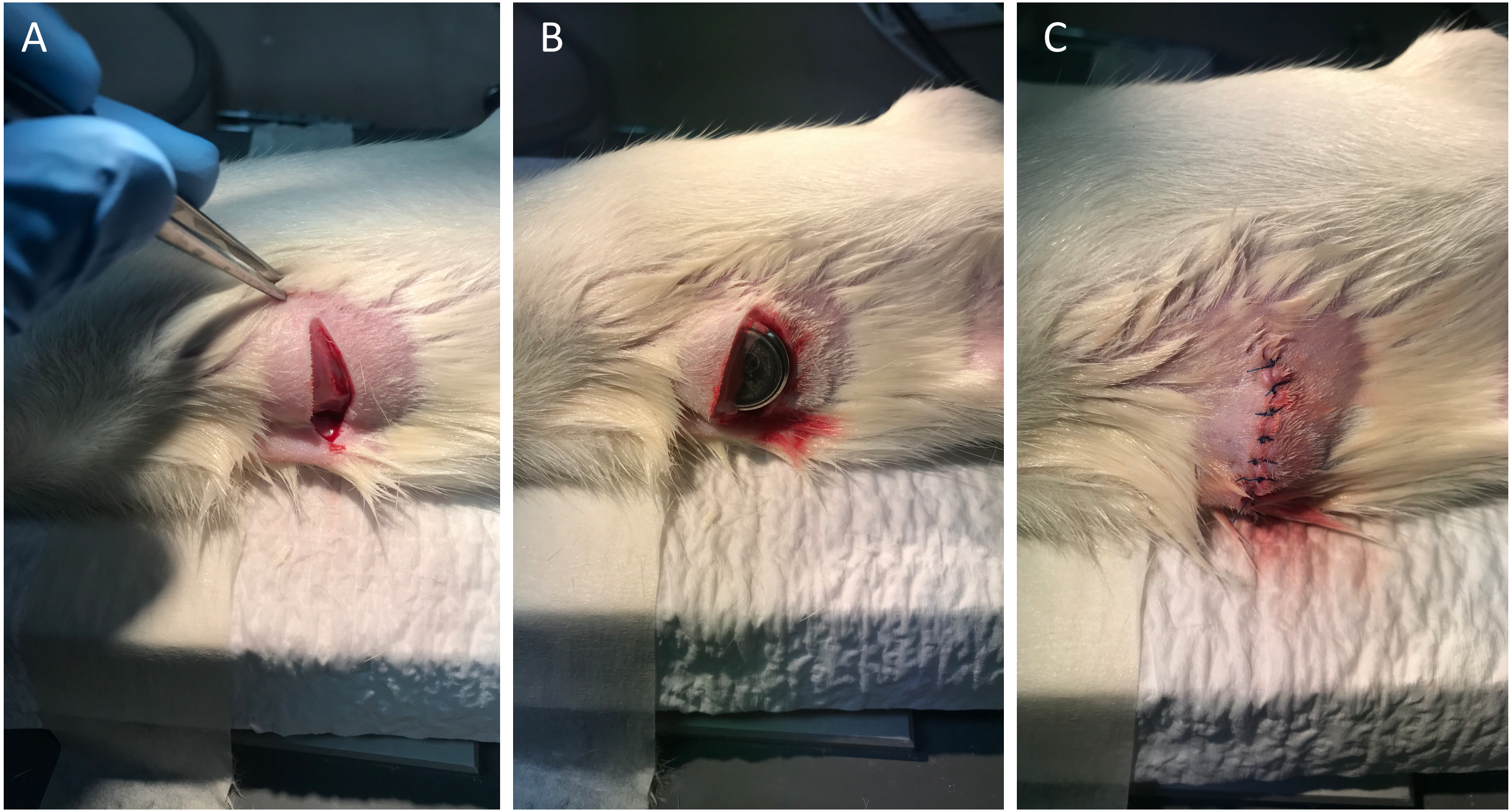

Figure 2: updated rapid cooling set up

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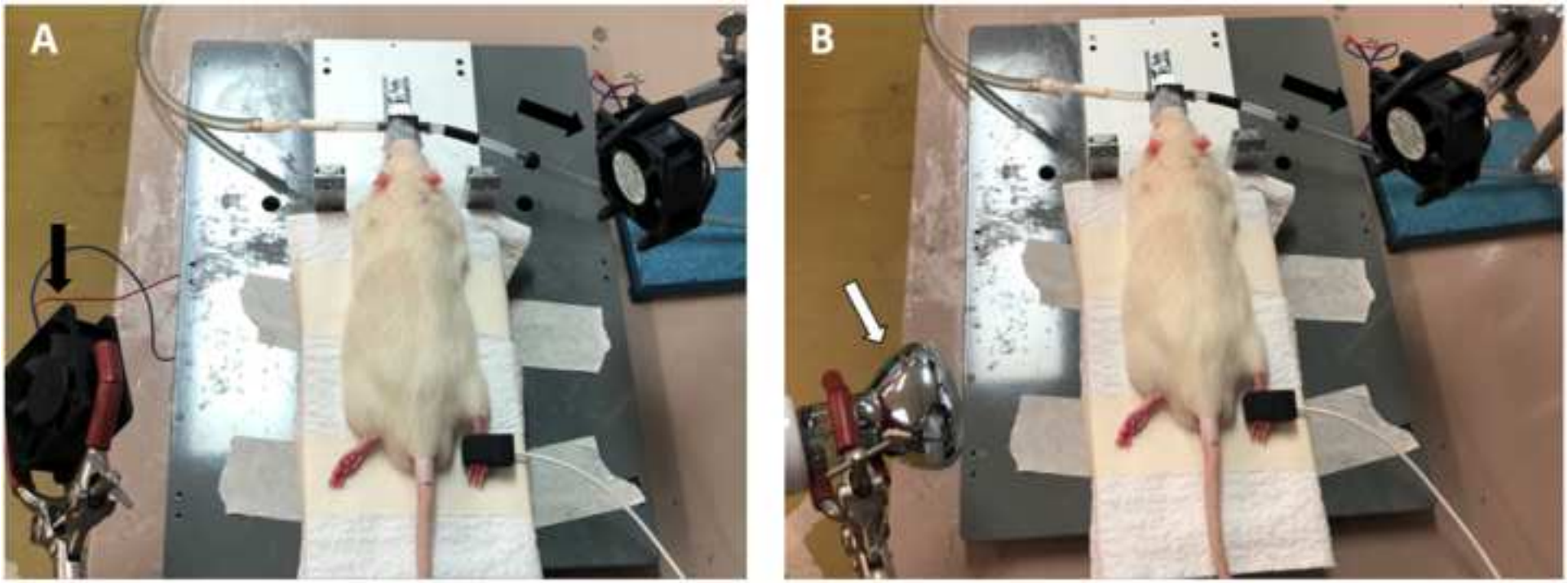

Figure 3: updated representative data

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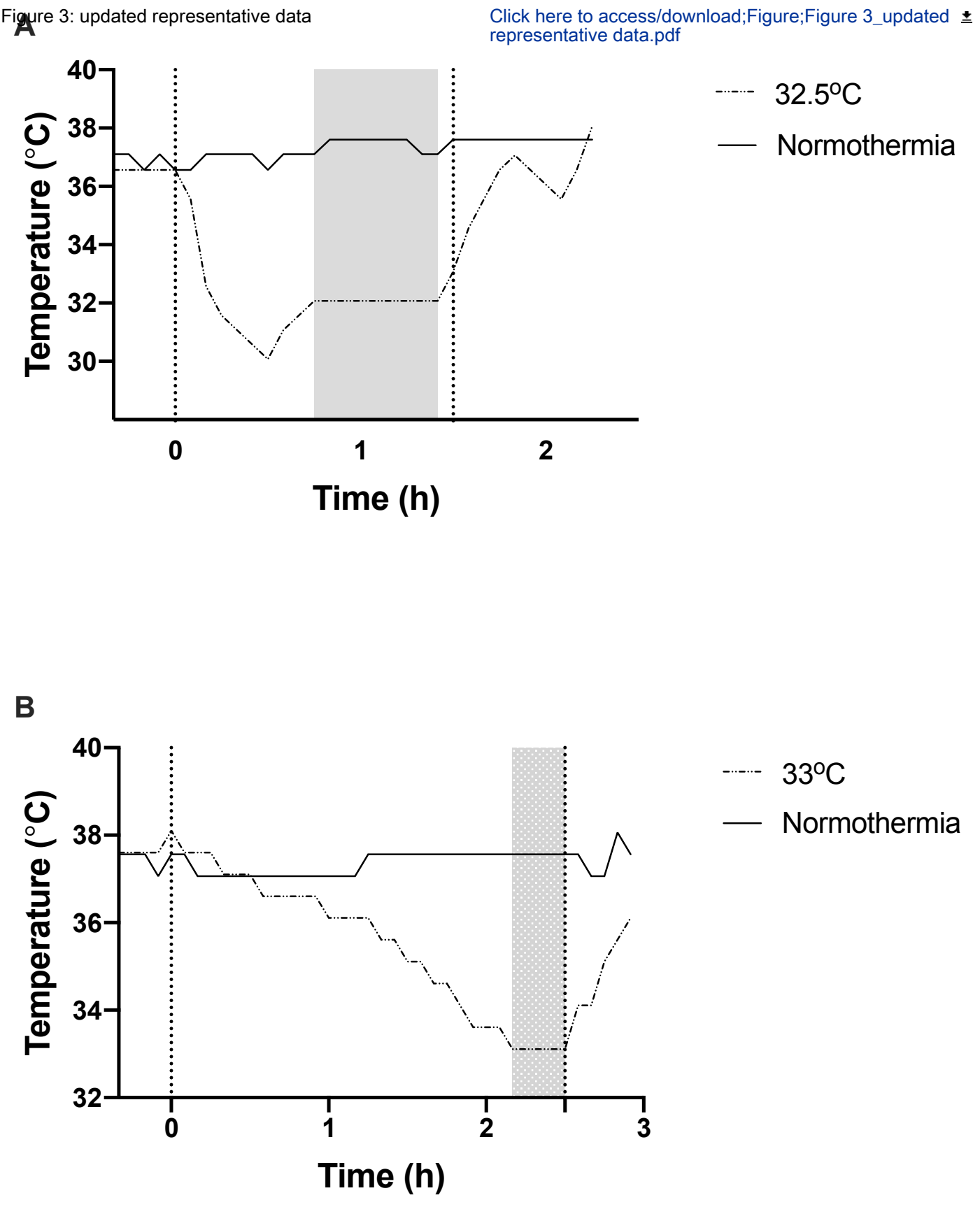

Name	Company	Catalog Number	Comments
Absolute ethanol	ThermoFisher Scientific/ Ajax Finechem	AJA214-20LPL	Diluted with deionized water to give 70 % ethanol
Antiseptic solution (Chlorhexidine)	David Craig	A2957	
Anaesthetic (Marcain)	Aspen	PS13977	
Brushless fan motor	Sirocco	YX2505	2 x 12 V/130 mA
Heat lamp	Reptile One	AC220	240 V 50/60 Hz
Heat pad	FHC, Inc	40-90-2	
Rectal probe	FHC, Inc	40-90-5D-02	
Temperature controller	FHC, Inc	40-90-8D	
Temperature Datalogger	Maxim	DS1922L-F5	

Rebuttal letter (JoVE62325)

Manuscript title: Short-Duration Hypothermia Induction in Rats: Models for Studies Examining Clinical Relevance and Mechanisms

We would like to thank the journal editor and reviewers for taking the time to review our manuscript and for providing such helpful and constructive comments. We have carefully taken the comments into consideration when preparing the revision and we hope that the revised manuscript is now suitable for publication in your journal. Responses to comments made by the editor and reviewers are reported below.

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a. The manuscript has been proofread for spelling and grammar.

2. Please provide complete addresses of the affiliations.

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3. Please provide an institutional email address for each author.

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4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

a. The use of personal pronouns has been removed from the text. However, we are unclear on the reason for this? It is our understanding that most style guides now recommend limited use of personal pronouns where appropriate, in order to avoid the ambiguity in meaning that can occur from the use of the passive voice/ dangling modifiers?

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7. Please mention if there is any age or sex specific bias for the animals used.

a. Further details of animals used has been provided.

8. Line 122: Please specify the details regarding the speed of the fan. Is there any specific setting used?
a. Details regarding the speed of the fan has been provided.

9. Please include a one line space between each protocol step and highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.
a. One-line space added to step 2 and 3 which were missing the space. Steps for inclusion in the protocol section of the video have been highlighted in yellow.

10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not use the &-sign or the word "and" when listing authors. Please do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations
a. We apologise for these errors. We used the JoVE plugin available for endnote but it must not have been up to date. The correct format has been applied.

11. Figure 2: Please label the equipments/ instruments in the figure.
a. Figure 2 has been retaken to address review comments and labels have been added.

12. Figure 3: Please use standard abbreviated forms for units included in the parenthesis (replace "hours" to "h" in the X-axis). Please define the dotted perpendicular lines in the Figure Legend.
a. Standard abbreviation has been applied to Figure 3.
b. The dotted perpendicular lines have been defined in the Figure Legend of Figure 3.

Reviewer's comments:
Manuscript Summary:
This is a very simple and well described methodology to induce hypothermia in rats, which would be useful for other laboratories wishing to establish the procedure.

Reviewer #1
Minor Concerns:
Does the data logging device provide a real time read-out of body temperature. If so, can't this alone be used to monitor and control animal body temperature.

Why are both the data logger and rectal probe required for animal body temperature monitoring. May be this could be explained.

a. The datalogger device that we have in our lab is not capable of providing real time read-out of temperature. We have clarified this by adding the following to the manuscript at *line 107-116*:

"NOTE: The datalogger device used in this protocol was not capable of providing real time read-out of body temperature. Read out is possible once the datalogger has been removed

from the animal and connected back to the computer. As a result, the rectal temperature probe is used to provide real time information during the cooling and rewarming process. Additionally, the rectal probe is also vital to this method because the surgical heat mat on which the animal is placed during the procedure is regulated by the rectal probe system. The datalogger also serves a valuable purpose of providing temperature data in freely moving, awake rats and was important for ensuring that normal body temperature was maintained after rewarming. Therefore, both temperature monitoring devices are important for this protocol."

Some further context on how "The gradual cooling model has important potential applications for the use of hypothermia in ischaemic stroke treatment" would be useful. E.g., would patients need to be anethetized or is it trying to replicate the cooling rate achieved with endovascular catheters, etc

a. We thank the reviewer for highlighting the ambiguity of this statement. The intention is to replicate cooling at a rate similar to that of clinical trials of hypothermia for ischaemic stroke. *Line 267-283:*

"The gradual cooling model described above has important potential applications for the use of hypothermia in ischemic stroke treatment. Long duration hypothermia and their resulting complications pose a challenge to elderly stroke patients. Moreover, the shivering response makes it difficult to achieve target temperature in some patients [10]. While anti-shivering medication can help to reduce the shivering response, short-duration gradual cooling could more effectively ameliorate the issue. Having a shorter cooling period is also likely to reduce the incidence of pneumonia often reported in trials. Another potential benefit of this short-duration method is that the speed of rewarming might not be as important when compared to long duration cooling. Very early clinical studies of long duration cooling in stroke patients with large infarcts found that fast rewarming led to large elevations in intracranial pressure (ICP), which worsened outcome and was often fatal. This led to the development of gradual rewarming paradigms, which further extended the overall duration of cooling. Short-duration cooling only maintains target temperature for a short period and may less likely result in rebound ICP. Previous work which has investigated hypothermia treatment for ICP elevation, using a similar rapid cooling and rewarming protocol as the ones described here, have shown no rebound ICP elevation after rewarming[23,24]."

Reviewer #2

Manuscript Summary:

The authors describe two basic methods for inducing hypothermia in rodents. One is the use of a fan and alcohol and the other is anesthetic (isoflurane)-induced cooling. The manuscript summarizes some of the issues in the field (clinical problems; issues with translational relevance in animal models). The figures show the basics of the setup (device implantation, and external cooling). Use of therapeutic hypothermia is commonly done in animal research (stroke, cardiac arrest, TBI, etc.), and these methods are applicable to those fields.

Major Concerns:
1. Both methods are widely known and have been commonly used for decades. Thus, there is absolutely no novelty with these approaches. There are also existing videos and detailed papers that allow one to conduct this type of research. For instance, data sciences int. provides video tutorials to illustrate how to implant devices. Some of that should be cited, especially where greater detail is provided (e.g., a standardized operating procedure for a temperature device implant).

a. We appreciate that there are existing videos and papers outlining methods for hypothermia induction and monitoring. However, there is none that outlines these particular methods, particularly in the case of the gradual cooling protocol- which may have much more clinical relevance for translational research, since it induces cooling at a rate achievable in patients, without the need for skin cooling (less frequently used in patients) which may have additional physiological effects.

2. There are other approaches to short-term cooling that can avoid some of the issues raised in the introduction. For instance, a cooling blanket could be used instead of alcohol spray. It would have been useful to have that comparison done (or at least discussed). Are these methods described here more accurate, easier to implement, more affordable, safer? This should at least be discussed.

a. **Reviewer 2 raises a valid point and it is true that other approaches of inducing hypothermia exist. The methods described in this manuscript are more clinically translatable -in terms of rate of cooling- and less invasive than other short-duration methods. They are also more affordable than the use of a cooling blanket.**

Line 79-83
Line 91 & 92

3. The use of a telemetry device is not needed during short term cooling. A calibrated rectal temperature probe would suffice. Furthermore, the placement of a telemetry device in the S.C. position is not as good as an I.P. placement (with respect to measuring "core" temperature and to avoid confounds such as with a heating pad). Nonetheless, this reviewer appreciates the value of using implanted devices (eg., post-surgical monitoring), which could be further expanded in this protocol report.

a. As the reviewer correctly points out, telemetry devices are useful even in short-term cooling as they provide vital information during the rewarming process. After hypothermia and rewarming, the rats are returned to their cages (half-placed over a heating pad) and are no longer connected to a rectal probe. Inadvertent post-operative hypothermia is very well recognised in animals and in patients, therefore we believe it is vital that we ensure that there are no fluctuations in temperature during rewarming as it could confound experimental outcomes. This is also the reason why post-recovery warming to 37°C is necessary as we want to ensure that we are not causing double hypothermia (or hypothermia to the 'normothermia group').

Protocol 3.4.1:

”For longer-term studies that require animal recovery, animals should be kept in a cage that is placed half over a heat mat to allow the animal to thermoregulate and to avoid inadvertent post-operative hypothermia.”

b. I.P vs S.C comparisons have been performed by our group and we found that I.P placement is more invasive and carries greater risk of adverse impacts on the animal. ***Protocol 1.6.1, 1.8***

4. Core temperature and brain temperature dissociate during anesthesia and with these methods. This has been shown in the late 80s and early 90s. Sometimes these temperature dissociations can be as much as 10 degrees C (e.g., during global ischemia). This issue was not discussed. Do these methods lead to different effects on brain/core temperature relationships during induction, maintenance or rewarming from TH?

a. The reviewer is correct that the brain tends to have lower temperatures relative to the core under general anaesthesia. We have previously conducted skin cooling studies in rats where we measured brain temperature, skin temperature and rectal temperature. We found baseline brain temperature to be between 1-1.5°C lower than rectal temperature. When skin cooling was initiated, we found that, although these temperatures were not identical, they tracked together closely during induction, maintenance and rewarming. Focal brain cooling may lead to different effects on brain/core temperature relationships. However, given the significantly greater challenges of achieving it in bigger-brained humans, this may not be best studied in rodent models and is beyond the scope of our study.

5. Rates of cooling and rewarming have to be considered, and while both can be manipulated with these approaches, the authors still have to account for those issues and whether the models/methods shown here can actually model what would be done in patients (who would not be rapidly rewarmed from systemic cooling). Is a few hours of cooling under anesthetic clinically relevant when current clinical protocols are 12 hr or more at target temperature + time to cool + time to rewarm? There is some arguments for brief cooling, including the fact that some local cooling methods could last ~30 min to a few hours in patients (e.g., intra-arterial cold saline, nasopharyngeal cooling). However, these methods described here are for systemic cooling. On this point, there is also an easy way to induce local cooling in rats (not discussed) - simply wrapping the head with a water cooling blanket while maintaining body temperature.

a. We agree, and indeed the entire point of the protocol is to address this. Pneumonia and immunosuppression are major concerns in long duration hypothermia. Surface cooling methods (including localised ones) can induce shivering which was one of the contributing factors to unsuccessful outcome of the EUROHYP-1 trial.
Line 57-59, line 270-274
b. As a result of the challenges faced by current clinical models, an alternate paradigm is required. This short-duration, gradual cooling reflects the cooling rates that are achievable in humans (but with a shorter duration of cooling and shorter duration at target temperature- as has been proposed for future clinical trials) and demonstrates that a therapeutic outcome can still be achieved.

6. The animal literature on temperature parameters is not adequately discussed (e.g., studies comparing various depths and durations of cooling). They argue for the "adaptation of short-duration hypothermia for use in clinical trials." Many animal studies have questioned the utility of short duration cooling, and these should be discussed to give a broader context thereby allowing the reader to better select a protocol to study. For instance, studies in the early 90s showed that brief postischemic cooling provided only transient benefit - longer cooling was needed to ensure enduring benefit and that depended upon other factors (e.g., insult severity and intervention delay). The complexity of these issues seems to be glossed over in this introduction (e.g., need for intervention delays, confounds with anesthetics and so on).

a. There are many controversies in the interpretation of the large hypothermia literature. However, we did not feel a methods paper was the appropriate place in which to attempt to summarise these. For example, although there are some studies questioning the utility of short-duration cooling, in fact the largest meta-analysis of the experimental literature revealed a very much greater weight of evidence for the benefits of short duration cooling (van der Worp 2007). As the reviewer alludes to, methodological issues with sustained hypothermia in animals (and need for prolonged anaesthetic) add further complexity to the interpretation of the studies to which the reviewer refers, and there are many other issues such as comorbidities etc. which would take many changes to do justice to.
We have provided a number of reviews (Kurisu and Yenari 2018, Dumitrascu et al., 2016, Hemmen and Lyden, 2009 and Wu et al., 2020) in the introduction (*line 76*) which provide in-depth information on temperature parameters in the context of animal studies and potential human applications. This should provide the readers with the broader context necessary to determine the best protocol for their needs. We have also added further evidence (*line 283)* for the use of short-duration hypothermia by citing various studies from our group, which have utilised the paradigm and have shown robust benefit after stroke.

Minor Concerns:
1. The figures of the animal setup look messy and dirty. I would not recommend showing them. There is too much extraneous materials in the field of view (e.g., screws). Sterility would be compromised by the fans and lack of draping (e.g., if ongoing procedures were needed). Would the fans contribute to corneal drying? Would alcohol get in the rat's eyes? Is there a risk of having ethanol vapors being generated near electronics (fire hazard)? Why not use water instead? It will also cause evaporative cooling but with less risk. Alternatively, a cooling blanket would work (e.g., water flow through blankets are commercially available and commonly used for rodents).

a. The animal set up figure has been updated to account for the clutter present in the original figure.
b. Both the fan and ethanol spray application are positioned so only the lower back of the rat is cooled and alcohol does not reach anywhere near the rat's eyes or face. *Protocol 2.2:*

“Attach a 60 mm 12 v/130 mA fan to each retort stand ensuring that the fans are aimed towards the lower back of the rat. The distance between the clamp and the rat is approximately 20 cm.”
c. Corneal drying can occur during the intraoperative period. For this reason, eye ointment is applied at the start of our procedures.
d. The ethanol is sprayed directly onto the back of the rat and the sprays are applied very sparingly so the risk posed by ethanol vapours is minimal. *Protocol 2.5.1*:
“Ethanol is used as a preferred solution over water because it has a faster rate of evaporation and therefore results in more rapid hypothermia induction**.”**
e. It is true that cooling blankets have been commonly used and are also very effective. However, the ethanol evaporation method used by our group has provided very reliable decreases in body temperature within a desired time frame.

2. A servo controlled system would provide greater precision of control and also would allow for recording cooling input (e.g., how long a cooling blanket was used or how much heat had to be applied).

a. The main part of the protocol (gradual cooling) does not require any active cooling at all and would therefore not benefit from the use of a servo controlled system.
b. For ethanol evaporation (rapid cooling), we believe that part of the appeal of this paper for many readers will be that we have shown that very precise control can be achieved with low-cost methods, and that precisely controlled delayed cooling can be achieved without the need of any cooling input at all.

3. Was an antiseptic solution applied before incisions were made? Was the surgical procedures done aseptically (autoclaved instruments, etc.)?

a. Antiseptic solution was applied prior to incisions being made. Surgical tools were also sterilised prior to surgery.
Protocol 1.3 and protocol 1.4

4. The abstract claims that "TH has not shown efficacy in clinical trials". However, there are several clinical trials that did show efficacy in cardiac arrest in adults and after hypoxic-ischemic injury in neonates.

a. Thank you for pointing this out. The successful clinical trials relating to cardiac arrest and hypoxic ischaemic injury were mentioned in the introduction but the claim made in the abstract should have specified that the majority of trials have not been successful- with a particular focus on hypothermia to treat stroke (or traumatic brain injury).
Line 26-28:

“Despite strong pre-clinical evidence, TH has not shown efficacy in clinical trials of most neurological disorders. The only successful trials employing therapeutic hypothermia were related to cardiac arrest in adults and hypoxic ischemic injury in neonates.”

5. The validity of these methods (vs. others) with respect to cooling methods in humans has not been adequately covered (briefly in the intro). There are numerous reviews on this topic that could be cited as the topic is perhaps too complex for this brief protocol.

a. It is true the topic is very complex and we are therefore unable to cover everything in this methods protocol. Reviews covering this have been cited. ***Line 76.***