bio-protocol

Published: Jul 05, 2022

Experimental Models for Cold Exposure of Muscle *in vitro* and *in vivo*

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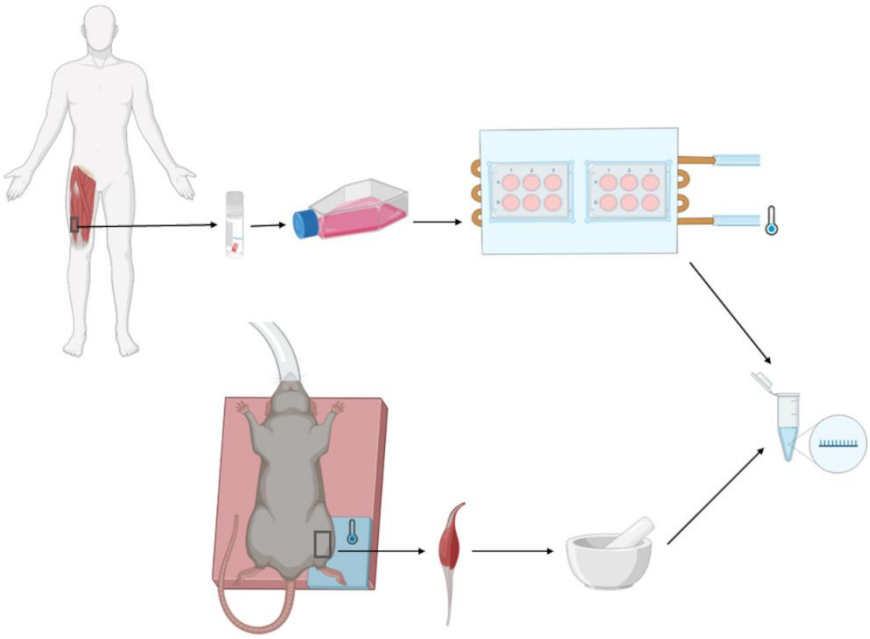
Abstract

Work in cold environments may have a significant impact on occupational health. In these and similar situations, cold exposure localized to the extremities may reduce the temperature of underlying tissues. To investigate the molecular effects of cold exposure in muscle, and since adequate methods were missing, we established two experimental cold exposure models: 1) *In vitro* exposure to cold (18°C) or control temperature (37°C) of cultured human skeletal muscle cells (myotubes); and 2) unilateral cold exposure of hind limb skeletal muscle in anesthetized rats (intramuscular temperature 18°C), with contralateral control (37°C). This methodology enables studies of muscle responses to local cold exposures at the level of gene expression, but also other molecular outcomes.

Keywords: Cold exposure, Skeletal muscle, Myotubes, *In vitro,* mRNA, Protein expression

This protocol was validated in: J Therm Biol (2021), DOI: 10.1016/j.jtherbio.2021.102930

Graphical abstract:



Background

Occupational cold exposure is reported by 14% in surveys of Norwegian employees/workers (SSB LKU-A, 2019). Technically, cold working conditions are defined by ambient temperatures lower than +10°C (ISO15743:2008, 2008). The impact of a cold working environment at the level of workers' tissues and cells is largely influenced by physical properties like air movement and humidity, but also behavioral factors like clothing and posture (Makinen and Hassi, 2009).

Exposure to cold temperatures has been associated with chronic pain in some studies (Vale *et al*., 2017; Farbu *et al*., 2019), while shorter durations of cold exposure may alleviate pain and inflammation (Bouzigon *et al*., 2016). Cold exposure localized to the extremities may reduce the temperature of underlying tissues (Saltin *et al*., 1968). The molecular changes and mechanisms occurring in muscle under cold conditions are currently unknown. To our knowledge, methods for cold exposure of cultured muscle cells and local cold exposure of muscles in anesthetized rats have not been established earlier, although it has been documented that cold exposure of short duration affects muscle gene expression (Egecioglu *et al*., 2018; Jaworska *et al*., 2018).

The current protocol may be used to elucidate the molecular effects of cold exposure in the short term. For example, gene expression in muscle, and muscle cell metabolism and secretion. The method could also be extended to study longer-lasting cold exposures, as well as other tissues and cell models.

Materials and Reagents

In vitro materials and reagents

1. Corning® CELLBIND® 6-well Plate (Corning Inc., catalog number: 734-1210)
2. Dulbecco's Phosphate Buffered Saline (DPBS wo/Ca^{2+} and Mg^{2+}) (Gibco®, Invitrogen, Gibco Life Technologies, catalog number: 14190169)
3. DMEM, low glucose, GlutaMAX (Gibco®, catalog number: 21885025)
4. Insulin, Actrapid penfil 100 IU (Novo Nordisk A/S, catalog number: 014398)

5. Fungizone® (250 µg/mL amphotericin B) (Gibco®, Invitrogen, Gibco Life Technologies, catalog number: 15290026)
6. Penicillin/Streptomycin (1,0000 U/mL, 10 mg/mL) (Gibco®, Invitrogen, Gibco Life Technologies, catalog number: 15140122)
7. Fetal bovine serum (FBS) (Gibco®, Invitrogen, Gibco Life Technologies, catalog number: 10500064)
8. HBSS wo/Ca^{2+} and Mg^{2+} (Hyclone™, GE Healthcare, catalog number: SH30588.01)
9. Trypanblue 0.4 % solution (Sigma-Aldrich™, catalog number: T10282)
10. Bovine serum albumin (BSA) (Sigma-Aldrich™, catalog number: A8412)
11. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich™, catalog number: D2650)
12. Biopsies, *musculus vastus lateralis*, from four male donors (age 24–29)
13. Proliferation medium (see Recipes)
14. Differentiation medium (see Recipes)

In vivo materials and reagents

1. Female Lewis rats, 20 weeks old (150–200 g) (LEW/OrlRj, Janvier Labs, France)
2. Isoflurane (ESDG9623C, Baxter International Inc., US)
3. Ethanol (Kemetyl AS, catalog number: 600068)
4. Heparin 5000 IE/mL (LEO Pharma AS, catalog number: 009165-07)
5. Micropore™ Surgical tape (3M Health Care, catalog number: 1535-1)
6. RNA-Solv® Reagent (Omega Bio-Tek, catalog number: R6830)

Equipment

In vitro equipment

1. Countess™ automated cell counter, counting chamber slides (Gibco®, Invitrogen™, Gibco Life Technologies, catalog number: C10227/C10228)
2. Water bath (Hetofrig, catalog number: CB11)
3. CO_2 Water-Jacketed incubator (Nuaire, model: NU-4500)
4. Microscope and cellSens Entry Software (Olympus, model: CKX41)
5. Cell scraper 24 cm (Sarstedt Inc., catalog number: 83.3951)
6. Thermal block: Aluminum block strung with perfusion tubes of copper, HI-CONTACT™ 6-PASS COLD PLATE (part No 416101u00000g, Aavid Thermoalloy SRL, Italy)
7. Stainless steel insulated flexible thermocouple temperature probe (Type-K 228-7445; RS Components AS, Norway)
8. USB TC-08 data logger (Pico Technology, catalog number: PP222) connected to a portable PC using the PicoLog software
9. Swip Shaker (Edmund Bühler GmbH, model: SM25-B)

In vivo equipment

1. Miniplus® 3 Peristaltic Pump (Gilson Inc., catalog number: GFAM00051)
2. Homeothermic blanket control unit (Harvard Apparatus Ltd., catalog number: 50-7137)
3. Rat Blanket Only, 15 × 20 cm (Harvard Apparatus Ltd., catalog number: 50-7214)
4. Harvard Apparatus Vaporizer (Baxter International Inc., catalog number: 34-0387)
5. Cooling plate EchoTherm (Torrey Pines, catalog number: IC25XR)
6. Surgical tools:
   Spring scissors (Fine Science Tools, catalog number: 15000-08)
   Fine scissors (Fine Science Tools, catalog number: 14058.09)
   Forceps (Fine Science Tools, catalog number: 11252-00)
   Forceps (Fine Science Tools, catalog number: 11006-12)
7. Haldenwanger™ Porcelain Spouted Mortar (Fisher Scientific, catalog number: 12373328)

Analysis equipment

1. 2100 BioAnalyzer (Agilent Technologies, model: G2938C)
2. StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, model: 4376592)
3. Mastercycler® nexus (Eppendorf® AG, model: 6333)
4. NanoDrop™ 8000 Spectrophotometer (Thermo Scientific™, model: ND-8000-GL)

Software

1. PicoLog software (PicoLog 6.2.4, Pico Technology, UK)

Procedure

A. Culturing of human skeletal muscle cells

1. Harvesting of biopsies of *m. vastus lateralis* from healthy volunteers, satellite cell isolation, and passaging have been described earlier (Lund *et al*., 2017).
2. Store isolated satellite cells from biopsies in cryo vials at -196°C (1×10^6–2×10^6 cells/vial).
3. Seed myoblasts in 6-well Corning® CELLBIND® plates (passages 3 and 4). Use 2.0 mL of proliferation medium per well and a cell concentration of 50,000 cells per mL, Incubate at 37 °C and 5% CO_2.
4. At 80–90% confluency (after one week), replace medium with 2.0 mL differentiation medium to facilitate myotube formation. Incubate for 6–7 days.

B. Incubator setup

1. Set up a parallel incubator system with both cooling (18°C) and regular (37°C) conditions in the same incubator. An overview is shown in Figure 1.

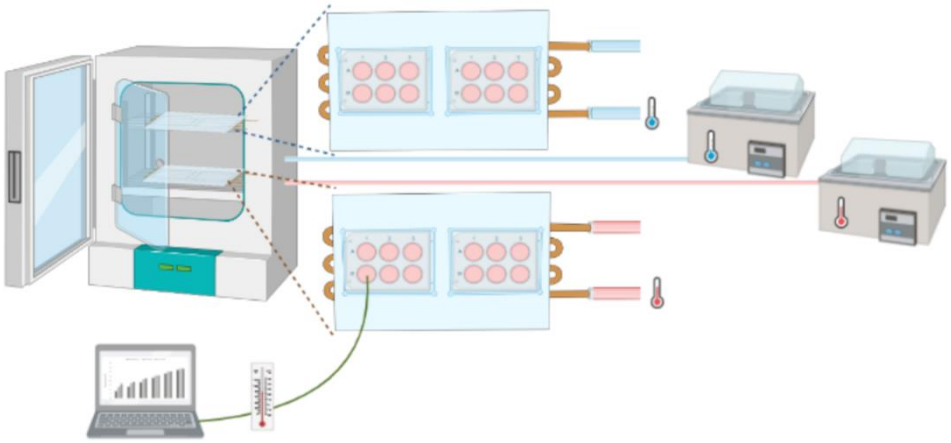


Figure 1. Setup of *in vitro* exposure of myotubes in CO_2 incubator with parallel exposure of cells to either warm or cold temperature.
Thermal block (highlighted with dashed lines, either blue indicating cold exposure or red indicating control conditions of 37°C) temperatures are controlled via connected water circulators (Control = red tubing; cold = blue tubing). Culturing conditions (the actual exposure temperature) are monitored with a thermocouple probe and PicoLog software. Styrofoam insulation is not shown but should be mounted around both tubing and thermos blocks. Figure created in BioRender (biorender.com).

a. Use a regular CO_2-incubator with openings for connecting tubes. Set at 37 °C and 5% CO_2.
b. Set up water circulators holding respective temperatures. The temperature of the cold circulator must be considerably lower than the desired exposure temperature, as the returning water heats up through the incubator. An outline of how to establish the correct temperature is described under section B.2, Method validation.
c. Install two temperature regulated blocks in the incubator. To ensure sufficient temperature regulation of the thermo block, an aluminum block strung with perfusion tubes of copper (Figure 2) was mounted on the shelves of the incubator. Allow tight connection between surface of the culture wells and the thermo block by milling grooves into the aluminum thermo block (Figure 2A).

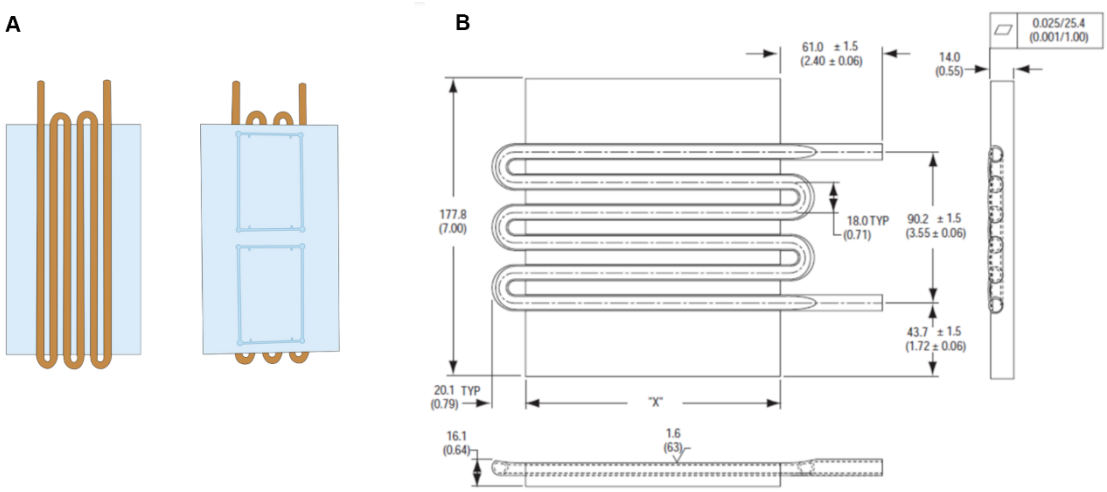


Figure 2. Thermo block.
A) Back and front sides of the aluminum thermo block (light blue), strung with serpentine copper tubes (brown) and with grooves milled into it (dark blue), allowing a tight contact to form between the thermo block and the culture plate wells. B) The copper tubing and its dimensions (mm) in the thermo block.

d. Cool or heat the thermo block by perfusion with either cold or hot water from water circulators (outside the incubator). Connect water circulators with thermo block through silicon tubing.
e. Use styrofoam for insulation of thermo block, culture plates, and tubes.

2. Method validation
   a. Add 2.0 mL of medium to each well in three 6-well culture plates. Place the 6-well plates onto the thermo block.
   b. Perforate the plate lid and place a thermocouple temperature probe immersed in the well medium of each well.
   c. Monitor the temperature in the culture wells.
      Connect thermocouple temperature probe to a USB TC-08 data logger, and monitor temperature data on a portable PC using the PicoLog software. Verify the temperature needed in the circulators for desired conditions.
   d. Measure the culture well temperature every 5th minute for 60 min to establish the required time for temperature stabilization after adjustment of the circulator temperature.
   e. Measure the culture well and thermo block temperature every 30 min (time to reach temperature stabilization in our lab). Set water circulator at 8 °C with temperature increment of 5 °C, resulting in a curve of six measurements.
   f. Adjust the temperature of the water circulator according to the acquired equation, to obtain the desired temperature in the cell medium before exposure of cells.
3. *In vitro* Cold exposure of cells

a. Incubate cells established in section A.7 at 18°C well temperature or 37°C (control) for 18 h.
b. Let cells recover at 37°C for 3 h.
c. Harvest cells by careful aspiration of medium followed by (for the purpose of gene expression analysis) addition of 500 µL Isol-RNA Lysis Reagent to each well. If other products than RNA are desired, use a suitable lysis reagent.
d. Detach cells with a cell scraper and shake the plate for 5 min to ensure complete lysis. Transfer the suspension to appropriate tubes and store at -80°C.

4. Continue with RNA isolation according to the procedure recommendations of the lysis manufacturer, or for other preferred downstream analyses.

C. *In vivo* cold exposure of muscle

1. Anesthesia
   a. Anesthetize the rat by placing it in an anesthetizing box. Close the lid and initiate sedation with Harvard Apparatus Vaporizer with 2.0 L (O_2)/min and 5% Isoflurane flow. Monitor to ensure complete sedation, 2–3 min.
   b. Place the rat on a table, in supine position. Redirect the anesthesia to an inhalation mask and adjust flow to 0.5 L/min and 3% Isoflurane.
   c. Regularly (every 15 min) inspect the absence of withdrawal reflexes to ensure complete sedation during the entire experiment. Reflexes are inspected by pinching the paws with a tweezer.
   d. Insert a rectal probe with feedback to a heating pad (Homeothermic blanket control unit), which maintains the core temperature at 36–37°C.
2. Cold exposure of muscle
   a. Shave the posterior legs of the rat
   b. Place the hind limb on the heating pad, at 37°C. Fixate limbs with surgical tape, with the left hind limb on a cooling plate holding 10°C, as shown in Figure 3. Keep limb on the cooling plate for 1 h. After thermal exposure, reheat the muscle by placing it on the heating pad for an additional 2 h.

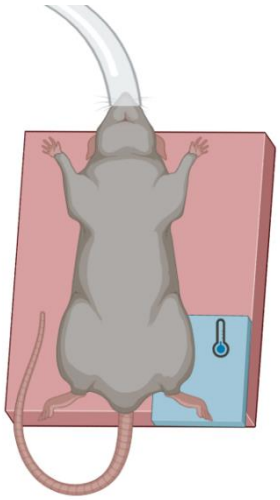


Figure 3. Setup of *in vivo* cold exposure.
Isoflurane anesthetized (mask) rat placed in supine position on a heating pad (red), with left hind limb fixed with surgical tape to a cooling plate (blue); the right hind limb serves as a contralateral control. Rectal probe and vaporizer are not shown. Figure created in BioRender (biorender.com).

3. Perform transcardial perfusion by a surgical thoracotomy.
   a. Spray the chest and abdomen with 70% ethanol.
   b. With a surgical scissor and tweezer, make a 5–7 cm transverse incision through the skin and abdominal

wall, at the abdominal center.

c. Make a horizontal incision through the diaphragm to fully identify the heart.
d. Inject 0.1 mL (50 mg) of heparin into the left ventricle.
e. Use a scissor to cut the rib cage laterally on both sides, starting from the lower ribs and up toward the clavicle. Use an artery clamp on the *xiphoid processus* and tilt the anterior rib cage surface up away from the heart.
f. Perform a small incision in the pericardium to uncover the heart muscle
g. Make a small incision on the apex, approximately 2–3 mm, into the left ventricle. Insert a perfusion needle through the left ventricle, 5–10 mm into the aorta. Fix the needle position with a clamp.
h. Make an incision in the right atrium to secure drainage and initiate the perfusion. Use Hank´s balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. Perform perfusion with Gilson Miniplus® 3 Pump at 20 mL/min. Terminate prefusion when the liver changes color from red to light brown.

4. Harvest *m. gastrocnemius* and snap-freeze in $N_2(\ell)$. Store at -80°C for further analysis.
5. Homogenize tissue with a mortar and pestle in $N_2(l)$.
6. Continue, for example, with RNA or protein isolation.

Data analysis

The *in vitro* temperatures in respective parts of the incubator were validated by assessing the time to reach a stable temperature, compliance between culture wells and thermo block, and the relation between culture well, thermo block, and water circulator temperature. In our specific setup, a stable temperature was observed after 30 min. Thereafter, the relation between thermo block and culture wells was examined at six different temperatures of the water circulators (Figure 4).

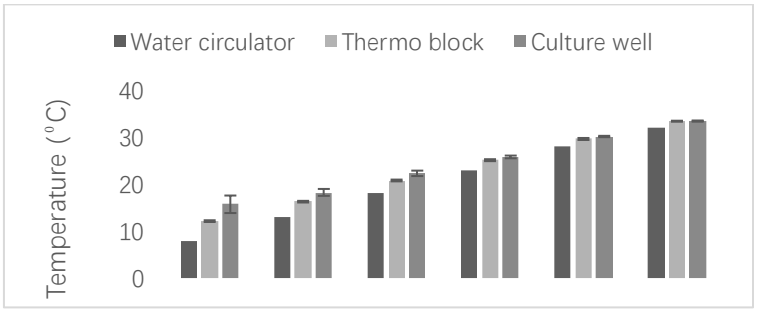


Figure 4. Temperature in thermo block and culture wells after 30 min at six different water circulator temperatures.
Mean + SEM values are shown for 18 culture wells in three 6-well plates (N = 18).

In our specific setup, the relation between the temperature in the water circulator and culture well (Figure 5) gave the following equation: $y = 0.75x + 9.01$
The equation was used to determine the water circulator temperature to obtain a culture well temperature of 18°C.

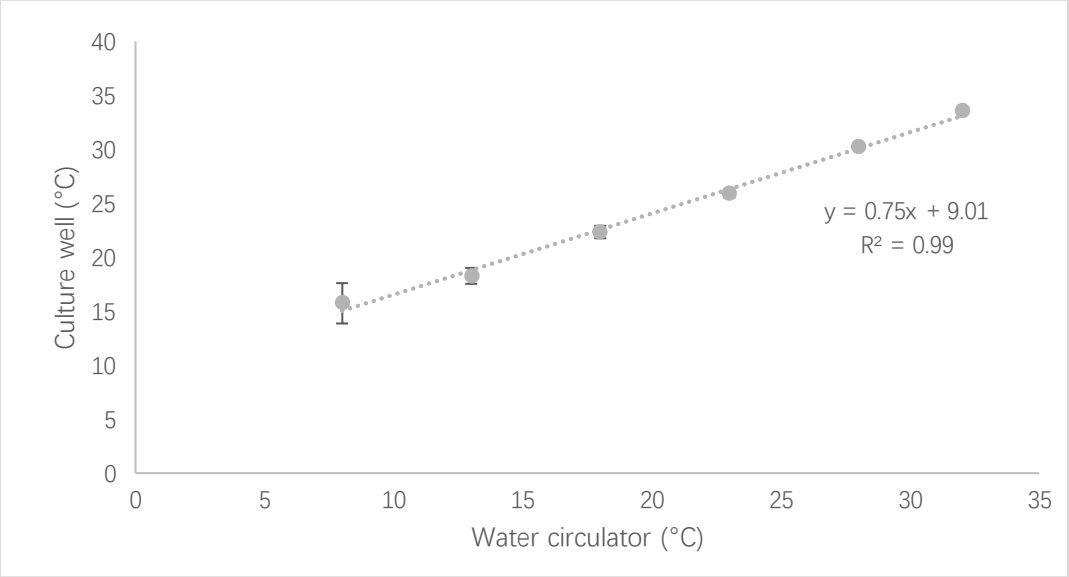


Figure 5. Linear relation ($R^2 = 0.99$) between culture well and water circulator temperature. Mean + SEM values are shown for 18 culture wells in three 6-well plates (N = 18).

Our previous results (Krapf *et al*., 2021) indicate that these novel methods can be used to study cold exposure in skeletal muscle. To this end, we have shown that myokine production is regulated by intramuscular temperature in vivo and in vitro. Down-stream analyses included gene expression at the mRNA level as well as protein secretion into the culture media. Furthermore, we have studied effects of direct cold exposure and rewarming on glucose and fatty acid metabolism in cultured human myotubes (manuscript in review). This was achieved using labeling with isotope tracers and measuring CO_2 production. The current protocol may be utilized to gather new insight into the biological impact of direct cold exposure and temperature changes to tissues and cells.

Recipes

1. Proliferation medium

Reagent	Amount
Dulbecco's modified Eagle's medium (DMEM) w/Glutamax (w/1.0 g glucose)	500 mL
FBS	50 mL
Penicillin/Streptomycin, 10,000 units/mL	2.5 mL
Fungizone (250 µg/mL amphotericin B)	2.5 mL
Total	555 mL

2. Differentiation medium

Reagent	Amount
Dulbecco's modified Eagle's medium (DMEM) w/Glutamax (w/1.0 g glucose)	500 mL

FBS	10 mL
Penicillin/Streptomycin, 10,000 units/mL	2.5 mL
Fungizone (250 µg/mL Amphotericin B)	2.5 mL
Insulin (25 pmol/L)	21.5 µL
Total	536.5 mL

Acknowledgments

This protocol was derived from our previously published paper (Krapf *et al.*, 2021).

Competing interests

The authors have no financial and non-financial competing interests.

Ethics

The animal studies were executed in accordance with regulations and approved by the Norwegian Food Safety Authority (FOTS, ID9483). Human muscle biopsies were obtained with informed consent from all participants, and all research was performed in accordance with all relevant guidelines and regulations with approval from the National Committee for Research Ethics, Norway (ref. no. 2011/2007 REK sør-øst B).

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