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Letter to the Editor

Cold shock therapy promotes hair growth in association with upregulation of cold-inducible RNA-binding protein and vascular endothelial growth factor

Cold shock, characterized by a sudden temperature drop, reportedly induces various physiological cellular responses [1]. In dermatology, cryotherapy applies extremely low temperatures, typically using liquid nitrogen, to rapidly freeze and subsequently thaw skin tissue [2], selectively destroying abnormal skin cells or inducing localized immune responses.

In the treatment of alopecia areata (AA), cryotherapy promotes the recovery of AA patches and potentially modifies the immunological mechanisms implicated in AA pathogenesis [3,4]. Cryotherapy-induced inflammation may trigger vasodilatation, thereby promoting hair regrowth [5]. Furthermore, cold exposure has been established to activate sympathetic nerves, creating a niche that regulates hair follicle stem cell (HFSC) activity and promotes hair growth [6]. Cooling also stimulates the expression of vascular endothelial growth factor (VEGF) [7], leading to endothelial cell proliferation within the hair follicles (HFs) [8]. Despite extensive research on cryotherapeutic applications for hair growth, the specific temperatures required and the underlying biological mechanisms that mediate cryotherapeutic efficacy in promoting hair growth remain unclear.

In an anagen induction study approved by the Seoul National University Hospital Institutional Animal Care and Use Committee (No. 21–0134-C1A0), we applied controlled cold shock to the dorsal skin of 7-week-old C57BL/6 mice using a cryo-device (TargetCool®, RecensMedical, Inc.), which provided direct control of the temperature at the target tissue surface, set to 5 °C and 0 °C, to induce the anagen phase. The back was divided into four sections with different exposure durations for a comprehensive evaluation of the treatment effects. Each section was carefully handled to prevent unintended exposure to the device. In addition, 2 % minoxidil (MNX) was administered topically as a positive control. Post-treatment skin surface temperature was measured after the application of the cryo-device.

Applications at 5 °C and 0 °C for 60 and 30 s, respectively, significantly induced anagen (Fig. 1A). However, the anagen induction score was significantly higher in the 5 °C-treated group (Supplementary Fig. 1) [9]. In the 5 °C group, the pre-application skin temperature was 36.3 ± 0.1 °C, which was reduced to 34.5 ± 0.7 °C upon the 30-s treatment. In the 60-s-treated section, the temperature was 33.3 ± 0.5 °C. No changes occurred in the MNX group with a maintained temperature of 36.1 ± 0.2 °C. In the 0 °C group, the baseline temperature was 36 ± 0.1 °C. Upon 30- and 60-s treatments, the skin temperatures were 33.5 ± 1.2° C and 30.7 ± 1.7 °C, respectively. Maintaining post-treatment skin surface temperature between 32–34 °C was found to be crucial for anagen induction (Fig. 1B). Skin temperatures below 32 °C, as for the 0° C 60-s treatment, were suboptimal for anagen induction in the mouse skin. (Fig. 1C and D). Subsequently, we immunostained the sectioned skin with the vascular marker CD31. Near-HF vessel formation occurred upon 5 °C 30- and 60-s and 0 °C 30-s treatments as well as in both the 5 °C- and 0 °C-treated MNX sections (Fig. 1E and G). CD31 and VEGF mRNA levels were significantly upregulated in the 5 °C 60-s treatment site. Moreover, cold-inducible RNA binding protein (CIRP) and RNA-binding motif protein 3 (RBM3) mRNAs were upregulated in these sections (Fig. 1F and H), which are known to be activated at cold temperatures, particularly at 32 °C [10].

At 32 °C, *in vitro* analysis using outer root sheath cells (ORSCs) showed no significant differences in cell viability upon incubated at this temperature for 12–48 h (Fig. 2A). For cold shock to the ORSC, we performed incubation for 2 h at 32 °C, repeated six times, expressions of CIRP and RBM3 were significantly increased (Fig. 2B). VEGF was upregulated, correlating with the *in vivo* results (Fig. 2C). Pro-inflammatory cytokines (IL-1β and IL-6) were downregulated after cold shock, and CXCL1, an upregulated chemokine in AA, was also downregulated (Fig. 2D). *Ex vivo* HF organ culture was conducted for 6 days, with follicles incubated at 32 °C for 2 h per day, resulting in significant hair shaft elongation after the cold shock (Fig. 2E). Ki-67-positive cells significantly proliferated after the cold shock (Fig. 2F). Collectively, cold temperature of 32 °C could promote hair growth both *in vitro* and *ex vivo*.

We demonstrated that controlled cold shock at 5 °C significantly induces the anagen phase, increasing VEGF and cold-inducible protein levels *in vivo*. Consistent with recent findings on the role of cold exposure in promoting hair regrowth through sympathetic nerve activity regulating HFSC at 5 °C [6], our study confirmed that a cold shock at this temperature ultimately led to anagen induction. This study highlights the importance of precise temperature control in HF dynamics.

Post-treatment skin surface temperatures between 32–34 °C is a crucial condition during this therapy, as temperatures below 32 °C were found to be suboptimal for anagen induction. Specifically, CIRP and RBM3, activated at 32 °C [10], could be central to the cryotherapeutic mechanism in HFs. Moreover, the increased VEGF expression would likely enhance hair regrowth through improved

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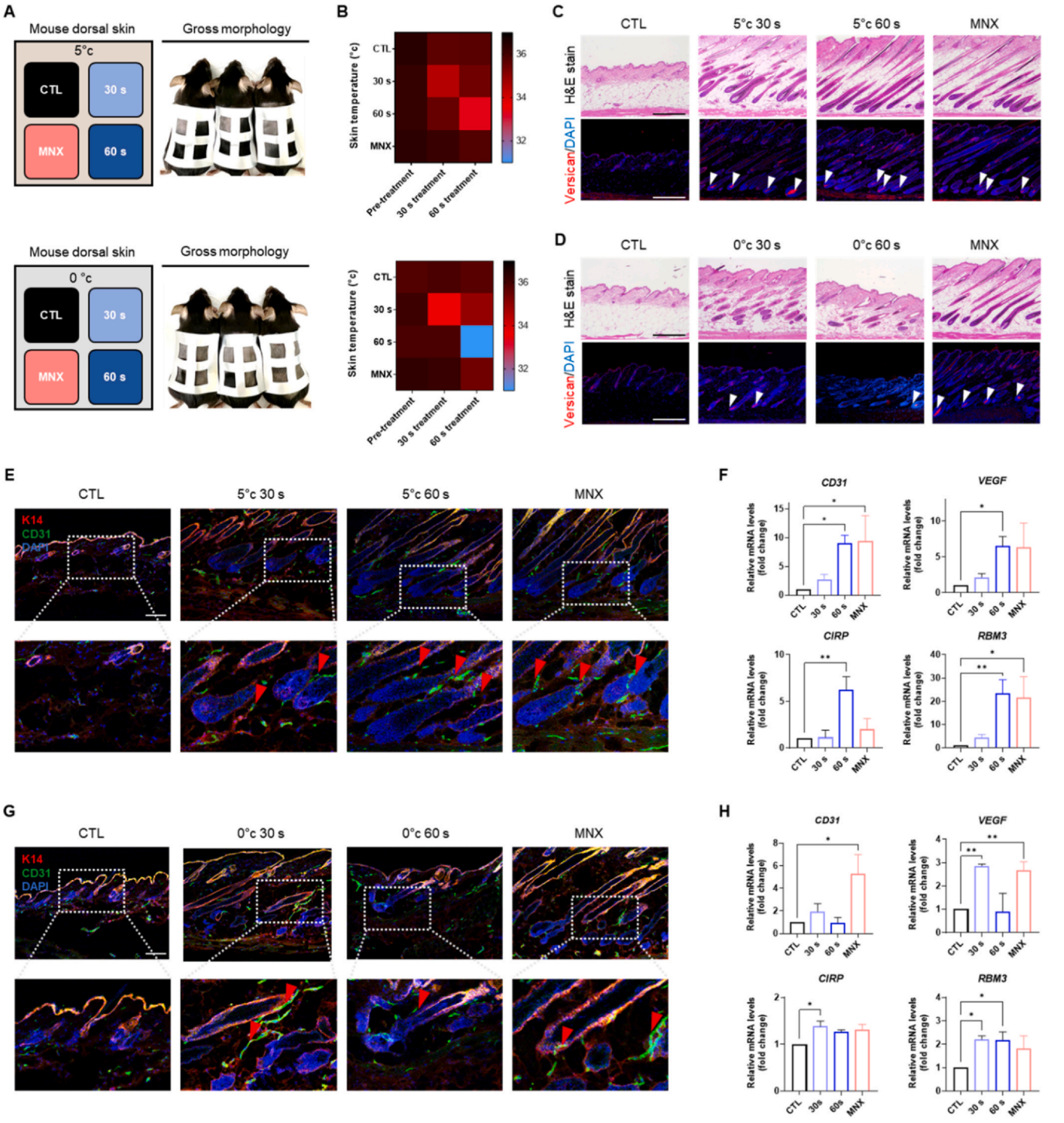

Fig. 1. Cold shock therapy induces anagen phase *in vivo*. (A) Dorsal skin of mice applied to a cold shock therapy at 5 and 0 °C in four distinct groups: control, 30-s exposure, 60-s exposure, and MNX treatment (n = 5–7 for each group). (B) Skin surface temperatures measured for each section pre- and post-treatment. (C and D) Versican staining revealing rapid anagen induction in the 5 °C- and 0 °C 30-s treatment groups. White arrows; versican-stained dermal papilla. Scale bar: 100 μm. (E) CD31 presence both in the 5 °C- and MNX-treatment groups. Red arrows: vasculature. Scale bar: 10 μm. (F) Significantly increased CD31, VEGF, CIRP, and RBM3 mRNA levels in the 5 °C 60-s group (n = 4 tissues/mouse). One-way ANOVA with Dunnett's test compared to the control. *, $p < 0.05$; **, $p < 0.01$. (G) Upon the 0 °C treatment, CD31 was present in the 30-s- and MNX-treated sections. Scale bar: 10 μm. (H) Significantly increased VEGF mRNA levels in the 30-s and MNX groups. CIRP and RBM3 were significantly increased in the 30-s-treated section (n = 4 tissues/mouse); One-way ANOVA with Dunnett's test compared to the control. *, $p < 0.05$; **, $p < 0.01$. All values are represented as the mean ± SEM. CTL, control; MNX, minoxidil; VEGF, vascular endothelial growth factor; CIRP, cold-inducible RNA binding protein; RBM3, RNA-binding motif protein 3.

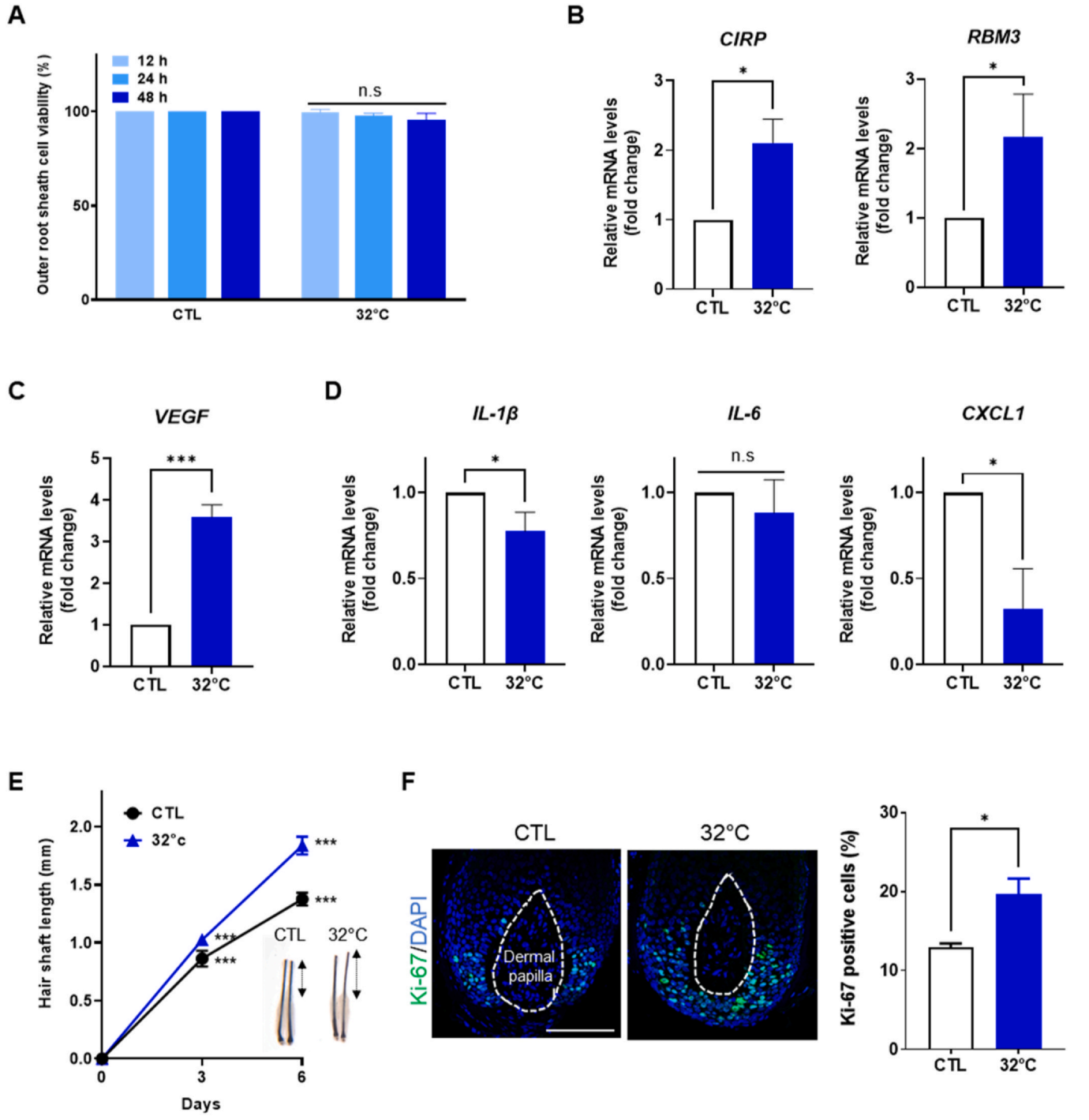

Fig. 2. Effects of cold shock therapy *in vitro* and *ex vivo*. (A) ORSC viability was evaluated using the Cell Counting Kit-8 assay. ORSCs were incubated at 32 °C up to 48 h (n = 3 independent experiments). Two-way ANOVA with Dunnett's test compared to the control. n.s., not significant. (B) After incubating the ORSCs at 32 °C for 2 h, repeated six times, CIRP and RBM3 levels were significantly upregulated. (C) VEGF levels were significantly increased after the cold shock in ORSCs. (D) Pro-inflammatory cytokines were down-regulated with treatment at cold temperatures (n = 3 independent experiments); Unpaired t-test. *, $p < 0.05$; ***, $p < 0.001$; n.s., not significant. (E) *Ex vivo* cultured human HFs were subjected to cold shock at 32 °C for 2 h per day. The hair shafts were elongated after cold shock treatment compared to the control (n = 12 HFs from two independent donors/group). Two-way ANOVA with Dunnett's test compared to the control. ***, $p < 0.001$. (F) Ki-67 indicated cell proliferation with cold therapy. Quantitative analysis of Ki-67 in the matrix region, normalized to DAPI-stained cells, indicating a significant increase after cold temperature treatment (n = 3 HFs/group). Unpaired t-test. *, $p < 0.05$. Scale bar: 1 mm. All data are represented as the mean ± SEM. CTL, control; CIRP, cold-inducible RNA binding protein; RBM3, RNA-binding motif protein 3; VEGF, vascular endothelial growth factor.

vascularization. Conclusively, our findings contribute to future cryotherapeutic treatment development not only for AA but also for other hair loss conditions, underscoring the necessity for further investigating the specific roles of CIRP and RBM3 in HF biology.

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CRediT authorship contribution statement

Seunghee Lee: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Sanseul Kim:** Investigation, Formal analysis. **Sungjoo Tommy Hwang:** Investigation, Resources. **Gun-Ho Kim:** Resources, Funding. **Ohsang Kwon:** Conceptualization, Validation, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing interest

Gun-Ho Kim is an inventor of the precision cooling technology used to develop the study device, which was licensed to RecensMedical

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jdermsci.2024.08.001.

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Seunghee Lee [a,b,c,d], Sanseul Kim [a,b,c,d], Sungjoo Tommy Hwang [e], Gun-Ho Kim [f,g,*], Ohsang Kwon [a,b,c,d,**]

[a] *Department of Dermatology, Seoul National University College of Medicine, Seoul, South Korea*
[b] *Laboratory of Cutaneous Aging and Hair Research, Biomedical Research Institute, Seoul National University Hospital, Seoul, South Korea*
[c] *Institute of Human-Environment Interface Biology, Medical Research Center, Seoul National University, Seoul, South Korea*
[d] *Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, South Korea*
[e] *Dr. Hwang's Hair-Hair Clinic, Seoul, South Korea*
[f] *Department of Biomedical Engineering, Ulsan National Institute of Science and Technology, Ulsan, South Korea*
[g] *Department of Mechanical Engineering, Ulsan National Institute of Science and Technology, Ulsan, South Korea*

*Correspondence to: Department of Biomedical Engineering, Ulsan National Institute of Science and Technology, 50 UNIST-gil, 112dong 801-7ho, Ulsan, 44919, South Korea

**Correspondence to: Department of Dermatology, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 110-744, South Korea

E-mail addresses: gunhokim@unist.ac.kr *(G.-H. Kim),* oskwon@snu.ac.kr *(O. Kwon).*

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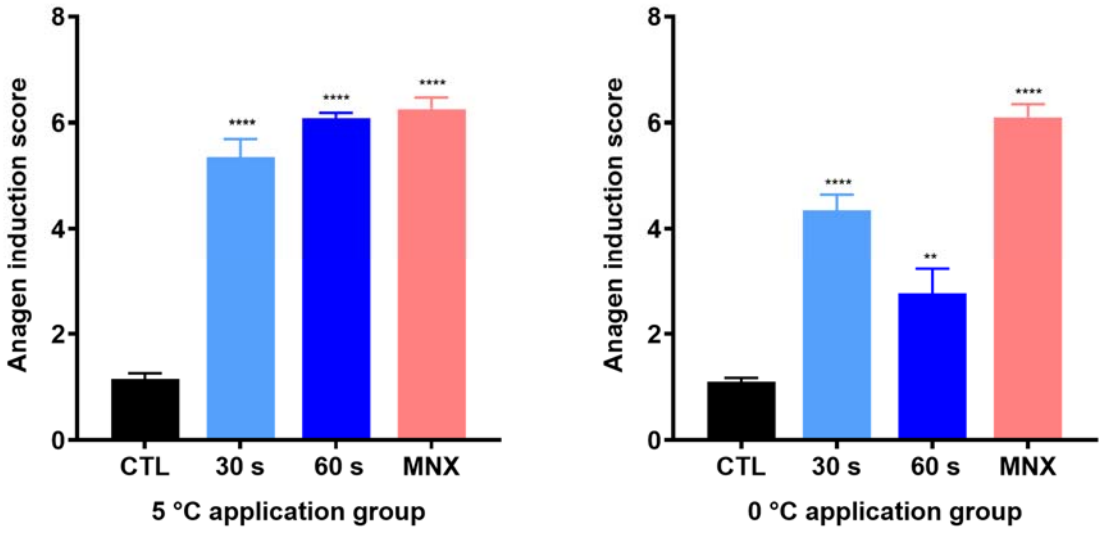

Suppl. Fig. 1. Anagen induction score after cold therapy treatment. (A) Application at 5 °C for 30 s and 60 s significantly induced anagen compared to the control. (B) Application at 0 °C for 30 s also induced anagen significantly. A predefined scoring system was utilized: telogen = 1, anagen I–VI = 2–7, based on a previously reported method; One-way ANOVA with Tukey's test compared to the control group. **, $p < 0.01$; ****, $p < 0.0001$. All values are represented as the mean ± SEM. CTL, control; MNX, minoxidil.