ABSTRACT: Vasospastic episodes in hand–arm vibration syndrome are more prevalent among power-tool workers in cold climates. To test whether cold enhances vibration-induced damage in arteries and nerves, tails of Sprague-Dawley rats were vibrated at room temperature (RT) or with tail cooling (<15°C). Cold vibration resulted in a colder tail than either treatment alone. Vibration at both temperatures reduced arterial lumen size. RT vibration generated more vacuoles in arteries than cold vibration. Vibration and cold induced nitration of tyrosine residues in arteries, suggesting free-radical production. Vibration and cold generated similar percentages of myelinated axons with disrupted myelin. Cold with and without vibration caused intraneural edema and dilation of arterioles and venules with blood stasis, whereas vibration alone did not. The similarities, differences, and interactive effects of cold and vibration on nerve and artery damage indicate that temperature is involved mechanistically in the pathophysiology of hand–arm vibration syndrome.

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EFFECTS OF TEMPERATURE ON VIBRATION-INDUCED DAMAGE IN NERVES AND ARTERIES

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Hand–arm vibration syndrome (HAVS) involves peripheral neuropathy and vasospastic episodes of digits triggered by cold. It afflicts workers after years of exposure to hand-transmitted vibration from power tools. In the late stages of the disease, the symptoms become irreversible, with finger biopsies showing loss of axons.[27] Animal models simulating hand–arm vibration have demonstrated that cumulative exposures of 200–800 h at 60-Hz frequency and 56.9-m/s^2 acceleration induce detachment of myelin from the axolemma at the nodes of Ranvier, axonal loss, accumulation of membrane whorls in the paranodal region, and widening of the Schmidt–Lantermann incisures in nerves of the rat tail.[7] After 5 days of rat hind-limb vibration at 82 Hz, nonmyelinated axons exhibited disorganized neurotubules and neurofilaments and increased smooth endoplasmic reticulum density.[21] These data indicate that neural degeneration begins early.

An imbalance in arterial vasoregulatory factors favoring sympathetic hyperactivity and vasoconstriction is proposed to cause HAVS.[26] In the rat-tail model of hand–arm vibration, a 4-hour period of vibration at 60 Hz and 49-m/s^2 acceleration generated endothelial damage, internal elastic membrane discontinuities, and smooth-muscle-cell vacuoles in the ventral artery, which were prevented by pretreatment with nifedipine, a calcium-channel blocker preventing vasoconstriction.[8,10] Vibration-induced vascular structural damage was similar to that caused by exogenous application of potent vasoconstrictors, norepinephrine and epinephrine.[17,18,29] Vibration-induced vascular degeneration appears to be an early event.

The vascular dysfunction of HAVS has a higher prevalence among workers using similar tools in colder regions than in tropical climates.[12,32] Workers using power tools in warm temperatures reported sensorineural symptoms such as tingling and numbness, whereas those in the colder regions also experienced vasospastic episodes.[12,32] Patients with HAVS show increased sensitization to cold, with low temperatures triggering the finger-blanching component of the syndrome. Cold provocation testing is utilized clinically to evaluate the aberrant vasoregulatory responses of the hands in HAVS. These obser-

Abbreviations: EC, endothelial cells; HAVS, hand–arm vibration syndrome; IEM, internal elastic membrane; MD, myelin disruption; RT, room temperature

Key words: cold injury; free radicals; hand–arm vibration; myelin disruption; vacuoles

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vations indicate that vibration and cold are interactive and contribute to development of the disease.

Many studies have investigated the pathophysiology of HAVS, but few have sufficiently addressed the contribution of temperature in the evolution of the disease. Vibration and cold separately can cause cold sensitization. Yu et al. demonstrated that rabbits exposed to whole-body vibration (60 Hz, 51 m/s^2) in a 4°C environment exhibited slower recovery of depressed skin temperature and blood flow than animals vibrated at room temperature.[33] Serum assays of patients with HAVS demonstrated increased free-radical activity and soluble vascular adhesion molecule-1 levels and decreased interleukin-8 levels.[19,20] These are indications of cellular stress caused by oxidative damage induced by reactive oxygen species. Cold-induced ischemia–reperfusion injury also caused increased free-radical production as measured by increased nitrotyrosine in many disease models.[14,28] It is as yet unknown whether acute vibration produces free radicals.

The present study employed the rat-tail vibration model to investigate interactions of vibration and cold by comparing structural damage to arteries and nerves caused by vibration at room temperature (RT, 25°C) and at cold temperatures (<15°C).

METHODS

Animals and Test Procedures. Male Sprague-Dawley rats (250–300 g) were randomly assigned to RT (n = 10), RT-vibration (n = 11), cold (n = 12), and cold-vibration (n = 12) groups. As performed previously, nonanesthetized rats were restrained in nonvibrated tube cages, and their tails rested on a vibrating platform, which was vertically accelerated by a B&K motor driven by a function generator and voltage amplifier (Simpson Electric, Elgin, Illinois).[8–10] Rats in the vibration-treated groups were exposed continuously for 4 h to horizontal sinusoidal waves of 60 Hz at 49 m/s^2, non–frequency-weighted, acceleration. This frequency is within the range experienced by workers using pneumatic power tools in the automobile industry.[25] Rats in the RT group were handled similarly, but were not subjected to vibration. Tails of rats in the cold-treated groups were wrapped in a layer of gauze and sandwiched between two more layers of gauze on the vibrating platform. The tails were cooled by placing ice next to the tail on the aluminum platform, avoiding direct contact of ice with the skin. Melted ice-water soaked into the gauze and flowed into a collecting tray by capillary action. Ice was replenished as needed to maintain cooling for 4 h. Thermocouples were attached to the lateral side of the C-6 tail segment, with the sensors in contact with the skin to record skin temperatures continuously throughout the experiment. Animal treatment, surgery, and husbandry procedures were approved by our institutional review board and complied with the Laboratory Animal Welfare Act.

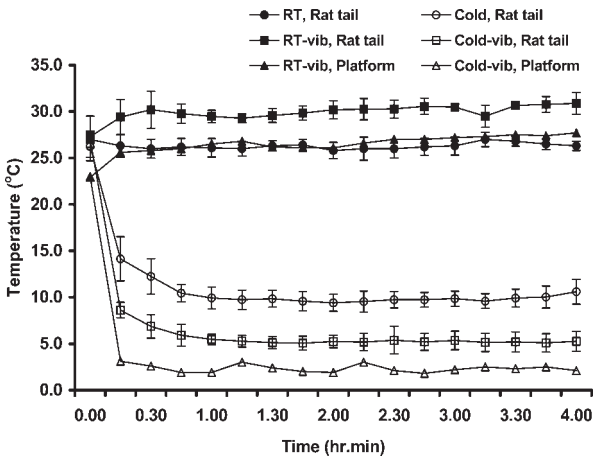

FIGURE 1. Temperature (°C) of the proximal tail skin during the 4-hour vibration period for the RT, RT-vibration, cold, and cold-vibration groups. Temperature remained constant for RT, increased slightly for RT-vibration, fell to a constant low level for the cold group, and decreased even lower for the cold-vibration group. The temperature of the platform increased with RT-vibration and decreased with cooling. The error bars are mean ± SD.

Tissue Processing and Analysis. Tail segments C-5, C-6, and C-8 were immersion-fixed in 4% paraformaldehyde, cryoprotected in buffered, graded sucrose solutions, and frozen in liquid nitrogen for light microscopy. Segment C-7 was immersion-fixed in 2% glutaraldehyde and 4% paraformaldehyde, postfixed in 1.3% osmium tetroxide, dehydrated, and embedded in epoxy resin for ultrastructure analysis. Lumen size of arteries was determined by the ratio of the lumen circumference to internal elastic membrane length as measured utilizing version 1.28v Image J software (National Institutes of Health, Bethesda, Maryland) in toluidine blue–stained semithin cross-sections (0.5 μm). The total number of vacuoles, 2–12 μm in size, was counted for each artery cross-section. Axons with disrupted myelin were identified in semithin cross-sections (0.5 μm) of nerves. Disrupted myelin exhibited darker toluidine blue staining, increased thickness, and distorted shape. The total number of myelinated axons per semithin cross-section and the percentage of axons with disrupted myelin were determined. The cross-sectional areas of the arterioles in nerves were also determined using Image J software. Intraneural edema was defined as an increase above normal in the area between myelinated axons in 40× images of semithin cross-sections of nerves. Area was defined by employing

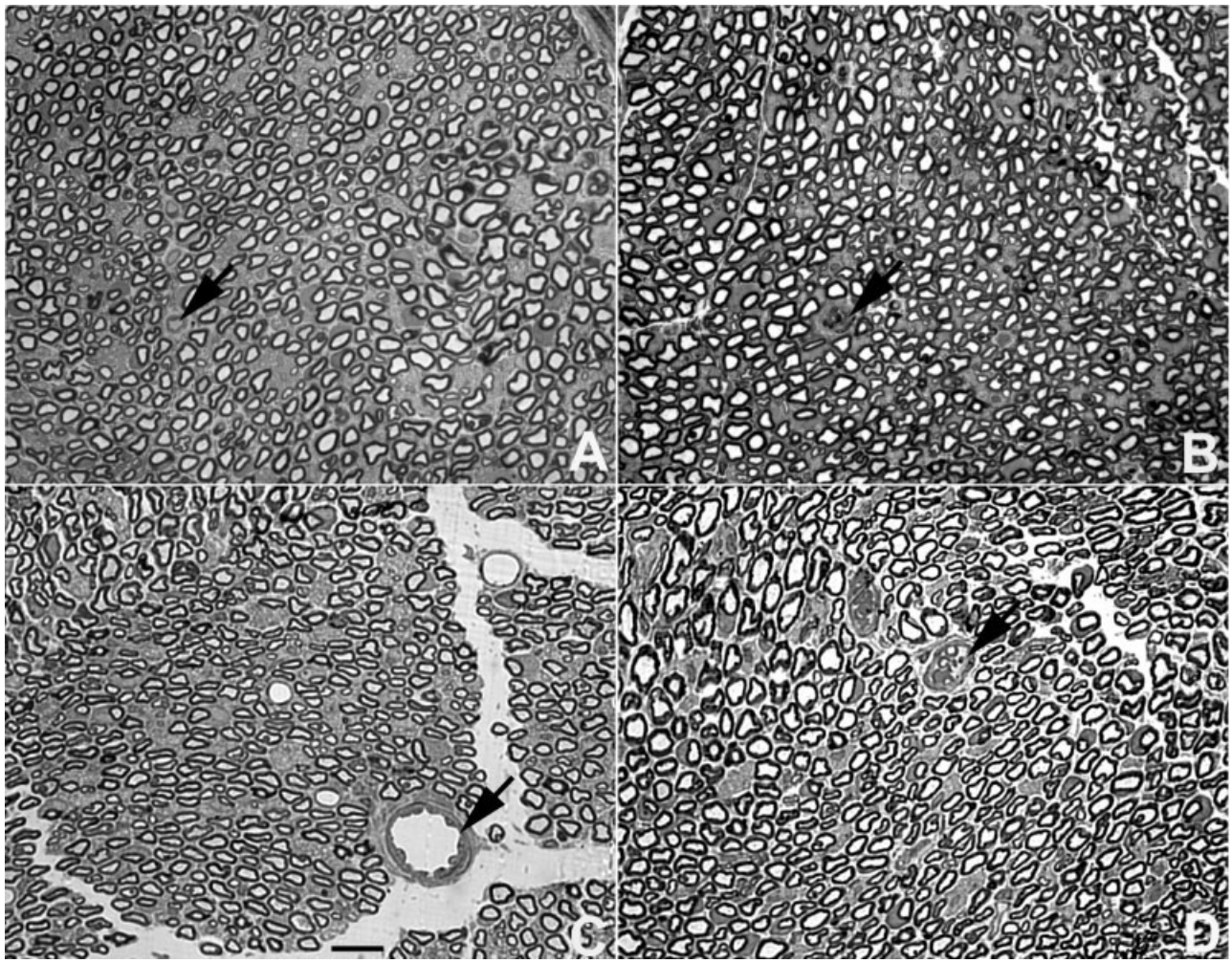

FIGURE 2. Toluidine blue–stained cross-sections (0.5 μm) of ventral tail nerves. **(A)** The RT group exhibits uniformly stained myelinated fibers and a normal-sized arteriole (arrow). **(B)** Vibration at RT results in mild edema, indicated by wider spaces between nerve fibers. **(C)** Cold generates severe edema and arteriole dilation (arrow). **(D)** Cold-vibration exposure produces extensive edema and dilated arterioles (arrow). Bar in **C** = 40 μm for all panels.

the exclusion thresholding procedure of MetaMorph 5.2 (Universal Imaging Corp., West Chester, Pennsylvania).

Statistical Analysis. A one-way analysis of variance was used to compare means, followed by Student–Newman–Keuls testing. Differences were considered significant at $P < 0.05$, and values are presented as mean ± SD for temperature and mean ± SEM for artery and nerve morphometric analysis.

Immunohistochemistry. Cryostat cross-sections (6 μm) of arteries were immunostained with primary rabbit polyclonal antibodies directed against nitrotyrosine (1:250 in phosphate-buffered saline; Upstate, New York, New York), goat anti-mouse, biotinylated secondary (Molecular Probes, Eugene, Oregon), and avidin-linked Alex fluor-455 tertiary (Molecular Probes) antibodies. Primary antibody was omitted for the negative control, and sections were treated with peroxynitrite to induce nitration of tyrosines before immunostaining as a positive control.

RESULTS

Temperature Changes. Room temperature was regulated at 25 ± 1°C. Core body temperature was stable at 34.5 ± 0.5°C throughout the 4-h restraint and tail treatments. Four of the 24 rats that were cold-treated showed mild periorbital porphyrin excretion. There were no other overt signs of stress. The temperature of the rat-tail skin in the vibration group showed a nonsignificant net gain of 3.6 ± 1.0°C above that of the RT group (Fig. 1). Over the

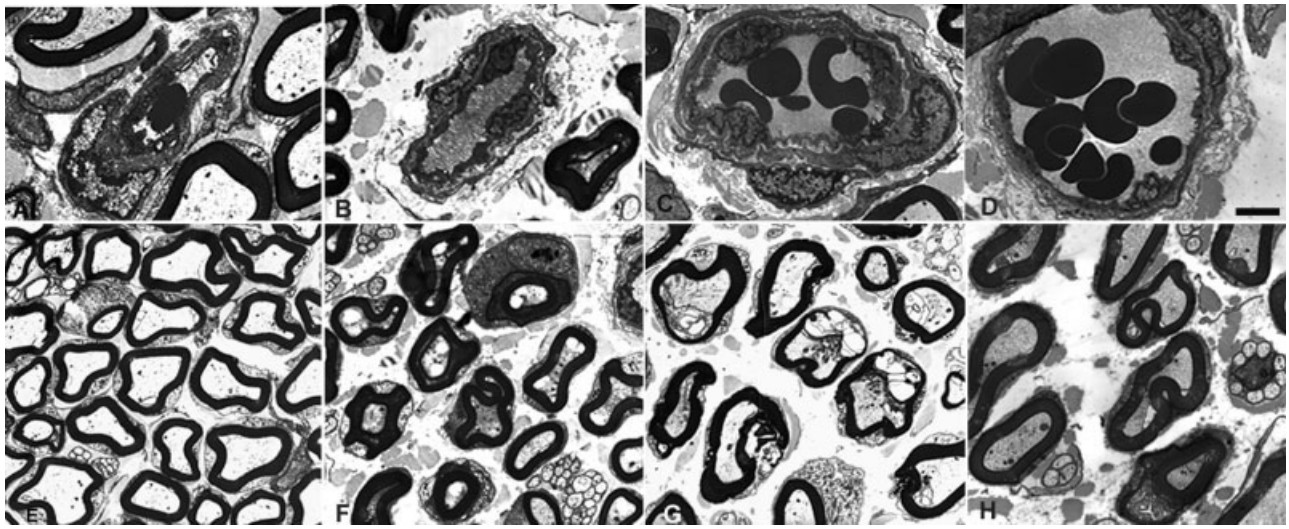

FIGURE 3. Electron micrographs of nerve cross-sections. The arterioles in the RT **(A)** and RT-vibration **(B)** groups are similar in size and contain few red blood cells. The arterioles of the cold **(C)** and cold-vibration **(D)** groups are markedly dilated, with clumped and misshapen red blood cells in the lumen. **(E)** The myelin is tightly wrapped and uniformly encircles the axons in the RT group. **(F)** In the RT-vibrated rat, the myelin shows multiple focal areas of decompaction, and some edema is present. **(G)** Cold treatment causes severe decompaction of myelin and marked edema. **(H)** In the cold-vibration group, the myelin is disrupted and edema is present. Bar in **D** = 3 μm for **A**–**D** and 3.6 μm for **E**–**H**.

4-h test period, tail temperature declined 15.6 ± 0.5°C in the cold group and 21.8 ± 0.4°C in the cold-vibration group (Fig. 1). During RT vibration, the vibrating platform warmed slowly at similar rates as the rat-tails (Fig. 1). During cold-vibration exposure, the platform temperature was significantly lower than the tail skin temperatures in the cold or cold-vibration groups (Fig. 1).

Structural Alterations. In semithin cross-sections of tail nerves, the regions between bundles of nerve fibers were greater in the cold and cold-vibration groups than those of RT and RT-vibration (Figs. 2 and 3, Table 1). The intraneural arterioles and venules in the cold and cold-vibration groups were significantly larger in cross-sectional area than in the RT group (Figs. 2 and 3, Table 1). Clumps of misshapen red blood cells were frequently found within the large-diameter arterioles. This was indicative of red blood cell stasis (Figs. 2 and 3, Table 1). The RT-vibration, cold and cold-vibration groups exhibited higher numbers of myelinated axons with focally disrupted myelin sheaths, which were wider and stained darker with toluidine blue (Figs. 2 and 3, Table 1). The percentages of axons with disrupted myelin were similar in the RT-vibration, cold, and

Table 1. Vibration and cold effects on nerve and artery.

Parameters	RT	RT-vibration	Cold	Cold-vibration
Number	10	11	12	12
Total myelin disruption (%)	5.0 ± 0.6	25.4 ± 3.5*	21.7 ± 2.2*	21.8 ± 1.6*
Perivascular myelin disruption (%)	9.9 ± 3.1	35.0 ± 5.3*	47.3 ± 3.2*	45.0 ± 3.3*
Edema area (%)	8.9 ± 4.4	11.5 ± 3.4	25.8 ± 3.4†	36.6 ± 3.2†
Cross sectional area of arteriole in nerve (μm^2)	117.5 ± 15.8	144.5 ± 44.9	592.1 ± 279.1†	771.6 ± 150.1†
Lumen size	50.1 ± 4.2	40.6 ± 2.7‡	56.4 ± 4.7	43.8 ± 2.7‡
Vacuoles in smooth muscle cells	6.8 ± 2.6	42.8 ± 9.7§	8.0 ± 4.1	12.0 ± 7.2
Vacuoles in endothelial cells	0.1 ± 0.1	17.6 ± 4.4*	14.1 ± 3.3*	13.6 ± 2.4*

Comparison of room temperature (RT), RT-vibration, cold, and cold-vibration groups. All groups were tested using Student–Newman–Keuls test and considered significant at $P < 0.05$.
**Significant difference vs. RT.*
†Significant difference vs. RT and RT-vibration groups.
‡Significant difference vs. RT group and cold group.
§Significant difference vs. RT, cold, and cold-vibration groups.

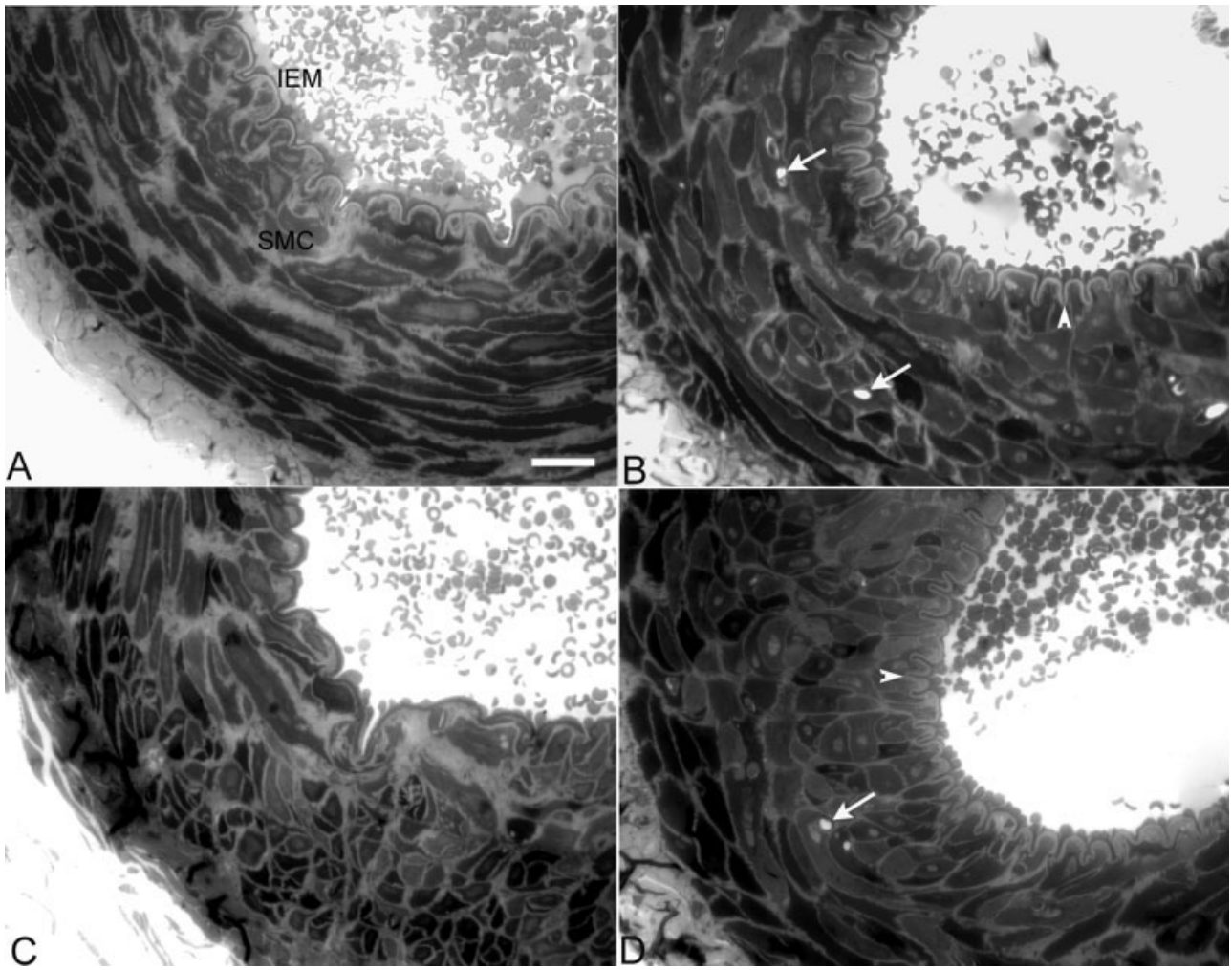

FIGURE 4. Toluidine blue–stained cross-sections (0.5 μm) of ventral tail arteries. **(A)** The RT group exhibits a moderately folded internal elastic membrane (IEM) and smooth muscle cells (SMC) with no vacuoles. **(B)** When vibrated at RT, the endothelial cells are pinched between the tight folds of the IEM (arrowhead) and protrude into the lumen. The smooth muscle cells are compact and contain vacuoles (arrows). **(C)** The cold-treated artery exhibits a moderately folded internal elastic membrane, and the SMC lack vacuoles. **(D)** Cold-vibration treatment causes tight folding of the IEM (arrowhead) and some vacuole formation. Bar in **A** = 40 μm for all panels.

cold-vibration groups, although the myelinated axons of the cold and cold-vibration groups exhibited more focal regions of thickening than those of RT-vibration (Figs. 2 and 3, Table 1). Abnormal myelinated fibers were the most prevalent in the perivascular regions of the cold and cold-vibration groups (Table 1).

Arteries from rats vibrated in both temperatures had smaller lumens than the RT or cold groups, and the endothelial cells were pinched between the tightly folded internal elastic membranes, causing the cells to bulge into the lumen (Fig. 4, Table 1). RT-vibration, cold, and cold-vibration groups had significantly more endothelial vacuoles when compared to RT ($P < 0.05$; Table 1). RT-vibration produced significantly ($P < 0.05$) more vacuoles in vascular smooth muscle cells than cold-vibration (Fig. 4B, Table 1). As with vacuoles induced by vibration at room temperature, some of the double-membrane–limited vacuoles were connected via narrow necks to the parent cell in the cold-vibration group (Fig. 5).[9]

Immunohistochemistry. Compared to the RT group, immunoreactivity for nitrotyrosine was more intense and extensive after cold treatment and following vibration at room and cold temperatures. Immunoreactivity was localized to the endothelial and smooth muscle cells and the extracellular matrix (Fig. 6). Omission of the primary antibodies eliminated immunostaining in the test groups.

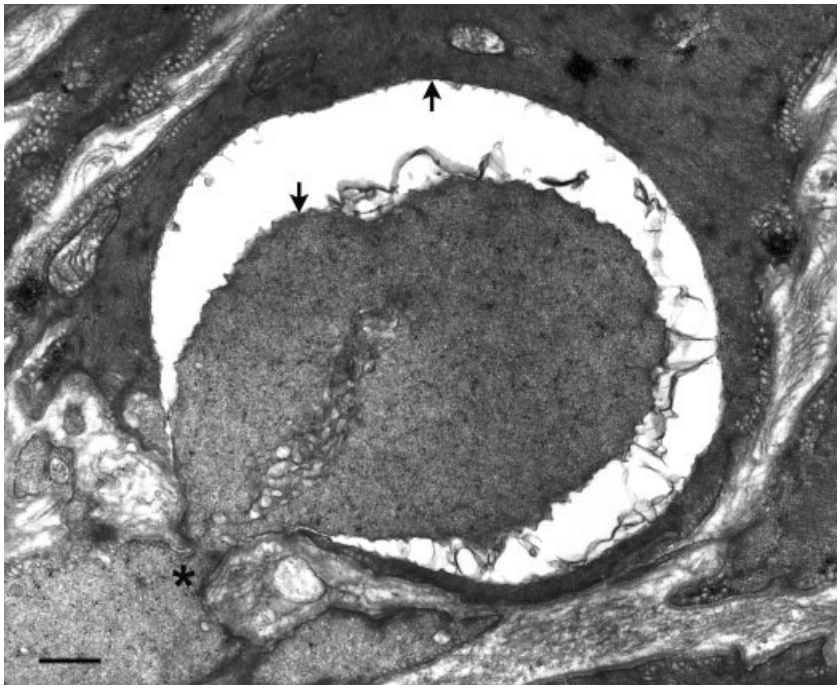

FIGURE 5. The vacuole formed in a vascular smooth muscle cell during cold-vibration treatment is double-membrane-limited (arrows) and is connected to the parent cell by a narrow stalk (asterisk). The inner and outer membranes are disrupted. The electron lucent area may represent artifactual movement of water during fixation. Bar = 0.6 μm.

DISCUSSION

Effects of Vibration and Temperature. It is well documented that, during continuous vibration of the finger for 30 min, blood flow is reduced and maintained at a low level with no significant alteration in skin temperature.[6,31] However, it is unknown whether this reduction in flow persists during longer periods of vibration. Prolonged vasoconstriction would be expected to cause ischemia and a fall in skin temperature. During 4 h of vibration at 25°C, the temperature of the proximal tail skin increased a few degrees above RT. RT-vibration–induced vasoconstriction was expected to reduce blood flow to the tail and result in cooling. Heating of the tail by the vibration platform during acceleration may have masked the cooling effects of vasoconstriction.

Cooling the nonvibrated tails with ice brought the proximal skin temperature down to 14.1 ± 0.7°C by 15 min, whereas cooling the vibrating tail lowered the temperature an additional 6°C. The temperature of the platform, when cooled, is comparable to temperatures experienced by workers using power tools during winter in colder regions of the world, which report higher incidences of the vasospastic component of HAVS.[2,5,23] Cold-induced changes in skin temperature result from blood flow fluctuation through the arteriovenous anastamoses.[3] Vibration-induced vasoconstriction and the concomitant blood-flow decrease are mainly evident in the arteries and arterioles. A combination of reduced flow in the arteries and the arteriovenous anastomoses may account for the lower temperature achieved during cold-vibration. These results suggest that cold temperatures contribute to the development of HAVS.

Structural Changes in Nerves. The disparity in the effects of vibration and cold was most apparent in the morphology of the tail nerves. Whereas both RT-vibration and cold produced comparable percentages of damaged myelinated fibers, the type of damage was dissimilar. Cold treatment evoked intraneural edema, and arterioles were dilated and often occluded by clumped red blood cells. The edema and vascular occlusion data were similar to the findings for the sciatic-nerve ligation model of ischemia–reperfusion and cold injury of the sciatic nerve.[16,22] Low temperatures can directly compromise stability of cell-membrane lipids by causing phase separation.[1,24] The myelin sheath, comprised of multiple layers of membrane rich in lipids, may be susceptible to the direct damaging effects of cold. Thus, cold may act via at least two mechanisms: alteration of lipid physicochemical properties and by causing ischemia–reperfusion injury. Vibration at room tem-

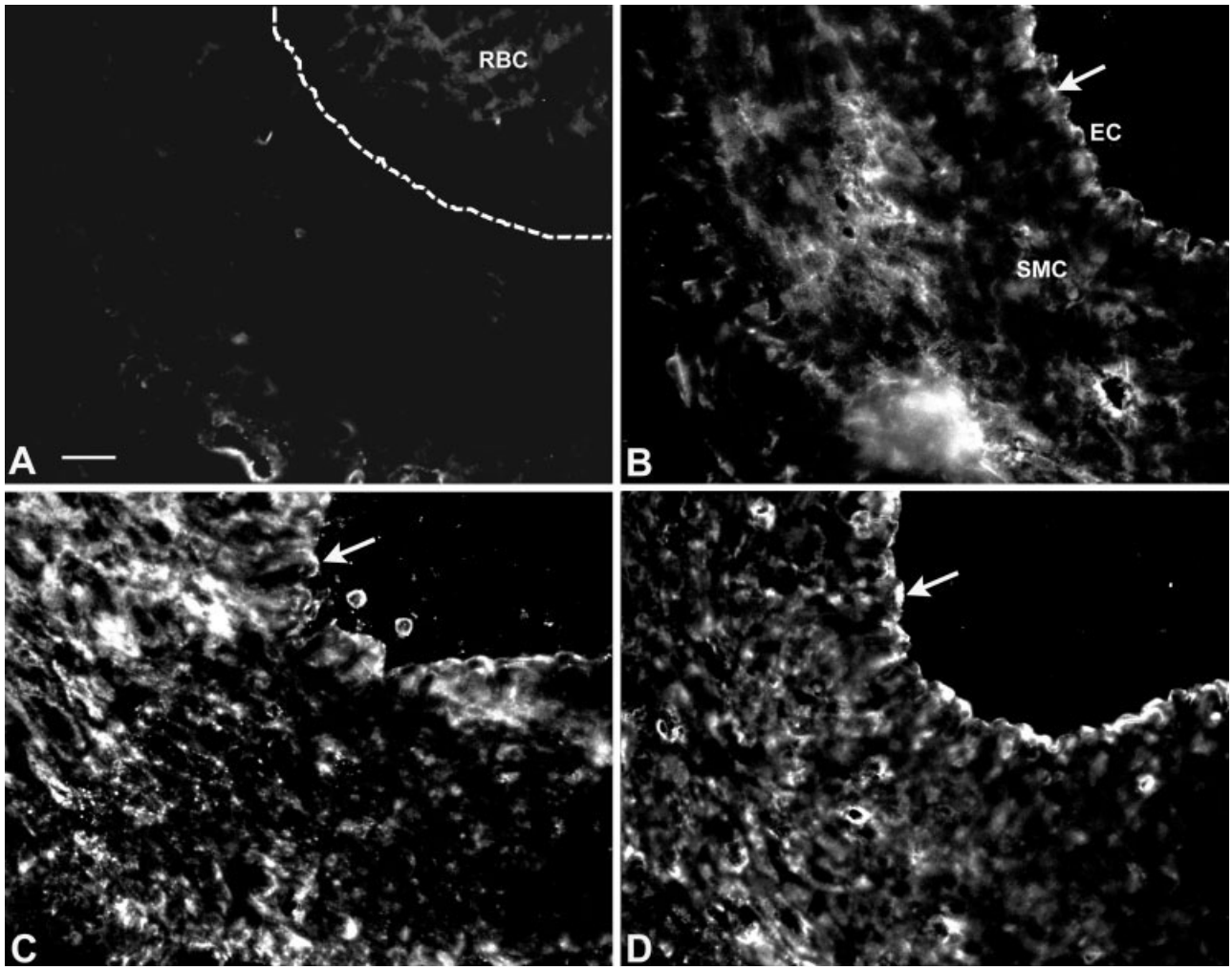

FIGURE 6. Cryostat cross-sections (6 μm) of arteries immunostained with anti-nitrotyrosine antibodies. The RT group **(A)** shows little immunoreactivity, whereas RT-vibration **(B)**, cold **(C)**, and cold-vibration **(D)** animals exhibit intense immunostaining for nitrotyrosine in both the tunica intima and tunica media. The endothelial cell (EC) layer is indicated by a dashed line for RT, and arrows point to immunoreactive ECs for the other conditions. Bar in **A** = 40 μm for all panels.

perature produced little nerve edema and a relatively uniform distribution of disrupted myelinated fibers in contrast to the perivascular-enhanced prevalence of myelin-damaged fibers in both cold-treated groups. Mechanical acceleration stresses may have caused unraveling of the myelin sheath, exposing more proteins for the toluidine blue to stain and account for the darker staining myelin. In addition, nitrosylation and nitration of myelin integral membrane proteins, which cross-link the membranes, may disrupt cross-linking and allow decompaction of myelin.[4] The effects of vibration on nerve blood flow are unknown, but vibration appears to activate vasoconstriction in arteries via the somatosympathetic pathway.[26] Blood flow in the vasa nervorum is likely to decline in response to vibration as occurs in digital arteries. If correct, ischemia–reperfusion may also be a contributing factor in causing myelin disruption in RT-vibration. The early symptoms of HAVS are tingling and numbness. Disruption of the myelin of large axons, which convey touch, pressure, and vibration sensation, is consistent with the sensations experienced by workers using power tools for the first time.

Endothelial and Smooth-Muscle-Cell Vacuoles. Our previous studies of vibration at room temperature showed that the formation of vacuoles in vascular smooth muscle cells was directly correlated with vasoconstriction, that is, contraction of smooth muscle cells.[9] The present findings for tails vibrated in cold temperatures are somewhat divergent, because, in spite of small arterial lumens, there were few smooth-muscle-cell vacuoles. When smooth muscle cells contract vigorously, cell-

surface protrusions emerge at multiple sites.[11,30] The mechanism of vacuole formation is unknown, but the process is envisioned to depend not only on the extent of cell shortening but also on the rate and force of contraction of vascular smooth muscle cells. At low temperatures, smooth muscle cells shorten more slowly and with less force.[15] If vascular smooth muscle cells undergo shortening at a slower rate and generate less force, fewer vacuoles may be formed. Vacuoles in vascular smooth muscle cells generated by vibration at room and low temperatures have similar morphology, indicating a similar mechanism of vacuole formation. Cooling appears to have a beneficial effect on vibration-induced artery damage because smooth muscle cells are less vacuolated.

Nitrotyrosine Formation. Protein tyrosine nitration and *S*-cysteine nitrosylation are posttranslational modifications of proteins requiring nitric oxide and reactive oxygen species.[13] A large body of literature suggests the generation of reactive oxygen species and reactive nitrogen species in ischemia–reperfusion injury in various organ systems.[14] The reduction in plasma thiol levels in HAVS patients is consistent with free-radical activity.[20] In the current study, cold and vibration for 4 h in both temperatures produced nitration of arterial tissue, indicating free-radical generation. Nitration may modify protein function and alter cellular responses to cytokines, interferons, and growth factors.[14] In vitro nitration of isolated optic nerves produces dramatic decompaction of myelin similar to that observed in the tail nerves.[4] The nitration is thought to disrupt cross-linking of the proteolipid proteins, leading to myelin decompaction.[4] Nerve tissues were consumed in analysis before the nitrotyrosine staining method was operational in our laboratory. Whether nitrotyrosine formation is occurring in vibrated tail nerves remains to be examined. Free-radical generation of nitrotyrosine warrants further research for understanding the pathophysiology of HAVS.

In conclusion, our results demonstrate that there are similarities between the effects of vibration and cold on peripheral arteries and nerves. Both treatments induced nitration, implying free-radical generation. The vasoconstriction of the tail artery and interstitial edema and blood stasis in the nerves indicate that ischemia–reperfusion damage may be occurring. Vibration at both temperatures results in smooth-muscle shortening, smaller lumen sizes, and formation of intracellular endothelial and smooth-muscle-cell vacuoles in arteries. Given the overlap in the damaging effects of cold and vibration, cold temperature may be incriminated at a mechanistic level in the pathophysiology of HAVS.

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