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## Frequency-dependent changes in mitochondrial number and generation of reactive oxygen species in a rat model of vibration-induced injury

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### ABSTRACT

Regular use of vibrating hand tools results in cold-induced vasoconstriction, finger blanching, and a reduction in tactile sensitivity and manual dexterity. Depending upon the length and frequency, vibration induces regeneration, or dysfunction and apoptosis, inflammation and an increase in reactive oxygen species (ROS) levels. These changes may be associated with mitochondria, this study examined the effects of vibration on total and functional mitochondria number. Male rats were exposed to restraint or tail vibration at 62.5, 125, or 250 Hz. The frequency-dependent effects of vibration on mitochondrial number and generation of oxidative stress were examined. After 10 days of exposure at 125 Hz, ventral tail arteries (VTA) were constricted and there was an increase in mitochondrial number and intensity of ROS staining. In the skin, the influence of vibration on arterioles displayed a similar but insignificant response in VTA. There was also a reduction in the number of small nerves with exposure to vibration at 250 Hz, and a reduction in mitochondrial number in nerves in restrained and all vibrated conditions. There was a significant rise in the size of the sensory receptors with vibration at 125 Hz, and an elevation in ROS levels. Based upon these results, mitochondria number and activity are affected by vibration, especially at frequencies at or near resonance. The influence of vibration on the vascular system may either be adaptive or maladaptive. However, the effects on cutaneous nerves might be a precursor to loss of innervation and sensory function noted in workers exposed to vibration.

### KEYWORDS

Hand-arm vibration; vascular dysfunction; peripheral nerve injury; neuropathy; biomarkers

### Introduction

Occupational exposure to hand-transmitted vibration has been associated with the development of cold-induced vasospasms that result in blanching in the fingers and hands, a reduction in tactile sensitivity, pain and paresthesia, and diminished grip strength and manual dexterity (Krajnak 2018). The vascular symptoms are often referred to as vibration white finger (VWF), and the vascular and sensorineural/sensorimotor symptoms combined are referred to as hand-arm vibration syndrome (HAVs; Griffin 1990, 2012). Epidemiological studies in humans (Bovenzi 1994, 2012; Burström 1994), experimental studies using animal (Curry et al. 2005; Krajnak et al. 2012b, 2013, 2010) and computational models (Wu et al. 2003b, 2006a; Wu, Welcome, and Dong 2006b) demonstrated that influence of vibration on peripheral vascular and sensorineural systems are frequency dependent. Exposure to

vibration at or near the resonant frequency is associated with a greater risk of developing HAVs (Bovenzi 2010a, 2010b, 2012), or vibration-induced vascular (Bovenzi 2010a; Krajnak et al. 2012b, 2010) or sensorineural dysfunction (House et al. 2009; Krajnak et al. 2012b).

Decreasing or elevating vibration exposure may result in a reduction in symptoms if there is an early diagnosis of vascular and/or sensorineural dysfunction (Cherniack, Clive, and Seidner 2000; Cherniack et al. 1990; Krajnak et al. 2014; Petersen et al. 1995). However, a reliable physiological and/or biological marker to detect vibration-induced dysfunction early has not been identified, and further the vascular and sensorineural system may still recover. Several investigators indicated that the sensorineural system may be more sensitive to the influence of vibration exposure, and therefore, responses to various physiological tests of sensory function and pain were examined (House et al. 2009; Krajnak et al. 2012b, 2013;

Loffredo et al. 2009; McGeoch, Gilmour, and Taylor 1994). However, the peripheral vascular system also seems to display a role in early responses to vibration exposure when challenged, as evidenced by peripheral blood flow (Krajnak et al. 2010). Although the response of peripheral blood vessels to phenylephrine, a neurotransmitter that induces vasoconstriction, returns to baseline levels after exposure to a single bout of vibration, it takes up to 10 days for the response to acetylcholine, a vasodilating neurotransmitter, to return to baseline levels (Krajnak et al. 2014). Based upon the results of these experiments, understanding how vibration exposure affects both physiological and biological functioning of the peripheral vascular and sensorineural systems might provide additional information that may be used to (1) determine how vibration affects specific tissues, and (2) identify specific tests or biomarkers for early detection of vibration-induced dysfunction or injury.

One of the reasons vibration-induced changes in vascular and sensorineural function may be enhanced and easier to detect in response to various challenges may be related to the influence of vibration on the metabolic activity and functioning of cells within specific tissues. Several investigators reported that vibration produces a vasoconstriction in exposed appendages, such as fingers, reducing blood flow to the region and activating hypoxia-induced cellular signaling concomitant with inflammatory and oxidative stress associated pathways in both vascular smooth muscle and endothelial cells (Curry et al. 2005; Krajnak et al. 2012b).

Mitochondria plays a major role in mediating oxidative activity, and therefore any change in their number or activity might exert an effect on the functioning of the exposed tissue. The goal of this study was to use an established model of hand-transmitted vibration to determine if, and the manner by which, vibration exposure alters the mitochondrial number and oxidative activity. Previous studies demonstrated that the effects of vibration on the peripheral vascular system was frequency dependent (Bovenzi 2012; Curry et al. 2005; Krajnak et al. 2012b, 2010), with the most prominent effects occurring at the resonant frequency of approximately 250 Hz. Effects of vibration on peripheral nerves also

tend to be more pronounced at the resonant frequency. However, the sensorineural system seems to be more sensitive to vibration with effects on function and indicators of injury and inflammation occurring at all exposure frequencies (Krajnak et al. 2012a). In the short-term, (i.e., less than 10 days of exposure) these changes may be adaptive and allow the tissue to be more resilient to subsequent exposures (Krajnak et al. 2012a, 2012b). However, longer-term exposures (i.e., 10 days and greater), and associated increases in inflammation and oxidative stress were associated with physiological and morphological changes indicative of dysfunction (Krajnak et al. 2016, 2012b). Based upon the results of these previous studies (Krajnak et al. 2012a, 2016, 2012b), it was postulated that the influence of vibration exposure on mitochondrial expression in peripheral blood vessels and nerves may be frequency-dependent with the greatest responses occurring at or near the resonant frequency. This hypothesis was tested by examining the effects of vibration on markers associated with the mitochondrial number and oxidative stress on the ventral tail arteries and nerves, and on cutaneous arterioles, nerves and sensory end organs. Studies in humans suggested that small arterioles, sensory nerve endings and sensory end organs in the skin (Bovenzi 2002; Bovenzi, Griffin, and Ruffell 1995, 2000; Brammer et al. 1987a; Brammer, Taylor, and Lundborg 1987b; Maeda and Griffin 1993) may exhibit consequences of vibration sooner than other structures, because the shear and bending stress that occurs in response to vibration exposure in the exposed skin is greater than that noted in more protected structures within the body or appendage (Wu et al. 2003a, 2003b, 2006a, 2006b)

## Methods

### Animals

Male Sprague Dawley rats (weighing 250–300 g) were obtained from Hilltop Breeders (PA) at 6 weeks of age. Animals were housed in an AAALAC International accredited facility, on a 12:12 hr. light/dark cycle (lights on 0600 hr), with food and water available *ad libitum*, for 1

week prior to beginning the experiment. All procedures were approved by the CDC-Morgantown Institutional Animal Care and Use Committee and were in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Institutes for Health Guide for the Care and Use of Laboratory Animals.

### Exposures

After 5 days of acclimation to the facilities, animals were weighed and randomly assigned to one of 5 groups; cage controls, restraint controls, or vibration exposed at 62.5, 125, or 250 Hz ( $n = 6$  rats/group). All rats underwent 4 days of acclimation to restraint in a Broome-style restrainer. Acclimation involved placing the animals in the restrainer for progressively longer lengths of time over a 4-day period (30 min, 1, 2 and 4 hr., respectively). Once acclimated to restraint, animals were exposed to their assigned treatment; cage control rats (controls) stayed in their home cage which was moved into the lab during the exposures. However, controls were not exposed to vibration or the sound produced by the shakers. Vibration exposed rats were placed in Broome style restrainers and had their tails secured to a platform mounted on a shaker that was in a sound attenuating chamber. Tails were restrained using 4–1 cm elastic straps that kept the animal's tail on the platform but did not put pressure on the tail (Welcome et al. 2010). Rat tails were then exposed to vibration at frequencies of 62.5, 125, or 250 Hz (amplitude  $49 \text{ m/s}^2$ ) for 4 hr/day for 10 consecutive days. Animals were returned to their home cage after each exposure. Rats in the restraint control group (restraint) were also placed in Broome style restrainers and had their tails secured to platforms mounted onto isolation blocks. Restraint rats were placed in the chambers with vibration exposed rats, but not exposed to vibration. Twenty-four hour after the last exposure, rats were anesthetized with pentobarbital (100 mg/kg i.p.) and exsanguinated. The ventral tail artery, ventral tail nerves and 300–500 mm section of skin from the C14-15 region were dissected, frozen in Tissue-Tek O.C. T. compound (VWR, Radnor PA) in cryomolds, wrapped in labeled foil and stored at  $-80^\circ\text{C}$  until sectioning.

### Tissue preparation

Embedded tissue was removed from the cryomold and the extra tissue was trimmed away. Frozen sections ( $20 \mu\text{M}$ ) were cut on a cryostat and thaw mounted onto charged slides (Fisher brand Super frost Plus, Pittsburgh, PA). Cross-sections of the ventral tail artery (VTA) and the ventral tail nerve (VTN) were both collected. To ensure that each slide contained sections throughout the range of the dissected tissue, the first section was placed on slide one, the second on slide two and so on until sections had been placed on 10 slides. Then, the process was repeated until there were 4–6 sections on each slide. Using this procedure, each section on a single slide was  $200 \mu\text{m}$  apart.

### Mitochondrial staining

To roughly estimate the mitochondrial number, Heidenhain's hematoxylin stain was used. This particular hematoxylin stains chromatin, mitochondria, striations in skeletal muscle and myelin (Kudryavtseva et al. 2016; Wilson and Gamble 2002). The staining procedure was performed using one slide from the VTA, VTN, and skin. Each slide was fixed in 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS, pH 7.4) for 5 min, rinsed  $4 \times 5$  min in distilled water ( $\text{dH}_2\text{O}$ ), and then stained. To perform the staining, sections were first incubated in a 5% ferric ammonium sulfate solution (in  $\text{dH}_2\text{O}$ ) for 1 hr., rinsed  $4 \times 5$  min in  $\text{dH}_2\text{O}$ , incubated in Heidenhain's hematoxylin (0.5% hematoxylin, 10 ml 100% ethanol in 100 ml  $\text{dH}_2\text{O}$ ) for 1 hr, rinsed, and re-incubated in the ferric ammonium sulfate solution 30 min. Slides were then rinsed  $2 \times 1$  min in  $\text{dH}_2\text{O}$ , dehydrated in an ascending series of 70%, 90%, and 100% ethanol, and after air-drying were cover slipped using Permount Mounting medium (VWR).

### Mitotracker

One slide from the skin, artery, and nerve were stained using Mitotracker Orange CMTMRos (Invitrogen, Waltham, MA). This specific mitotracker stain enters the cell and is oxidized to its fluorescent form which is then sequestered in active mitochondria. To perform staining on sections, the

mitotracker stain was diluted to a 1 mM stock solution in dimethyl sulfoxide (DMSO) per the manufacturer's instructions, pipetted into vials and stored at  $-20^{\circ}\text{C}$  in a light-tight box. Just prior to staining, the stock solution was thawed and diluted to 500 nM using 0.1 M phosphate-buffered saline (PBS). The sections on each slide were circled using a pen that produced a liquid barrier, and 100  $\mu\text{l}$  diluted mitotracker was pipetted onto each slide. Slides were incubated in diluted mitotracker for 30 min at  $37^{\circ}\text{C}$ , rinsed  $3 \times 5$  min in PBS, incubated in 4% paraformaldehyde in 0.1 M PBS for 10 min, dried, coverslipped using Prolong with DAPI (FisherScientific), and stored in a light-tight container at  $4^{\circ}\text{C}$ .

### Microscopy

Slides were examined using an Olympus microscope BX63 and photomicrographs made at a magnification of 20 x using DP73 camera and cellSense version 510 (Fisher Scientific, Indianapolis IN). To quantify staining, bright field images of sections stained with Heidenhain's hematoxylin, and photomicrographs from each section were taken using a 20X objective and controlling for lighting. Pictures of the skin sections were taken and organelles in the ventral portion of the skin (i.e., the region directly in contact with the vibrating sources) were photographed, and included arterioles, nerves, sensory receptors and hair follicles (pictures taken,  $n = 3\text{--}4$  organelles/section). The total number of nerves, arteries, and sensory receptors was also counted in each skin section. Photomicrographs of the VTA and the left VTN were also taken. Mitotracker staining was quantified in a similar manner except that slides were visualized using fluorescent microscopy at a wavelength of 554–576 nm. Pictures ( $n = 4\text{--}5$ /animal) were saved and densitometry was performed using ImageJ (<http://wsr.imagej.net/distros/>). Each artery, nerve, or sensory organ was outlined, and a density threshold was set. Any staining above the set threshold was identified as labeling and the ImageJ program measured both the area and intensity of this staining. In the large arteries, the outer perimeter of the endothelial cell layer, along with the outer perimeter of the VTA was outlined. Staining area and intensity of the vascular smooth muscle were calculated by subtracting measures of the endothelial cell layer from the vascular

smooth muscle, and the area and intensity of labeling in the endothelial cell layer were determined using measures collected from the outlined endothelial cell region. For analyses, the average area for each structure or organelle outlined along with the density of the labeled area was utilized for analyses. Differences with a  $p \leq 0.05$  were considered significant. In the skin, the number of arteries, nerves, and hair follicles was also counted employed as an indicator of alterations in vascularization, innervation, or number of hairs in the skin.

### Statistical analyses

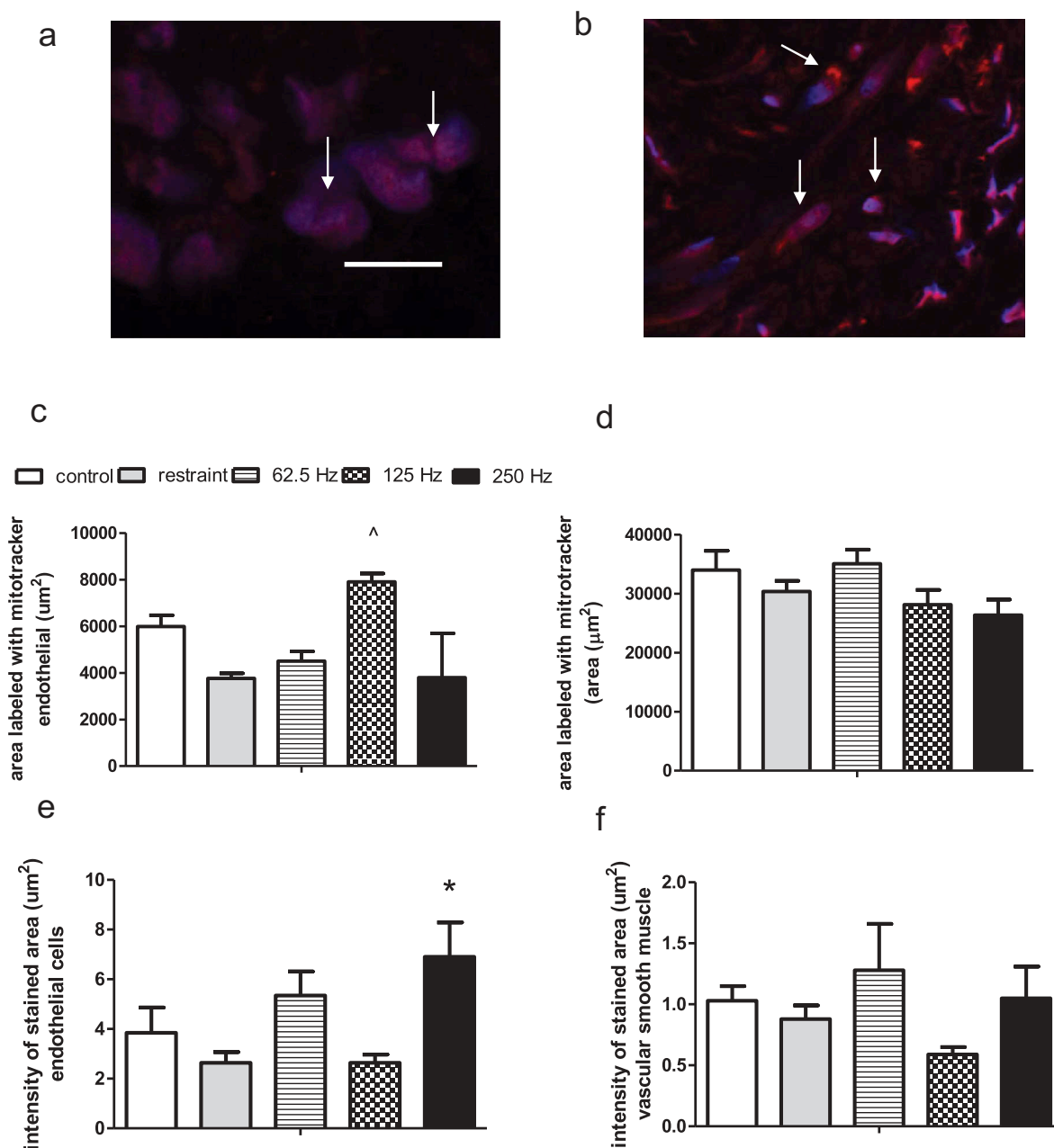
JMP 13.2.0 (2016 SAS Institute Inc, Cary, NC). Changes between groups were analyzed using one-way ANOVAs. Pairwise comparisons of were performed using Student's t-tests controlled for family-wise error. Differences were significant if  $p < .05$ .

## Results

### Ventral tail arteries (VTA)

Confocal images of the mitotracker staining in the endothelial cell layer and the vascular smooth muscle (VSM) of an artery obtained from an animal exposed to vibration at 250 Hz are illustrated in in [Figure 1a,b](#). These photomicrographs demonstrate that multitracker staining in the endothelial cells was primarily near the nucleus of the cell, with the red mitotracker staining surrounding the blue nuclear stain in [Figure 1a](#), while staining in the VSM cells was in both the cytoplasm and around the nucleus (i.e., the outline of the total cell can be seen; [Figure 1b](#)). The intensity or brightness of the staining was indicative of the level of oxidative activity (i.e., brighter staining indicates greater oxidative stress). When the mitotracker labeling was quantified in endothelial cells, exposure to vibration at 125 Hz resulted in an increase in the mitotracker area labeled and in the intensity of labeling with exposure to vibration at 250 Hz ([Figure 1c,e](#)). In the VSM, there was no marked effect of treatment on the area using mitotracker labeling ([Figure 1d](#)). Although there appeared to be a reduction in the intensity of mitotracker labeling in VSM from





**Figure 1.** Confocal photomicrographs show mitotracker staining in the VTA (a-b). The area and intensity of the labeling are indicative of the number of functional mitochondria in the tissue. Labeling in endothelial cells (a) from the ventral tail artery (250 Hz exposure) was primarily found around the nucleus, while labeling in the VSM (b) was noted in both cytoplasm and around the nucleus. The arrows indicate cells stained with mitotracker and the bar is 60 μm. Quantification of mitotracker staining showed that the area labeled with mitotracker was increased in the endothelial cells of arteries from animals exposed to 125 Hz vibration (c), however mitotracker staining was not affected by treatment in the VSM (d). The intensity of mitotracker was significantly increased in the endothelial cells with exposure to 250 Hz vibration (e) and appeared to be reduced with exposure to 125 Hz in VSM, but this difference was not significant (f). Differences \*  $p < .05$ .

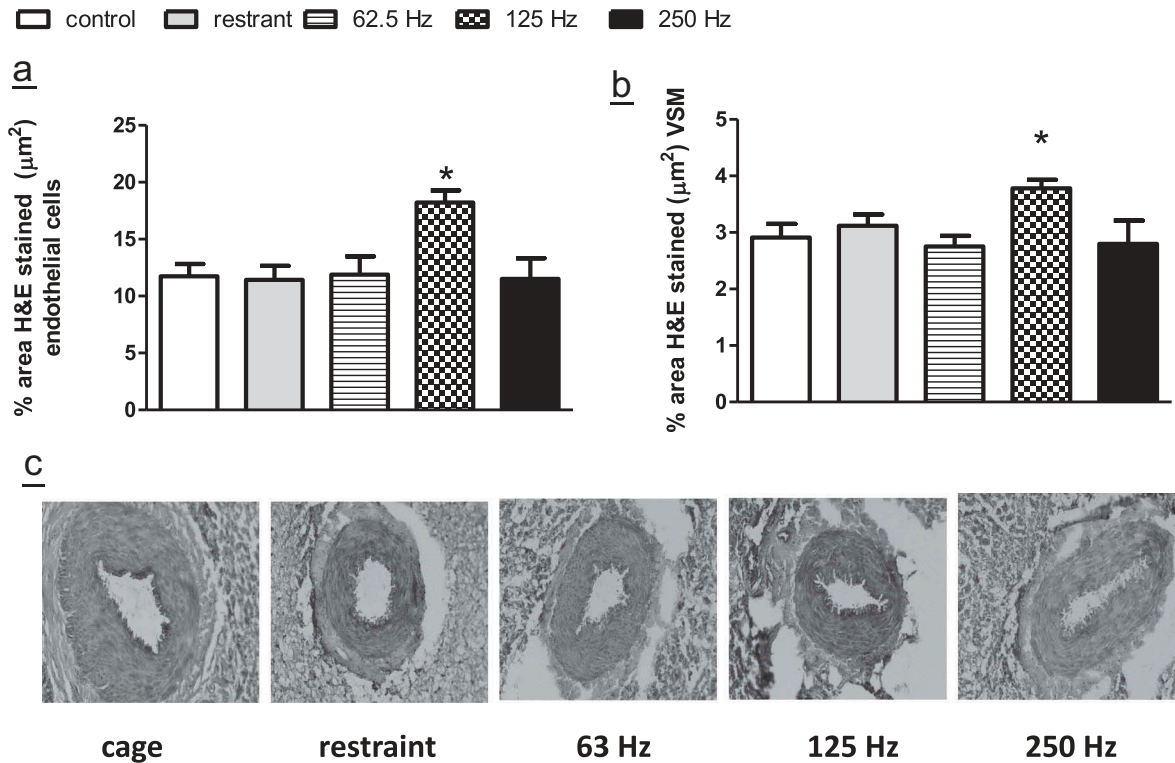
arteries exposed to vibration at 125 Hz, this difference was not significant (Figure 1f).

In the VTA, there was a significant decrease in the endothelial cell area both at 125 and 250 Hz, but because of the variability, this reduction was only significant at 125 Hz. There were no marked

exposure-related differences in the smooth muscle area (Table 1). When mitochondrial density was estimated using hematoxylin staining, there was a significant rise in staining density in both endothelial and smooth muscle layer at 125 Hz (Figure 2a-c).

**Table 1.** Area ( $\mu\text{m}^2 \pm \text{sem}$ ) of the vascular smooth muscle and the endothelium of the ventral tail artery. Exposure to vibration at 125 Hz resulted in a reduction in the area of both the smooth muscle and endothelium (\* $p < .05$ ).

Area by region ( $\mu\text{m}^2$ )	Cage	Restraint	62.5 Hz	125 Hz	250 Hz
endothelium	1130.54 (126.78)	1207.35 (149.20)	1002.94 (113.51)	699.78 (30.61)*	528.89 (215.9)
Smooth muscle	3603.48 (399.8)	3381.7 (251.82)	3785.27(334.98)	2685.56 (150.71)*	4079.3 (757.98)



**Figure 2.** Vibration affected hemotoxylin staining of mitochondria in the VTA. Exposure to vibration at 125 Hz resulted in an increase in the hemotoxylin stained area in both endothelial cells (a) and VSM (b) as compared to the labeling in cage controls in the (VTA). Light level photomicrographs showing the hemotoxylin labeling in arteries from animals exposed to different conditions are presented (c).

### Ventral tail nerve (VTN)

There was no marked effect of vibration on the hemotoxylin labeled area, or in the area labeled with mitotracker in the VTN (Figure 3a,b). Hemotoxylin stained nerves from each condition are presented in Figure 3c. There was an elevation in mitotracker staining density in nerves from animals exposed to vibration at 125 Hz (Figure 3d, which was primarily located in the myelin sheath (Figure 3e)

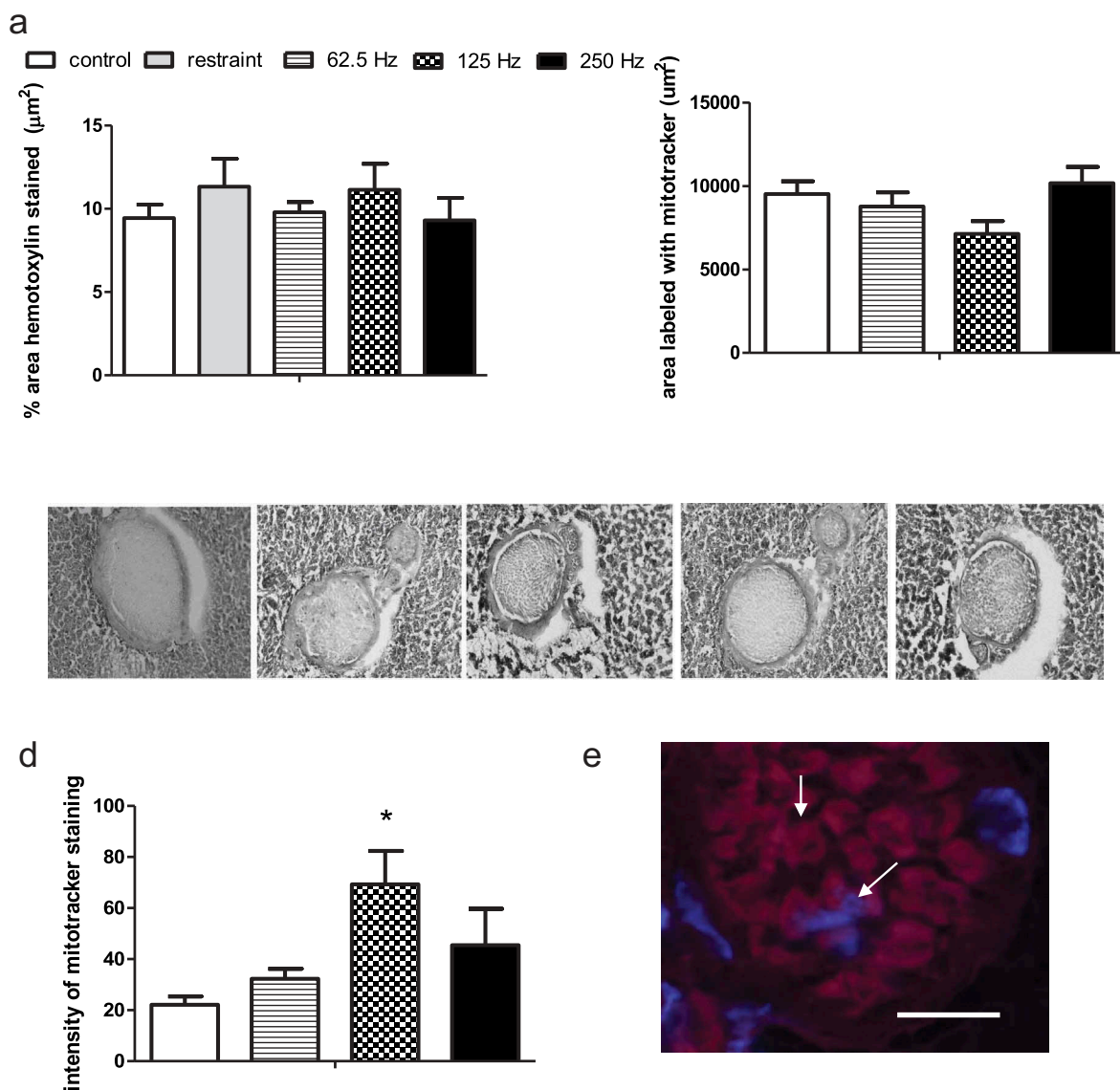
### Cutaneous arterioles ( $\leq 200 \mu\text{m}$ internal diameter)

There was no marked effect of vibration exposure on the number of arterioles in the skin (Table 2).

There also was no marked effect of restraint or vibration on the total area or in the % hemotoxylin stained area in the arterioles (Figure 4a,b). In the endothelial cells, the area or the intensity of the mitotracker staining was not significantly affected by exposure (Figure 4c,d). In the VSM, there was no significant effect of vibration on the area labeled with mitotracker staining, and although the intensity of labeling seemed to be elevated with exposure to vibration at 125 Hz, this difference was not significant (Figure e,f).

### Cutaneous nerves

Exposure to vibration at 250 Hz resulted in a reduction in a number of nerves in the skin



**Figure 3.** Exposure to restraint or vibration did not affect the cross-sectional area stained with hemotoxylin (a and c) or mitotracker labeling (b) in the VTN. However, the intensity of mitotracker staining was significantly increased in nerves from rats exposed to vibration at 125 Hz as compared to cage controls and rats exposed at 62.5 Hz. (d; Differences \*  $p < .05$ ). Based on the pattern, staining was primarily seen in the myelin sheath surrounding the nerves and not within the nerve fibers (e). Arrows show mitotracker labeled cells and the bar = 60  $\mu\text{m}$ .

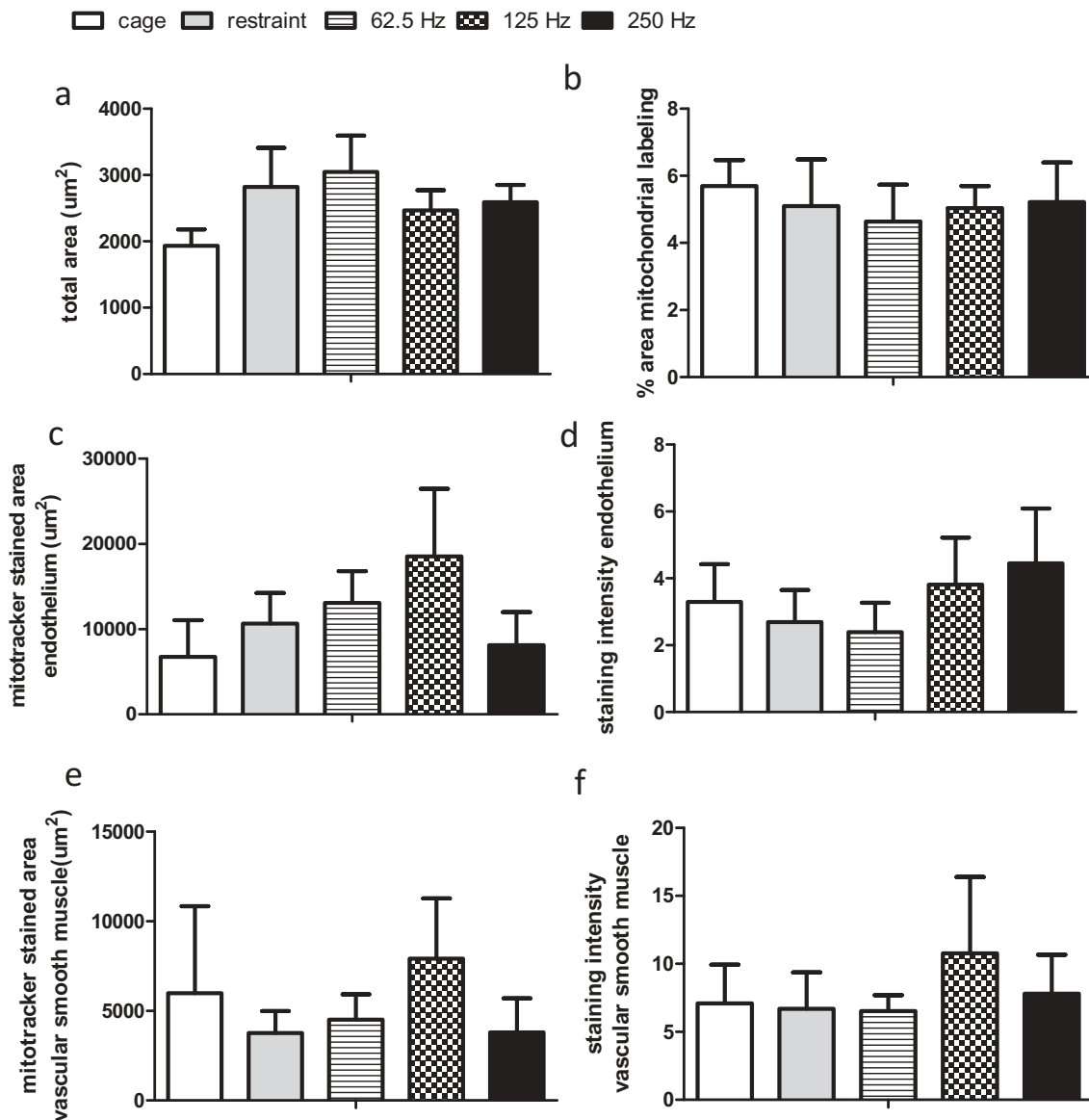
**Table 2.** The mean ( $\pm$ sem) number of organelles per skin section. Exposure to vibration at 250 Hz resulted in a reduction in the number of nerves in the skin (\* $p < .05$ ).

Number of organelles in skin	Cage	Restraint	62.5 Hz	125 Hz	250 Hz
arterioles	8.76 (1.26)	7.86 (0.80)	8.89 (1.12)	7.42 (0.81)	7.00 (1.11)
nerves	8.29 (0.83)	7.25 (0.78)	8.17 (0.90)	7.00 (0.78)	5.33* (0.09)
Sensory receptors	11.00 (1.48)	12.31 (1.38)	13.17 (1.59)	12.13 (1.39)	12.67 (1.60)

(Table 2). Exposure to vibration at 62.5 Hz produced a decrease in the total area of small cutaneous nerves and an elevation in % hemotoxylin staining in cutaneous nerves of rats exposed to vibration at 250 Hz (Figure 5a,b).

The mitotracker stained area in the nerves was diminished by exposure to restraint and vibration at all frequencies (Figure 5c). However, the intensity of the staining was not significantly affected by exposure (Figure 5d).





**Figure 4.** Exposure to vibration did not significantly affect the total cross-sectional area of the arteries (a), the % area stained with hemotoxylin (b), the area of the endothelial (c) and VSM (e) labeled with mitotracker, or the intensity of mitotracker labeling in the endothelium (d) and VSM (f).

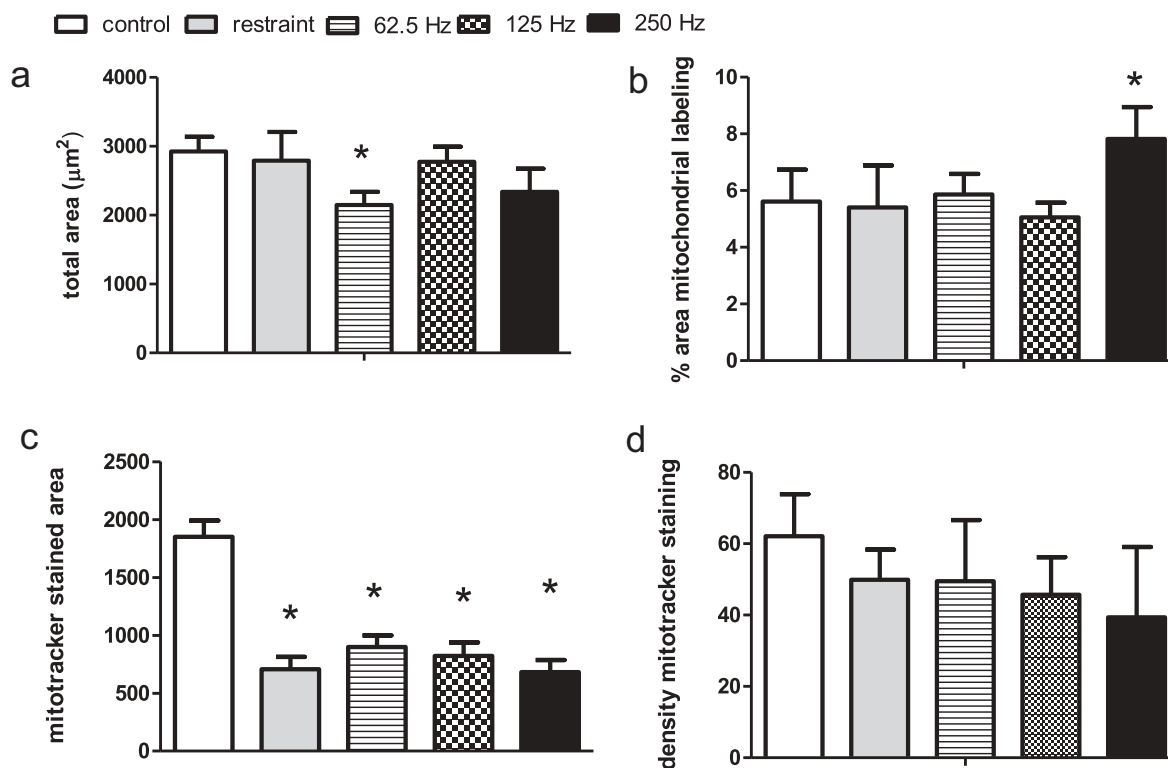
### Sensory receptors

The number of sensory receptors (Table 2) was not markedly altered by exposure. There was a reduction in the total area of the sensory receptors in the skin of rats exposed to vibration at 125 Hz (Figure 6a). Although restraint and vibration, particularly at 62.5 Hz, appeared to diminish hemotoxylin staining in sensory receptors, the differences were not significant (Figure 6b). Vibration also appeared to result in an increase in the mitotracker labeled area, but these responses were not significant (Figure 6c). However, when the intensity of mitotracker staining was analyzed,

there was a significant fall in the intensity of staining in sensory receptors in restrained animals, and a rise in staining intensity in animals exposed to vibration at 125 Hz (Figure 6d).

### Discussion

Repeated exposures to vibration were noted to increase oxidative stress in both peripheral vascular and sensorineural systems in a frequency-dependent manner (Curry et al. 2005; Krajnak et al. 2012a, 2012b, 2009; Raju et al. 2011). Cellular stressors, such as vibration, were found



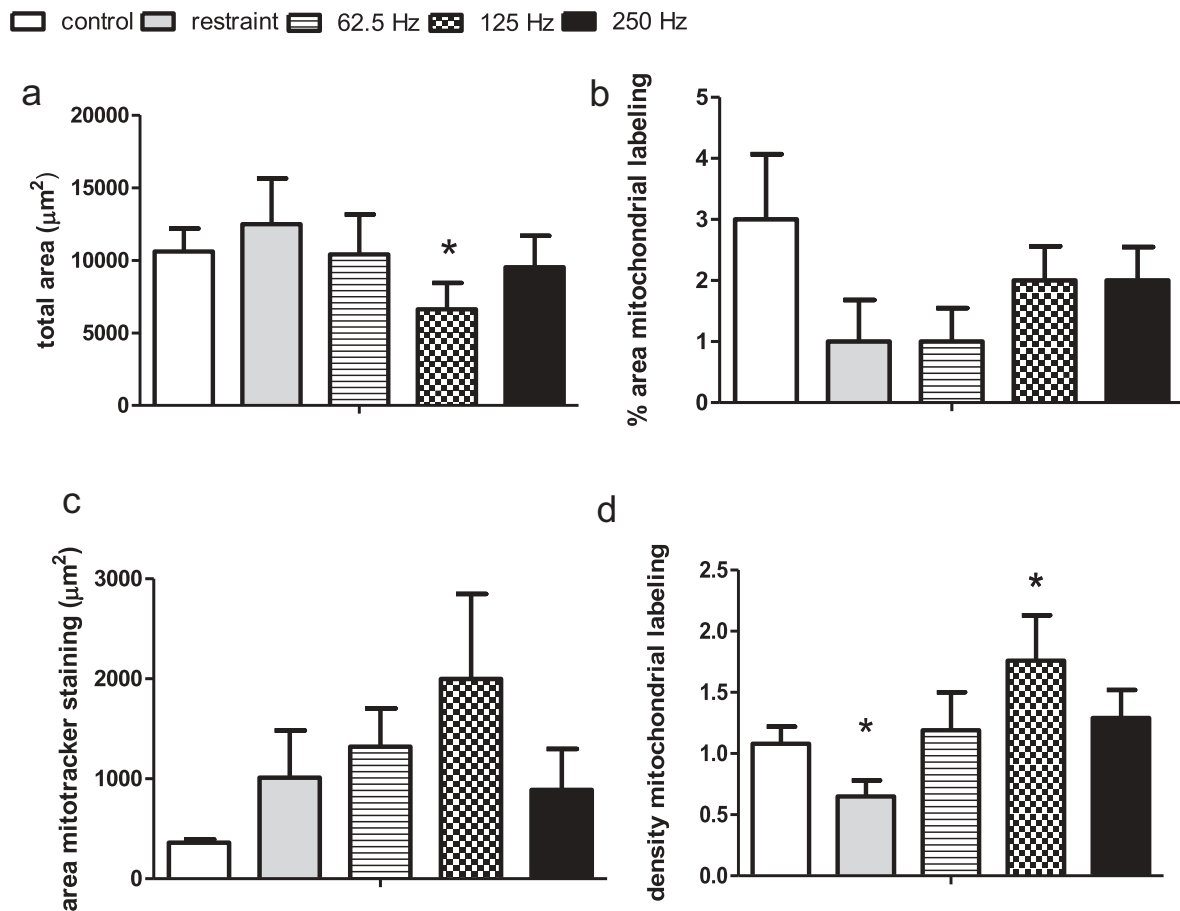
**Figure 5.** The total cross-sectional area of the nerves was lower in the skin of animals exposed to vibration at 62.5 Hz (a) and the % hematoxylin stained area was increased in nerves from animals exposed to 250 Hz (b). The area of mitotracker staining in nerves was significantly reduced by restraint exposure and by exposure to vibration at all 3 frequencies (c; \*  $p < .05$ ). Although the intensity of mitotracker staining tends to decrease with increases in the frequency of the vibration exposure, none of these differences were significant.

to enhance mitochondrial-induced production of reactive oxygen species (ROS) (Ohno and Ikenaka 2019; Persson et al. 2016; Roman-Pintos et al. 2016; Sajic et al. 2018; Smith and Gallo 2018; Song et al. 2011). The aim of this experiment was to determine if vibration-induced changes in ROS were associated with alterations in mitochondrial number. Data demonstrated that both the estimated number of mitochondria and intensity of mitotracker staining exhibited frequency-dependent effects in the endothelial layer of VTA and VTN. There were also frequency-dependent alterations in mitochondrial number and generation of ROS in cutaneous hair follicles, nerves, and sensory receptors.

One of the reasons mitochondria were examined in this study is because of their role in cellular function and metabolism. Mitochondria are not only the primary producers of cellular energy but, under physiological conditions, ROS may be generated which can enhance vasodilation and promote cellular growth. However,

under more extreme cellular stress, mitochondria produce ROS and alter cellular metabolism and functions resulting in apoptosis (Kanaan and Harper 2017). Mitochondria also plays a role in axonal repair in the nervous system by promoting axonal extension and regrowth of severed or injured axons by increasing energy production such as ATP, which is critical for actin filament guidance of growth cones during development and after injury (Smith and Gallo 2018).

A number of factors influence mitochondrial number and activity. For example, aging is associated with a fall in the number of mitochondria in endothelial cells as well as a reduction in expression of genes that are expressed under normal physiological conditions. This decrease is partially responsible for endothelial cell dysfunction and calcification (Ungvari et al. 2008). In the nervous system, disease, exposure to toxic chemicals or injury, affects mitochondrial number and activity, and may play a role in development of neurological diseases, myelin associated diseases, neuropathies, and chronic pain



**Figure 6.** Exposure to vibration at 125 Hz resulted in a significant reduction in the cross-sectional area of cutaneous sensory receptors (a). Although vibration and restraint also appeared to reduce the H&E staining in the receptors, none of the changes were statistically significant (b). The area labeled with mitotracker in the sensory receptors was higher with all exposures, but none of the differences were statistically significant (c). However, the intensity of mitotracker staining was reduced in sensory receptors of restrained animals and an increase in receptors of animals exposed to 125 Hz (d; \*  $p < .05$ ).

(Duggett et al. 2016; Englezou et al. 2012; Ino and Iino 2017; Ohno and Ikenaka 2019; Park et al. 2006; Persson et al. 2016; Prior et al. 2017). Since exposure to vibration was reported to be a cellular stressor in peripheral arteries (Hughes et al. 2009; Krajnak et al. 2006), it is possible that it is also a stressor for other cell types and that this exposure affects mitochondrial number and/or activity, thereby contributing to the injury or dysfunction induced by the exposure.

In this study, mitochondrial number and generation of ROS were assessed using hemotoxylin staining, and mitotracker labeling. The ferric ammonium sulfate employed as part of the hemotoxylin staining procedure is an oxidant, and preferentially accumulates in mitochondria, striations in skeletal muscle, chromatin, and myelin (Kudryavtseva et al. 2016; Wilson and Gamble

2002). Based upon the tissue and staining pattern, it is conceivable that the majority of the staining in arteries and sensory receptors was in mitochondria, although some staining may have occurred in condensed chromatin. In the nerves, the staining pattern suggests that the staining was of mitochondria in the myelin sheath surrounding the nerves. Therefore, this stain was used as a general marker to estimate the mitochondrial number in each tissue analyzed. Mitotracker staining was utilized to identify functional mitochondria.

Previously Welcome et al. (2008) demonstrated that the resonant frequency of the rat tail was in the same range of that as the human finger (between 100 and 300 Hz). Krajnak et al. (2010, 2012a; 2012b), Sakakibara et al. (2002) and Bovenzi, Lindsell, and Griffin (2000) found that the physiological and cellular effects on measures

of peripheral vascular and sensorineural health and function are also frequency-dependent, with dysfunction and injury to the system being greatest within the resonant frequency range. Animal, human, and computational investigations demonstrated that physical stress and strain on tissues is greatest in the resonant frequency range (Krajnak et al. 2012b, 2010; Wu et al. 2006a, 2006b). In the current study, the total area of the endothelium was generally lower in arteries from rats exposed to vibration at 125 and 250 Hz compared to arteries from rats in the cage control and restraint groups. However, these differences were only significant in animals exposed to vibration at 125 Hz. This reduction in the total endothelial area may be attributed to maintained vasoconstriction in response to vibration at these frequencies. Krajnak et al. (2010) examining the influence of vibration in the same animals found that vasoconstriction, as estimated by the perimeter of the lumen or the endothelial cell area, was more pronounced at 250 than 125 Hz. The differences in the results may be due to the fact that arteries display pulsatile activity and contain certain regions that are more constricted than others. Therefore, natural changes in diameter along the length of the blood vessel may have led to different findings in the area of the VTA. Regardless, these findings are consistent with observations of other studies showing that exposure to vibration, especially at or near the resonant frequency, produce vasoconstriction (Bovenzi 1988; Bovenzi, Franzinelli, and Strambi 1988; Curry et al. 2005; Krajnak et al. 2010; Sakakibara et al. 2002). There was also a decrease in the area of vascular smooth muscle in ventral tail arteries of rats exposed to vibration at 125 Hz. Several investigators previously demonstrated that vasoconstriction induced by cold and the vibration is partially due to the effects of norepinephrine on the  $\alpha_2$ C-adrenoreceptor and an elevation in the generation of ROS (Hughes et al. 2009; Krajnak et al. 2006). The elevation in ROS, and inhibition of endothelial-induced vasodilation, is mediated through enhanced mitochondrial activity (Bailey et al. 2005). It was also reported that prolonged vasoconstriction induced pharmacologically or by certain environmental exposures such as cold or vibration, produced smooth muscle remodeling,

hypertrophy, and in some cases abnormal growth (Bailey et al. 2005; Curry et al. 2005; Harper et al. 1982; Krajnak et al. 2012b, 2010; Sakaguchi et al. 2011; Vayssairat et al. 1982).

The VTA did not exhibit a change in the mitochondrial-labeled area, but there was an increase in the intensity of the mitotracker labeling at 125 Hz. These results are consistent with previous findings showing that exposure to vibration within the resonant frequency range results in changes in tactile sensitivity, oxidative stress, and a reduction in myelination of nerves (Krajnak et al. 2012a, 2016, 2013; Loffredo et al. 2009). Similar alterations in sensory function and nerve morphology were noted in workers diagnosed with vibration white finger (House et al. 2009; Stromberg, Dahlin, and Lundborg 1998; Takeuchi, Takeya, and Imanishi 1988; Virokannas 1995). The results of these investigations are also similar to those displaying changes in mitochondrial number and oxidative stress may be attributed to exposure to toxic chemicals, axotomy, or injury resulting in neuropathies, pain, and demyelination of exposed nerves (Duggett et al. 2016; Englezou et al. 2012; Ino and Iino 2017; Ohno and Ikenaka 2019; Persson et al. 2016). However, mitochondria also plays a critical role in neural degeneration. Because the exposure to vibration was relatively short in this study, it is not clear if enhanced oxidative activity and inflammation reported previously by Krajnak et al. (2010; 2012a), and an increase in mitotracker staining intensity at 125 Hz, might lead to regeneration, or myelin loss and whether degeneration is maintained. However, a longer exposure to vibration at 125 Hz decreased myelin basic protein staining in the ventral tail nerve and a reduction in the sensitivity of A $\beta$  (large-myelinated) nerve fibers to electrical stimulation (Krajnak et al. 2016). Therefore, the enhanced oxidative stress seen in nerves in this study may have led to degeneration and dysfunction if the exposures had been longer.

Measurements of mitochondrial density and staining intensity from small arterioles of the skin responded differently to vibration than the ventral tail artery. There was no marked effect of vibration on the overall level of mitochondrial staining as detected using hematoxylin staining. There also were no marked exposure-related

differences in the staining intensity in either VSM or endothelial cell layer. There was more variability in these measurements, and this in part may be due to the fact that the size and location of the arteries in the various layers of skin was different. Additional studies focusing specifically on skin vascularization may provide more details of cellular and physiological mechanisms underlying vibration-induced changes in skin blood flow.

In contrast, nerves and sensory receptors in the skin exhibited significant changes in response to vibration exposure. Exposure to vibration at 250 Hz produced a fall in a number of nerves but did not markedly alter the number of sensory receptors. Based upon % area of hematoxylin staining, there was a significant elevation in mitochondrial number. However, when the mitotracker stain was employed, a reduction in mitochondrial number in restraint and all vibration exposed groups was detected. A decrease in the area stained with mitotracker indicates that there was a reduction in the number of functioning mitochondria. Because the restraint straps placed minimal pressure on the tail, but rather prevented the tail from moving, it is possible that having to maintain that static posture resulted in a fall in blood flow which subsequently diminished the number of functional mitochondria (Bellin et al. 2006; Krenz et al. 2002; Murphy and Steenbergen 2008; Song et al. 2011; Ungvari et al. 2008). Evidence indicates that reductions in a number of functional/active mitochondria may be associated with apoptosis and degeneration of numerous tissues, including peripheral nerves (Duggett et al. 2016; Ino and Iino 2017; Persson et al. 2016).

Vibration-induced changes in cutaneous nerves were accompanied by alterations in receptors size and mitochondrial labeling. Exposure to vibration at 125 Hz decreased the average size of the sensory receptors (i.e., area) in the region of the skin that was in contact with the vibrating platform, and a rise in mitotracker intensity. This was unexpected because the primary sensory receptor affected by vibration is the Pacinian corpuscle, and these sensory receptors are most sensitive to stimulation at 250 Hz (Lundström et al. 1995; Sato 1961). However, it is possible that the nerves that innervate Pacinian corpuscles degenerated, and therefore, the receptors exhibiting mitochondrial

activation were the Merkl disks and Meissner corpuscles (Ribot, Vedel, and Roll 1988; Sato 1961). It is also possible that repetitive exposure to vibration shifts the sensitivity of Pacinian corpuscles to vibration or makes them sensitive to other stimuli by altering their connections in the spinal cord. Previous investigators demonstrated that exposure to vibration at or near the resonant frequency initiates inflammation and changes in the expression of peptides, such as calcitonin-gene relate peptide, that are involved in sensation and pain (Duggett et al. 2016; Englezou et al. 2012; Krajnak et al. 2016; Richner et al. 2014). Regardless the increased intensity of staining suggests that there was an enhanced oxidative stress in sensory receptors exposed to vibration at 125 Hz and this elevation in sensitivity may have resulted in either retraction or death of the nerves.

The results of these studies demonstrate that vibration induces changes both in the total number of mitochondria, and in a number of mitochondria that are active in exposed tissues. Mitotracker CMTMRos was used in the current experiment because it labels mitochondria in frozen tissue sections, and thus the frequency-dependent effects of vibration on mitochondria number in tissue sections adjacent to those were used to assess oxidative stress and expression of inflammatory factors after exposure to vibration at various frequencies was determined. However, because this stain labels cells based upon the membrane potential of the mitochondria, it is not an estimate of all mitochondria. Future studies might use the other mitotracker stains that stain all mitochondria, or other methods, to assess mitochondria number and activity. The change in number of active mitochondria in specific tissues may contribute to the increase in the concentration of ROS reported in previous studies (Krajnak et al. 2012a, 2010). Because mitochondria plays a role in energy production, cellular remodeling and apoptosis, vibration-induced alterations in their number and activity might play a significant role in the development of vascular and sensorineural changes that are associated with HAVs. Cellular stressors are one of the major regulators of mitochondrial cell number and function (Bailey et al. 2005), and vibration along with cold both serve as cellular stressors that might alter pathways associated



with mitochondrial function (Bailey et al. 2005; Hughes et al. 2009; Krajnack et al. 2006). Reducing exposure to those cellular stressors, either through use of anti-vibration devices (Dong et al. 2004; Paddan and Griffin 1997; Xu et al. 2011) or by diminishing time spent working with a vibrating hand tool, may block activation of cellular pathways that lead to physiological dysfunction associated with hand-transmitted vibration (Cherniack et al. 1990; Krajnack et al. 2014).

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## Disclaimer

“The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.”

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