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BRIEF COMMUNICATION

Light-Dark Patterns Mirroring Shift Work Accelerate Atherosclerosis and Promote Vulnerable Lesion Phenotypes

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BACKGROUND: Despite compelling epidemiological evidence that circadian disruption inherent to long-term shift work enhances atherosclerosis progression and vascular events, the underlying mechanisms remain poorly understood. A challenge to the use of mouse models for mechanistic and interventional studies involving light-dark patterns is that the spectral and absolute sensitivities of the murine and human circadian systems are very different, and light stimuli in nocturnal mice should be scaled to represent the sensitivities of the human circadian system.

METHODS AND RESULTS: We used calibrated devices to deliver to low-density lipoprotein receptor knockout mice light-dark patterns representative of that experienced by humans working day shifts or rotating shift schedules. Mice under day shifts were maintained under regular 12 hours of light and 12 hours of dark cycles. Mice under rotating shift schedules were subjected for 11 weeks to reversed light-dark patterns 4 days in a row per week, followed by 3 days of regular light-dark patterns. In both protocols the light phases consisted of monochromatic green light at an irradiance of 4 $\mu W/cm^2$. We found that the shift work paradigm disrupts the foam cell's molecular clock and increases endoplasmic reticulum stress and apoptosis. Lesions of mice under rotating shift schedules were larger and contained less prostabilizing fibrillar collagen and significantly increased areas of necrosis.

CONCLUSIONS: Low-density lipoprotein receptor knockout mice under light-dark patterns analogous to that experienced by rotating shift workers develop larger and more vulnerable plaques and may represent a valuable model for further mechanistic and/or interventional studies against the deleterious vascular effects of rotating shift work.

Key Words: atherosclerosis ■ circadian disruption ■ mice ■ shift work

The central clock synchronizes peripheral molecular clocks to the day-night cycle, and light-dark patterns reaching the retina are the primary entraining environmental cue to the 24-hour solar day.[1] Circadian disruption resulting from disrupted light-dark patterns, such as that experienced by shift workers, leads to metabolic and cardiovascular disorders, and compelling epidemiological data have linked shift work to a higher incidence of atherosclerosis-related vascular events such as myocardial infarction and stroke.[2] Unfortunately, as a 24-hour society we cannot eliminate shift work, and there is an urgent need to understand the underlying mechanisms and develop preventive strategies for at-risk populations. Studies in mice have significantly advanced our understanding of the pathogenesis of a vast number of diseases, and mice are a common preclinical model for translational research. However, knowledge from lighting interventions in mice cannot be easily translated to humans, because, although mice and humans share some of the same photoreceptors, their sensitivities to light are very different.

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In most species the sensitivity of the circadian system to light is generally irradiance dependent, that is, once above threshold, the higher the irradiances at the cornea, the greater the response until it reaches a saturation point. In humans exposure to the normal indoor light in office environments at night induces intermediate levels of melatonin suppression.[3] However, mice are 3000 to 10 000 times more sensitive to optical radiation than humans. Typical indoor light in laboratory facilities, for example, is above saturation for the mouse circadian system.[4] Moreover, the spectral sensitivity in nocturnal rodents peaks close to 500 nm, whereas the spectral sensitivity of acute melatonin suppression peaks at 460 nm in humans.[4] In agreement with other studies, previously we determined that an irradiance ≈0.1 μW/cm^2 of green light (peak λ=519 nm) is below threshold for activation of the murine circadian system, and saturation of the response was achieved at ≈10 μW/cm^2. An irradiance of 4 μW/cm^2 elicited an intermediate response that would be equivalent to that experienced by most humans in indoor work environments at night.[3–5] A subsequent study on glucose homeostasis demonstrated that mice subjected to rotating shift schedules (RSS) using 4 μW/cm^2 of green light develop glucose intolerance.[6] To investigate the effects on atherosclerosis development, here we have implemented a similar shift work paradigm on atherosclerosis-prone low-density lipoprotein receptor knockout mice. In line with epidemiological and longitudinal studies in human shift workers, we found that mice under RSS develop accelerated atherosclerosis and unstable plaque phenotypes, and identified endoplasmic reticulum (ER) stress-induced apoptosis as a putative underlying mechanism.

METHODS

The data and methods that support the findings of this study are available from the corresponding authors upon reasonable request.

Mice and Lighting Interventions

Low-density lipoprotein receptor knockout mice were purchased from the Jackson Laboratory (stock No: 002207). Female mice were used to allow comparisons to previous studies done in polychromatic light. Each experimental group consisted of 11 mice in order to have an 80% chance of detecting 30% differences between groups at $P<0.05$. At 8 weeks of age, mice were randomly divided into 2 groups and housed in 2 separated rooms. In 1 room the mice were exposed to a pattern of 12 hours of light and 12 hours of dark, similar to that experienced by a dayshift (DS) worker (Figure 1A). In the second room, mice were exposed to a reversed light-dark pattern similar to that experienced by a rotating-shift worker working 4 days in a row per week, followed by a light-dark pattern experienced by a dayshift worker (Figure 1A). These RSS were repeated for 11 consecutive weeks. Illumination for every cage interior was provided by software-regulated light emitting diodes that provided green irradiances (peak λ=519 nm) of 4 μW/cm^2 on the cage floor. This was the only biologically meaningful light stimulus for mice, whether lights were on during the day or during the night. Ambient room lighting was turned off during the entire experiment, and caretakers used red flashlights to navigate the room. Mice were fed a western diet (Harlan TD.88137) containing 21% wt/wt milk fat and 0.20% wt/wt cholesterol through the duration of the study. At 19 weeks of age mice were fasted overnight, a blood sample was obtained from the retro-orbital venous plexus under anesthesia with isoflurane, and mice were immediately euthanized by cervical dislocation. The mice in the RSS group were in their light phase the night just before euthanasia. All animal protocols were approved by the Rensselaer Polytechnic Institute Institutional Animal Care and Use Committee.

Plasma Lipids and Atheroma Analyses

Hearts were dissected by a parallel cut under the tip of the atria and immediately embedded in optimal cutting temperature compound and stored frozen at −80°C. Cryosections spanning the entire aortic sinus were stained with oil red O similarly as we previously described.[7] The size of atherosclerotic lesions was determined in 4 specific regions: (1) the region where the aortic valve contains 3 complete leaflets; (2) the region where the leaflets begin to emerge from the leaflet base; (3) the region where the leaflet base begins to bulge; and (4) the ascending aorta ≈200 μm from region II. Lesion composition in the regions with 3 complete leaflets was analyzed using Masson's trichrome and hematoxylin-eosin to quantify collagen and necrosis, respectively. Additional sections were immunostained with anti-Mac3 (Lamp2/Mac3 SC-19991 or Lamp2/Mac3-Alexa647 SC-19991AF647, Santa Cruz Biotechnology), anti-Bmal1 (NB100-2288, Novus Biologicals), and anti-GRP78/BiP (anti-glucose-regulated protein 78/binding immunoglobulin protein; ab21685, Abcam). A TUNEL (terminal deoxynucleotidal transferase–mediated biotin–deoxyuridine triphosphate nick-end labeling) assay (12156792910, Roche) was used to identify apoptotic cells. Plasma cholesterol and glucose were determined using Cholesterol E (Wako Diagnostics) and glucose hexokinase (Thermo Fisher Scientific), respectively.

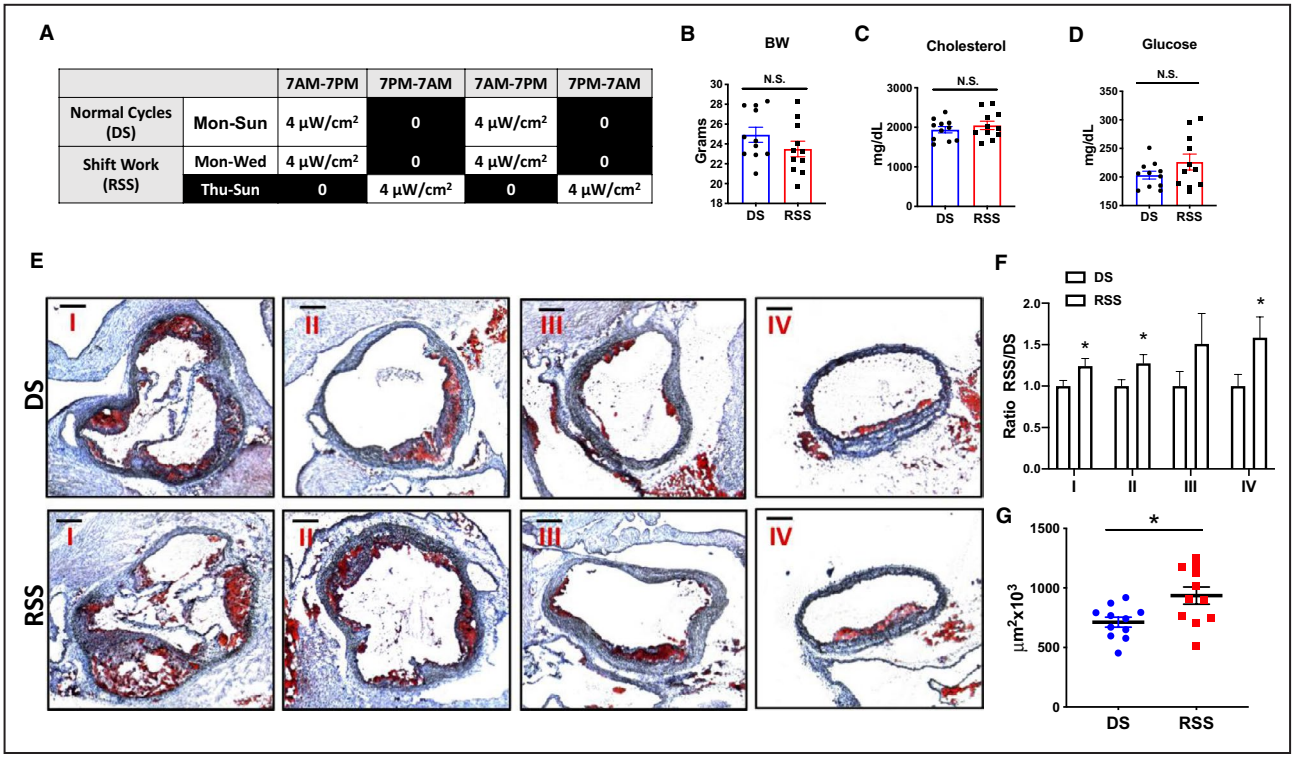

Figure 1. Light-dark patterns mirroring shift work accelerate atherosclerosis.
A, Diagram summarizing the lighting conditions experienced by mice maintained under regular day shifts (DS) and rotating shift schedules (RSS). **B** through **D**, Fasting body weight (BW) (**B**), plasma cholesterol (**C**), and plasma glucose (**D**) at the time of euthanization (19 weeks of age) were similar in mice maintained under DS and under RSS. **E**, Representative oil red O images of the aortic sinus in (I) the region where the aortic valve contains 3 complete leaflets; (II) the region where the leaflets begin to emerge from the leaflet base; (III) the region where the leaflet base begins to bulge; and (IV) the ascending aorta, ≈200 µm from region (II). **F**, Quantification lesion size in regions I to IV. **G**, Total lesion size determined by adding lesions in regions I to IV. Bar=200 µm. N=11 mice per group. Comparisons were performed using *t* test except for lesions in regions III and IV, which were analyzed using Mann Whitney *U*. N.S. indicates not significant. **P*<0.05.

Statistical Analysis

Data were plotted and analyzed using GraphPad Prism Software and are presented as mean± SEM. Analyses were performed using 2-tailed unpaired *t* test when the data followed a normal distribution or using Mann-Whitney *U* if the distribution was not normal.

RESULTS

Mice Under RSS Develop Larger Atherosclerotic Lesions and Vulnerable Phenotypes

There were no significant differences between groups in fasting body weight (24.93±0.76 g in DS versus 23.49±0.76 g in RSS), total plasma cholesterol (1938±80 mg/dL in DS versus 2048±105 mg/dL in RSS), and plasma glucose (203±23 mg/dL in DS versus 226±46 mg/dL in RSS) (Figure 1B through 1D). However, supporting that shift work-induced circadian disruption is also proatherogenic in mice, the atherosclerotic lesions at the aortic sinus were larger in mice under RSS than in DS (control) mice (712±42×10^3 µm^2 in DS versus 936±72×10^3 µm^2 in RSS). These differences were consistent through the entire sinus (Figure 1E through 1G). Interestingly, besides an increase in lesion size, the analysis of sections stained with oil red O used for lesion quantification suggested important morphological differences between groups, and lesions of mice under RSS seemed more necrotic. To better characterize lesion compositions, we stained sections consecutive to those stained with oil red O with hematoxylin-eosin, Masson's trichrome, and the macrophage marker Lamp2/Mac3. Quantification of acellular areas in sections stained with hematoxylin-eosin confirmed that necrotic cores were significantly larger (≈2-fold) in the RSS group (Figure 2A). In addition, Masson's trichrome showed a significant reduction in collagen deposition (Figure 2B). Mac3 staining showed that macrophages were the predominant cell type (>50% of lesion area), and macrophage content was similar in lesions of both experimental groups

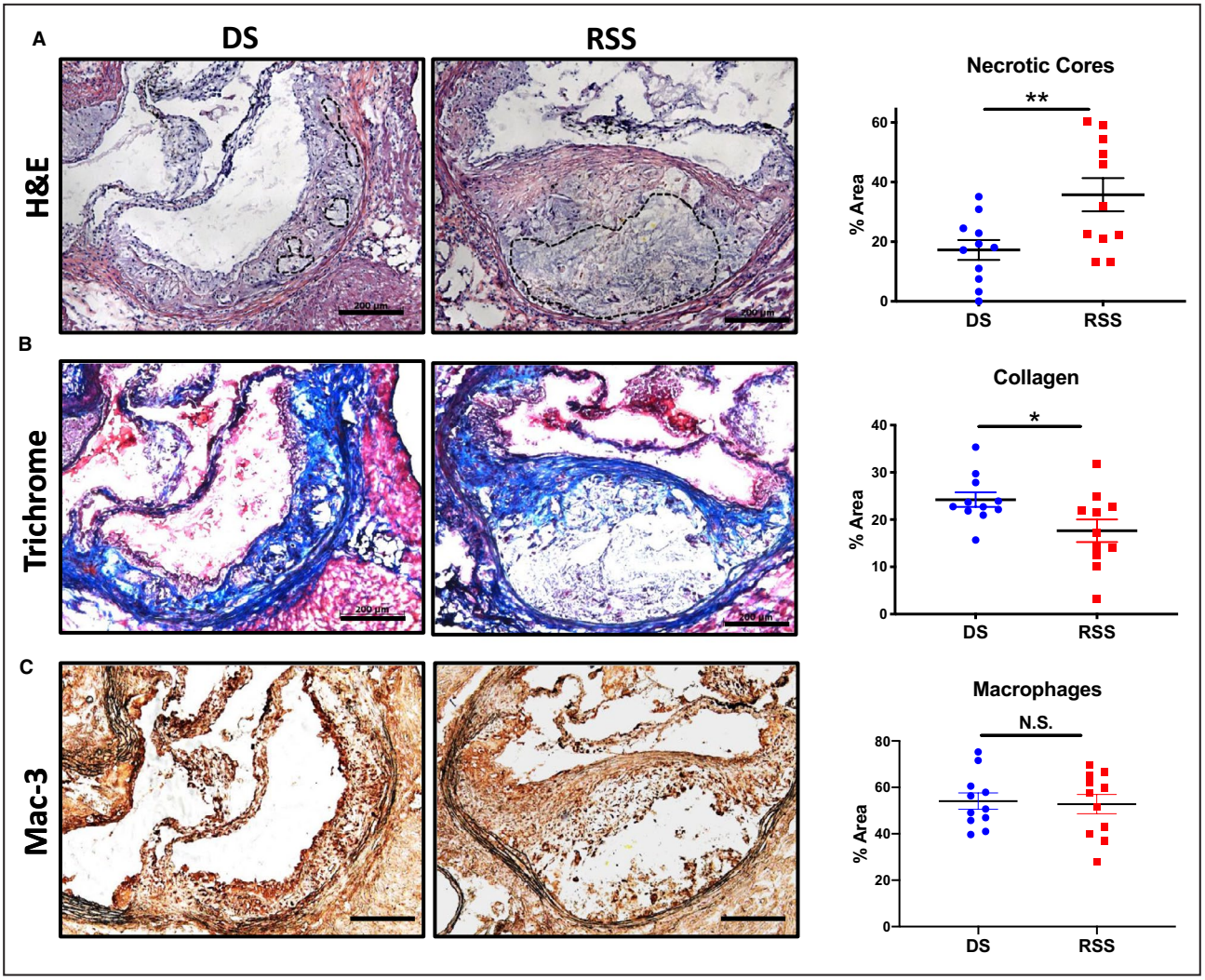

Figure 2. Mice under RSS develop vulnerable lesion phenotypes.
A, Representative images of hematoxylin-eosin staining with necrotic cores outlined by dotted line and quantification of areas of necrosis. **B**, Representative images of Masson's trichrome staining, which stains collagen in blue, and quantification of collagen-positive areas. **C**, Mac-3 immunostaining (brown) and quantification of Mac3-positive areas. DS indicates day shift; N.S., not significant; and RSS, rotating shift schedules. Bar=200 μm. N=11. **P*<0.05, ***P*<0.01, *t* test.

(Figure 2C). Thus, the RSS paradigm increased lesion size but also reduced collagen content and induced a marked increase in lesion necrosis.

Rotating Shift Schedules Disrupt the Foam Cell's Molecular Clock and Induce ER Stress and Increased Apoptosis

In other tissues, changes in light patterns have been shown to alter the expression of the molecular clock, including Bmal1, the core transcriptional activator of the circadian loop.[1] However, the effect of light on arterial foam cells remains poorly understood. As seen in Figure 3A, the intensity of Bmal1 immunostaining in Mac3-positive areas within atherosclerotic lesions was significantly reduced in lesions of mice under RSS, indicating that changes in light patterns also affect the molecular clock in foam cells. The development of necrosis within atheroma is normally caused by excessive foam cell apoptosis that exceeds the phagocytic clearance capacity of neighboring cells.[8] We carried out TUNEL staining, focusing the analysis on regions of lesions that were still cellular and mainly composed of macrophages. As seen in Figure 3B, the number of TUNEL-positive cells was ≈4-fold higher in mice subjected to RSS, indicating that circadian disruption from RSS paradigm increases foam cell apoptosis. ER stress is a main catalyzer of cell death within atheroma.[8] Prolonged circadian disruption often activates adaptive stress responses, and a number of studies, including our own in pancreatic ß-cells, have associated circadian rhythm perturbations and disruption of the molecular clock with increased ER stress.[9,10] We assessed the expression of GRP78/BiP, an ER

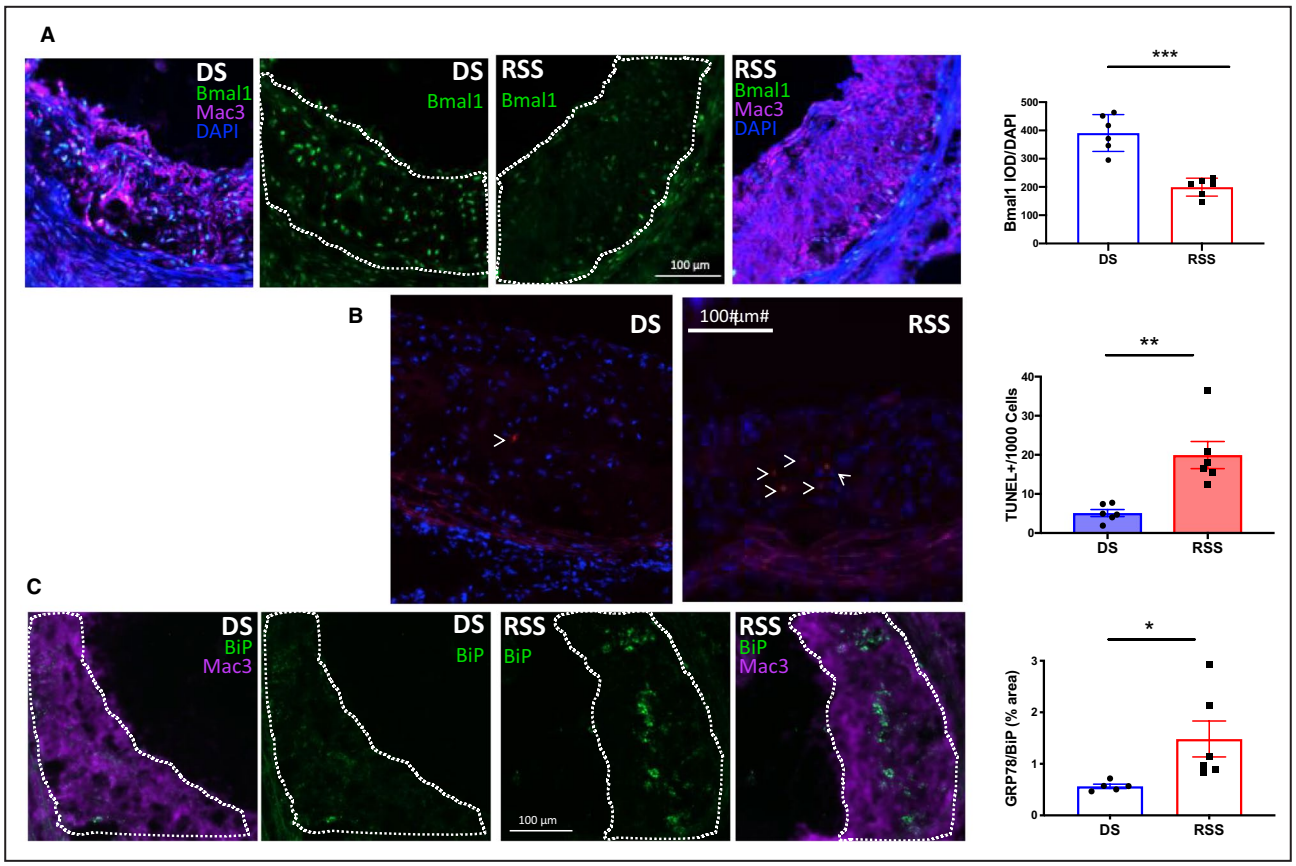

Figure 3. RSS induce ER stress and apoptosis.
A, Representative images of immunostaining for Bmal1 and coimmunostaining for Bmal1, Mac3, and 4′,6-diamidino-2-phenylindole (DAPI), and quantification of Bmal1 in macrophage-rich regions of lesions of mice maintained under DS (n=6) and RSS (n=6). **B**, Representative images of TUNEL (terminal deoxynucleotidal transferase–mediated biotin–deoxyuridine triphosphate nick-end labeling) staining (red nuclei, arrowheads) and quantification of TUNEL positive cells within lesions. N=6 per group. **C**, Representative images of immunostaining for GRP78/BiP and co-immunostaining for GRP78/BiP and Mac3, and quantification of BiP in macrophage-rich regions of lesions of mice maintained under DS (n=5) and RSS (n=6). BiP indicates binding immunoglobulin protein; DS, day shift; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; and RSS, rotating shift schedules. Bar=100 µm. **P*<0.05, ***P*<0.01, ****P*<0.001, *t* test.

chaperone commonly used to monitor ER stress, and found that levels were ≈3-fold higher in macrophage-rich regions within the plaques of mice in the RSS paradigm group (Figure 3C). Altogether, these data suggest that changes in light patterns disrupt the molecular clock in foam cells, leading to ER stress and enhanced necrosis.

DISCUSSION

In this study we maintained low-density lipoprotein receptor knockout mice under 12 hours of light and 12 hours of dark schedules (DS/control) or under RSS paradigm mirroring those experienced by shift workers. We found that the RSS paradigm accelerates atherosclerosis development, reduces fibrillar collagen, and increases ER stress, apoptosis, and areas of necrosis within lesions. To date, we were able to identify only 1 report on the effects of simulated shift work on atherosclerosis development in mice.[11] In this study, weekly alternating light-dark cycles using polychromatic light increased atherosclerosis by about 2-fold in female APOE*3-Leiden mice.[11] Given that the sensitivities to light of the human and murine circadian systems are very different, by demonstrating that irradiances that were previously calculated to exert an intermediate level of circadian disruption that mimics that experienced by human shift workers are sufficient to enhance atherogenesis, the present study validates and adds a critical layer of understanding on the effects of RSS on atherosclerosis development in mice.

In humans most ischemic vascular events are triggered by the disruption of a vulnerable atherosclerotic lesion, and an increase in lesion size is not necessarily paralleled by the development of unstable phenotypes.[12] Lesions with large necrotic cores, particularly when necrosis is accompanied

by a reduction in fibrillar collagen that confers tensile strength and reinforces the plaque structure, are considered more vulnerable.[12,13] These 2 parameters were impaired in plaques of mice under RSS, which contained significantly less collagen and a marked increase in necrosis, indicating that, as in humans, prolonged circadian disruption in mice results in structural defects that predispose to plaque rupture. Previously we found that a similar shift work paradigm induced glucose resistance.[6] In this study plasma glucose in mice under RSS was ≈15% higher, yet differences did not reach statistical significance. However, to study atherosclerosis mice were under prolonged western diet feeding, and given that fasting plasma glucose was relatively high in both experimental groups, suggesting a strong effect of the diet, it is difficult to determine whether the lack of significance in plasma glucose is real or related to low statistical power. Although the focus of our study was on the analysis of atheroma, changes in light-dark patterns induce neural and hormonal changes that can affect multiple organs and tissues.[1] It is possible that these systemic disturbances could have indirectly affected atherosclerosis development. Determining the relative contribution of systemic and local factors to the proatherogenic effects of shift work is a complex task that will require analysis of mouse models with tissue-specific clock deficiency and/or gain of function. However, to our knowledge this is the first study to prove that the molecular clock of foam cells is affected by light-induced circadian disruption. Together with a significant increase in ER stress and macrophage apoptosis in the lesions of the RSS paradigm group, these data suggest that arterial foam cells are circadian responsive and that circadian disruption such as that experienced by shift workers augments the ER stress response, eventually resulting in increased cell death and development of larger lesions with more vulnerable phenotypes.

Notwithstanding recent progress, there are still many mechanistic unknowns and an urgent need to develop preventive strategies for shift workers who are at higher risk of atherosclerotic vascular diseases. This study provides further evidence and mechanistic insight on the proatherogenic effects of circadian disruption stemming from shift work and identifies pathways that are amenable for therapeutic intervention. Prospective studies in humans have shown that shift work increases both the extent and the severity of coronary and carotid atherosclerosis.[14,15] By demonstrating that low-density lipoprotein receptor knockout mice under our shift work paradigm also develop larger lesions with more vulnerable phenotypes, this study identifies an experimental platform that may prove very valuable for future mechanistic and interventional studies.

ARTICLE INFORMATION

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Disclosures

None.

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