

Oxidative Stress Induces Endothelial Dysfunction and Endothelial Cell Damage in Rapid Eye Movement (REM) Sleep Deprivation Animal Model

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ABSTRACT

INTRODUCTION: Rapid eye movement (REM) sleep deprivation causes oxidative stress, leading to endothelial dysfunction, a predictor of cardiovascular disease. It is still unclear what causes endothelial dysfunction in patients with sleep disorder. This study evaluates the effects of REM sleep deprivation on the endothelium in the rat model of REM sleep deprivation. **MATERIALS AND METHODS:** Sprague-Dawley male rats (N=28) were divided into four groups (n=7); (1) free-moving control (FMC), (2) REM sleep deprivation (REMsd), (3) tank control (TC), and (4) sleep recovery (SR). The inverted flowerpot technique was utilised to develop REM sleep deprivation. Bodyweight gain (BWg), food consumption (Fc), and systolic blood pressure (SBP) were evaluated. The descending thoracic aorta was isolated to assess oxidative stress markers, in vitro functional study, and histomorphological examination. **RESULTS:** REM sleep deprivation showed a decrease in BWg significantly despite a significant increase in Fc, increased SBP, increased oxidative stress markers, caused endothelial dysfunction and endothelial cell damage. In REM sleep-deprived rats, there was a significant reduction in antioxidant markers, including total antioxidant capacity (TAC), superoxide dismutase (SOD) activity, catalase (CAT), and glutathione (GSH), while the levels of malondialdehyde (MDA) were significantly increased. The REM sleep-deprived rats displayed altered vascular function, including impaired vasorelaxation and hypercontractility. Histomorphology of the endothelium in REM sleep-deprived rats revealed features of endothelial damage. **CONCLUSION:** REM sleep deprivation is suggested to be linked to endothelial dysfunction due to endothelial damage. These changes are proposed to result from increased oxidative stress. Sleep recovery reduced the harmful effects following REM sleep deprivation.

Keywords

Oxidative stress, sleep deprivation, antioxidants, endothelial dysfunction, hypertension

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INTRODUCTION

Oxidative stress reflects a disequilibrium between antioxidants and reactive oxygen species (ROS).¹ ROS play important physiological and pathological roles; they include superoxide radicals (O₂^{•-}), hydrogen peroxide (H₂O₂), hydroxyl radicals (•OH), and singlet oxygen (¹O₂), all of which are primarily generated as metabolic by products.² Increased ROS production can harm important cellular components such as membranes, lipids, proteins, and deoxyribonucleic acid (DNA).³ Oxidative stress causes various diseases, including cardiovascular disease (CVD), diabetes, and cancer.⁴ To counteract oxidative

stress, the human body produces a variety of antioxidants, enzymatic and non-enzymatic components.⁵

Sleep has two stages; rapid eye movement (REM) and non-rapid eye movement (NREM). Previous studies on humans and animals had demonstrated the relationship between sleep deprivation and oxidative stress.^{6,7} The functions of sleep include muscle repair, hormone regulation, and energy recovery processes.¹ The function of REM sleep is not well understood, however, it has been linked to increased brain activity, random movements of

eyes, vivid dream, quiet muscle tone, and lessened homeostatic regulation of the body.⁸ It is proposed that oxidative stress generated during awakening is eliminated during sleep, implying that sleep serves as an antioxidant.⁹ Oxidative stress is linked to REM sleep deprivation associated with appetite behaviour and memory disturbance.¹⁰⁻¹² This has drawn the interest of researchers to study whether oxidative stress produced during REM sleep deprivation is linked to CVD risk factors.

Previous animal studies had shown the link between REM sleep deprivation and endothelial dysfunction, an early indicator of CVD development.¹³⁻¹⁵ Endothelial dysfunction can be defined as the reduced vasodilatory capacity or any changes that impact the vasoprotective homeostatic function of the endothelium.¹⁶ The dysfunction could be due to the damage to the vascular endothelium. Oxidative stress is known to induce endothelial dysfunction and endothelial cell damage.¹⁷ ROS have been shown to cause apoptosis in endothelial cells and increase monocyte adhesion, responsible for endothelial damage.¹⁸ REM sleep deprivation causes lipid peroxidation in the blood and aorta of rats.^{14,19} Moreover, oxidative stress impaired the endothelium-dependent vasorelaxation due to decreased protein expression of endothelial nitric oxide synthase (eNOS), which is essential for nitric oxide (NO) synthesis.¹⁵

Rodents, including rats and mice, are the preferred animal models in sleep research.²⁰ Various animal models of REM sleep deprivation were used, including platform technique (single, double or multiple), disk-over-water, gentle handling, and electrical stimulation.^{13-15,19,21-23} Each REM sleep deprivation technique has its advantages and disadvantages.²⁵ Platform techniques, commonly used inverted flowerpot, were widely used because the equipment is readily available, simple, and inexpensive.²⁵ However, multiple platforms techniques have been reported to cause social conflicts among the rats because many rats were placed in the tank with multiple platforms.²⁶ Other methods such as disk-over-water and electrical stimulation are invasive as surgical procedures needed to be done to implant the electrodes through the skull for electroencephalogram monitoring. This study determined the effects of REM sleep deprivation on

oxidative stress status and whether the oxidative stress affected the vascular endothelium.

MATERIALS AND METHODS

Animals

The study included twenty-eight (N=28) Sprague-Dawley (SD) male rats (8–10-week-old, 180-250 g), obtained from Animal Research and Service Centre (ARASC), Universiti Sains Malaysia, Malaysia. They were maintained in standard cages under temperature control (23 ± 1 °C), 60-70 per cent humidity and 12 hr light/12 hr dark cycles. Food pellets and tap water were available *ad libitum*. Ethical clearance was obtained from the Animal Ethics Committee USM (USM/IACUC/2018/(914)(114) that followed the institutional guidelines. Before the study commenced, animals were acclimatised for three days. After euthanasia with sodium pentobarbitone (Dose:100 mg/kg I.P.), isolation of the descending thoracic aorta was performed for oxidative stress parameters, *in vitro* functional study, and histomorphological study.

Experimental design

There were four study groups (each group, n=7); Group 1: free-moving control group (FMC), Group 2: REM sleep deprivation group (REMsd), Group 3: REM sleep deprivation group (TC), and Group 4: sleep recovery group (SR). In REMsd, TC and SR groups, rats were kept in a dry tank model for 72 hr individually before the procedure of REM sleep deprivation. The adaptation exposed and familiarised them with the environment of the glass tank. FMC rats were maintained individually in a standard cage for 72 hr before the experimental period.

Model of REM sleep deprivation

The inverted flowerpot technique was utilised as the REM sleep deprivation model in this study.^{14,19,27} Rats were put in this setting for 72 hr. In a glass tank (30 × 60 × 30 cm), two platforms of 6.5 cm diameter were placed, 1 cm above a pool of water. This technique is based on the

muscle atonia that will develop during REM sleep stage, which causes the rats to fall into the water, preventing the REM sleep component from developing. This method was validated by electroencephalogram (EEG) that REM sleep is completely abolished.²⁸ Water bottles and food pellets were placed on a grid on top of the chamber, available *ad libitum*. The tank was transparent, lightproof and covered with a wire mesh.

Tank control

The tank setting and experiment duration were comparable to the model of REMsd group, except the diameter of the platform was wider, 13.5 cm.¹⁴ This control group eliminates other non-specific factors such as isolation, immobilisation and aquatic environment that may impact the experiment results.^{19,27} NREM and sleep stages were well preserved when using this platform.

Sleep recovery

Rats were allowed to sleep for 72 hr following 72 hr of REM sleep deprivation. They were kept in standard cages singly. This group was developed to see if the harmful effects of REM sleep deprivation could be reduced by sleep recovery.

Physical changes parameters

Before and after the adaptation and experimental periods, food consumption (Fc) and body weight (BW) were measured. Systolic blood pressure (SBP) were monitored after the adaptation period and after the experimental period. The Fc was calculated as the difference in the consumption of food by rats normalised as grams/day/kilogram of body weight. The value was then taken to the 0.67 power ($\text{g/day/kg}^{0.67}$) to eliminate the differences in the metabolic rate among the rats.²⁹ SBP was taken from the tail (Tailcuff plethysmography, Labquip).

Oxidative stress markers measurement

The aorta was homogenised using a sonicator LWI-TEC-209 (Models S-450D). The sample was then centrifuged at

16,000 rpm for 15 min, temperature of 4 °C. After that, the supernatant was put into a 0.5 mL centrifuge tube. Enzyme-linked immunosorbent assay kits were used to measure the markers as followed the instructions by the manufacturer. The malondialdehyde (MDA), total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) levels were measured using Malondialdehyde Assay (Elabscience Biotechnology Inc.), Total Antioxidant Capacity (T-AOC) (Elabscience Biotechnology Inc.), Superoxide Dismutase Assay (Elabscience Biotechnology Inc), Catalase Assay (Bioassay Systems, California, USA), and Reduced Glutathione (GSH) Colorimetric Assay Kit (Elabscience Biotechnology Inc.) respectively.

***In vitro* functional study**

In vitro functional study was performed using a multichannel myograph (Danish Myo Technology Model 620M, Denmark). The aortas were cut into ring segments (3 mm each) and mounted horizontally between parallel hooks in four separate chambers filled with Krebs Hanseliet buffered solution and continuously aerated with carbogen (95% O₂, 5% CO₂) at 37 °C. Rings were initially loaded with basal tension, 1.50 g, through incremental application for 30 minutes, then equilibration for another 30 minutes before the studies began.²¹ During the experiment, the changes in isometric tension (in grams) were recorded by a LabChart programme (version 7.0) (ADIInstruments Ltd, Oxfordshire, United Kingdom).

The viability of the rings was assessed by exposure to KCl (40 mM) two times. Rings with contractile response greater than 50% maximal contraction by KCl were included for the following protocols.²¹ To determine endothelial-dependent relaxation response, rings were pre-contracted with phenylephrine (PE, 1 μM).¹³ A series of cumulative acetylcholine (ACh) were added once the tension plateau to PE developed. The relaxation response to ACh was presented as the percentage of the contractile response induced by PE. The equation is; $(\text{tension PE} - \text{tension ACh}) / \text{tension PE} \times 100\%$ (the changes of reactivity percentage to dosage ACh after 1 μM PE). The endothelium was considered functionally intact when the relaxation of greater than 80% of the pre-contracted

tone.³⁰ The contractile responses were assessed by cumulative addition of PE (1 nM-10 mM). The contractile responses were normalised to 40 mM KCl contractility.

Histomorphological study

The histomorphology of the aorta was examined using a scanning electron microscope (SEM). A 1 cm segment of the descending thoracic aorta was immediately fixed in McDowell-Trump for 24 hr at 4 °C. After that, it was washed with 0.1 M phosphate buffer three times for 10 minutes at a time. The sample was then fixed in 1% osmium tetroxide, incubated for 2 hr at 4 °C, washed with 0.1 M phosphate buffer for 10 minutes, and dehydrated in a series of ascending acetone concentrations. Finally, the sample was air dried and mounted onto nickel stubs and sputter-coated with gold and platinum before observing under SEM.

Data analysis

Data are represented as mean (standard deviation) and analysed using GraphPad Prism (version 8 for Windows, GraphPad Software, La Jolla, California, USA). Two-way repeated-measures ANOVA, with a within-subject factor of time (two levels, adaptation and experiment) were used to analyse data for physical parameters. One-way ANOVA, followed by post hoc Bonferroni test, was used to analyse oxidative stress parameters and maximum contractile response. Relaxant responses were represented as a mean reduction in tension from the induced precontraction \pm SEM. The contractile responses were represented by the mean increases in tension above the resting level (1.5 g) \pm SEM. The two-way repeated-measures ANOVA was used for both. A value of $p < 0.05$ was considered statistical significance.

RESULTS

Levels of physical parameters and oxidative stress markers following REM sleep deprivation

Table I represents the levels of physical parameters and oxidative stress markers. The results of physical changes

during the adaptation period were not significant. Fc levels in REMsd group increased significantly during the experiment compared to FMC, TC, and SR ($p < 0.05$) groups. In contrast, BWg levels were significantly decreased in REMsd group compared to other groups ($p < 0.001$). SBP levels in REMsd group were significantly increased compared to other groups ($p < 0.001$).

Levels of MDA increased significantly in REMsd group compared to FMC ($p < 0.01$), TC ($p < 0.01$), and SR ($p < 0.05$) groups. Levels of TAC were significantly decreased in REMsd group compared to FMC ($p < 0.001$), TC ($p < 0.001$), and SR ($p < 0.01$) groups. SOD activity was significantly decreased in REMsd group compared to FMC ($p < 0.001$), TC ($p < 0.01$), and SR ($p < 0.05$) groups. Meanwhile, levels of CAT were significantly decreased in REMsd group compared to FMC ($p < 0.01$), TC ($p < 0.05$), and SR ($p < 0.01$) groups. Levels of GSH decreased significantly in REMsd group compared to TC and SR ($p < 0.05$) groups, but not significantly different compared to FMC group.

Table I: Levels of physical parameters and oxidative stress markers in experimental groups during the experimental period.

Parameters	FMC (n=7)	REMSd (n=7)	TC (n=7)	SR (n=7)
Physical parameters				
Food consumption (g/day per kg ^{0.67})	17.40 (2.14) ^a	21.39 (2.39)	17.13 (3.35) ^a	17.46 (1.43) ^a
Body weight gain (g)	20.43 (4.35) ^c	-23.43 (23.89)	26.86 (9.46) ^c	28.86 (6.34) ^c
Systolic blood pressure (mmHg)	90.00 (5.11) ^c	144.0 (4.06)	95.29 (6.42) ^c	90.29 (7.73) ^c
Oxidative stress markers				
MDA (ng/ml)	16.57 (5.23) ^b	32.68 (10.78)	19.59 (1.22) ^b	17.21 (5.86) ^a
TAC (mmol/L)	0.74 (0.06) ^c	0.57 (0.06)	0.76 (0.06) ^c	0.70 (0.06) ^b
SOD (U/ml)	240.6 (12.77) ^c	120.0 (30.33)	163.8 (29.96) ^b	173.70 (18.25) ^a
CAT (ng/ml)	71.55 (17.19) ^b	25.80 (10.96)	63.15 (13.08) ^a	67.73 (20.09) ^b
GSH (µg/ml)	19.01 (0.63)	9.83 (5.17)	27.23 (8.70) ^a	28.82 (13.65) ^a

Data represent the mean (standard deviation), n=7 per group. ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ compared to REMsd group. Abbreviations: FMC: free-morning control group, REMsd: REM sleep deprivation group, TC: tank control group, SR: Sleep recovery group, malondialdehyde: MDA, total antioxidant capacity: TAC, superoxide dismutase: SOD, catalase: CAT, and glutathione: GSH.

Changes in endothelial function following REM sleep deprivation

The endothelium-dependent relaxation was impaired in the REMsd group, as shown in Figure 1. A pre-contracted aortic ring was subjected to an endothelium-dependent

relaxant agent, acetylcholine (ACh). A healthy endothelium was relaxed when exposed to ACh. The percentage (%) reduction in the contraction of the aortic ring from the REMsd group was less than 50% and significantly lower compared to other groups ($p < 0.05$, main effect). This indicates REM sleep deprivation impaired endothelial function.

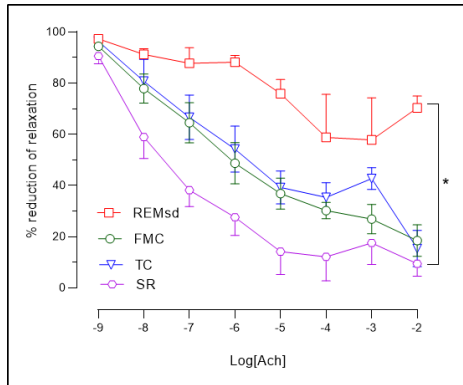


Figure 1: Effect of REM sleep deprivation on Acetylcholine (ACh)-induced vasorelaxation of the descending thoracic aortic rings from all groups, $n=4$. The results are expressed as a percentage (%) reduction in contraction when the aortic rings are exposed to serial concentrations of ACh. The percentage (%) reduction in the contraction of the aortic ring from the REMsd group was less than 50% and significantly lower compared to other groups ($*p < 0.05$, main effect). Abbreviations: FMC: free-moving control group, REMsd: REM sleep deprivation group, TC: tank control group, SR: Sleep recovery group

The contractile response of aortic rings from the REMsd group was significantly increased compared to other groups ($p < 0.05$ main effect) (Figure 2) and significant higher E_{max} ; REMsd 281.3 (44.97), FMC 140.1 (15.58), TC 91.31 (12.54), and SR 162.2 (7.80), as shown in Figure 3. Thus, REM sleep deprivation showed alteration in vascular function that was characterised by impaired vasorelaxation and hypercontractility with increased maximal contractile response to PE.

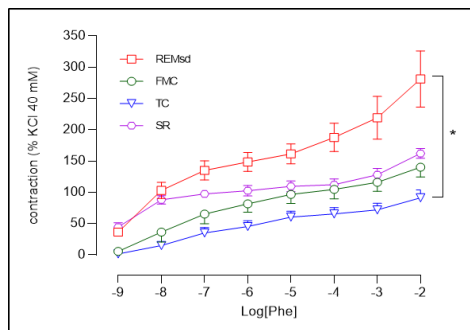


Figure 2: Contractile responses of descending thoracic aortic rings to phenylephrine from all groups, $n=4$. The contractile response of aortic rings from the REMsd group was significantly increased compared to other groups ($*p < 0.05$, main effect). Abbreviations: FMC: free-moving control group, REMsd: REM sleep deprivation group, TC: tank control group, SR: Sleep recovery group

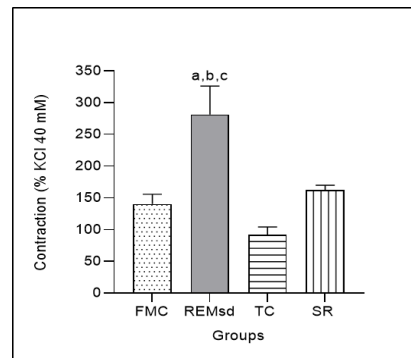


Figure 3: Contractile responses of the descending thoracic aorta to phenylephrine at 1 mM (E_{max}) in experimental groups. E_{max} was significantly higher in REMsd group 281.3 (44.97), compared to other groups: FMC 140.1 (15.58), TC 91.31 (12.54), and SR 162.2 (7.80). $*p < 0.05$ REMsd vs SR, $^b p < 0.01$ REMsd vs FMC, $^c p < 0.001$ REMsd vs TC. Abbreviations: FMC: free-moving control group, REMsd: REM sleep deprivation group, TC: tank control group, SR: Sleep recovery group

Histomorphological changes of the aorta following REM sleep deprivation

Figure 4 represents the transverse sections of the aorta from all study groups. In the FMC, TC, and SR groups, the endothelial surface appeared smooth, and the arrangement of endothelial cells were regular while the intercellular clefts were not dilated. However, in REMsd group, the endothelial surface was rough with dilated intercellular clefts. There was a distorted arrangement of the intercellular clefts, which had lost the parallel arrangement. Fibrin networks with red blood cells were also observed.

DISCUSSION

The pathogenesis of CVD has been linked to oxidative stress.³¹ Although endothelial dysfunction is one of the first recognisable signs of CVD development, the association between endothelial dysfunction with oxidative stress in REM sleep deprivation is poorly understood. We have demonstrated previously that endothelial dysfunction was associated with endothelial damage and decreased endothelial nitric oxide synthase expression in REM sleep-deprived rats.^{15,19} This study investigated the role of oxidative stress in the pathogenesis of endothelial dysfunction in the REM sleep-deprived animal model.

The REMsd group demonstrated a significant decrease in body weight gain despite increased food consumption

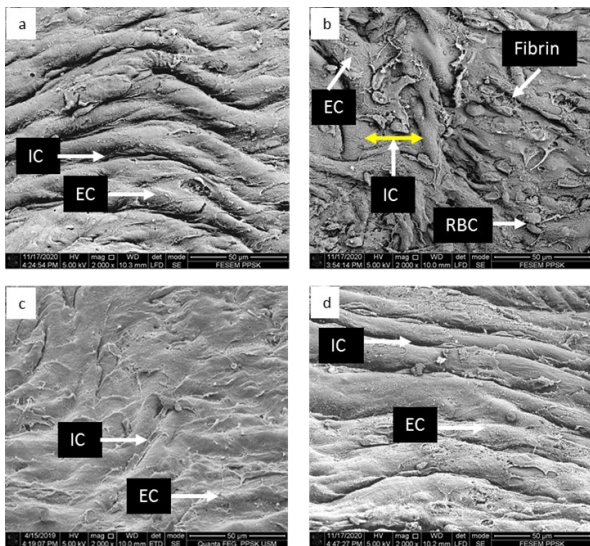


Figure 4: Transverse-sectional photomicrographs of descending thoracic aorta endothelium from (a) FMC, (b) REMsd, (c) TC, and (d) SR at 2000x magnification (Scanning Electron Microscope). The endothelial surface is regular in FMC, TC and SR groups. In REMsd group, endothelial surface appears rough, the arrangement of endothelial cells is irregular, and the intercellular clefts are dilated. Fibrin networks with red blood cells are also observed in the REMsd group. IC; intercellular cleft, EC; endothelial cell, RBC; red blood cell. Abbreviations: FMC: free-moving control group, REMsd: REM sleep deprivation group, TC: tank control group, SR: Sleep recovery group

(Fc). The findings were consistent with several previous studies that indicated the inverted flowerpot technique induced REM sleep deprivation in this study.^{14,19,27,32} A negative energy balance was demonstrated in rats following REM sleep deprivation, meaning that their expenditure exceeded their calorie intake. This condition could be caused by increased resting metabolic rate during REM sleep deprivation or increased energy expenditure during the experiment.^{29,33} In addition, exposure to the tank environment may cause anxiety and mobilisation of the animals, resulting in reduced body weight gain.³⁴ Stress may not be due to the water inside the tank as the bodyweight did not reduce in the tank control group. No reduction in the body weight gain was observed in the sleep recovery group; probably, the natural sleep pattern was restored following sleep recovery.³⁵

Hypercontractility, endothelial dysfunction, inflammation, calcification, and fibrosis have been attributed to hypertension-associated vascular damage.^{36,37} Because hypertension is linked to endothelial dysfunction, thus increased systolic blood pressure (SBP) in the REMsd group in this study suggests REM sleep deprivation may induce endothelial dysfunction.^{13,15} Oxidative stress and inflammation are involved in the pathogenesis of

endothelial dysfunction associated with hypertension.³³ Increased SBP was demonstrated in rats that underwent 72-hours, 96 hours, 114 hours, and five days of REM sleep deprivation compared to control.^{13,15,38-40}

In this study, reduced antioxidant markers in the REMsd group may enhance oxidative stress. For example, reduced SOD leads to increased superoxide anion, one of the major ROS. SOD, the essential antioxidant defence in all cells exposed to oxygen, catalyses the dismutation of superoxide anion into oxygen and hydrogen peroxide.⁷ Meanwhile, GSH is an antioxidant capable of scavenging ROS, including hydrogen peroxide and superoxide anion.⁴¹ ROS have been reported as mediators in the mechanism of vasoconstriction activated by urotensin-II, endothelin-1, and angiotensin II that modulate the vasomotor system.⁴² The bioavailability of nitric oxide (NO), a major vasodilator, is strongly influenced by redox status. Low levels of intracellular ROS play an essential role in the normal redox signalling that maintain vascular integrity and function. However, in the presence of increased ROS, it induced vascular dysfunction and remodelling via oxidative damage.⁴² Furthermore, our findings revealed REM sleep deprivation-induced lipid peroxidation indicated by a significant increase in MDA levels in the aorta of the REMsd group. MDA is one of the polyunsaturated fatty acids peroxidation products in the cells. In lipid peroxidation, oxidants attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs).⁴³ This process is initiated by various oxidants, including hydroxyl radicals, superoxide anion and hydrogen peroxide.⁴⁴

In vitro functional study showed that REM sleep deprivation leads to alteration of vascular function that was characterised by impaired vasorelaxation, hypercontractility, and increased maximal contractile response to phenylephrine. A previous study using a mouse model of chronic mild dyslipidaemia reported that hypercontractility of the aorta could be due to a direct consequence of decreased NO production.⁴⁵ Besides, high calcium levels in the smooth muscle cells of the blood vessels have been attributed to hypercontractile response to phenylephrine.⁴⁶

The histological changes of the endothelium were assessed using a scanning electron microscope (SEM). SEM is the best method available to evaluate endothelial integrity and ultrastructural examination as it permits large areas to be examined.⁴⁷ The endothelium from the REMsd group only demonstrated features of endothelial damage, which include a rough surface of the endothelium, irregular arrangement of endothelial cells with dilatation of intercellular clefts. An irregular arrangement of endothelial cells is a known feature of endothelial dysfunction.⁴⁸ ROS generated from REM sleep deprivation leads to intercellular gap formation and changes to the endothelial cell shape.⁴⁹ The fibrin formation may suggest endothelial damage as fibrin only forms when there is damage to the endothelium. Damage to the endothelium is the initiating factor for vascular diseases such as hypertension and atherosclerosis.⁴⁷

CONCLUSION

This study provides compelling evidence that oxidative stress is induced by REM sleep deprivation, causing functional and structural damage to the endothelium. Oxidative stress is an essential regulator of vascular changes related to hypertension in REM sleep deprivation. It is suggested that REM sleep deprivation is a cardiovascular risk factor.

DECLARATIONS OF INTEREST

No conflicts of interest.

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