

Original Article

Effect of REM sleep deprivation on gastric mucosa

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Abstract: Sleep deprivation is considered a risk factor for human health. Stomach lesions and formation of peptic ulcers have been reported in sleep-deprived animals. Ischemic damage of gastric mucosa can be monitored through impedance spectroscopy (IS). The aim of this study was to assess the effect of rapid eye movement sleep deprivation (REMD) on gastric mucosa through IS, confocal endomicroscopy (CEM), Light Microscopy (LM) and Scanning Electron Microscopy (SEM) in a rat model. There were 6 rats by group: control, REMD for 24 h, REMD for 48 h, and REMD for 96 h. Animals were raised in groups and sleep deprived in a water tank containing 3 platforms. IS measurements, CEM images, and gastric mucosal samples were collected at the end of REMD period. Fluorescein and acriflavine stains were applied for CEM analysis. Then biopsies were processed by standard histological techniques. Impedance and fluorophore infiltrations increased along with REMD time, showing the greatest changes at 48 h, indicating an inflammation process. LM and SEM showed epithelial erosion zones with focal distribution are exposed, which denote epithelial cell loss caused by lethal damage. ANOVA test was used to evaluate variations between groups. Significant changes were observed for all impedance parameters among groups ($P < 0.05$). At 96 h there is a mucosal epithelium showing ischemia adaptation after sleep deprivation. The results suggest that there is an effect in gastric mucosa caused by REMD, showing the initial phases of the acute inflammatory response.

Keywords: Gastric mucosa, bioimpedance, REM deprivation, confocal endomicroscopy, gastropathy

Introduction

Sleep is a biological requirement for survival, allowing for physical and mental recovery, especially after sickness. On the other hand, sleep deprivation is considered a risk factor for human health with no specific impairments defined [1]. It has been associated with nutritional and metabolic abnormalities, host defense failure and systemic bacterial infection [2-4]. Significant attention has been given to the effects of sleep loss on physiology [5], gene expression [6], immune function [7, 8], and stress response [9, 10]. The most prominent features of sleep deprivation in rats are increased food intake with progressive weight loss, thermoregulatory changes, debilitated appearance, and lesions on the tail and plantar surfaces [11]. Furthermore, chronic sleep restriction or sleep deprivation can limit blood delivery, leading to metabolic deficits with the

potential for neural trauma [12]. Results of studies on the effects of sleep deprivation in animals and humans include alterations in autonomic regulation of the heart [13], increase in arterial blood pressure [14], the risk of obesity, diabetes and cardiovascular disease increases [15]. Moreover, sleep deprivation was associated with the pathophysiology of some cardiovascular events [16].

The stomach is an organ predisposed to stress showing injury by a variety of stressors such as sepsis, burn injury, trauma, and multiple organ failure [17, 18]. Stomach injuries have been reported in sleep deprived animals [19], and Levenstein [20] reported peptic ulcer formation as a consequence of stress and sleep deprivation. Pathogenesis of stress-induced gastric lesions includes alterations of gastric luminal factors, gastric mucosal blood flow, gastric motility, superoxide generation, cell pro-

liferation [17], and the secretion of some gastrointestinal hormones that modulate gastric acid secretion and function [21]. However, as was reported by Guo et al [21], the pathogenesis of stress-induced gastropathy is complex and changes with different forms of stress. Mucosal ischemia, which weakens gastric mucosal defense and repair, as well as luminal acid, which plays an important role in the formation of gastric erosive lesions, are two key factors within the multifactorial pathophysiology of stress-related gastropathy [21, 22]. It has been reported that partial sleep deprivation (PSD) stresses the gastric mucosa and activates an adaptative response [17]. In addition, PSD may be a risk factor for integrity of the gastric mucosa [21]; however, the effect of REMD on gastric tissue has not been recently studied.

Bioimpedance in tissues has been studied for many years for multiple applications [23]. Impedance spectroscopy is the study of the passive electrical properties of biological tissues as a function of frequency. These properties are determined by the quantification of the tissue electrical response to the injection of an electrical current in a frequency range. The tissue acts as an electrical circuit composed by resistors, capacitors and inductors. The extracellular (EC) medium can be considered as an ionic solution. The intracellular (IC) space has similar ionic concentration to the concentration of the EC medium. The impedance of the IC and EC medium has conductive properties that provide the resistance of the tissue. The cell membrane separates the EC and IC, and control the chemical species exchange. The bilayer lipid membrane can be considered as an isolating material, that together with the IC and EC space behave as a capacitance, which store energy [23, 24]. Our group designed and constructed an impedance monitor to study the progression of ischemic injury in gastric mucosa with frequencies between 215 Hz and 1 MHz [25]. The gastric impedance monitor includes an impedance spectrometry probe and a nasogastric tube (ISP/NGT) [26], which has an array of four electrodes for impedance acquisition. The system displays impedance spectra average every ten minutes. This is a low cost instrument, easy to use, and it has the advantage to facilitate on line monitoring. Since different tissues exhibit different electrical parameters, bioimpedance can be used to characterize a specific tissue, as gastric mucosa. We development a mathe-

tical model based on Cole equation to characterize gastric tissue bioimpedance with some parameters [27]. We proposed that central reactance at low frequency X_L was the principal parameter that reflects gastric tissue edema caused by prolonged ischemia; furthermore, it only appears in live tissues. The gastric impedance monitor has been tested for more than 12 years by our research group, providing reproducible measurements under experimental and clinical conditions [28]. Also, we reported that bioimpedance increases during ischemia, and founded general alterations in cellular and tissue integrity by confocal endomicroscopy and light microscopy in an animal model [29]. This device could be used to evaluate the effect of sleep restriction on gastric tissue, and can be related to some microscopy techniques.

The objective of this study was to evaluate and quantify the effect of REMD on the gastric mucosa of rats, using impedance spectroscopy, confocal endomicroscopy, Light Microscopy (LM) and Scanning Electron Microscopy (SEM).

Material and methods

Animals

Adult male Wistar rats weighting 300-400 g obtained from the Animal Facility of the Autonomous Metropolitan University were used in this study. Animals were raised and maintained at in a controlled environment on a 12 h light/dark cycle. Food and water were available ad lib. All procedures used in the study followed international guidelines for the care and use of research animals. The Ethical Research Committee of the National Center for Medical Instrumentation and Imaging approved the experimental protocol.

Experimental design

Six rats were randomly assigned to each different experimental group: control (animals housed individually in standard, dry cages in the animal housing facility), REMD for 24 h (REMD24), REMD for 48 h (REMD48), and REMD for 96 h (REMD96).

REMD

REM sleep deprivation using the island technique is a stressful procedure that also induces

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a loss of slow wave sleep. The multiple platform technique was used [28] in order to reduce the stress associated with isolation, movement restriction, wetness, and muscle fatigue [30-34]. Furthermore, animals were raised in groups and sleep deprived along with their peers. As previously described, when the group has social stability, the release of ACTH and corticosterone due to stress, is significantly reduced [35]. Briefly, the REMD rats were placed in a water tank (70 × 50 × 45 cm) containing 3 platforms 7.6 cm in diameter and 5 cm high. The tank was filled with water to a level of 3.8 cm. The water tank was cleaned on a daily basis, during which time rats were placed in standard, dry holding cages and maintained in the wake state.

Collection of gastric measurements and biopsies

Under a 1.5 mL of an intraperitoneal anesthetic cocktail (0.05 mL ketamine -0.25 mL propi-
onilpromazine -0.1 mL xylazine, in 0.6 mL saline solution per mL), rats were attached on a dissection setting. Then 10 µL/g of fluorescein (Alcon Pharma, Novartis Pharmaceutical, Mexico) was injected in the tail vein. Afterwards the abdomen was opened and the stomach was exposed, and a 1 cm incision was made along the greater curvature. Then impedance measurements were taken during 10 minutes, a collection of endomicroscopy images was stored, and finally biopsies were obtained for LM and SEM analysis. For topical staining, a few drops of a 0.02% solution of acriflavine (Merck KGaA, Darmstadt, Germany) in saline were spreaded on the tissue surface, and excess dye was cleaned with a phosphate-buffered saline [29]. Once the experiment was concluded animals were gently sacrificed, in accordance with Mexican Official Standard (NOM-033-ZOO-1995).

Impedance spectra acquisition

The gastric impedance monitor meets quality standards, and includes the following parts: the impedance spectrometry probe and a nasogastric tube (ISP/NGT), the impedance spectrometer used to inject a current and to record the voltage of the tissue, and the computer with custom algorithms that we designed to process the resulting data to generate spectra automatically every 10 minutes. The ISP/NGT is a silicon

tube with four Ag/AgCl electrodes positioned at the distal tip, and it is connected to the impedance spectrometer. Impedance is the relation between the input voltage and the input current for a particular frequency. The resistance is the opposition to the flow of an alternating electrical current that runs through the two external electrodes. The two internal electrodes measure the magnitude and the phase shift between the voltage and the current to calculate resistance and reactance (components of the impedance).

The ion channels are porous structures that allow the flow of ions from outside to inside the cell or viceversa. The gap junctions allow the flow of ions from one cell to another one. The ion pumps are structures that force ions to flow through the membrane. All these structures are responsible of maintaining the hydrostatic cellular pressure, and generate a DC voltage across the membrane. Under ischemia these structures failure and yield to cellular edema, increasing resistance and reactance.

Bioimpedance spectra were handled to estimate central resistance and reactance at low frequencies (R_L and X_L respectively), and central resistance and reactance at high frequencies (R_H and X_H respectively), as the distinctive gastric impedance parameters of interest [27].

Confocal endomicroscopy images acquisition

Six sets of confocal endomicroscopy images were obtained for each animal. A confocal endomicroscopy EC-3870CIFK; Pentax, Tokyo, Japan was used for in vivo microscopic imaging of the gastric mucosa without the collection of biopsies, using intravenous fluorescein sodium 10% and topical acriflavine for labeling cytoplasm and nucleus respectively. The laser light and the detection system are aligned in the same focal plane. The blue laser light is focused at a selected depth in the gastric mucosa and then the reflected light is refocused on the detection system by the same lens. Only the fluorescing light within the focal plane of interest can be sensed, which improve the image quality and resolution. This technique allows serial cellular images of 500 × 500 µm in size with a resolution of 7 µm. The system has a depth controller from the mucosal surface down to a depth of 50 µm to clearly appreciate vasculature and cellular architecture.

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Table 1. Bioimpedance parameters: R_L (central resistance at low frequencies), X_L (central reactance at low frequencies), R_H (central resistance at high frequencies), and X_H (central reactance at high frequencies) for control and REMD groups. Data are presented as mean \pm SD. ANOVA was performed for each parameter

Impedance Parameters	Control Group	REMD24 Group	REMD48 Group	REMD96 Group	p value
R_L [Ohms]	68.31 \pm 12.28	83.51 \pm 17.52	108.47 \pm 7.70	93.54 \pm 15.63	< 0.01 ^a
R_H [Ohms]	43.33 \pm 3.85	45.00 \pm 8.38	55.67 \pm 4.56	44.09 \pm 9.82	< 0.01 ^a
X_L [-jOhms]	8.00 \pm 1.50	12.77 \pm 4.28	16.13 \pm 7.09	11.57 \pm 4.11	< 0.01 ^a
X_H [-jOhms]	9.87 \pm 1.54	14.17 \pm 5.72	16.00 \pm 2.78	18.88 \pm 3.71	< 0.01 ^a

^aStatistically significant ($P < 0.01$).

Confocal endomicroscopy image equalization, filtering, and binarization processing were used to improve image quality. The number of imaging fluorophore infiltrations was estimated, which suggest edema modifications, and they were averaged for each group. Analysis of confocal images was performed using a particular software proposed by our group [29].

Tissue collection and histological analysis

After imaging acquisition, pyloric antrum tissue samples were taken and fixed in formalin (10% buffered) [36]. The tissues were dehydrated and embedded in paraffin wax. Paraffin-embedded section of 5 μ m were stained with hematoxylin and eosin using standard histological techniques [37]. The tissue sections were analyzed under a clear field light microscope (Axioskope II, Carl Zeiss) and image analyzer (Axiovision 4.8, Carl Zeiss). Thirty microscopic fields per animal were chosen at random. Micrographs were taken with an AxioCam MRc5 (Carl Zeiss).

Eight fragments of the pyloric antrum tissue were fixed in 2.5% glutaraldehyde with 4% paraformaldehyde in a phosphate buffer solution (0.16 M at pH 7.2) and refrigerated for two hours. The fragments (Fg) were processed using the SEM technique. First, the Fg were rinsed in a phosphate buffer solution, post-fixed for 4 hours with 1% osmium tetroxide in a phosphate buffer solution. Fg were rinsed three times in a phosphate buffer solution and then passed through a graded series of 30%, 50%, 70%, 80%, 90%, 96% and two changes of 100% ethanol [38]. Fg were dried using the critical point method (CO₂ as the transition solvent). They were mounted on aluminum stubs and sputter-coated with gold, using SCD 050

ionizer [39]. The material was observed through a JEOL Scanning Electron Microscope at 10 Kv.

Statistical analysis

Statistical differences in impedance parameters between groups were determined with a one-way ANOVA followed by the post hoc Bonferroni test, or unpaired two-tailed Student's t-test (SPSS statistical package, version 12.0, SPSS Inc, Chicago, IL). A total of 280 to 300 endomicroscopy gray-scale images were collected for each group. The percentage of fluorophore infiltration was calculated for each original image, and also for the brightness transformation image. Matlab® software was used for image analysis. Data are showed as mean \pm standard deviation (SD). $P < 0.01$ indicates significant differences.

Results

Significant changes were observed for all impedance parameters among groups (**Table 1**). Statistically significant changes were observed for almost all pairs of experimental groups, except for R_H between control-REMD96 groups, R_H and X_L between REMD24-REMD96 groups and X_H between REMD24-REMD48 groups.

Resistance parameters increased mainly for the REMD48 group, indicating an inflammation process. Control and REMD24 groups have almost the same values, whereas REMD96 exhibits a decrease compared with the control and REMD24 groups. An increase in all bioimpedance parameters, especially in the REMD48 group, should be considered an indicator of gastric mucosal damage.

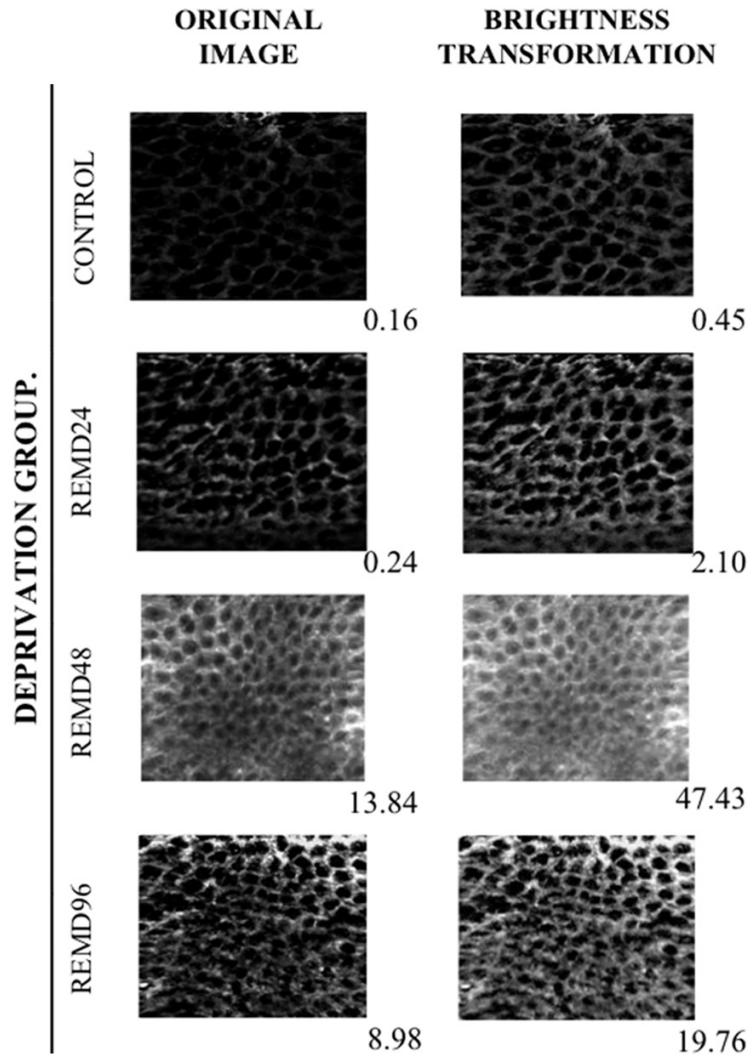


Figure 1. Confocal image processing for control and sleep deprivation (REMD) groups. Numbers at the right of each image represents the percentage of fluorophore infiltration. The left column presents original confocal images for each group, and the right column presents brightness transformation for each original image.

The number of fluorophore infiltrations increased with REMD time up to 48 h of REMD, and then decreased at 96 h (**Figure 1**). Impedance parameters increment in the REMD48 group is related to more fluorophore infiltrations.

The control group shows well delineated foveolas, with an appearance of uniform integrity. At 24 h, there is a loss in foveola delineation, as well as in tissue integrity in some areas. At 48 h, foveola delineation is preserved in some regions, with an increase in tissue filling interfoveolar spaces, as well as in superficial density,

indicating greater fluorophore infiltrations. At 96 h, there is reduction of area and of foveolar delineation, as well as a loss of tissue integrity in some regions.

The gastric mucosa of sleep-deprived subjects exhibits a change sequence, proper of the initial phases of the acute inflammatory response, as can be seen in the photomicrographs (**Figures 2 and 3**). In the REMD24 group, vasodilation and hyperemia by engorgement of mucosal capillaries with blood is observed. In the REMD48 group, epithelial erosion zones with focal distribution are exposed, which denote epithelial cell loss caused by lethal damage; moreover, they show edema. In the REMD96 group, epithelial zones with structural differences are displayed: there is epithelial thickening with surface cell flattening. At 96 h, basophilia in gastric gland base is present which suggests greater acid secretion.

Scanning electron micrographs showed differences between control and all experimental groups in surface mucous cells (MC), foveolae or gastric pits (F), and secretion vesicles (SV). At 24 h, the F are full of SV, and MC are normal. At 48 h, gastric superficial layers can be observed (deep layers are not observed in the gastric wall), also, focal ulcers with cellular detachment and erythrocytes are present. At 96 h, the gastric pit is wide with scant secretion vesicles (**Figure 4**).

Discussion

We propose that bioimpedance and, particularly, gastric reactance is an early indicator of ischemia and damage; and we developed some studies that confirm the prognostic and diag-

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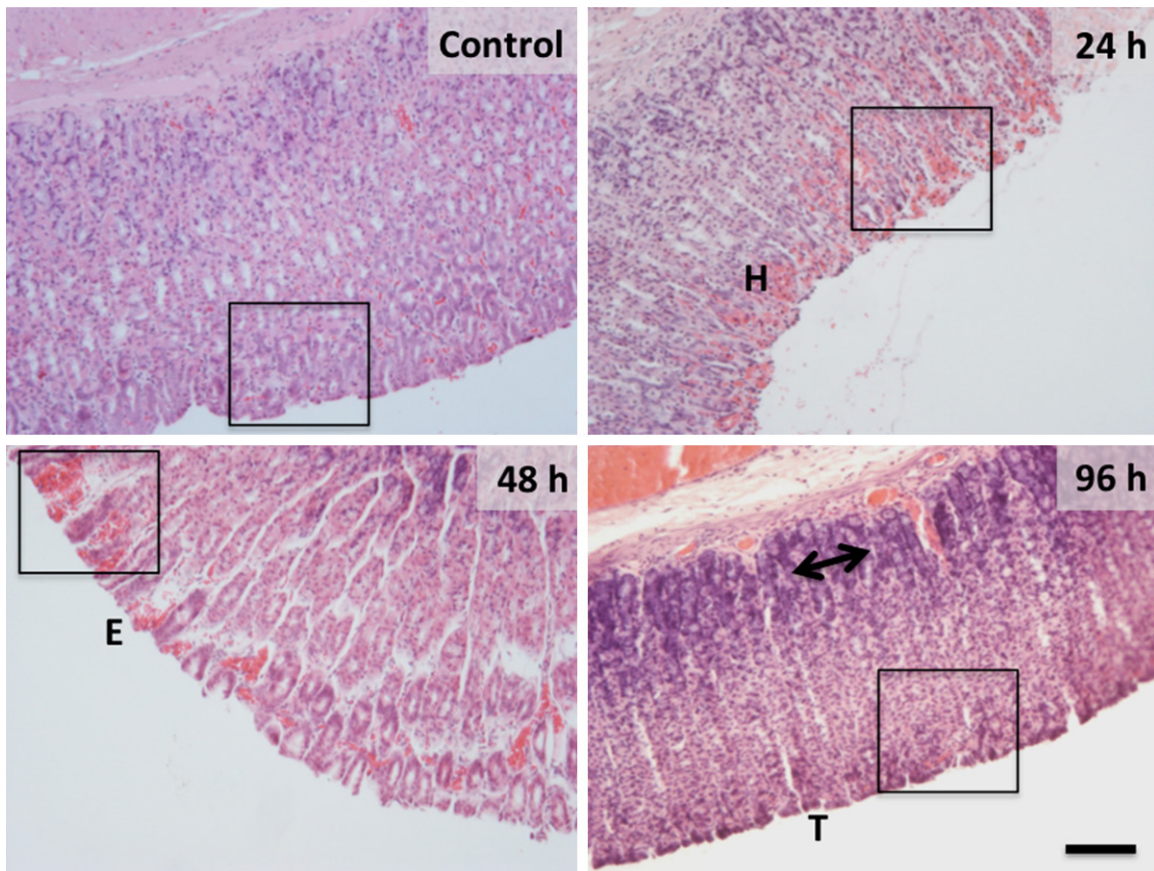


Figure 2. These photomicrographs show the histology of the longitudinal sections of rat gastric mucosa. Important changes can be observed between control and experimental groups (24 h, 48 h and 96 h): vasodilation and hyperemia (H) at 24 h, slight erosion (E) of epithelium at 48 h, and epithelial thickening (T) at 96 h. In this group basophilia at the gastric gland base (reversible arrow) can be seen. Framed regions were enlarged to show them in the next figure (H-E, bar 100 μ m).

nostic value of these measurements [28, 40, 41]. Nevertheless, this is the first time that bio-impedance and gastric tissue damage are linked to sleep deprivation.

This is a new animal model designed to quantify gastric injury caused by sleep deprivation using confocal endomicroscopy imaging, and to evaluate its relationship with impedance parameters, light microscopy and scanning electron microscopy images.

According to Suchecki et al [35], using rats that were bred together reduces the activation of the hypothalamus-hypophysis-adrenals axis. Tufik and his colleagues employed the method of EEG monitoring to discover that modified multiple platform method used to create sleep restriction led to a complete suppression of REM sleep during the 18 h sleep deprivation

period each day, which persisted throughout the whole restriction period [42].

From the pioneering papers of Jouvet et al [43], Cohen et al [44], Mendelson et al [45], the search for an optimal control group for REM deprived animals has produced several proposals. Among them, the large platform was proposed as an adequate control for the stressful situation inherent to the island technique. However, studies performing polygraphic recordings showed that during the first 3 days, the animals lose comparable amounts of REM in both the small and the large platform. In addition, the loss of slow wave sleep is similar in the two conditions. Thus, for a REM deprivation experiment lasting 4 days, the large platform is not a suitable control. According to the results of some other experiments with only one control group due to similar outcomes,

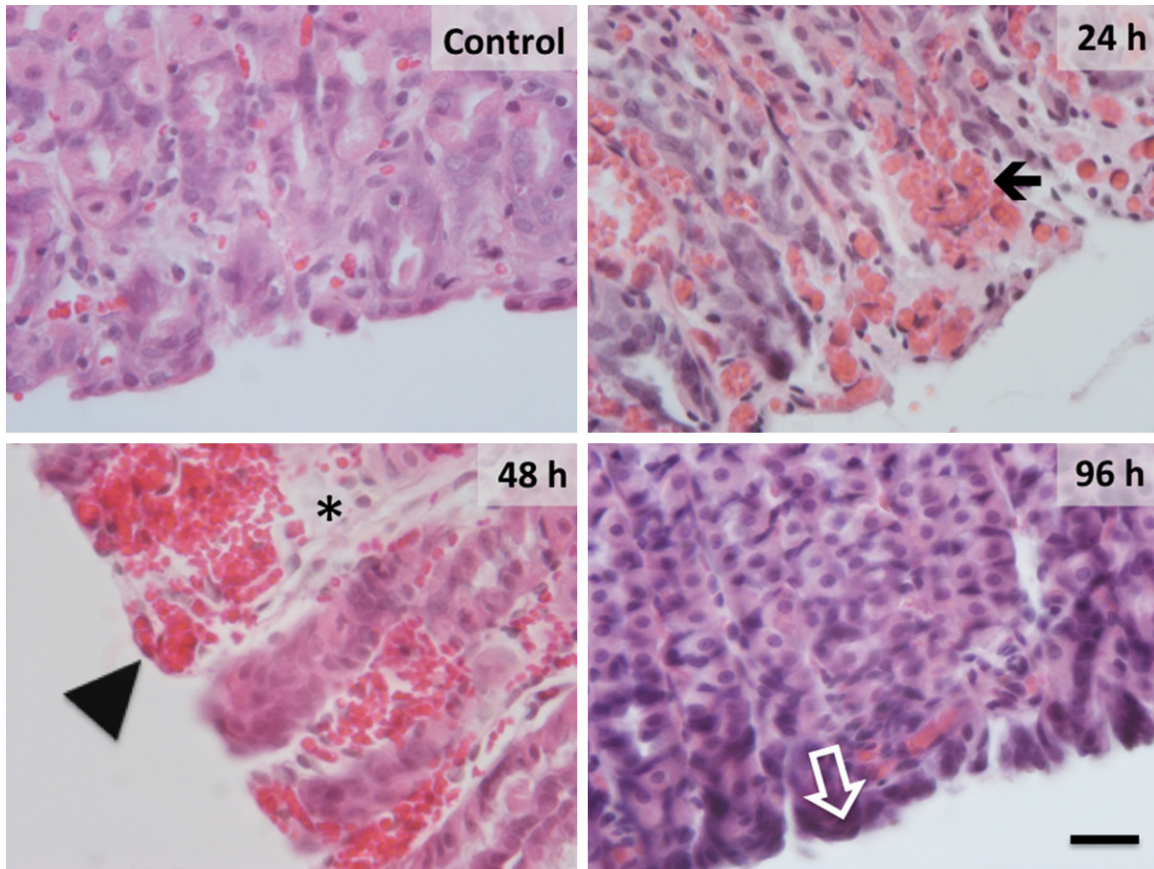


Figure 3. When the control group is compared with the REMD24 group (24 h) there is evident vascular dilation (arrow) in mucosal capillaries; at 48 h there is epithelial slimming (arrow head) and edema (*); 96 h shows normal vascular blood and surface cell flattening (unfilled arrow) (H-E, bar 25 μ m).

there was only one control group in the study (cage control).

Furthermore, Machado et al [46] did a polygraphic recordings using the multiple platform technique, using both small and large platforms. They reported a total loss of REM sleep during the four days of deprivation, as well as a significant decrease of REM sleep in the large platform group. Thus, there is no doubt about the effectiveness of the small platform to totally suppress REM sleep but the stress component of the technique and the participation of slow wave sleep loss remains to be elucidated.

As can be seen in **Table 1**, impedance parameters increased up until 48 h and then there is partial recovery at 96 h. The changes in central resistance at low frequencies (R_L) achieved in this work are similar to those reported previously in a gastric ischemia model [29]. All REMD groups show an increment that reflects tissue

edema, especially at 48 h, which exhibits almost 75% of increment with respect to the control group.

The changes in central reactance at low frequencies (X_L), which is the principal parameter that reflects gastric tissue edema caused by prolonged ischemia, shows a 100% increase in REMD48 with respect to the control group. The biological tissues, and specially the gastric mucosa, exhibit two frequency regions for the dielectric properties of the tissue. In particular, the β dispersion region is associated to the dielectric properties of the cell membranes, and their interactions with the intracellular and extracellular electrolytes. In this study, we describe gastric impedance changes observed at frequencies between 215 Hz and 1 MHz, which are included in the β dispersion region. Beneath ischemia, metabolic product accumulation is produced, ion permeability is reduced, pumps are closed, and cells are swelled. Those

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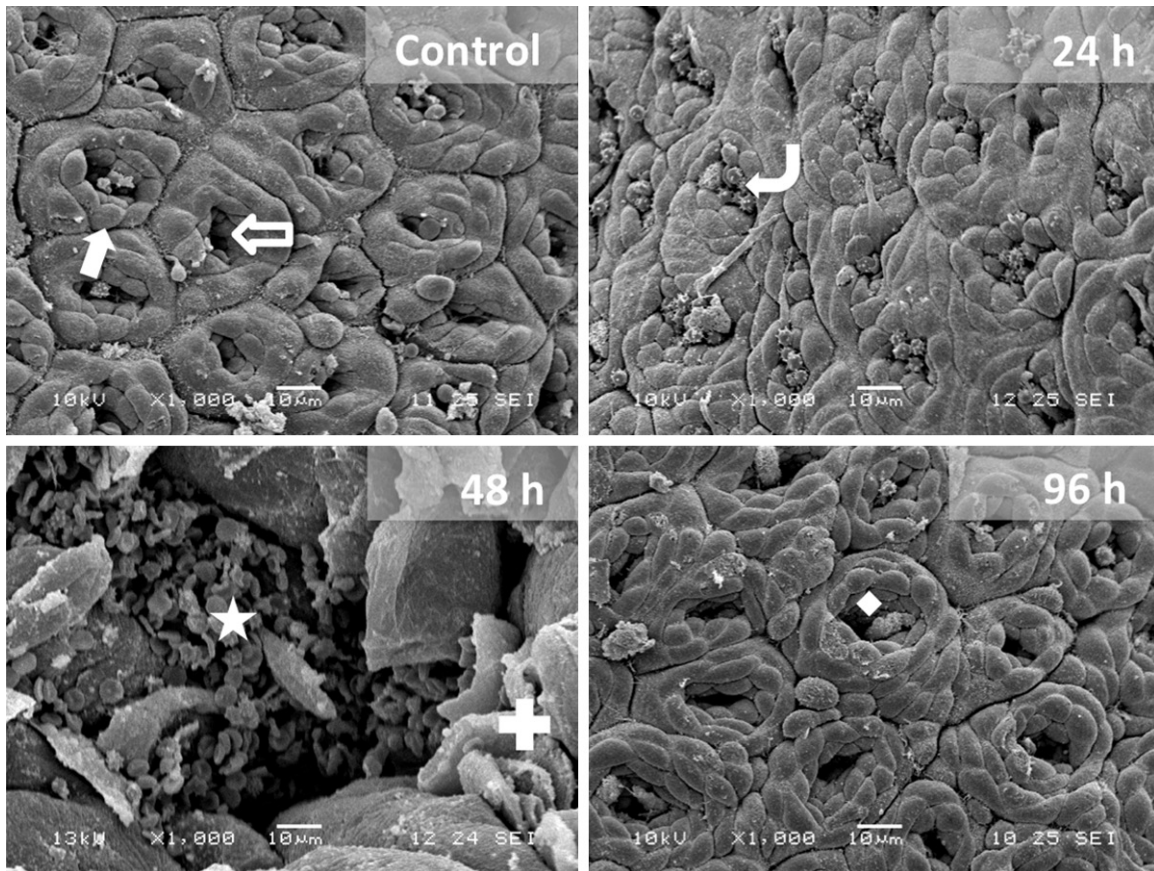


Figure 4. Scanning electron micrographs showing the control and experimental groups (24 h, 48 h, and 96 h) of rats. Mucous cells (arrow) and, foveolae (unfilled arrow) reveal some interesting differences: (a) In 24 h the gastric pits are full of secretion vesicles (curved arrow); (b) At 48 h we can see focal gastric ulcers with cellular detachment + and erythrocytes ★; at 96 h the gastric pit is wide with scant secretion vesicles (◆).

changes increase resistance and reactance, especially at low frequencies.

According with confocal endomicroscopy imaging processing, we developed an algorithm to indirectly quantify cell swelling by fluorophore infiltration accumulation. Nuclear staining was diminished in necrotic areas that were generated in the REMD48 group. The confocal endomicroscopy technique permits the identification of histological features of lamina propria, blood vessels and cells, basement membrane integrity, inflammatory cells, and necrotic areas through a “targeted” biopsy. However, some constraints of this technology has been reported, as the incapacity to generate a tissue record for extensive molecular categorization and analysis, suboptimal contrast agents, slight fields of view and shallow penetration, and lack of animal disease models and clinical validation studies [47]. Correlation between

optical biopses and histopathology are recently reported for different types of cancer and neoplasias [48, 49]. However, ischemic tissue lesions using confocal endomicroscopy remain unclear. In this case, we not just compare standard histology images with confocal images as reported in other studies [50], but also quantify indirectly tissue damage through infiltration accumulation. Our algorithm made a clear differentiation between control and inflammatory fluorophore infiltrations in the REMD48 and REMD96 groups (**Figure 1**).

At the histological level, vasodilation and hyperemia occur at 24 h. Leucocyte infiltration is not evident, which suggests that this cellular damage will be like the type described by Fenoglio-Preiser et al [51], where a certain type of cellular damage and its regeneration skipped some inflammatory process stages. At 48 h, forthright focal cellular damage with superficial

ulcerations and edema are observed. At 96 h, there is a mucosal epithelium showing ischemia adaptation after sleep deprivation.

The results obtained are consistent with the results reported by Guo et al (17) in terms of the damage caused by sleep deprivation. Differences should be the consequence of the sleep deprivation model type. They use a slow rotating drum to induce a total sleep restriction for 7 and 14 days. It is worth stressing that in this total deprivation model, cellular damage observed in epithelial cells (hyperemia and erosion) is gradual, reaching a maximum level, and then showing an ischemic condition of adaptation. The continuous stress condition can be associated with an increase in acid secretion at the base of the gastric gland. In the present study, however, we analyzed the impact of short term selective REM sleep deprivation which means a different physiological challenge.

The results of this study suggest that there is an effect in gastric mucosa caused by REM sleep deprivation, showing the initial phases of the acute inflammatory response. At 24 h of REMD there is a loss of tissue integrity in some areas, whereas at 48 h epithelial cell loss by lethal damage is apparent. These findings are related to impedance changes observed, as well as more fluorophore infiltrations showed in confocal endomicroscopy images in the REMD48 group. At 96 h of REMD, the results suggest the presence of ischemic adaptation of the mucosal epithelium, which could be related to a decrease in impedance parameters and fluorophore infiltrations.

As mentioned above, any REM deprivation technique has at least two confounding factors: the stress component and the loss of slow wave sleep. Recently, Arthaud et al [52] reported a two fold increase in corticosterone in mice submitted to REM deprivation. However, with the use of the multiple platform technique in this study, we minimize the stress component and the release of corticosterone [32]. However, the loss of slow wave sleep and its influence in the present results remains to be elucidated. Further studies are necessary in order to understand the effects of severe sleep restriction in the pathogenesis of gastropathy.

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Disclosure of conflict of interest

None.

Authors' contributions

Nohra E Beltran and Mario Garcia-Lorenzana contributed equally to this work; they designed and performed the research, analyzed the data, and wrote the paper. Elisheba Y Gomez performed the experiments and the confocal image analysis, Javier Velazquez-Moctezuma analyzed the data and wrote the paper.

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