

Mitigation of Light Pollution-Induced Oligospermia, Sexual Dysfunction, and Sperm DNA Damage Through Probiotics is Associated With TNF- α , IL- 1β Expression and Na⁺/K⁺ ATPase Activity

Leke Jacob Medubi¹, Oluwatoyin Ojuolape Medubi²

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Abstract

Background Anthropogenic light pollution, an integral part of modern life, has been implicated in male reproductive dysfunctions. This study aimed to determine if augmenting gut microbiota during light pollution protects against this dysfunction and by what mechanisms.

Methods Twenty sexually experienced Wistar rats weighing 170-190 g were randomized into the control (C), artificial light at night (ALAN), probiotics (P), and ALAN-P groups. The ALAN and ALAN-P groups were exposed to ALAN for 6 hours/night for 14 consecutive nights using white light of 2500 lm. Gut microbiota was supplemented in the ALAN-P group rats with a commercially available probiotic formulation of 13 bacterial species at 10⁷ colony-forming units every other day. Following euthanasia, right epididymal homogenates were processed for biochemical analyses, whereas left caudal epididymides were utilized for sperm parameter assessment.

Results Data analysis revealed a significant ($p < 0.05$) decrease in sperm concentration, progressive motility, Na⁺/K⁺ ATPase activity, and fertility index; increased sperm DNA fragmentation, DNA oxidation, redox status, TNF- α , and IL- 1β in the ALAN group relative to other groups, but with a significant improvement of these parameters in ALAN-exposed probiotics-treated rats.

Conclusion Light pollution impairs sperm concentration and quality through overexpression of proinflammatory markers, TNF- α , and the cytokine IL- 1β , as well as DNA fragmentation and methylation. However, treatment with probiotics mitigates inflammation and protects against oxidative stress and DNA damage during light pollution.

Keywords ATPase, Cytokines, Epididymis, Sexual behavior, Sperm quality

✉ Leke Jacob Medubi
jmedubi@unilag.edu.ng

1. Department of Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin, Ilorin, Nigeria
2. Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, Nigeria

1 Introduction

Circadian misalignment caused by chronic exposure to artificial light at night (ALAN) has been shown to affect psychological and metabolic functions, among others.^[1] It has recently been established that the gut microbiota plays crucial roles in spermatogenesis and male fertility.^[2-4] Studies demonstrate a strong connection between testicular function and the regulation of gut microbiota via the host.

metabolomes.^[5] Three weeks of supplementation with *B. longum* and *L. rhamnosus* improved sperm motility and reduced DNA fragmentation in asthenospermic patients.^[6-8] Furthermore, the administration of oligo-fructosaccharides plus *Lactobacillus* strains has been shown to improve sperm quality.^[9]

Experimentally, following supplementation with *L. rhamnosus*, obese rats exhibited improved sperm motility and increased Leydig cells.^[10] In addition, reduced sperm damage and improved motility have been demonstrated in infertile mice fed *S. cerevisiae* and *Lactobacillus* spp.^[11] Also, evidence suggests that pathogenic displacement of semen microbiota may cause dysbiosis, leading to infertility.^[12,13] This study aimed to determine if light pollution-induced reproductive dysfunction can be mitigated by probiotic supplementation.

2 Methods

All animal care procedures met the NIH guidelines (NRC 1985) and the guidelines and approval of the institutional ethics committee. Twenty sexually experienced Wistar rats (170-190) g were randomized into the C (control), ALAN, P (probiotics), and ALAN-P groups. The ALAN and ALAN-P groups were exposed to ALAN for 6 hours/night for 14 consecutive nights using white light of 2500 lm. Daily Probiotic, manufactured by Bactolac Pharmaceutical (Hauppauge, NY, USA), was purchased online and used for this study. Gut microbiota was augmented by feeding probiotics-treated groups with 10⁷ colony-forming units of probiotics dispersed in distilled water containing *Lactobacillus Plantarium*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus salivarius*, *Streptococcus thermophiles*, *Bifidobacterium Lactis*, *Bifidobacterium Brevis*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium longum*, and *Lactobacillus bulgaricus* every other day. Following euthanasia, 10% of the right epididymal homogenates (centrifuged at 3000×g for 15 min) were prepared at 0 °C in phosphate buffer solution (PH = 7.4) and used for biochemical assays. The left caudal epididymides were used for sperm parameter measurements.

Estimation of malondialdehyde (MDA), Reduced Glutathione (GSH Level, Glutathione Peroxidase (GPx), Glutathione-S-Transferase (GST), Superoxide dismutase (SOD), Catalase (CAT) Activities.

A previously described method^[14] was used in quantifying lipid peroxidation by mixing thiobarbituric acid and trichloroacetic acid-HCl with testicular homogenate. The GSH concentration was determined by a previously described procedure.^[15] while glutathione peroxidase (GPx) activity was assessed using modifications to a previous method.^[16] Briefly, 0.5 mL of 10% trichloroacetic acid was added to the reaction mixture containing the sample after it had been incubated at 37 °C for 3 minutes. Then, the mixture was centrifuged at 3000 rpm for 5 minutes. The absorbance was measured at 412 nm against a control. Based on glutathione-S-transferase activity on 1-chloro-2,4,-dinitrobenzene as the second substrate, glutathione-S-transferase activity was assessed as previously published.^[17] At 340 nm, the absorbance was measured against the blank after the assay's prepared medium and the reaction had run for 60 seconds at 37 °C. Superoxide dismutase (SOD) activity was measured as previously described,^[18] while catalase activity was estimated as previously described.^[19]

Measurement of Testicular Cytokines and Nuclear Oxidative Stress Marker

Testicular levels of 8-hydroxy-2-deoxyguanosine (8OHdG), Caspase-3, and interleukin-1 β (IL-1 β) tumor necrosis factor-alpha (TNF- α) were measured using rat-specific enzyme-linked immunosorbent assay (ELISA) kits (Elabscience, Wuhan, China) in accordance with the manufacturer's instructions. Briefly, for TNF- α , 100 μ L of standard or sample was added to the wells and incubated for 90 minutes at 37 °C. The liquid was then discarded, and immediately followed by the addition of 100 μ L of Biotinylated Detection Ab working solution to each well, which was further incubated for 60 min at 37 °C. The plate was aspirated and washed three times. Then, 100 μ L HRP conjugate working solution was added and incubated for 30 min at 37 °C, aspirated, and washed five times. Then, 90 μ L Substrate Reagent was added and incubated for 15 minutes at 37 °C, after which 50 μ L of Stop Solution was added. The optical density was immediately read at 450 nm. Following the manufacturer's instructions, 8OHdG, caspase-3, and IL-1 β were measured similarly to TNF- α .

Assessment of Spermatid DNA Fragmentation

Spermatid DNA fragmentation was determined by modifying the method previously described.^[20] Briefly, testicular tissue homogenized in ice-cold phosphate buffer solution (PBS) was centrifuged first to obtain homogenization-resistant steps 17-19 spermatids to form pellets containing native DNA and the supernatant

containing fragmented DNA was re-suspended in 200 μ L hypotonic lysis buffer to which 200 μ L perchloric acid (0.5 M) was added, followed by the addition of 2 volumes of a solution containing 0.088 M diphenylamine, 98% V/V glacial acetic acid, 1.5 % V/V sulfuric acid and 0.5% acetaldehyde solution. The samples were kept at 4 °C for 24 hours. The absorbance of light green/yellowish-green supernatant was read spectrophotometrically at 600 nm. DNA FI was determined as OD 600 supernatant X 100 OD 600 supernatant + OD 600 pellet.

Sperm Parameters

Using the caudal epididymis of each animal, sperm parameters were determined by adding 0.1 mL of the fluid to 1.9 mL of water to prepare an epididymal fluid ratio of 1:20. After thoroughly mixing the dilution, the improved Neubauer hemocytometer was used to determine the sperm concentration, motility, viability, and morphology with the aid of a Leica D750 microscope as formerly described.^[21] The daily sperm production (DSP) was determined based on the method described,^[22] which relies on homogenization-resistant spermatids.

Sexual Behavior Study

The sexual behavioral study was carried out a day after completing 14 consecutive days of exposure to ALAN, following the reported method.^[23] Each observation lasted for 30 minutes. Mount frequency and latency, intromission frequency and latency, and ejaculation frequency and latency were observed and recorded. All the animals except one from the ALAN group successfully mated. Furthermore, all the females that successfully mated during the sexual behavioral study were used for the fertility testing. On gestational day (GD) 15, the female underwent a cesarean section following euthanasia with chloroform. The uterine horns were recovered and dissected to record pregnancy status. In addition, the ovaries were recovered to determine the number of corpora lutea. The uterine horns of one rat (in the ALAN group) that showed no obvious signs of pregnancy were stained with approximately 9% ammonium sulfide to visualize early resorptions. Implants were counted, each classified as a live fetus, dead fetus, or resorption.

Quantitative Study Endpoints

For quantitative fertility assessment, the number of corpora lutea, the number of implantations, and the number of live embryos were determined. Furthermore, secondary variables were calculated, including preimplantation loss, percentage post-implantation loss, and fertility index determined as follows:

$$\text{Coupletory index (\%)} = 100 \times \frac{\text{Number of males that had evidence of mating with female}}{\text{Number of male mated}}$$

$$\text{Pre-implantation loss (\%)} = 100 \times \frac{\text{Number of corpora lutea} - \text{Number of implantations}}{\text{Number of corpora lutea}}$$

$$\text{Post-implantation loss (\%)} = 100 \times \frac{\text{Number of implantations} - \text{Number of live embryo}}{\text{Number of corpora lutea of implantations}}$$

Statistical Analysis

Data were subjected to one-way ANOVA with Bonferroni's post-test with the aid of Graphpad Prism 5.1 (Boston, MA, USA) and reported as mean \pm standard error of mean. Comparison of any two groups was considered to be statistically significant only when $p < 0.05$.

3 Results

Gut Microbiota Supplementation Mitigates ALAN-induced Disruption of Epididymal Redox Balance

As shown in Figure 1a, exposure to ALAN resulted in a significant elevation of MDA level in the epididymis compared with the control or Prbt groups; however, the level decreased significantly in the ALAN+Prbt group compared with the ALAN group. The activity of SOD (Figure 1b) is the statistical reverse of what was observed with MDA levels. In contrast, CAT activity remained significantly higher in both the ALAN and ALAN+Prbt groups compared with the control and Prbt groups (Figure 1c). Epididymal GSH levels (Figure 1d) were significantly reduced in the ALAN and ALAN+Prbt groups compared with the control and Prbt groups, but were higher in ALAN+Prbt compared with ALAN. The GPx and GST levels follow the same statistical pattern as GSH (Figure 1e and Figure 1f).

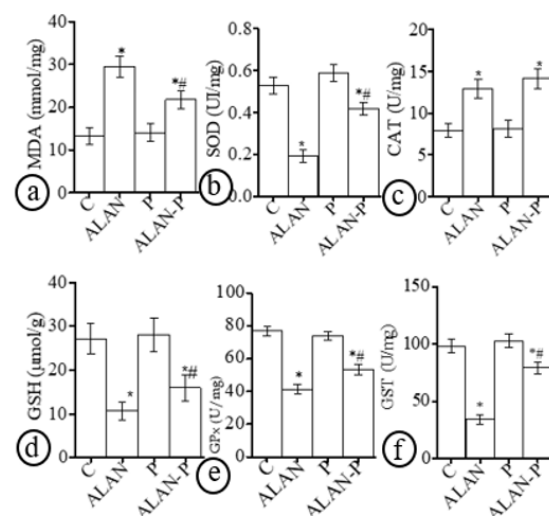


Figure 1 Epididymal redox status following exposure to ALAN and gut microbiota supplementation. * indicates a significant ($p < 0.05$) difference from other groups, while # shows a significant ($p < 0.05$) difference from ALAN. C: control; ALAN: artificial light at night; P: probiotics

Probiotics mitigate light pollution-induced overexpression of Cytokines, improve transmembrane ion pumps, and reduce DNA Fragmentation and Oxidation

Epididymal expression of proinflammatory cytokines, IL-1 β , and TNF- α is significantly overexpressed following ALAN exposure. Although they were not returned to the control levels, they were significantly reduced in the ALAN-P group relative to the ALAN group (Figure 2a and Figure 2b). Similarly, exposure to ALAN caused a significant decrease in the activity of epididymal Na⁺-K⁺-ATPase (Figure 2c); however, treating rats with probiotics during ALAN exposure significantly increased epididymal Na⁺-K⁺-ATPase in the ALAN-P group compared with the ALAN group. Similarly, epididymal H⁺-K⁺-ATPase activity was significantly reduced in the ALAN group but remained, statistically, at the control levels in the ALAN-P group. Furthermore, exposure to ALAN caused a significant increase in the percentage of DNA fragmentation in epididymal sperms (Figure 2d); however, treating rats with probiotics during ALAN exposure brought the amount of DNA fragmentation to a statistically similar level as the control. Also, the concentration of 8-OHdG was significantly elevated in the ALAN group but remained statistically at the control levels in the ALAN-P group (Figure 2e).

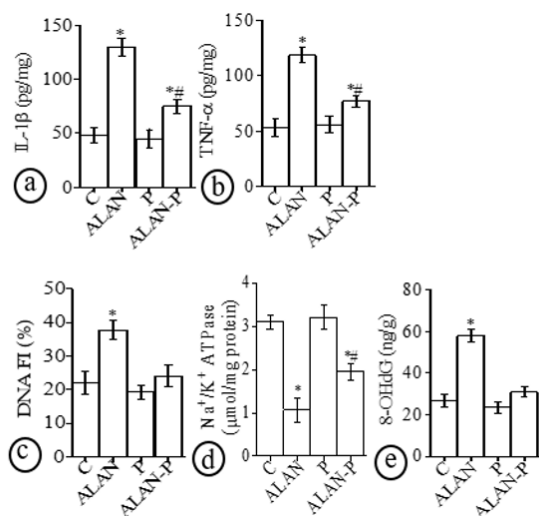


Figure 2 Epididymal cytokine expression, transmembrane ion pumps, DNA fragmentation, and DNA oxidative stress following ALAN exposure and gut microbiota supplementation. * indicates a significant ($p < 0.05$) difference from other groups, while # shows a significant ($p < 0.05$) difference from ALAN. C: control; ALAN: artificial light at night; P: probiotics

Probiotics Boost Sperm Quality During Exposure to Light Pollution

As shown in Table 1, light pollution exposure significantly decreases quantity, quality, and vitality compared with any other group. However, in the ALAN-P group, sperm

quality and vitality remained statistically at the same levels as the control. Only sperm concentration was significantly different in the ALAN-P group compared with the control.

Light Pollution-Induced Sexual Dysfunction Improved in Probiotic-Treated Animals

As shown in Figure 3a, Figure 3b, and Figure 3c, exposure to ALAN caused significantly increased mount, intromission, and ejaculatory latency compared with observations in other groups. However, the latency for these sexual behaviors was significantly reduced in the ALAN-P group compared with the ALAN group. Also, mount, intromission, and ejaculation numbers were significantly reduced in ALAN groups compared with other groups (Figure 3d, Figure 3e, and Figure 3f). However, these parameters were significantly increased in the ALAN-P group compared with the ALAN group, so much so that the ejaculation number was not statistically different from the control.

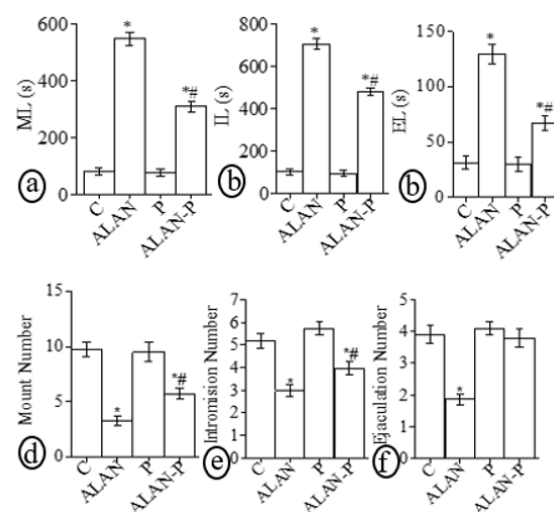


Figure 3 Sexual behavioral indices following exposure to ALAN and gut microbiota supplementation. * indicates a significant ($p < 0.05$) difference from other groups, while # shows a significant ($p < 0.05$) difference from ALAN. C: control; ALAN: artificial light at night; P: probiotics; ML: mount latency; IL: intromission latency; EL: ejaculatory latency

Preimplantation Loss in Female Fertilized by Light-polluted Males

There was a significant preimplantation loss in female rats mated with ALAN-exposed male rats, but this was significantly reduced in the ALAN-P. There was no significant post-implantation loss in any group, and the copulatory competence of the ALAN group was only 80%, while it was 100% for the other groups (Table 2).

Table 1 Epididymal sperm parameters following ALAN exposure and probiotic treatment.

	C	ALAN	P	ALAN-P
Sperm conc ($\times 10^6/ml$)	89.79 \pm 7.91	67.24 \pm 8.78*	93.01 \pm 8.38	71.30 \pm 6.61*
Progressive motility (%)	76.91 \pm 4.22	55.39 \pm 5.72*	75.94 \pm 6.05	76.02 \pm 5.81
Vitality (%)	94.51 \pm 8.28	79.31 \pm 7.92*	96.47 \pm 7.87	92.46 \pm 6.08
Normal morphology	85.39 \pm 6.79	67.08 \pm 8.29*	87.09 \pm 7.43	81.02 \pm 6.39

* indicates a significant ($p < 0.05$) difference from other groups, while # shows a significant ($p < 0.05$) difference from ALAN. C: control; ALAN: artificial light at night; P: probiotics

Table 2 Fertility analysis following light pollution and probiotic treatment.

	C	ALAN	P	ALAN-P
Couplelatory index (%)	100.00	70.00	100.00	100.00
Preimplantation loss (%)	17.49 \pm 2.08	49.62 \pm 3.46*	16.22 \pm 2.68	26.84 \pm 3.07*#
Pos-implantation loss (%)	9.74 \pm 0.52	10.37 \pm 0.83	10.22 \pm 0.49	9.83 \pm 0.59

* indicates a significant ($p < 0.05$) difference from other groups, while # shows a significant ($p < 0.05$) difference from ALAN. C: control; ALAN: artificial light at night; P: probiotics; ML: mount latency; IL: intromission latency; EL: ejaculatory latency

4 Discussion

A state of oxidative stress exists when there is an imbalance between natural cellular/tissue oxidants and antioxidants in favor of oxidants. Oxidative stress is damaging to cells, tissues, and organs.^[24,25] Data analysis in the current study shows a significantly elevated MDA level in the ALAN group, while the antioxidant system, comprising SOD, CAT, GSH, GST, and GPx, was significantly decreased. This partly explains the ALAN group's reduced progressive motility. Besides, it has been reported that oxidative stress is one of the major factors likely to cause elevated sperm chromatin/DNA damage,^[26] which possibly explains the observed significant increase in 8-OHdG concentration and DNA fragmentation in the ALAN group. There is ample experimental evidence that oxidative damage permanently occurs to the lipid bilayer of cellular membranes, proteins, and DNA.^[27] A significantly elevated level of 8-OHdG in this study shows the detrimental effect of ALAN on the epididymal spermatozoa.

In the current investigation, sperm concentration, vitality, and progressive motility decreased significantly in the ALAN group, suggesting that light pollution negatively impacts the basic and most important indices of male fertilization capacity. Moreover, only progressively motile spermatozoa are relevant in natural fertilization. A decrease in any of these sperm parameters clearly indicates that ALAN impairs fertility. Studies have shown a link between increased levels of cytotoxic ROS and loss of sperm concentration, motility, and other parameters.^[28] Because sperm motility is directly related to mitochondrial impairment, defective sperm mitochondria are implicated in impaired sperm motility in humans.^[29,30] Furthermore, there is strong evidence that elevated mitochondrial ROS generation induces DNA fragmentation and a decline in sperm motility and

viability.^[2,29,31]

Significant to the current study is the observed improvement in sperm parameters, redox status, and DNA integrity in the animals treated with probiotics during exposure to light pollution. The link between male reproductive function and gut microbiota has been empirically established. Previous studies^[32] show that gut microbiota significantly influences the expression of many cytokines in different tissues. In the current study, epididymal expression of TNF- α and IL-1 β was significantly reduced in the ALAN-P group compared with the ALAN group, suggesting that cytokine-mediated elevated free radical generation or DNA fragmentation is significantly reduced in the probiotic-treated group during ALAN exposure, which may explain the improved epididymal sperm parameters.

The presence and significance of Na⁺/K⁺ ATPase in spermatozoa have been demonstrated.^[33,34] The significant decrease in Na⁺/K⁺ ATPase activity in the ALAN group in this study suggests the dysregulation of sperm ion and membrane potential due to exposure to light pollution. The downregulation of Na⁺/K⁺ ATPase activity in the ALAN group partly explains the reduced progressive motility seen in the group, as adequately functioning Na⁺/K⁺ ATPase, particularly the $\alpha 4$ isoform, is essential to sperm motility.^[35-37] Spermatozoa flagellar function has been shown to depend on an adequate activity of Na⁺/K⁺ ATPase, as it determines sperm kinematics.^[38] It is clear from the data in this study that the reduced sperm motility in the ALAN group is associated with the downregulation of sperm Na⁺/K⁺ ATPase. Equally, reduced Na⁺/K⁺ ATPase activity in the ALAN group is associated with reduced fertilization following mating and possibly accounts for the significant difference in the number of corpora lutea and implantation. Adequately expressed and functional Na⁺/K⁺ ATPase is critical to sperm capacitation, a necessary condition for fertilization.

^[39] The downregulation of Na⁺/K⁺ ATPase observed in this study could be tied to increased epididymal oxidative stress, as it has been previously reported that oxidative stress can oxidize the Na/K-ATPase and impair its activity.^[40]

A natural conception requires a male with normal sexual behavior and competence, absent which, natural delivery of spermatozoa into the female reproductive tract is impossible. Rat sexual behavior has been studied, and its essential elements have been identified.^[41,42] The current study shows that exposure of rats to light pollution significantly raised mount, intromission, and ejaculation latency. The normal male sexual moves toward a female are believed to be influenced by testosterone levels under the control of the executive decision-making center of the frontal cortex.^[42,43] Considering the reported impairment of testosterone and cognitive capacity during stress, such as disrupted circadian rhythm, it can be inferred that the current observation of significantly delayed mounting of the female may be linked to light-pollution-induced hormonal dyshomeostasis. Increased latency may waste mating opportunities and reduce the success rate of ejaculation. Animals treated with probiotics had significantly reduced mount, intromission, and ejaculation latency, suggesting they were better prepared for sexual engagement. Due to increased latency in mount, intromission, and ejaculation, animals in the ALAN group had few mount, intromission, and ejaculation numbers. Impressively, however, treatment with probiotics significantly increased mount, intromission, and ejaculation; the number of ejaculations in the ALAN-P group statistically approached that of the control, suggesting that treatment with probiotics during ALAN exposure aids ejaculation success.

With approximately 30% preimplantation loss above the control, it is within the bounds of the current body of evidence to infer that the quality of embryos that resulted from ova fertilized by spermatozoa from light-polluted male rats was degraded, further concretizing the evidence for reduced sperm quality secondary to exposure to light pollution. The detection of a significantly higher level of DNA fragmentation in the ALAN group supports the inference of poor-quality embryo-mediated preimplantation loss. In humans, it has been shown that sperm DNA quality influences *in vitro* fertilization (IVF) success rate.^[44,45] Furthermore, poor sperm DNA quality has been linked to lifestyle factors.^[45] One implication from the current study is the possibility of categorizing apparent infertility in this situation as idiopathic infertility were it to manifest in humans. Importantly, data in this study show that post-implantation losses were statistically the same across all the groups, suggesting that the preimplantation natural screening was sufficient to eliminate light pollution-induced differentials in embryo quality.

5 Conclusion

Light pollution impairs sperm concentration and quality through overexpression of proinflammatory markers, TNF- α , and the cytokine IL-1 β , as well as DNA fragmentation and methylation. Male sexual function is significantly affected, in addition to impaired reproductive capacity. However, treatment with probiotics mitigates inflammation and protects against oxidative stress and DNA damage, leading to a better reproductive potential during light pollution.

Declarations

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Artificial Intelligence Disclosure

The authors confirm that no artificial intelligence (AI) tools were used in the preparation of this manuscript.

Authors' Contributions

All authors contributed equally to this study.

Availability of Data and Materials

The data that support the findings of this study are available upon request from the corresponding author.

Conflict of Interest

The authors have no conflict of interest.

Consent for Publication

Not applicable.

Ethical Considerations

The proposal for this study was approved under the Code of Ethics CMUL/ACUREC/01/19/643. The researchers adhered to all the principles recommended by the National Research Council.

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