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ORIGINAL ARTICLE

Testicular Morphological Alterations in 2.45 GHz Wi-Fi Exposed Rat Pups and The Mitigating Effects of Edible Bird Nest

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ABSTRAK

Aplikasi Wi-Fi telah menjadi keperluan harian untuk individu bagi semua peringkat umur termasuk kanak-kanak. Walau bagaimanapun, peranti Wi-Fi memancarkan radiasi-radiofrekuensi yang sensitif kepada organ yang sedang melalui perkembangan, terutamanya testis. Namun, hanya segelintir kajian menilai kesan Wi-Fi terhadap testis yang sedang melalui perkembangan. Oleh itu, kajian ini dilakukan untuk menilai kesan dedahan Wi-Fi terhadap perubahan histomorfometri testis pada anak tikus Sprague Dawley yang sedang membesar. Kajian ini turut memberikan suplementasi sarang burung walit (EBN) untuk mengurangkan kesan Wi-Fi yang dijangkakan. Sebanyak 30 ekor (n=30) anak tikus berumur tiga minggu dibahagikan sama rata kepada lima kumpulan (n=6): Kawalan, EBN, Wi-Fi 1, Wi-Fi 2 dan EBNW. Hanya Wi-Fi 2 dan EBNW menerima dedahan terhadap Wi-Fi aktif. Manakala EBN dan EBNW menerima suplementasi EBN 250 mg/kg. Dedahan Wi-Fi dan suplementasi EBN diberikan selama 14 minggu berturut-turut. Hasil menunjukkan bahawa kumpulan Wi-Fi 2 mempunyai sel spermatogonia yang terpisah dari membran dasar, disorientasi sel germa, kehadiran tubul kosong dan kawasan edema di antara tubul seminiferus. Penurunan signifikan dalam diameter tubul seminiferus dan ketinggian sel germa turut dicatatkan. Sebaliknya, suplementasi EBN telah mengekalkan sel-sel di dalam tubul seminiferus kumpulan EBNW menjadikannya kelihatan normal dan utuh. Selain itu, kumpulan Wi-Fi+EBN menunjukkan peningkatan signifikan dalam diameter tubul seminiferus. Tiada perubahan signifikan direkodkan bagi indeks spermatogenesis di antara semua kumpulan. Kesimpulannya, dedahan Wi-Fi dari usia muda menyebabkan kemerosotan histomorfometri testis. Manakala, suplementasi EBN melemahkan kesan Wi-Fi terhadap ciri-ciri tertentu histomorfometri testis.

Kata kunci: *Indeks spermatogenesis; histologi testis; ketinggian sel germa; radiasi radiofrekuensi*

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ABSTRACT

Wi-Fi applications are now a daily necessity for people of all ages including children. However, radiofrequency-radiation emitted from the Wi-Fi devices is sensitive to their developing organ, especially the testis. Nonetheless, sparse study has evaluated the Wi-Fi effect on the developing testis. Hereby, this study evaluated the effect of Wi-Fi exposure on the histomorphometry changes of the testis in the growing Sprague Dawley pups. This study also incorporated edible bird nest (EBN) supplementation to attenuate expected Wi-Fi effects. A total of 30 (n=30) three-week-old pups were divided equally into five groups (n=6): Control, EBN, Wi-Fi 1, Wi-Fi 2, and EBNW. Only the Wi-Fi 2 and EBNW were exposed to active Wi-Fi. Meanwhile EBN and EBNW received 250 mg/kg of EBN supplementation. Both Wi-Fi exposure and EBN supplementation were conducted for 14 consecutive weeks. Findings showed that the Wi-Fi 2 group demonstrated detachment of the spermatogonia cells from the basement membrane, disorientation of the germ cells, presence of empty tubules and mild oedematous area in between seminiferous tubules. Significant decreases in seminiferous tubule diameter and germ cell height were also noted. Supplementation of EBN preserved the cells in the seminiferous tubule of the EBNW group as it appeared normal and intact. Besides, the EBNW group showed a significant increase in the seminiferous tubule diameter. No significant changes were recorded for the spermatogenesis index between all groups. In conclusion, Wi-Fi cause degenerative changes in developing testis while EBN supplementation attenuates the Wi-Fi effects on certain histomorphometry features of the testis.

Keywords: Germ cell height; radiofrequency radiation; spermatogenesis index; testis histology

INTRODUCTION

The ubiquity of Wi-Fi, accelerated by the COVID-19 lockdown, has amplified its usage across age groups. The increment of Wi-Fi users not only occurs among adults but also has grown significantly among children (Noaman 2019). It was reported that the worldwide lockdown had caused an increase of 500 and 155% in Wi-Fi users among children in the United States and Malaysia, respectively (MCMC 2020). Notably, children's exposure to Wi-Fi has expanded due to remote learning, especially during the pandemic (Derya & Adam 2020), and it stays as a modern lifestyle when the pandemic is over.

The concern regarding the Wi-Fi application is that it emits radiofrequency radiation (RFR) (Jaffar et al. 2019), the lowest energy in the electromagnetic spectrum (Yadav et al. 2021). RFR is applied in many broadcast and wireless communication applications, including cellular phones and Wi-Fi technologies. Given the vulnerable nature of the growing tissue in children, especially the reproductive organ (Atasoy et al. 2013), this age group is probably more susceptible to RFR than any other age group (Martens 2005). Therefore, this study conducted 2.45 GHz Wi-Fi exposure from pre-pubertal to adult age, which corresponds to the age range of the younger generation that uses the Wi-Fi application most frequently (Dasdag et al. 2015).

Three organs are reported to be the most vulnerable to RFR exposure which include the brain, testis and eyes (Singh & Kapoor 2014). However, the main interest of this current study is on the effect of RFR emitted by Wi-Fi devices on the testis of the children. To mimic the effect of Wi-Fi exposure on a growing human child, Sprague Dawley pups were used as an

animal model. Thereafter, this study assessed the alterations in testicular histomorphometry as it is imperative to provide insight into the stage of spermatogenesis in each seminiferous tubule. For this reason, it is a crucial factor in determining the male fertility status during their development (Adelakun et al. 2021).

Additionally, this study also incorporated edible bird nest (EBN) supplementation to attenuate possible Wi-Fi adverse effect on the testis histomorphometry. EBN originates from the saliva secretion of the male *Aerodramus Fuciphagus* swiftlet during the breeding season (Fan et al. 2022). This swiftlet is an insectivorous bird that mostly inhabits South China and Southeast Asia (SEA) (Aswir & Wan 2010; El Sheikha 2021). EBN is known for its various nutritional content including protein, carbohydrate, fat, ash and moisture (Hamzah et al. 2013). Besides, EBN also contains high sodium, calcium and other mineral components such as magnesium, potassium, phosphorus and iron (Quek et al. 2017).

EBN was chosen to be used in this study in addition to its nutritional composition since it demonstrated a remarkable potential for cell proliferation (Roh et al. 2012), contained several reproductive hormones including follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone (Ma & Liu 2012), and also exhibited antioxidative activity (Hou et al. 2015). All these features are vital in maintaining the proliferation of germ cells within the seminiferous tubule. Therefore, it may enhance mitogenic responses that may preserve the integrity of the testis histomorphometry against adverse effects of Wi-Fi exposure.

Hereby, the purpose of this study is to better understand how Wi-Fi exposure affects developing young testis and to develop strategies to counteract any potential negative effects. This study may therefore become a platform to predict the fertility status of children in the subsequent 20 to 30 years considering the trend of Wi-Fi application among children may persist until they reach active reproductive years. On the other hand, the implementation of EBN may help to increase the EBN's scientific and market value, particularly in enhancing male fertility in the advanced technology era.

MATERIALS AND METHODS

This study employed the same Wi-Fi exposure and EBN supplementation techniques as our earlier publication (Jaffar et al. 2021; Jaffar et al. 2022). The experimental setting applied was briefly outlined below.

Sample and Sample Size

This study involved 30 (n=30) male Sprague Dawley pups of 3-week-old which was divided randomly into five groups. The sample size was determined based on a Test Guideline OECD (2008). The groups consisted of Control, EBN, Wi-Fi 1, Wi-Fi 2 and EBNW group. Each group consisted of six (n=6) rats.

Experimental Schedules and Setting

All the animals were kept individually in separate cages (43 cm length x 16 cm wide x 29 cm height) without movement restriction. The animals in Control and EBN were not exposed to Wi-Fi. Only rats in the Wi-Fi 2 and EBNW group were exposed to an active Wi-Fi router. On the other hand, Wi-Fi 1 was exposed by a non-active Wi-Fi router. Wi-Fi 1 group was included in this study to exclude the background electromagnetic field emitted by the router itself. The Wi-Fi router was place at 20 cm distant from the cages. This distance was chosen in accordance with the minimum

distance recommendation for Wi-Fi devices from users by the Federal Communications Commission (FCC) of the United States (Means & Chan 2001). The exposure was done 24 hours daily for 14 consecutive weeks.

Meanwhile, animals in the EBN and EBNW groups received EBN supplementation of 250 mg/kg/day for 14 weeks. The dose of EBN applied in this study was based on our previous report (Jaffar et al. 2021).

Animal Euthanisation

After 14 weeks of Wi-Fi exposure and EBN supplementation, all animals were euthanised with ketamine-tiletamine-xylazine cocktail through intraperitoneal injection. Their testis was collected and evaluated for histomorphometry changes and spermatogenesis index.

All the animal procedures implemented in this study was approved by the Universiti Kebangsaan Malaysia Animal Ethical Committee (UKMAEC). The approval reference number for this study was FISIO/PP/2018/SITI FATIMAH/28-MAR./908-MAR.-2018-DEC.-2020 and FP/2023/FARAH HANAN/15-FEB./1307-FEB.-2023-JULY-2023-NAR CAT2.

Histology Assessment

Only the right testis was harvested, cleaned and fixed in 10% neutral buffered formalin (Merck, Germany). The left testis was immediately frozen for further molecular analysis. Following fixation, the right testis was divided in half and dehydrated using graded ethanol (70 to 100%) and three series of toluene. The tissue was subsequently paraffin-embedded. The paraffin-embedded testis was then sectioned into 3-µm thickness and stained with hematoxylin and eosin (H&E).

Histology assessment of the tissue section was single-blinded by masking the slide label. Evaluation of any histological changes of the testicular tissue section in each group was done by a histopathologist in Makmal Bioserasi, Universiti Kebangsaan Malaysia (UKM).

Measurement of Seminiferous Tubule Diameter and Germ Cell Height

For the evaluation of other histomorphometry indices, 20 transverse sections of the most circular-shaped seminiferous tubules were analysed. The pictures for each section were recorded under 20 magnification by using an Olympus BX53F (Olympus, Tokyo, Japan) bright field microscope. The measurement of the seminiferous tubule diameter and germ cell height were done in duplicate across the major and minor axes (Figure 1). All the measurement was done by using image J version 1.52a (National Institutes of Health (NIH), Maryland, USA)

Spermatogenesis Index

The testis was also evaluated for its spermatogenic potential according to the Johnsen score (Thanh et al. 2020). Approximately 20 seminiferous tubules were randomly indexed for each tissue section for the presence of the spermatogenic cells throughout the tubules and included layer of spermatogonia and spermatocyte, the appearance of late spermatids and the spermatozoa in the tubules. Each tubule was given a score from 1 to 10 based on the index criteria as in Table 1. The scoring for the spermatogenesis index was performed double-blinded by a histopathologist at Makmal Bioserasi, UKM.

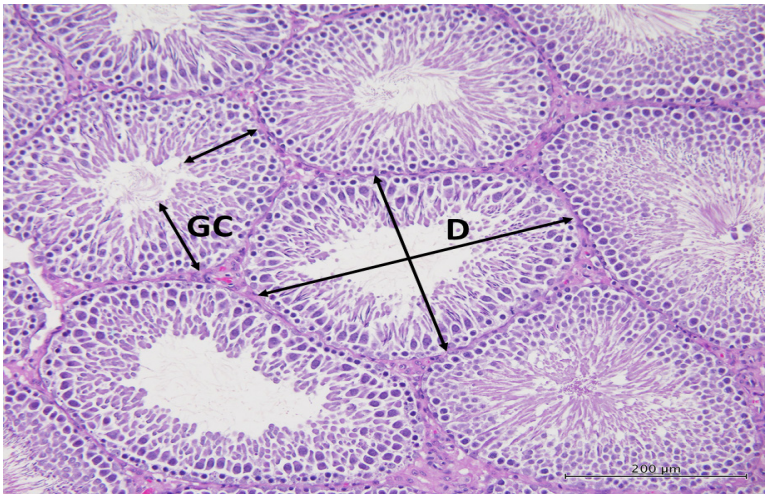

FIGURE 1: Seminiferous tubule in its most circular shape observed under 20x magnification. **D** represented the measurement of the diameter and **GC** represented the measurement of the germ cell height

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's post-hoc analysis was conducted as the normal distribution and variance homogeneity of the data followed the statistical test assumption. A p-value of <0.05 was considered statistically significant. This statistical analysis was performed using SPSS version 22.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Histological Changes of The Testis

Histological findings of the testis section in the Control and EBN group demonstrated a normal appearance of the seminiferous tubule. Both groups showed most of the seminiferous

TABLE 1: Johnsen scoring system for spermatogenesis index assessment (Thanh et al. 2020)

Score	Description
10	Complete spermatogenesis with many spermatozoa. Germinal epithelium organized in a regular thickness leaving an open lumen
9	Many spermatozoa present but germinal epithelium disorganised with marked sloughing or obliteration of lumen.
8	Only a few spermatozoa present in the section.
7	No spermatozoa but many spermatids present.
6	No spermatozoa and only a few spermatids present
5	No spermatozoa, no spermatids but several or many spermatocytes present
4	Only few spermatocytes and no spermatids or spermatozoa present
3	Spermatogonia are the only germ cells present
2	No germ cells but Sertoli cells present.
1	No cells in the tubular section

tubules have one to two cells thickness of spermatogonia residing at the basement membrane. The spermatogonia was stained pale with a large, rounded polygonal shape with a prominent nucleolus. The arrangement of cells is followed by large, rounded spermatocytes which have granulated nuclei, and later by early spermatids which consist of round and dense nuclei with minimal visible cytoplasm. The late spermatid appears with an elongated, thin nucleus, and is attached to the cytoplasmic bridge. In most of the tubules, the spermatozoa were visible in the lumen. Each seminiferous tubule was intact and close to each other with the Leydig cells tightly located in between the tubules (Figure 2A).

Only mild lesions were noted in both groups with the presence of vacuolated and pyknotic spermatogonia that was indicated by a very dense, small nucleus. Other most notable observations for the EBN group included the separation of some spermatogonia layer which subsequently created free spaces between the spermatogonia and the basement membrane (Figure 2B).

In the Wi-Fi 1 group, there were only very few presents of pyknotic spermatogonia with very mild homogenous pinkish edematous area between the tubules. Most of the tubules in the Wi-Fi 1 group showed a normal appearance (Figure 2C). On the other hand, the Wi-Fi 2 group showed vacuolation of spermatogonia and built up spaces between spermatogonia and its basement membrane. Notably, certain tubules showed disorientation of the germ cells and the absence of spermatozoa and spermatids. There were also mild homogenous pinkish edematous in between seminiferous tubules (Figure 2D).

Supplementation of EBN to the EBNW demonstrated improvement of the testis histology structure in which there was minor detachment of the cells from the basement membrane. Most of the tubules contained mild pyknotic spermatogonia and appeared normal and intact. There were still some tubules with germ cell disorientation. However, the edematous in between seminiferous tubules are still the most noticeable lesions in the EBNW group (Figure 2E).

Seminiferous Tubule Diameter and Germ Cell Height

Evaluation of the seminiferous tubule diameter demonstrated that the Wi-Fi 2 group (772.15 μm ± 9.71) showed a significant decrease in the diameter compared with the Control (896.68 μm ± 11.29, $p<0.001$), EBN (935.50 μm ± 14.46, $p<0.001$) and Wi-Fi 1 (937.38 μm ± 12.68, $p<0.001$) groups. EBN supplementation to the EBNW group (882.44 μm ± 10.23, $p<0.001$) caused a significant increase in the seminiferous tubule diameter compared with the Wi-Fi 2 group (Figure 3A).

Moreover, there was a significant decrease in germ cell height in the Wi-Fi 2 group (219.63 μm ± 3.31) compared with the Control (334.51 μm ± 5.72, $p<0.001$) and EBN (377.90 μm ± 8.60, $p<0.001$) groups. Interestingly, there was a significant decrease in the germ cell height in the Wi-Fi 1 (157.56 μm ± 2.62, $p<0.001$) and EBNW group (181.66 μm ± 2.92, $p<0.001$) compared with the Wi-Fi 2 group (Figure 3B).

Spermatogenesis Index

Scoring of the spermatogenesis index using the Johnsen score revealed that all groups had scores ranging from 8 to 9. However, the variations were not statistically significant among the experimental groups (Table 2).

DISCUSSION

Based on the recorded data, prolonged Wi-

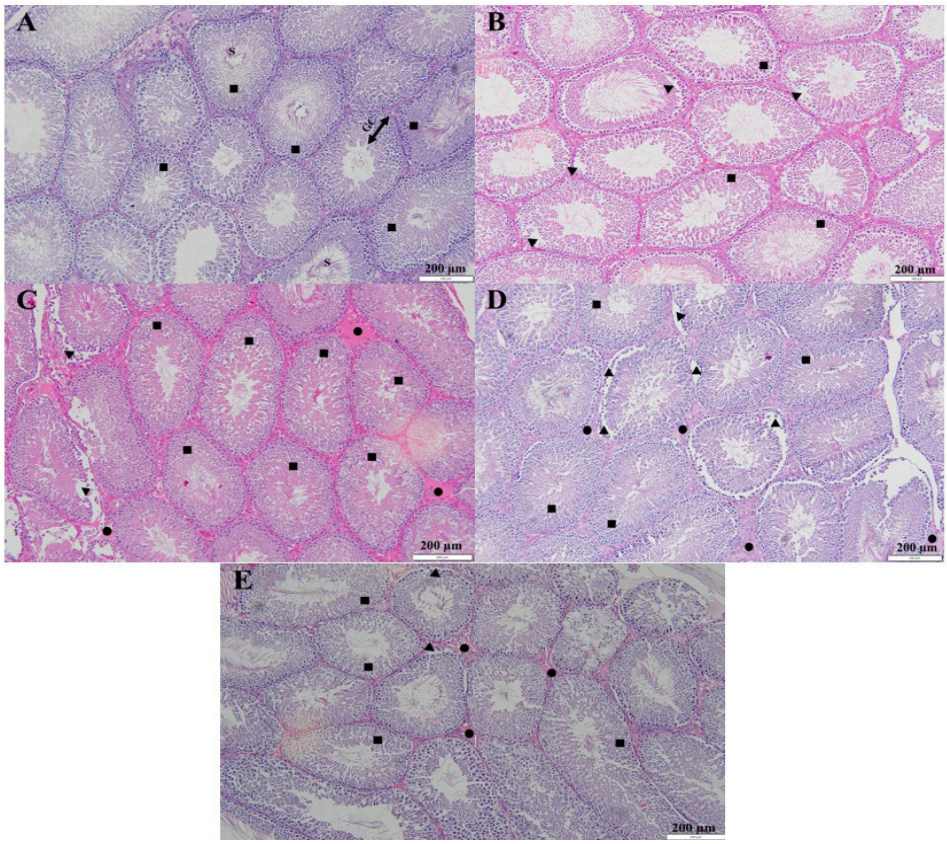

FIGURE 2: Haematoxylin and Eosin (H&E) staining of the testis sections for each experimental group. Figures were recorded under 10x magnification. (A) Control; (B) EBN; (C) Wi-Fi 1; (D) Wi-Fi 2; (E) EBNW group. **S** represented spermatozoa; **GC** represented germinal epithelium; ■ represented a normal arrangement of seminiferous tubule; ▲represented detachment/free spaces of the cells from the basement membrane; ● showed pink edematous lesion (shading different in Figure 1B to 1E)

Fi exposure from pre-pubertal to adult age demonstrated a prominent hazardous effect on the testicular histomorphometry. Among the alterations is spermatogonia vacuolation, which is an indicator suggesting degenerative injury to the seminiferous tubules (Cruceño et al. 2024). The presence of the vacuoles possibly leads to the building of free spaces between the basement membrane and the germ cells. These findings were aligned with previous studies that showed Wi-Fi exposure causes the appearance of highly irregular empty spaces in the seminiferous epithelium (Almášiová et al. 2018; Shahin et al. 2014), and the seminiferous tubule becomes irregular in shape (Šimaiová et al. 2018). The presence of free spaces may subsequently disrupt the molecular contact required for proper spermatogenesis and therefore decrease the spermatogenesis in the affected tubule. Ultimately, these changes may lead to the absence of spermatozoa and spermatids in the lumen as recorded in this study.

Moreover, Wi-Fi exposure also caused a significant decrease in the seminiferous tubule diameter and the germ cell height compared to the Control group. This finding is also consistent with previous studies which also

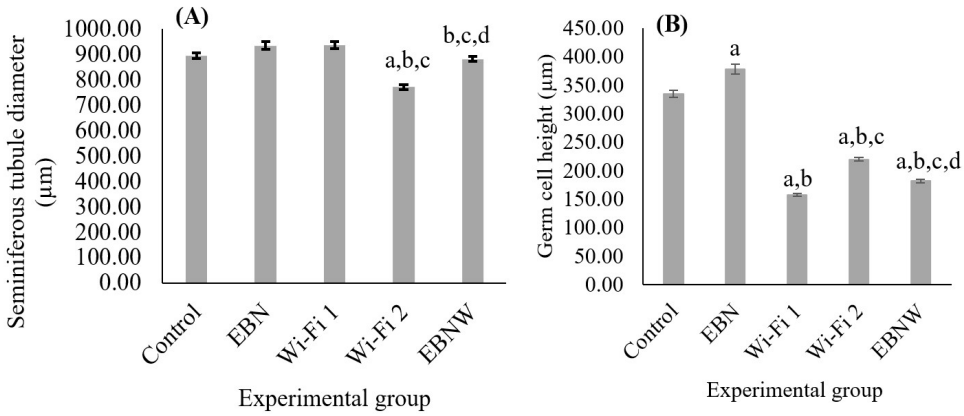

FIGURE 3: (A) Seminiferous tubule diameter. Data were presented as mean SEM (n=20). [a]showed a significant difference compared with the Control, [b]showed a significant difference compared with the EBN group, [c]showed a significant difference compared with the Wi-Fi 1, and [d]showed a significant difference compared with the Wi-Fi 2; (B) Measurement of the germ cell height of the seminiferous tubule. Data were presented as mean ± SEM (n=20). [a]showed a significant difference compared with the Control group, [b]showed a significant difference compared with the EBN group, [c]showed a significant difference compared with the Wi-Fi 1, and [d]showed a significant difference compared with the Wi-Fi 2

TABLE 2: Spermatogenesis index of each experimental group

Groups	Spermatogenesis index
Control	9.35 ± 0.09
EBN	8.99 ± 0.20
Wi-Fi 1	8.78 ± 0.25
Wi-Fi 2	8.72 ± 0.17
EBNW	9.11 ± 0.19

Data were presented as mean ± SEM (n=20). No significant difference between groups, p>0.05.

reported a significant decrease in the diameter of the seminiferous tubule following Wi-Fi exposure (Delavarifar et al. 2018; Shahin et al. 2014). The seminiferous tubule diameter area typically will increase during sexual maturation (Tesi et al. 2020) which is attributed to the proliferation and differentiation of germ cells and Sertoli cells (Rathi et al. 2005). Therefore, the reduced diameter of the seminiferous tubule and germ cell height reflects the failure of Sertoli and germ cell proliferation in that particular seminiferous tubule. These results further suggest that the seminiferous tubule's ability to mature was compromised in the Wi-Fi group, which resulted in the presence of empty tubules. As a result, this phenomenon suggests that spermatogenesis may be adversely affected.

Intriguingly, this study discovered that the sham exposing Wi-Fi (Wi-Fi 1) significantly reduced the height of germ cells in comparison to the Wi-Fi group. This result contradicted our initial assumption that the Wi-Fi router's background electromagnetic

field would not have a substantial impact on testicular histomorphometry. However, according to earlier research, low-frequency electromagnetic fields can also lead to seminiferous epithelial tubular atrophy, necrosis, and degeneration (Erpek et al. 2007), which in turn causes a decrease in seminiferous tubule (Bahaodini et al. 2015).

Despite the histomorphometry changes recorded in all the exposed groups, the spermatogenesis index based on the Johnsen score showed a non-significant decrease compared to the Control group. These results were in accordance with a previous study (Delavarifar et al. 2018) which discovered that exposure to Wi-Fi for two hours per day for four days did not significantly reduce the spermatogenesis index. In contrast, another study (Bilgici et al. 2018; Saygin et al. 2011) reported a significant decrease in the spermatogenesis index. Both of these studies applied 1-hour exposure for 28 to 30 days using a monopole antenna placed in close contact with the experimental animals. Therefore, the differences in experimental settings, such as exposure duration, antenna type, and proximity to the animal, may be the reason for the inconsistent spermatogenesis index findings across studies (Jaffar et al. 2019).

Supplementation of EBN to the Wi-Fi group (EBNW) demonstrated improvement of the testis histomorphometry in which there was no longer detachment of the cells from the basement membrane and appeared normal and intact. However, there were still some tubules with germ cell disorientation and oedematous area in between seminiferous tubules are still the most noticeable lesion in this group. These results indicate that EBN supplementation has a certain effect in preventing testicular tissue damage against RFR emitted by Wi-Fi exposure. As the RFR emitted by Wi-Fi exposure is also known to cause tissue damage through oxidative stress activity (Jaffar et al. 2019), the attenuation effect following EBN supplementation in this study may somehow be associated with its antioxidant content (Hamzah et al. 2013; Yida et al. 2015). A more recent study has demonstrated that these EBN antioxidant properties can significantly reduce total oxidant status and attenuate the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the testis of rats exposed to Wi-Fi (Jaffar et al. 2024).

The antioxidant properties of EBN could be attributed to a few of its constituents, namely ovotransferrin and lactoferrin (Hou et al. 2015). Both ovotransferrin and lactoferrin able to attenuate hydrogen peroxide (H_2O_2)-induced cytotoxicity, and decreased radical oxygen species (ROS) in SH-SY5Y cells (Hou et al. 2015). As H_2O_2 is one of the oxidants that can form following Wi-Fi exposure (Kamali et al. 2018), thus both of these EBN constituents may act as effective scavengers and subsequently minimising the damage caused by Wi-Fi exposure. Moreover, the presence of vitamins A, C and D (Ma & Liu 2012), certain minerals including zinc (Looi & Omar 2016) and its high protein content may also serve as a good source of natural antioxidants in EBN (Ghassem et al. 2017).

However, the reasons why EBN supplementation did not improve germ cell height and spermatogenesis index remain unclear. Several factors could contribute to these findings. For instance, prolonging the Wi-Fi exposure time and reducing the distance between the experimental animal and the Wi-Fi device may result in more significant alterations. Furthermore, this study did not examine how Wi-Fi exposure affects testicular growth at different phases of maturity. The EBN dosage may also need to vary depending on the developmental stage in order to prevent potential toxic effects. Therefore, to holistically

comprehend the Wi-Fi effects on the testicular health, future research should concentrate on these aspects. Moreover, optimising EBN dosages and exploring different developmental phases are necessary to better understand the full potential of EBN supplementation in mitigating Wi-Fi-induced testicular damage.

CONCLUSION

The long-term exposure of Wi-Fi from early childhood to adulthood in this animal model demonstrated detrimental effect on the testicular microscopic structure. The findings indicate that Wi-Fi exposure imposes tissue and cell damage on testicular tissue with no significant effect on the spermatogenesis index. Considering that Wi-Fi have been widely employed in residences, schools and other private spaces without time restrictions, this could contribute to the rising case of male infertility year by year. If this trend persists, it may decrease the reproductive potential in future generations which potentially lead to demographic challenges by 2030. Therefore, precautions and preventive measures should be considered. One of the approaches is by having EBN as supplementation. This study demonstrated that supplementation of EBN preserved the cells in the seminiferous tubule, maintaining its normal and intact appearance, and significantly increased seminiferous tubule diameter. These findings suggest that EBN may offer certain protective effects on testicular tissue against Wi-Fi exposure, particularly during testicular development. This scientific evidence highlights the potential for EBN to be incorporated into male therapeutic regimens. However, further research is needed to fully elucidate the biological impact of EBN as a potential strategy for improving male fertility, particularly among Wi-Fi users.

Ethical approval: All the animal procedures implemented in this study was approved by the National University of Malaysia Animal Ethical Committee (UKMAEC). The approval reference number for this study was FISIO/PP/2018/SITI FATIMAH/28-MAR./908-MAR.-2018-DEC.-2020 and FP/2023/FARAH HANAN/15-FEB./1307-FEB.-2023-JULY-2023-NAR CAT2.

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Conflict of interest: The authors declare no conflict of interest.

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