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The Impact of Mobile Phone Electromagnetic Waves on the Neurons and Blood Brain Barrier Integrity in the Chick Embryo

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The electromagnetic environment surrounding us has dramatically evolved over the past decade, with the proliferation of Wi-Fi, Bluetooth, and other wireless technologies becoming commonplace in our daily lives. Mobile phones emit non-ionizing low-frequency electromagnetic waves (EW). To examine the effects of EW on living cells, this study aims to explore the impact of cell phone EW on the developing brain in chick embryos. The fertilized eggs were allowed to develop under exposure to electromagnetic waves emitted by cell mobile. A cell phone was placed inside the incubator with 20 eggs and was called from outside on a precise schedule. The same number of fertilized eggs were placed in another incubator without a mobile phone and served as the control. Embryos were sacrificed on days 10 and 15, and the cerebral cortex and cerebellum were removed and sent for electron microscopy. In the control group, cerebral neurons appeared healthy, with a large, centrally placed nucleus, visible oligodendrocytes, and a less dense extracellular matrix. In contrast, neurons from the exposed group were smaller, fewer in number, with unclear nuclear margins, signs of shrinkage, and apoptosis and a dense extracellular matrix. In the cerebellum, the exposed group revealed a reduced number of Purkinje neurons and noticeable mitochondrial swelling. The blood-brain barrier remained intact in the control group but was compromised in the exposed group. We conclude that electromagnetic waves emitted by cell phones adversely affect the normal development of the brain in chick embryos.

Keywords: Blood Brain Barrier; Electromagnetic Waves; Mobile Phone; Neuron.

Mobile phones utilize non-ionizing low frequency electromagnetic waves (EW), causing rapid environmental pollution due to exposure to radio waves at frequencies of 800-900 Mega Hz. Excessive exposure to these EW may increase the production of Radical Oxygen Species (ROS). Excessive use of mobile phones by teenagers and pregnant mothers raises concerns due to their increased radiosensitivity. Teenagers developing bodies and the rapid division of embryonic cells in fetuses potentially heighten their vulnerability to electromagnetic waves. Cell phone exposure to prenatal and postnatal children resulted in behavioral problems [1]. These electromagnetic waves affect the brain and memory [2]. Xu demonstrated mitochondrial DNA and mitochondrial RNA level

in the neurons were affected by the electromagnetic fields [3-4]. High levels of electromagnetic waves in mother's bedroom during pregnancy may cause autism in the baby [5].

The effect of radio waves in the adult brain was also a point of interest for investigators due to its proximity to the brain and ears while talking on mobile phones. Reports mention increased headache, fatigue, irritability, stress, sleepiness, concentration difficulties, nausea, lack of appetite, blurred vision and depression[6-12]. Disturbances in sleep and alteration of synaptic plasticity, neurotransmitter release and life cycle of nerve cells occur because of the use of cellular phone [13-14]. Neuropsychiatric changes such as depression, somatization, obsessive compulsivity, phobic anxiety, paranoid ideation, sleeping disturbances were also reported in literature [14-18]. The increasing incidence of depression and increasing use of mobile phones seems to directly relate to each other suggests memory dysfunction, attention dysfunction, decreased motor function, decelerated reaction time and lowered neuromuscular strength [19]. Asad reported a positive relationship between anxiety and depression with smartphone addiction in South Korea [20]. In another study, Siddiqi also mentioned the excessive use of smartphones by medical students [21].

Salford reported that prenatal and postnatal exposure of radio waves stimulate neural cell death and prevents stem cells to change into adult neurons [4]. It is now understood that many neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis may be due to damage to mitochondria of the neurons [22-28]. Furthermore, Down syndrome [29-30], Huntington's disease [31], Friedreich ataxia; oxidative damage of mitochondria is most likely playing a major role in the pathogenesis of these diseases [32-34].

Not much research has been done on the negative side effects of EW on the nervous system. The study revealed more profound knowledge on the effects of EW exposure on the neurons, neuroglia, blood brain barrier and brain intercellular matrix.

Hypothesis

The radio waves affect the developing neurons and the neuroglia in the developing chick embryo.

Objectives

1. To expose the developing chick embryo with EW for 10 and 15 days starting from day zero.
2. To examine the morphological changes in the cerebral cortex and cerebellum at days 10 and 15 of embryo development in the control and exposed groups.
3. Observe the blood brain barrier in the exposed and control groups.

MATERIAL AND METHODS

Chicken fertilized eggs ('Cobb' *Gallus gallus domesticus)* were received from Sohar poultry. This animal model has been used extensively for research purposes [35].

An incubator (Model EH-35, Sino-PFE Company, China) with a capacity of holding 30 eggs at a time and with computerized control of temperature, humidity and air ventilation was utilized (Fig.1). Eggs were rotated ten times per day. Forty zero-day fertilized eggs were divided equally at random into control and exposed groups. In each group, 20 eggs were placed inside the incubator and the temperature was set to 37 degrees and humidity 50-60% by the control panel.

In the exposed groups, the eggs were subjected to EW released by a cell phone placed inside the incubator, while in the control group the eggs were unexposed to EW. A specific branded cell phone with a common cellular service provider was used, operating at 1800 MHz, with a power output of 0.47 W/kg and SAR of 1.10 W/kg (head). The intensity of the EW during the experiment was

Fig. 1. A 30-egg incubator

measured using a Tri Field Meter, model 100XE. The cell phone was activated for 5 minutes at a time, ten times daily, by calling from another cell phone outside the incubator, with no exposure periods in between. Calls were not made during the night. This resulted in a total daily exposure duration of 50 minutes, starting from day zero. The chick embryos were sacrificed on day 10 (500 minutes of exposure) and day 15 (750 minutes of exposure). The egg fully develops in 21 days, day 10 and 15 of development is a reasonably good interval to observe the effects on cells and had been used in other studies [35]. The eggshell was carefully served by scissors, the membranes were removed around the embryo, the chest wall then opened with sharp micro scissors, and the heartbeat was observed. The brain of the embryo was dissected and removed after opening the bones of the skull. The cerebral cortex and cerebellum of the brain were dissected, and specimens were removed and fixed for EM studies (Fig.2).

Control Group

20 eggs were incubated under the same environment except that the cell phone was placed without a battery to make sure that there is no EW emitted. The embryos were dissected at day 10 and

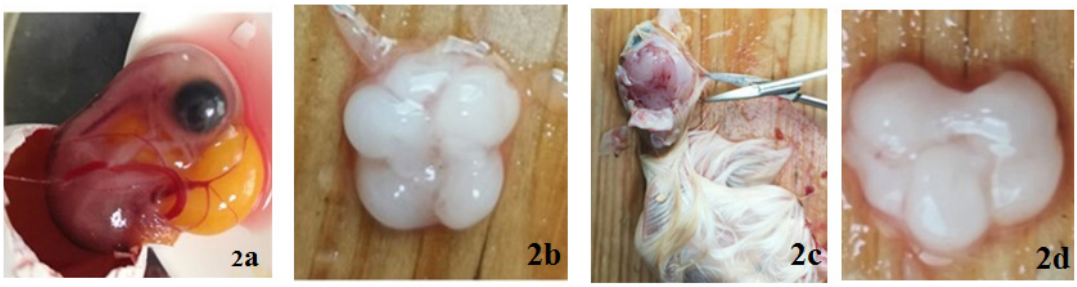

Fig. 2. Day 10 a) Removal of the embryo on day 10 b) brain at day 10. c) Opening of the skull of chick embryo on day 15 d) brain at 15

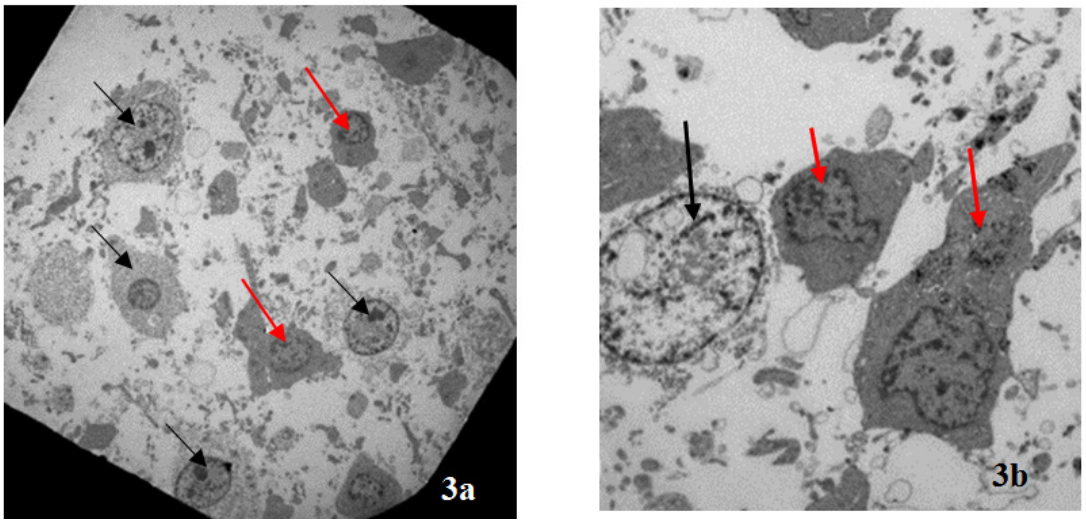

Fig. 3. Control group at Day 10: a) Transmission electron microscope (TEM) analysis shows multiple well developed healthy large pyramidal neurons (black arrow), neuroglial cells (red arrow) and thin extracellular matric (ECM). TEM original magnification X 1000 b) Transmission electron microscope (TEM) analysis shows a well-developed neuron (black arrow) surrounded by neuroglial cells (two oligodendrocytes marked by red arrows) in the cerebral cortex, and scanty ECM with nerve fibers which are not fully developed. TEM original magnification X 3000

15, brain was removed and specimens of cerebral cortex and the cerebellum were removed and fixed for EM study.

Electron microscopy (EM)

Brain specimens were fixed in glutaraldehyde for 5 hours, followed by washing in buffer solution. Further processing for the EM was done at EM Laboratory at Sultan Qaboos University, Oman. Same number of specimens of cerebral cortex and cerebellum in both the groups were taken to the EM lab for processing. Processing of the samples were done based on a protocol by Bozzola and Russell with a few changes. All samples were immediately placed on vials containing EM fixative (2.5% Glutaraldehyde) and left for 2 hours. Tissues were then washed twice using cacodylate buffer (PH 7.2-7.4) 10 minutes each, post fixation was carried using osmium tetroxide for one hour and dehydration was done using series of acetone starting from washing with distilled water, 25% acetone, 75% acetone, 95% acetone and 99.9% acetone. Every step was carried for 10 minutes. Tissues then were placed in 1:1 acetone to pure epoxy resin for one hour then in 1:3 acetone to pure epoxy resin for half an hour. Next it was placed in pure epoxy resin for one hour followed by fresh pure resin for half an hour. Embedding was carried out in size 00 embedding beam capsules (Agar) and polymerized in 70°C oven for 12 hours.

All tissue blocks were cut using Lieca ultramicrotome for semi thin sectioning (0.5 um) with glass knives to allocate the areas of interest using light microscopy then were again cut to have ultrathin sections (60-90 nm thick) with diamond knives and picked up on 300 mesh cupper grids. All sections were stained using uranyl acetate and lead citrate. Sections were then observed using JEOL JEM-1230 EX Japan transmission electron microscope and EM photos were subsequently analyzed.

RESULTS

Brain: 1) TEM of Cerebral cortex: Control group

a) Day 10: Cerebral cortex of the chick embryo showing large well-developed neurons (pyramidal neurons and oligodendrocytes). The nerve fibers can be seen occupying the space in between the neurons; however, many empty spaces could be seen in between the neurons (Fig.3a.b).

b) Day 15: Cerebral cortex of the chick embryo shows large well-developed neurons (pyramidal neurons) and oligodendrocytes and clear nucleus. The nerve fibers are well developed and organized

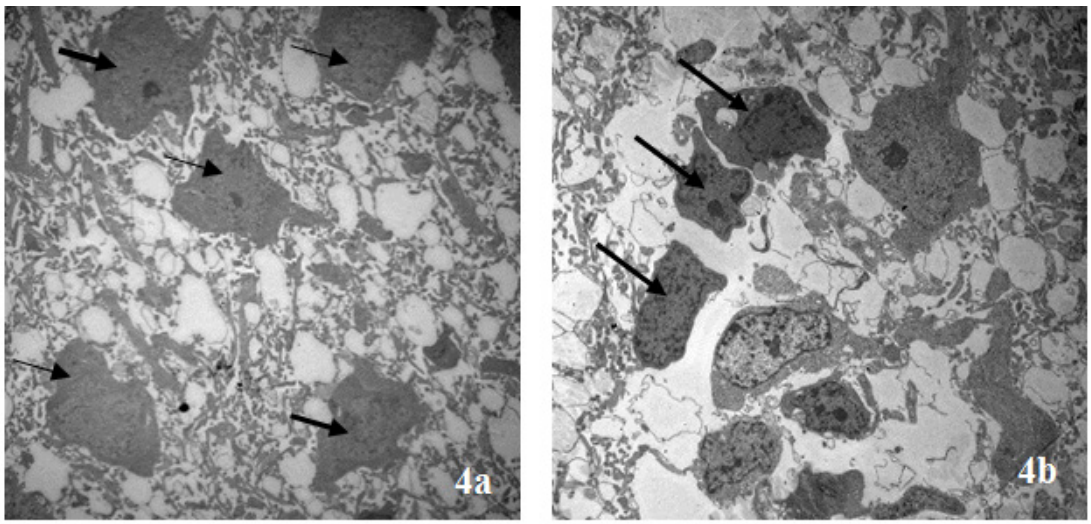

Fig. 4. Control group on Day 15: 4a) Transmission electron microscope (TEM) analysis shows many well-developed pyramidal neurons (arrow), nerve fibers and neuroglia and in the cerebral cortex. TEM original magnification X 2000; 4b) Transmission electron microscope (TEM) analysis shows well developed neurons surrounded by oligodendrocytes (arrows), and nerve fibers in the scanty ECM which is not fully developed. TEM original magnification X 3000

regularly with small gaps in between. (Fig.4 a, b).

Brain: 2) TEM of Cerebral cortex: Exposed group

a) Day 10: The neurons showing changes of apoptosis, chromatin condensation, cellular membrane not visible as compared to the control group. Neurons become shrunken and appear smaller than healthy neurons, with condensed cytoplasm and reduced organelle density. The loss of cytoplasmic volume is often accompanied by a distorted cellular shape and irregular contours. The extracellular matrix (ECM) becomes dense and dark and much fewer white gaps visible thus filling most of the matrix. Axons in the matrix are not clearly seen (Fig.5a).

b) Day 15: Shrunken and atrophied neurons with chromatin condensation and degenerative alteration were observed in the pyramidal neurons;

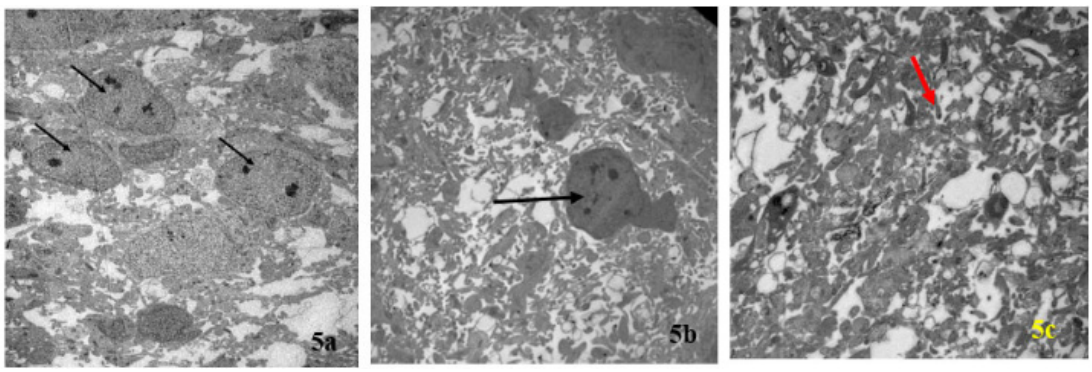

Fig. 5. Exposed group day 10 and day 15: a) Transmission electron microscope (TEM) analysis on day 10 shows multiple cortical neurons whose nucleus are not clearly visible and dense extracellular matrix as compared to the control. TEM original magnification X 3000; b) Transmission electron microscope (TEM) analysis on day 15 shows degenerating pyramidal neurons (arrow) present in the cerebral cortex. The extracellular matrix was much denser when compared to the control on day 15. TEM original magnification X 4000; c) Transmission electron microscope (TEM) analysis on day 15 shows dense extracellular matrix. The cortical neurons and the nerve fibers were not clearly visible as compared to the control. TEM original magnification X 3000

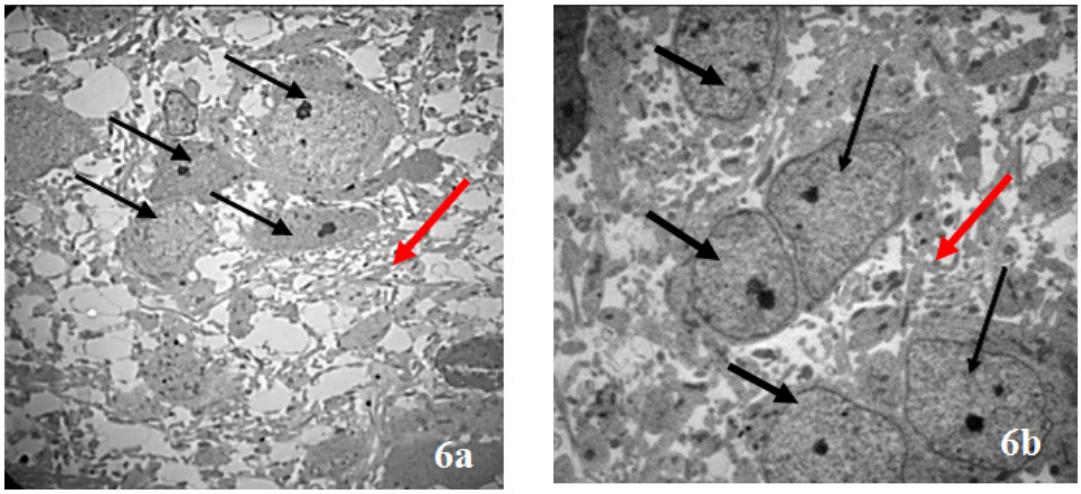

Fig. 6. Cerebellum: Control: a) Transmission electron microscope (TEM) analysis of control group on day 10 shows few large cerebellar (Purkinje) neurons (arrow) and light extracellular matrix containing nerve fibers (red arrow) in the less dense extracellular matrix. TEM original magnification X 4000; b) Transmission electron microscope (TEM) analysis on day 15 shows multiple large multiple well developed Purkinje neurons with rounded nucleus and nerve fibers (red arrow). The extracellular matrix shows clearly the nerve fibers. TEM original magnification X 8000

nucleus membrane blebbing was also apparent. The extracellular matrix (ECM) became very dense and disorganized when compared to the control group. (Fig 5b,c).

Brain: 3) TEM of Cerebellum: Control

a) Day 10: cerebellum shows multiple large neurons and patchy extracellular matrix with nerve fibres. These large neurons are most likely Purkinje cells. The extracellular matrix is less dense with empty spaces in between the neurons. (Fig.6a).

b) Day 15: Multiple large neurons with clear margins and prominent nucleoli can be seen. The extracellular matrix density is increased than day 10 (Fig 6b). The mitochondria in the neurons were oval or slender in shape (Fig.8a). A normal blood brain barrier can be seen with tight junctions between the capillary endothelia (Fig.9a.).

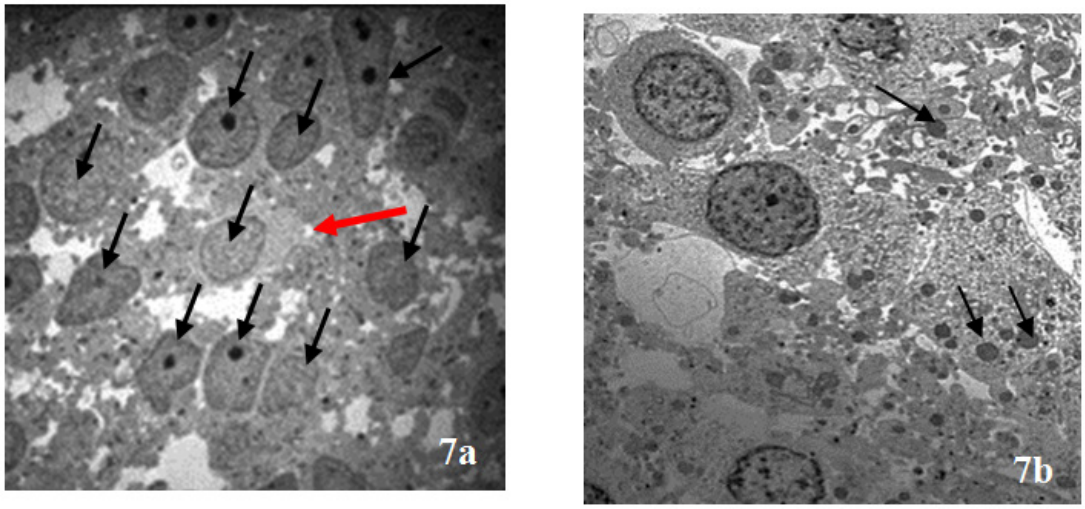

Fig. 7. Cerebellum: Exposed: a) Transmission electron microscope (TEM) analysis on day 10 shows an increased number of Purkinje neurons (arrow) with rounded nucleus, dense extracellular matrix (red arrow) with the nerve fibers and as compared to control. TEM original magnification X 5000; b) Transmission electron microscope (TEM) analysis on day 15 shows well developed rounded nucleus and darkly stained cytoplasmic stress granules (arrow) in the neurons. The extracellular matrix is much denser than the control. TEM original magnification X 8000

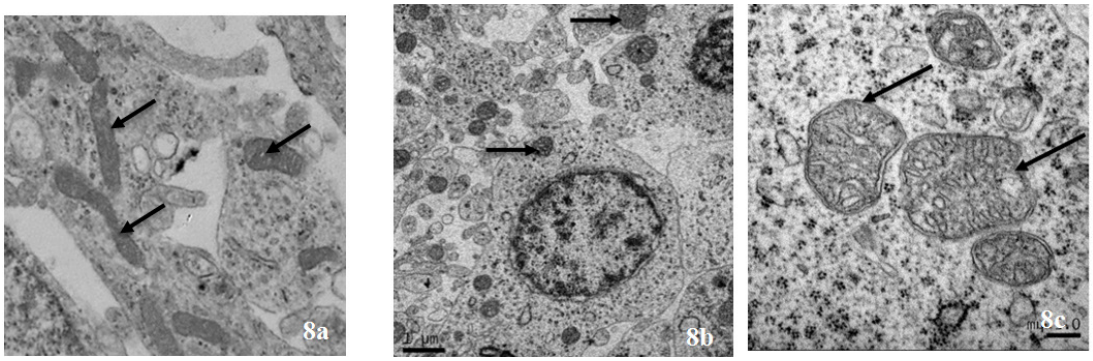

Fig. 8. Cerebellum: Exposed group Day 15: a) Transmission electron microscope (TEM) analysis in the control group on day 15 shows multiple elongated or oval shaped mitochondria (arrow) with cisterns present in the neurons (arrow) TEM original magnification X 40000; b) Transmission electron microscope (TEM) analysis in the exposed group on day 15 shows multiple rounded mitochondria (arrow) with cisterns in the neurons. TEM original magnification X 15000; c) Transmission electron microscope (TEM) analysis in the exposed group on day 15 shows swollen mitochondria showing cisterns and rupture of its membrane (arrow). TEM original magnification X60000

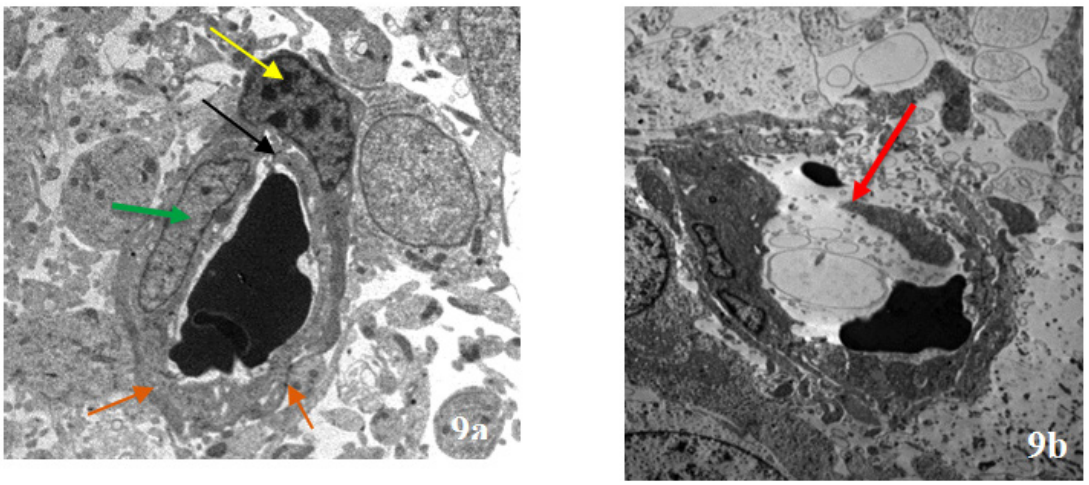

Fig. 9. a) The capillary shows well developed endothelial cell (green arrow) with tight junction (black arrow) and pericyte (yellow arrow) forming the blood brain barrier. The foot process of astrocytes can also be seen surrounding the capillary (orange arrow). TEM original magnification X 6000; b) TEM shows damage to the endothelial cell lining the capillary (arrow) and tight junctions cannot be seen. TEM original magnification X 6000

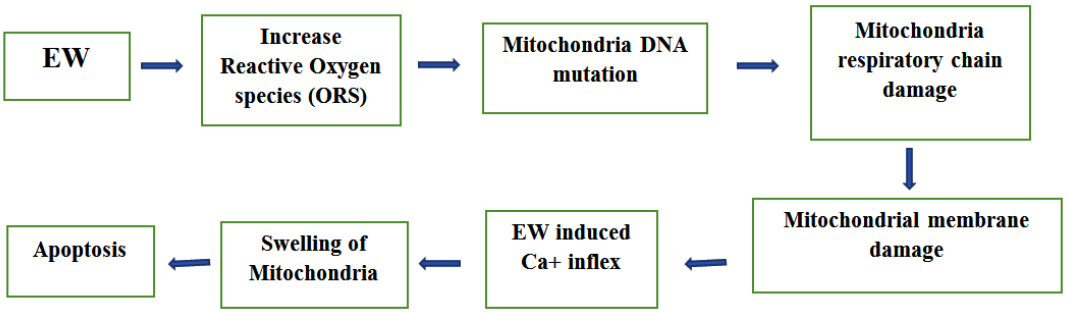

Fig. 10. The diagram showing the mechanism of how the ORS can lead to mitochondrial swelling and cell death

Brain: 4) TEM of Cerebellum: exposed group

c) Day 10: Multiple large neurons were seen and nucleus showing chromatin condensation. The extracellular matrix is much denser as compared to the control group and many black apoptotic granules in the extracellular matrix were observed (Fig 7a). b) Day 15: The neurons were shrunk, and chromatin condensation was apparent. The neurons show multiple rounded swollen mitochondria. (Fig.7b, 8b) At higher magnification the mitochondria can be seen swollen and irregular in shape and rupture of the inner and outer membranes was noted (Fig 8c). A blood brain barrier shows disruption of the tight junctions and damage of capillary endothelium (Fig.9b.).

DISCUSSION

EW adverse effects were studied in the chick embryo brain tissues on days 10 and 15 of its fetal development. The effects of the electromagnetic waves are classified as thermal and non-thermal. Thermal effects are now controlled by strict regulation on Specific Absorption Rate (SAR); this is a measure of the rate at which energy is absorbed per unit mass by the body when exposed to EW. Regulatory bodies like the FCC set limits on SAR to ensure safety (e.g., 1.6 W/kg in the U.S.). Non-thermal effects such as oxidative stress, damage to mitochondria, cell wall, DNA etc. need further research. This study explores the effects

of EM on the neurons, neuroglia and extracellular matrix in a developing chick embryo. In the control groups, neurons were well developed surrounded by oligodendroglia cells and the nerve fibers were showing increasing density from days 10 to 15. In the exposed groups, the neurons showed shrunken morphology with degenerative alteration, nucleus not clearly visible, extracellular matrix became very dense and disorganized as compared to the control. Oligodendrocytes were also not clearly visible.

Sadequl Islam reported darkly stained nuclei in degenerated neurons and decrease in healthy neurons when the chick embryo was exposed with electromagnetic waves [36]. On day 14 of chick development, the number of degenerated neurons with hyperchromatic deeply stained nucleus were significantly more in the group exposed than the healthy neurons in the control group ($p=0.05$).

Hasan [37] observed similar results and found fewer neurons in the exposed embryo. Apoptosis in a few neurons were also observed in the exposed group [38-41]. Radiation causes an increase in superoxide dismutase, catalase and malondialdehyde levels in the embryo.

Eser reported that radio waves caused morphological alterations in the cerebral cortex, brain stem and cerebellum in albino male rats [42]. They observed marked degenerative changes, decrease in cytoplasm and dark pyknotic nuclei and a smaller number of neurons in the EM exposed group versus the control group.

Further reported in literature that radio waves of 900 MHz have caused pyknotic neurons in the hippocampus, deeply stained granules in cerebellum [43-45]. This study also observed deeply stained granules in the cerebellum which are stress granules (SRs). These stress granules inhibit stress-induced apoptosis and help the cell to repair.

Romeo did a meta-analysis on the fact that electromagnetic waves exposure causes apoptosis and found a mixed result; however, many studied reported apoptosis as a result of electromagnetic exposure [46].

Islam also reported an increase of VEGF-A expression in the exposed group which mean inflammation and hypoxia leading to oxidative stress induced by the radio waves [36]. Vahid reported the electromagnetic radiation induced the entry of calcium via TEPV1 channel in hippocampus and dorsal root ganglia in rats resulting in apoptosis [47]. Absence of mitochondrial function, rise in amyloid beta expression and stimulation of apoptotic factors e.g. caspase-9 and -3 in the hippocampus after EMR-2450 MHz exposure was reported [48].

Blood brain barrier is another structure which can be affected by electromagnetic waves. Persson reported that radiation from cell phones can damage the blood brain barrier in rats allowing albumin to enter the brain [49]. This study observed very dense extracellular matrix due to which the nerve fibers cannot be identified. It seems that rupture of the blood brain barrier in the exposed group on day 15, resulted in leakage of albumin in the extracellular matrix giving it a dense matrix appearance. (Fig.5a, b).

Salford, L. G investigated the impact of microwave exposure from GSM mobile phones on rat brains, specifically looking at the blood-brain barrier and neuron damage and reported significant damage of the neurons of the cortex, hippocampus and basal ganglia because of albumin leakage through blood-brain barrier [50]. Stam reported damage of blood brain barrier by radio waves exposure in animal model however stated that in humans the evidence of damage is still not reported [51]. Leszczynski in his research focused on how mobile phone radiation can non-thermally activate HSP27 stress pathways in human endothelial cells, thereby increasing permeability of blood-brain barrier [52]. Nittby also reported the permeability changes in blood-brain barrier of rats after exposure to GSM-900 mobile phone radiation and found significant correlation of albumin excavation and exposure level [53]. Eberhardt, J. L. in his research provides an overview of how microwave radiation affects blood-brain barrier permeability, leakage of albumin into the extracellular matrix and resulting in neuron damage in rats[54]. Shabani also reported increased permeability of the blood-brain barrier in rats[55]. Gao examined albumin immunohistochemistry and Evans blue staining after exposure of rat's brain to electromagnetic pulses. Zonula occludens were evaluated using western blotting, results revealed increased permeability of blood brain barrier [56]. This research revealed that the blood brain barrier

in the cerebellum was damaged, and leakage of albumin was evident in the extracellular matrix of the exposed group.

This study also reported the degenerative changes in the cerebral cortical and cerebellum neurons after exposure to radio waves. This exposure also affected the mitochondria in the cerebellum neurons and observed deformity of the mitochondria which became rounded instead of oval or elongated. This agrees with other reports mentioned in literature. Mitochondrial DNA damage due to oxidative stress in primary cultured neurons were observed [3]. Increased permeability to calcium due to ROS production leads to mitochondrial swelling. Vicious cycle causing further increase in ROS triggers apoptosis (Fig.10). First sign of mitochondria cell membrane damage is its swelling, which increase with further increase production of ROS, and the mitochondria will become round. Oxidative stress cause mitochondrial DNA mutation, damage mitochondrial respiratory chain, alter membrane permeability and effect ca+ homeostasis and mitochondrial defense system [57]. Mitochondria became swollen and vacuolated in motor neurons in mice [58]. In this study, mitochondria were observed to be swollen in the cerebellum in the exposed group versus the control where they were oval and elongated. Permeability of mitochondrial membrane depends on communication between ca^{2+} and ROS system. A major cause of EM-induced calcium influx into the mitochondria is increased sympathetic activity [59]. ROS when stimulated will produce free O +2 on the mitochondrial inner surface. This free O+ 2 will invade thiol protein to open up the transition pores, increases membrane permeability and causes mitochondria to swell [60]. Mitochondria is the most sensitive organelles to oxidative stress and swells under such conditions [61].

To carry out highly specialized functions the neurons require more energy as compared to other cells. Mitochondria generate energy, and its dysfunction thus plays a major role in different neurodegenerative diseases. It is now understood that abnormal mitochondrial dynamics pertinent to neuronal synaptic loss and cell death may be a cause of Alzheimer's disease, Parkinson's disease, and Huntington's disease [48]. Abnormal mitochondrial morphology was reported in mutants of Parkinson's disease-related genes.

Mitochondria are unique organelles within cells, distinct from the nucleus, as they contain their own DNA known as mitochondrial DNA. They are capable of synthesizing their own RNA and proteins, and has the mitochondrial respiratory chain on its inner membrane [62]. This chain comprises of five complexes—namely I, II, III, IV, and V. The first four complexes are accountable for converting adenosine diphosphate (ADP) into adenosine triphosphate (ATP)[63].

1-5% oxygen is converted into ROS under normal conditions; mitochondria is a primary source of intracellular ROS [64]. Complex III is the major site of ROS production under typical metabolic conditions [65]. Elevated ROS levels can damage these complexes and other mitochondrial macromolecules, including lipids, proteins, and DNA [66]. Damage to mitochondrial DNA exacerbates oxidative stress, creating a harmful cycle of ROS generation and eventually leading to apoptosis, which can disrupt mitochondrial energy production [67,68].

Increased permeability of the mitochondrial inner membrane disrupts mitochondrial calcium homeostasis [60]. Elevated calcium levels enhance the production of superoxide radicals, propagating a cycle of damage. Excessive calcium accumulation leads to osmotic swelling and damage of the outer mitochondrial membrane [69]. Increased ROS production further compromises membrane integrity, boosts calcium uptake, and triggers apoptosis and cell death [46, 70].

Calcium ions must enter the neuron's cytosol for neurotransmitter release [5]. Neuronal signaling relies on the release of neurotransmitters, which is initiated by a brief influx of calcium into the cytosol. Exposure to radio waves can cause membrane leaks, elevating intracellular calcium concentrations. This heightened calcium level causes the cell to be overly responsive, releasing more neurotransmitters and leading to increased brain activity [71-74]. Consequently, the brain can become overloaded with excessive signals, potentially resulting in concentration issues and attention deficit hyperactivity disorder (ADHD).

CONCLUSION

We concluded that electromagnetic waves have produced damage to the cerebral and

cerebellar neurons and shown increased density in the extracellular matrix of the developing chick embryo because of damage to the blood-brain barrier. Brain blood-barrier damage and leakage of albumin are the most probable causes of the increased density in the extracellular matrix and neuronal damage. Swelling of the mitochondria and damage to its membrane might be due to an increase in oxidative stress caused by electromagnetic waves. To bridge our findings with broader health implications and an increase in the neurodegenerative disease in old age, further research is needed to understand how the observed cellular changes found in this study due to exposure of electromagnetic waves could translate into the development of neuro degenerative diseases.

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Conflicts of Interest

The author(s) do not have any conflict of interest.

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Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and with the main author. The raw data that support this study is available at the EM laboratory at Sultan Qaboos University, Oman, which are available from the 1st author upon reasonable request.

Ethics Statement

This article was approved by the research committee of the college of medicine and health sciences, National university of science and technology.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Author's contribution

Prof. Najam Siddiqi: Writing the proposal, doing the experiment, collecting the data, analyzing the data, writing the paper. Dr Faisal Moin: Analyzing the data, writing the paper, collection of the references, and putting them in order. Mr. Mohammed Al Kindi: Processing the EM mesh for EM, Taking the pictures and analyzing the data.

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