

Role of radio-frequency electromagnetic waves in causing oxidative stress

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Introduction

Mitochondria are the only organelles inside the cell except for the nucleus, which contains DNA called mitochondrial DNA and which can synthesize its own RNA and proteins (Douarre et al., 2012). Inner membrane contains the mitochondrial respiratory chain. The respiratory chain consists of five complexes, namely I, II, III, IV, and V, out of which the first four complexes constitute the respiratory electron-transport chain and catalyze the phosphorylation of adenosine diphosphate to adenosine triphosphate (Sas et al., 2007). More than 80 proteins comprise these complexes and 13 of them are encoded by mitochondrial DNA (Chen et al., 2005). Moreover, 1%–5% of oxygen is converted to ROS in normal conditions, and the majority of intracellular ROS is produced by the mitochondria (Wei et al., 2001). Complex III is the main site of the production of ROS under normal metabolic conditions (Finkel & Holbrook, 2000). An increase in ROS would directly attack these complexes and macromolecules within the mitochondria including lipids, proteins, and DNA (Andreazza et al., 2010). Mitochondrial DNA if damaged will increase oxidative stress and lead to a vicious cycle of ROS leading to apoptosis (Van Houten et al., 2006). This can lead to the shutdown of mitochondrial energy production (Ghezzi & Zeviani, 2012). Mitochondrial inner membrane permeability increases, which affects mitochondrial Ca homeostasis; an increase in Ca levels causes an increase in the production of superoxide ion radicals (Joshi et al., 2005), causing a vicious cycle. Mitochondria becomes overloaded with Ca resulting in osmotic swelling and rupture of the outer mitochondrial membrane (Ghezzi & Zeviani, 2012). ROS production increases membrane permeability and increases Ca uptake, which initiates apoptosis and cell death (Ross, 2012). Smartphones use electromagnetic waves. Excessive exposure to these low-frequency radio waves may affect the mitochondria by increasing ROS production. There has been great concern about the rising use of mobile phones by children and the possibility of affecting unborn babies during pregnancy since the embryonic cells in the fetus and children are more radiosensitive than adults. The aim of this study is to observe the changes in the mitochondria of different cells in the

embryo of a chick after exposure to radio waves. Heat shock proteins can be used as biomarkers to detect cytotoxicity and environmental stress affecting normal cellular functions. In this study, we used levels of HSP70 mRNA and protein expression to quantitate the severity of stress induced by irradiation of breed 0-day fertilized chicken eggs embryo. Cells respond to a variety of stimuli in which the precise biological effect reflects the nature of the applied stress that depends on the duration of the exposure. Among these effects are altered transcription of specific genes and translation of various proteins including heat shock protein 70 (HSP70). Heat shock proteins play a major role in maintaining cellular homeostasis, particularly in situations of stress. They are constitutively expressed but can be upregulated by cell stresses including heat (hence the name “heat-shock protein”), oxidative stress, infection, and so on (Brenu et al., 2013). HSPs play an important role in immunity and inflammation. Recent work indicates that they modulate the function of regulatory T cells. These proteins have the potential to interact with a wide array of proteins and protein factors to facilitate proper folding patterns of specific proteins in addition to potentiating downstream effects. HSP70 translocates into the plasma membrane following cellular stress and is released into the extracellular environment in a membrane-associated form that activates macrophages (Vega et al., 2008). Extracellular heat shock proteins (eHSPs) in general help to modulate the immune system by activating the neutrophils (Ortega et al., 2009), chemotaxis (Ortega et al., 2009), and cytokine production by the immune cells (Horn et al., 2007). It also increases enzymatic activities of ornithine decarboxylase and cytochrome oxidase both of which measures to combat sudden stress trauma (Byus et al., 1988). Hypothesis: The radio waves produce stress to the cells and damage the mitochondria present in the hepatocytes, cardiomyocytes, and neurons. Objectives: 1. Expose the developing chick embryo to radio waves from a mobile phone for a period of 10 and 15 days. 2. Observe mitochondria in hepatocytes, cardiomyocytes, and neurons after 10 and 15 days of development and compare with the control group. 3. Study the production of HSP70.

Material and methods

Animal experiment: “Cobb” (*Gallus gallus domesticus*) breed 0-day fertilized chicken eggs were acquired from Sohar Poultry Company S.A.O.G. (PO Box 2808, Ruwi, Postal code 112, Sultanate of Oman), by applying prefixed inclusion and exclusion criteria. The chick embryo model was previously extensively used as an animal model to assess the effects of electromagnetic waves. A 30-egg incubator (Egg Incubator Model EH-35, Sino-PFE Company, China) with automatic temperature, humidity control, and forced air ventilation was used. It was also equipped with special egg holders with automatic egg rotation capability, which was fixed at 10 rotations per day. The mobile phone was placed in the center of the incubator under the egg holder so that the farthest egg was within a radius of 16 cm. The temperature was set at 37°C and the humidity at 50%–60%. Moreover, 30 eggs were placed at one time in the egg holders (Fig. 5.1).

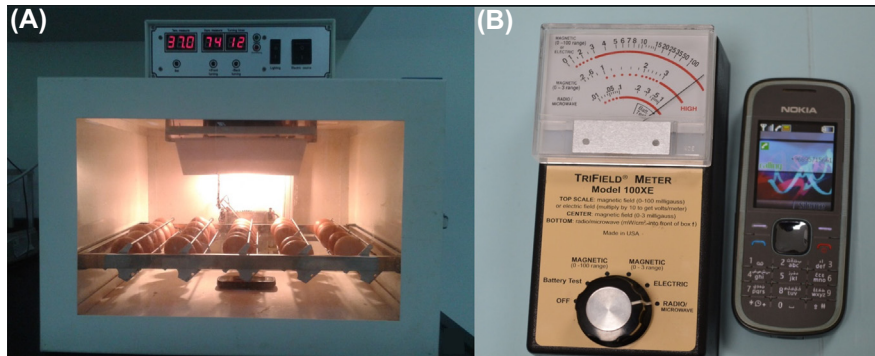


FIGURE 5.1

Incubator. (A) A 30-egg incubator. (B) TriField meter showing high levels of electromagnetic waves from mobile phone during call receiving mode.

For each experiment, 40 fertilized eggs were randomly divided into two groups: the control and the experimental group. One incubator was used carrying 20 eggs at a time: one wave of the exposed group experiment and one wave of the control group. Freshly fertilized chicken eggs were exposed to radiofrequency waves (RFW) emitted by a mobile phone during embryonic development and compared with the control eggs, which were not exposed to RFW. A popular mobile phone and service provider was selected with 1800 MHz frequency, power of 0.47 W/kg body, and SAR 1.10 W/kg (head). A TriField Meter, model 100XE was used to detect the strength of RFW of the mobile phone during the experiment (Fig. 5.1). *Experimental group*: 20 fertilized eggs were incubated in the incubator with the mobile phone in silent mode with the vibration mode disabled. The distance of all the eggs from the mobile phone was maintained within one wavelength (approximately 16.5 cm) of the emitting 1800 MHz frequency electromagnetic waves. The mobile phone was rung from another mobile phone for 5 min, 10 times daily with an exposure-free period in between the calls. No calls were made at night. The total daily exposure duration was 50 min in each 24 h starting from day 1. Ten eggs were sacrificed on day 10 (total exposure time 500 min) and 10 eggs at day 15 (total exposure time 750 min). For the control group, 20 eggs were placed in the incubator, and 10 eggs were sacrificed on days 10 and the other 10 eggs at day 15 of development.

On the scheduled day of sacrifice, a small hole was first made in the egg shell and then a portion of the shell was carefully cut by scissors and removed. The embryo was dissected from the membranes and its survivability was noticed by either movement of the limbs or beating of the heart. The chest wall was opened with the help of sharp microscissors and observed the heart beating. *Control group*: 20 eggs were incubated in the same conditions in the same incubator. The mobile phone was turned off and the battery was removed and placed in the middle of the incubator. The embryos were examined just as in the experimental groups on days 10 and 15 (Fig. 5.2).

**FIGURE 5.2**

Chick embryo. The chick embryo after removal of the shell but still inside the membranes.

Electron microscopy

The heart, liver, and brain were removed and fixed in glutaraldehyde for 5 h, and then washed and placed in a buffer before it was taken to the electron microscopy lab for processing. Five specimens from day 10 and day 15 each for each organ in the exposed group and the same number in the control groups were selected for electron microscopy preparation. Specimens were sent to the Department of Pathology, Electron microscopy laboratory, Sultan Qaboos University, Muscat. *Heat shock protein (HSP 70)*: The developing embryos were removed at 10 and 15 days, and liver and heart tissues were dissected, fixed in formalin, homogenized, and processed for the estimation of HSP70 mRNA and protein concentration. After tissue homogenization, total RNA was extracted and HSP70 mRNA was quantitated. Gradient-based ultracentrifugation was employed to fractionate tissues into various cellular organelles after which cells were lysed and HSP70 concentration was assessed.

RNA extraction

RNA was extracted from the supernatant of the homogenized tissue using the Qiagen RNeasy Mini Kit (QIAGEN, CA, USA), and concentration was determined. cDNA

synthesis was performed by using a High-Capacity cDNA Reverse Transcription Kit (Applied BioSystems, Austin, TX). cDNA was then quantified using a Nanodrop reader (Thermo-Fisher Scientific, USA) (Beltrame et al., 2015). Quantitative RT-PCR TaqMan reagents were used to perform qRT-PCR. qRT-PCR was then carried out in the ABI 7500 Fast real-time PCR machine (Applied Biosystems, Austin, TX). The relative expression level of HSP70 was calculated using the comparative delta Ct method by normalizing the cycle threshold values of HSP70 with those of GAPDH (Wu-Fienberg et al., 2014). *Protein analysis:* Differential centrifugation (Beckman J2- 21 and ultracentrifuge, USA) was used to separate organs from the sacrificed chick embryos. Protein quantitation was then carried out using standard electrophoretic techniques. ELISA analysis was then performed using an HSP70 antibody obtained from SimpleStep (abcam, Cambridge, UK).

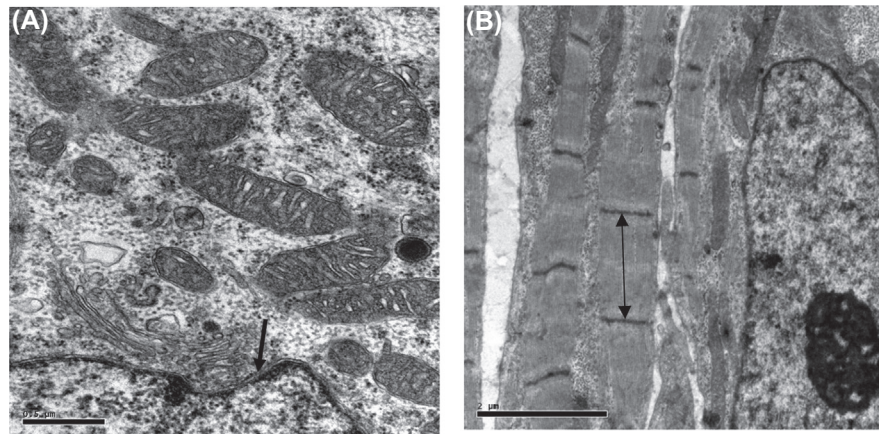
Results

Cardiomyocyte: 1. Control group: At day 15, the cardiomyocyte showed well-developed normal oval mitochondria, showing the cristae and double membrane. The nucleus can also be seen within the nuclear membrane. The cell cytoplasm was seen full of actin-myosin filaments. Many intercalated discs can also be clearly seen. Cytoplasm also shows the Golgi apparatus and scattered ribosomes. 2. Exposed group: The mitochondria's normal oval shape became flat and elongated and the double-layered membrane can be seen broken; however, the cristae were visible. The intercalated discs were also seen as damaged. The ribosomes were scattered among the actin and myosin filaments whose arrangements were damaged (Figs. 5.3 and 5.4).

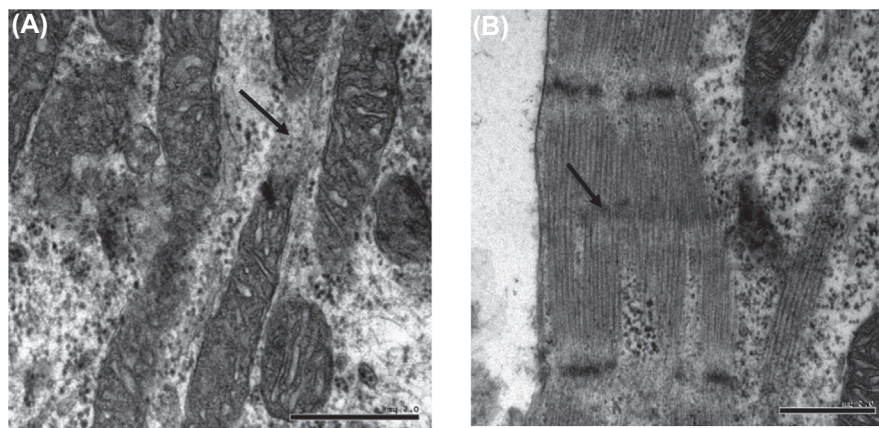
Hepatocytes: 1. Control Group: On both day 10 and 15, the cell cytoplasm was full of mitochondria, which were oval and showed the crista and the inner and outer membranes. Rough endoplasmic reticulum and nucleus can also be seen. 2. Exposed Group: the cells showing elongated or swollen mitochondria, some degenerated and rupture of the membranes. Some mitochondria were dumbbell shaped and surrounded by fragments of rough endoplasmic reticulum. The crista was not clearly seen (Figs. 5.5 and 5.6).

Brain: 1. Control Group: The specimens were taken from the cerebral cortex. It showed well-developed neurons and oligodendrocytes and developing axons. The neurons were less developed on day 10 with less fibers; however, on day 15, the axons were regularly organized and showed well-developed neurons. Mitochondria were oval showing the crista and the inner and outer membranes. 2. Exposed Group: on day 10, a few neurons were observed, which were smaller. At day 15, the axons were denser and seemed irregularly arranged, the neurons were few and smaller in size. The mitochondria were seen as more rounded in shape and the crista was not clearly seen (Figs. 5.7–5.9).

Heat shock protein: Liver and heart tissue expression levels of HSP70 mRNA were quantitated using real-time PCR (qRT-PCR). Analysis was done after 10 and

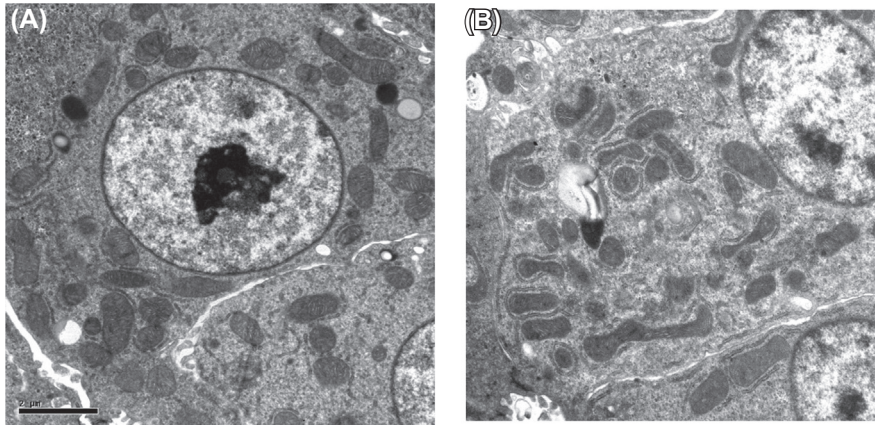
**FIGURE 5.3**

Heart: Control group, day 15. (A) Mitochondria with well-formed cristae, Golgi apparatus, and nuclear membrane can be seen (*arrow*). (B) Well-formed sarcomere (*double arrow*), nucleus, nucleolus, and few mitochondria.

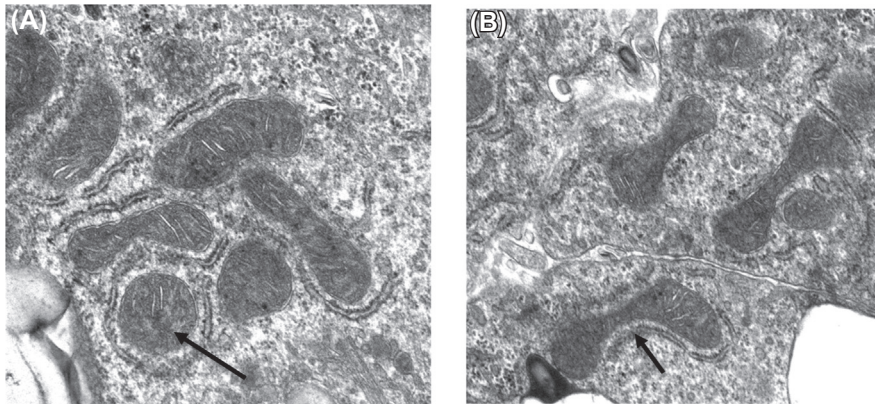
**FIGURE 5.4**

Heart: Exposed group, day 15. (A) Mitochondria changed their shape and became thin and long as compared to the control, damage can also be observed (*arrow*). (B) Damage to the Z-lines can be observed (*arrow*).

15 days of exposure to RFW. mRNA levels remained almost unchanged for the control samples while a significant increase in the expression of HSP70 mRNA in the exposed groups was observed. The liver tissue expression of mRNA was more significant particularly after longer exposure to the physical stimulus (Fig. 5.10). The amount of mRNA in the heart tissue decreased steadily with longer exposure to RFW radiation. Under normal circumstances, levels of mRNA in various tissues

**FIGURE 5.5**

Liver: Control group, day 15. (A) Day 15 control showing well-formed nucleus, oval mitochondria surrounded by a layer of rough endoplasmic retinaculum, canaliculi, and few fat vacuoles. (B) Exposed group: the mitochondria become dumbbell shaped and myelin figures were seen.

**FIGURE 5.6**

Liver: Exposed group, day 15. (A) A few mitochondria were swallowed (*arrow*); others were elongated or dumbbell shaped in the process of degeneration and marked derangement of the crista. (B) Dumbbell-shaped mitochondria surrounded by fragments of rough endoplasmic retinaculum (*arrow*).

were equal and increased only after adequate exposure to the stress stimulus. It is interesting to notice that mRNA levels in all tissues were identical demonstrating the universal role HSP70 plays in cellular hemostasis across all tissue types. Both heart and liver tissues are increasingly expressing higher levels of HSP70 mRNA as exposure to RFW is prolonged further (Fig. 5.11). Higher levels of HSP70

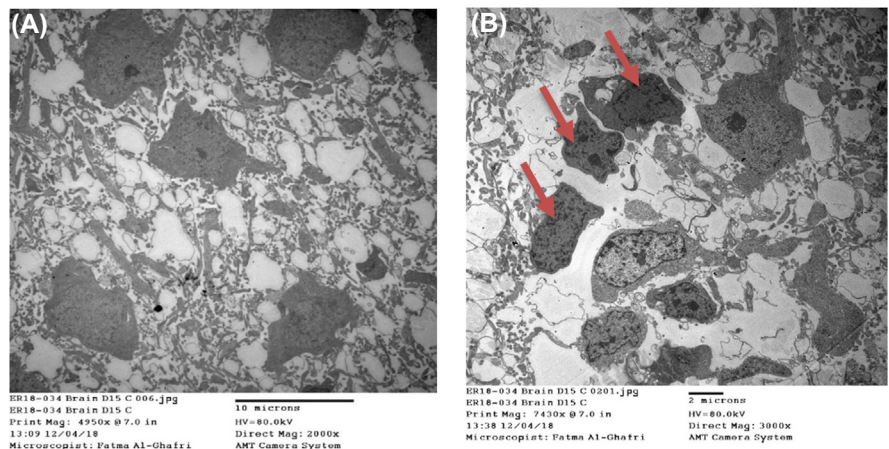


FIGURE 5.7

Brain: Control group, day 15. (A) The cerebral cortex of the brain showing enlarged neurons, neuroglia composed of fibers. (B) The control group showing neurons and oligodendrocytes (*arrow*) and fibers.

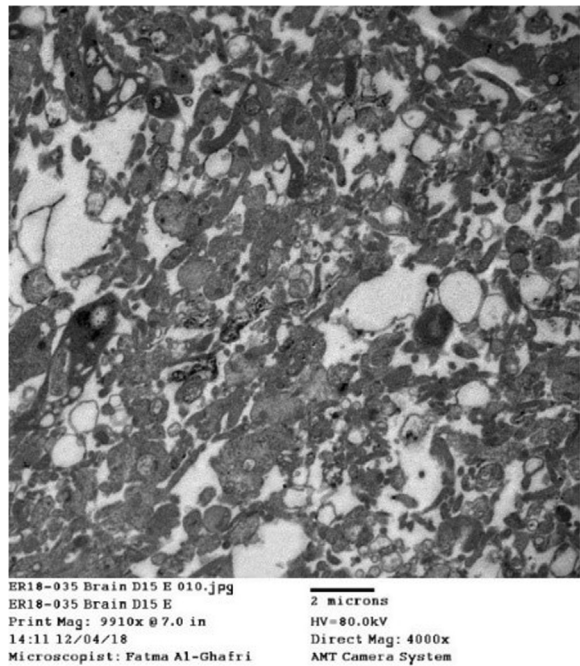


FIGURE 5.8

Brain: Exposed group, day 15. The cortex of the brain showing few small small neurons, and the neuroglia is very dense.

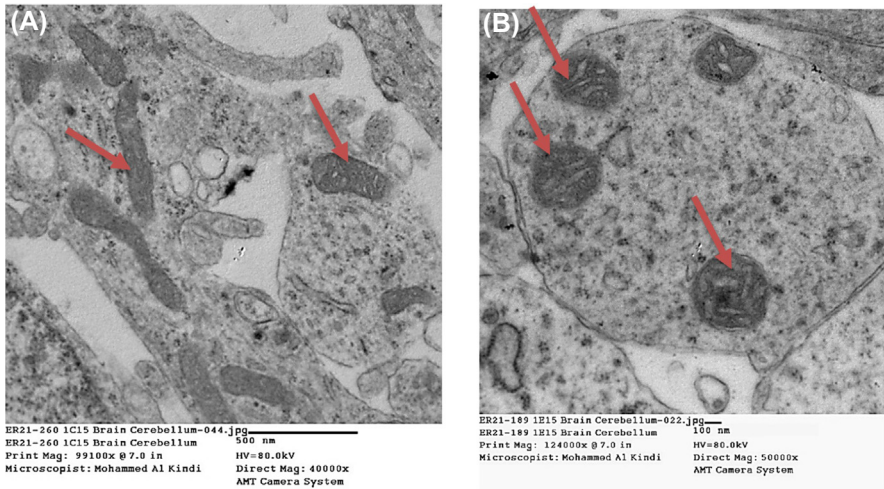


FIGURE 5.9
Brain: Mitochondria, day 15. (A) Control group: The cerebral cortex neurons showing oval and elongated mitochondria (arrow). (B) Exposed group: The mitochondria are swollen and rounded in shape (arrow).

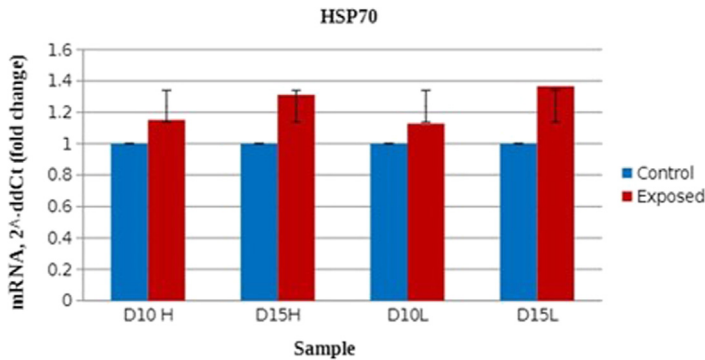


FIGURE 5.10
Quantitative real-time PCR: mRNA expression analysis after 10 and 15 days of exposure, respectively (H: heart, L: liver).

mRNA are produced in the liver tissue than in the heart tissue emphasizing the pivotal role the liver plays in survival mechanisms. Such augmentation to express higher amounts of HSP70 mRNA is an attempt to synthesize more amounts of the protein to help offset the stress stimulus (Fig. 5.10).

Discussion

The effects of radio waves were studied in a developing chick embryo at day 10 and day 15 of its development. Electron microscopy of the hepatocytes, cardiomyocytes,

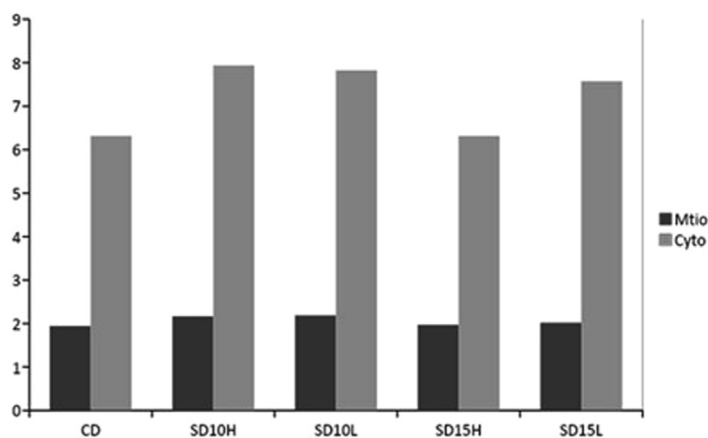


FIGURE 5.11

HSP70 expression in different cellular compartments. CD: Control; SD10H and SD15H: Heart tissue exposed for 10 and 15 h, respectively; SD10L and SD15L: Liver tissue exposed for 10 and 15 h, respectively.

and neurons was conducted, and the structure of the mitochondria was observed in the exposed group and compared with the control group. The control group, an oval mitochondrion, was seen in all the cells on both day 10 and day 15. The crista, inner and outer membrane, can be seen clearly. In the exposed groups, there was an increase in the number of mitochondria; they were swollen, elongated, dumbbell shaped, and in the process of degeneration. In the hepatocytes, fragments of rough endoplasmic reticulum surrounded the mitochondria, in the cardiomyocytes, mitochondria shape changed from oval to elongated and showed areas of degeneration while in the neurons in the cerebral cortex, the mitochondria were swollen. Oxidative stress induced by H_2O_2 leads to an increased number of mitochondria and mtDNA in human lungs (Lee et al., 2000). Mitochondria are the source of free radicals produced in response to electromagnetic wave exposure in human sperm. A potential causative mechanism was electron leakage from the mitochondrial electron transport chain which causes oxidative DNA damage. It has been reported that the permeability of the mitochondrial membrane is dependent on the interaction between Ca^{2+} and ROS system. Increased sympathetic activity is considered a primary cause of RFW-induced calcium influx into the mitochondria (Lahijani et al., 2009). ROS when stimulated on the inner surface of mitochondria will produce free oxygen radicals, which invades thiol protein and causes transition pores in the membrane to open thus increasing its permeability and causing mitochondria swelling (Lahijani et al., 2009). Mitochondrial swelling was also observed by Attia and Yehia (2002) in rat hepatocytes in response to electromagnetic field exposure. Voyedovodskayanv et al. (1981) reported that paramagnetic particles are naturally located in the mitochondria of liver cells which make the hepatocytes sensitive to impose magnetic fields leading to modification of hemostasis by altering mitochondrial respiration

in the hepatocytes. Moreover, [Gorczyńska and Węgrzynowicz \(1991\)](#) reported that mitochondria are the most sensitive organelle to stress-generating factors and observed mitochondrial swelling in such conditions. Oxidative damage to the mitochondrial DNA in primary cultured neurons was also reported ([Xu et al., 2010](#)). Hence, this study observed changes in the shape of the mitochondria which is similar to other reports mentioned in literature. Production of ROS causes an increase in permeability to Ca which leads to swelling of the mitochondria. Further increase in ROS leads to a vicious cycle which triggers apoptosis. Swelling of the mitochondria is the first sign of damage to the cell membrane, and with continued ROS production the mitochondria become elongated, or dumbbell shaped and ultimately broken and degenerated. Heat shock protein70 (HSP70) is a multifaceted chaperon protein that is conserved across species, and it is involved in many cellular metabolic processes. Its prime role as an antistress agent has been exploited heavily to further elucidate its mode of action and identify its cellular distribution ([Krause et al., 2015](#); [Tytell, 2005](#); [Whitham & Fortes, 2008](#)). In this study, HSP70 was investigated for its cellular organelle distribution and pattern of differential expression under stress conditions induced by RFW. Previous studies have demonstrated the overexpression of HSP70 mRNA under various stress conditions but have not explained the magnitude and the subcellular compartmental localization of the protein modification. Moreover, to investigate the differential tissue and cellular mode of expression of HSP70, an inexpensive and readily available chick embryo experimental model was used, which proved to be reliable for this type of experiment. Liver and heart tissues exposed to RFW have shown a significant increase in levels of mRNA and HSP70. RFW significantly altered the pattern in which HSP70 is expressed and translated in almost all studied tissues. There was an 18% increase in the expression of mRNA in the heart tissue after 10 days of exposure compared to the control group. This level of mRNA expression was more evident with prolonged exposure to RFW reaching up to 32% after 15 days compared to the control unexposed group. Similar results were observed with the liver samples where 15% and 38% increases were calculated after 10 and 15 days of exposure, respectively. Moreover, the magnitude of increase of mRNA after 15 days as opposed to 10 days was 14% in the heart tissue and 23% in the liver tissue. This corresponds to an increase rate of approximately two folds and two folds and a half in the heart and liver tissues, respectively, after longer exposure to the stimulus. Differences in mRNA levels after response to the effector stimulus may be attributed to the difference in copy number in each tissue giving an insight into the role this protein plays under specified conditions. This increase in mRNA was not paralleled by an equivalent increase in HSP70 protein concentration in both tissues and under all given experimental conditions. Levels of mRNA and HSP70 in the liver tissue were higher than in the heart tissue demonstrating the important role the liver plays in combating cellular stress using HSP70 as one of many mechanisms employed to attenuate stress effect. Total protein in both tissues remained almost unchanged for both exposed and control groups possibly because cells were under stress conditions and to survive needed to quench the effects of stress by activating more of the housekeeping genes. HSP70 is an

antiapoptotic (Rérole et al., 2011) agent that will promote cellular survival. Initially, when cells are exposed to stress stimuli, they respond in part by producing surplus amounts of HSP70 mRNA by activating the heat shock transcription factors (HSFs). Moreover, the stimulation of HSP70 expression is dependent on various genetic and environmental factors that orchestrate their effects to control stress shock (Shamovsky & Nudler, 2008). This stimulation can last as long as the stimulus is active and may generate excessive amounts of HSP70 mRNA to the extent that it will start competing with the housekeeping genes mRNAs for a place on the cytoplasmic ribosome for translation. This competition and the limited number of cellular ribosomes result in lesser amounts of HSP70 mRNA translation into a fully functional protein. Moreover, HSP70 gene transcription is active albeit processing into messenger RNA is slow due to the excessive number of housekeeping genes being transcribed at the same time. This suggests that the translation efficiency of HSP70 mRNA under stress conditions is intensely modified for as yet unclear reasons. In recent studies (Tytell, 2005; Whitham & Fortes, 2008), it was observed that the ratio between extracellular HSP70 (eHSP70) and intracellular (iHSP70) may indicate the severity level of the stress applied (Krause et al., 2015). Our study and unpublished data have demonstrated increasing concentrations of eHSP70 as the stress stimuli sustain for longer periods, particularly in the liver tissue. Cells undergo structural and morphological alterations at different levels including the cell membrane which becomes leakier expelling HSP70 to the extracellular side. In addition, membrane-bound HSP70 might also become detached and therefore shed into the extracellular space (Jones et al., 2011). It is possible to postulate from the above that the role of eHSP70 is to act as an antiinflammatory agent and possibly help in immune response modulation and cytokines recruitment. To evaluate the effect of the stress stimuli in inducing expression of HSP70 in specific cellular compartments, we proposed to examine cytoplasm and mitochondria as an example in both heart and liver tissues. Our results have shown considerable variations within the cellular compartments of the same tissue and across different tissues that were examined under increasing exposure to RFW. In the mitochondria of the heart, there was a decrease of 10% in the concentration of HSP70 after 15 days of exposure compared to exposure for 10 days while in the liver the decrease was only 7% between the two exposure intervals. Mitochondria are involved directly in the process of apoptosis initiation, and this is opposite to the potential role of HSP70 as an antiapoptotic protein even with longer exposure. Moreover, HSP70 genes are not part of the mitochondrial genome therefore, it depends on the cytoplasm for its HSP70 load. The cytoplasmic HSP70 levels have shown a similar trend although more pronounced than in the mitochondrial. The calculated drop of HSP70 concentration in the heart was 26% between 10 and 15 days of exposure and it was a mere 4% in the liver tissue. In conditions of stress, the liver activates several housekeeping genes to voice survival signals and to maintain its role as a major protein synthesis organ, the housekeeping genes mRNA will occupy much of the locations on the ribosomes for translation resulting in less HSP70 being synthesized. Continuous synthesis of mRNA will continue if the effector is active leading to an accumulation of HSP70 mRNA

surpassing the capacity of cellular ribosomes and instigating deformations of the cell membrane where HSP70 will become translocated to the extracellular space. Translocation of HSP70 has two dual purposes: serves to activate the immune system and at the same time reduces cellular molecular pressure that would alleviate apoptotic signals. More studies need to be conducted to further elucidate the mechanism of HSP70 as an antiapoptotic protein and to additionally verify the significance of the differential distribution across organelles and the role extracellular HSP70 might play in attenuating the immune system in response to RFW.

Conclusion

It is apparent from this study that electromagnetic wave exposure has induced many changes to the hepatocytes, cardiomyocytes, and neurons of developing chick embryos, especially to the mitochondria and increase in the levels of HSP70. Hence, it is scientifically documented that these electromagnetic waves are causing stress to the living cells. Further studies should be carried out to fully understand the mechanism of apoptosis because of this mitochondrial damage when exposed to RFW.

Acknowledgments

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