International Journal on Emerging Technologies 10(3): 337-344(2019)

ISSN No. (Print) : 0975-8364
ISSN No. (Online) : 2249-3255

Occupational Exposure to Extremely Low Frequency-Electromagnetic Fields (ELF-EMFS) and Light at Night (LAN) and Risk of Breast Cancer in Human Subjects

Kavitha Varak[1], Surender V.[1], Ahuja Y.[2] and Tiwari R.[3*]

[1]UGC Research Unit, Bhavan's New Science College, Hyderabad, Telangana State, India.
[2]Genetics Unit, Vasavi Medical and Research Centre, Hyderabad, Telangana State, India.
[3]Dean, Global Institute of Engineering Technology, Moinabad, Telangana State, India.

(Corresponding author: R. Tiwari)
(Received 26 June 2019, Revised 29 August 2019 Accepted 25 September 2019)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: Electromagnetic Fields (EMFs) are used for many purposes on this planet for human benefits. From the past many years researchers are extending their research towards this area. Few recent studies on occupational exposure of EMFs have mostly focused on Extremely Low Frequency (ELF) fields. The reports given by the researchers are controversial and also there is no proper conclusive information on the effect of ELF-EMFs on the night shift working women. Thus the main objective of our research was to study the effect of ELF-EMFs and Light At Night (LAN) on the estradiol hormone in night shift working women. Other parameters like DNA integrity, oxidative stress and gene expression were also considered for the study.
Samples from 400 night shift working women were collected and estradiol levels were estimated in their sample by performing Chemiluminescence Immunoassay (CLIA). Using Single Cell Gel Electrophoresis (SCGE) method DNA damage was studied. The level of plasma Malonicdialdehyde (MDA) the oxidative stress and Nitric Oxide (NO) level were estimated. The melatonin levels were assayed for 50 samples with higher estradiol levels and RT-PCR was performed for 10 samples which had lesser melatonin and higher lyestradiol values. Our results signify that the level of estradiol was increased ($p<0.0001$) in samples compared to the controls. DNA damage also increased ($p< 0.0001$). Same results were observed during the plasma MDA level demonstrations. Melatonin levels were significantly suppressed in the samples ($p<0.0001$) compared to controls. RT-PCR analysis on gene expression slightly implicated the risk of breast cancer. Our results envisage that ELF–EMFs and LAN increase the estradiol levels with decrease in melatonin levels, which may lead to DNA damage, oxidative stress and thereby risk of breast cancer in the women working during the night shifts.

Keywords: CLIA, DNA Damage, ELF-EMFs, Estradiol, LAN, MDA, MNT, Melatonin, NO, RIA, RT-PCR.

Abbreviations: ELF-EMFs–Extremly Low Frequency–Electromagnetic Fields, LAN-Light At Night, MDA-Malonic Dialdehyde, RIA-Radio Immune Assay, RT-PCR-Real Time-Polymerase Chain Reaction, CLIA-Chemiluminescence Immunoassay, MNT- Micronuclei Test.

I. INTRODUCTION

In 21st century most of us are depending on the wireless communications like cell phones, internet, satellite devices, Wi-Fi, personal systems etc. Due to this humans as well as other living beings are continuously exposed to the EMFs day and night in an extreme way [1, 2]. In recent years a large number of studies are held on this topic but the results are controversial and largely unanswered [3, 4]. However ELF-EMFs and LAN are hypothesized to be responsible for the changes in hormonal configurations leading to development of cancer in women, particularly night shift workers.

The EMFs affect the basic life processes, like growth and development. The possible association between ELF-EMFs, leukemia and breast cancer has been discussed widely since 1979 [5] particularly when the epidemiological study suggested a possible link between ELF-EMFs and childhood leukemia along with other reports [6] of similar association. WHO and IARC have already declared that ELF-EMFs are carcinogenic to humans in group 2B category [7]. Endocrine system of the body is also affected by these fields, especially the stress hormones and sex hormones [8].

ELF-EMFs are quite pivotal in the electromagnetic spectrum. Literature on epidemiological and laboratory studies on their genetic effects is controversial and inconclusive [9]. It has been found that the technical literature is conflicted regarding EMF exposure and health effects. While many studies conclude that there are no adverse health effects from established acceptable levels of EMF exposure [10], others conclude that adverse health effects may result from long-term exposure to high level EMFs [11]. However, it is the general consensus of the majority of the scientific community that scientific studies, to date, have suggested that the existence of harmful effects from environmental levels of exposure has not been substantiated but remains a possibility [12, 13].

Genetic effects of these ELF-EMFs in the laboratory studies have been conjectured to be thermal [14]. The *in-vitro* and *in-vivo* studies have demonstrated the ability of EMFs to cause epigenetic modifications [15]. At low frequency waves, EMFs have the effect on the nervous system due to their intensive sensitivity [16-18]. Not only the nervous system they also affect the psychological conditions. Due to the heat released by them the temperature of the body also raises.

The health is seriously affected when there is longer exposure to the waves than the short exposure [19, 20]. The effect of the waves ranges from cellular mechanism level in the body to tissue level, nuclei, and mitochondrial, reproductive systems and other hormone levels [21]. In hormones especially, melatonin and estradiol hormone levels are affected by these fields due to the long term exposure of body and particularly during night [22].

Estradiol is a steroid hormone that is necessary for the normal development and growth of the breasts, menstrual cycle and reproductive organs essential for perpetuation of species [23, 24]. Recent analyses have shown a positive link between estradiol and night shift work [25-29]. Also the observational studies [30] reported higher estradiol levels in night shift women workers.

Higher levels of estrogens have been associated with breast cancer risk in women [31, 32]. Thus estradiol may be implicated in breast cancer risk because of its role in stimulating breast cell division during the critical periods of breast growth and development [33]. It is important to understand that night shift workers are more likely to be obese and to have unhealthy life style which may also contribute to risk of breast cancer and sex hormone metabolism [34, 35].

There is mounting evidence of an association between night shift work and breast cancer risk which is of increasing concern [36, 37]. The reason for incidences of increased breast cancer among night shift working women may be due to decrease in melatonin level and increase in reproductive hormone synthesis (estradiol) in response to LAN [38]. Same concept has been hypothesized in other findings [39, 44]. The mechanism where ELF-EMFs and LAN exposure could increase cancer risk lie in the possible oncostatic property of melatonin and its circulating levels [45-47].

The main objective of this project was to study the effect of ELF-EMFs and LAN on the estradiol hormone and risk of breast cancer in the night shift female workers.

Our study was planned to evaluate the genotoxic effects, hormonal levels and risk of breast cancer and implicate the possible health effects associated with exposure to ELF-EMFs and LAN in the occupational environments.

The subjects selected for the study were females working in night shifts exposed to ELF-EMFs and LAN. Samples from 400 night shift working women were collected and estradiol levels were estimated in the samples by performing Chemiluminescence Immunoassay (CLIA) [48]. Using Single Cell Gel Electrophoresis (SCGE) method DNA damage was studied [49, 50]. Measuring the level of Plasma Malonicdialdehyde (MDA) the oxidative stress and Nitric Oxide (NO) level were estimated [51, 52]. The Melatonin levels were assayed by Radio Immuno Assay (RIA) [53] and risk of breast cancer was assayed by RT-PCR method [54]. The chromosomal aberrations by Micronuclei Test (MNT) [55, 56].

II. MATERIALS AND METHODS

A. Participants

The women (n=400) working in the night shifts for about 5 years, from different hospitals and software companies situated in various locations in Hyderabad, were considered for the study. The control population (n = 200) included individuals who were not working in night shifts. The screened control population was age matched with similar socioeconomic status. The subjects did not have any family history of genetic anomaly or major illness. Informed consent was taken from all the participants.

B. Proforma/Questionnaire

Age, gender, diet, exercise and recent medical history were used as criteria for the selection of both exposed and control populations. Detailed questionnaire on work characteristics, psychologically perceived stress and non-specific symptoms experienced by exposed populations were responded by 94% of participants. Questionnaire on subjective symptoms related to EMF exposure included self assessment of non-specific symptoms such as headache, dizziness, tinnitus, visual impairments, sleep disturbances and menstrual cycle.

C. Sampling

After receiving informed consent, 7 ml peripheral blood was collected in the early hours between 6 am to 8 am from each volunteer by venipuncture into a sterilized disposable syringe. 1ml of whole blood was allowed to clot to collect the serum. The remaining 6 ml of blood was heparinized and both the samples placed in ice to prevent exogenous damage. The samples were processed for estimating estradiol by CLIA, DNA damage by SCGE, oxidative stress by LPO and NO, Chromosomal Aberrations by MNT- Micronuclei Test, melatonin assay and RT-PCR.

D. Estradiol assay- Chemiluminescence Assay (CLIA)

Plasma estradiol was quantitatively measured by Chemiluminescence Assay (CLIA) [48]. The E2 EIA is based on the principle of competitive binding between E2 in the test specimen and E2- HRP conjugate for a constant amount of rabbit anti-Estradiol. In the incubation, goat anti-rabbit IgG coated wells were incubated with 25 µl E2 standards, controls, samples, 100 µl Estradiol-HRP Conjugate Reagent and 50 µl rabbit anti-Estradiol reagent at room temperature for 90 minutes. A solution of chemiluminescent substrate was then added and read Relative Light Units (RLU) with a Luminometer. The intensity of the emitting light was found to be proportional to the amount of enzyme present and was inversely related to the amount of unlabeled E2 in the sample. Quantification of unknown samples was achieved by comparing their activity with a reference curve prepared with known calibrators.

E. Alkaline Comet assay (SCGE)-

A sensitive test to study the degree of DNA damage was used in this study [49]. The cells from each sample were mixed with low melting point agarose and embedded on a layer of normal melting point agarose, precoated on a micro slide. On top, a third layer of low melting point agarose was spread.

The slides were treated overnight in freshly prepared, chilled lysing solution containing high salt concentration and detergents. DMSO was added before use and pH was adjusted to 10 at 40℃. The slides were removed from the lysing solution, and kept in alkaline electrophoresis unit for 30 minutes. On electrophoresis fragmented DNA produced by single strand breaks, moved towards anode to form characteristic comets. The slides were neutralized with Tris buffer and rinsed with distilled water. All of these steps were conducted in dim light to prevent additional DNA damage. The slides were air-dried and stored. Before staining, they were left

in fixing solution for 10 minutes, washed several times with water and dried thoroughly. Silver staining method was used to visualize the nature of DNA. [50] The slides were screened under a bright field transmission microscope. Comet tail length, which is an estimate of DNA damage was measured for 100 cells per treatment using ocular micrometer.

F. Lipid Peroxidation-Thiobarbituric Acid Reactive Species (TBARS) Assay

MDA is the stable end product of lipid peroxidation and an efficient parameter for lipid oxidative damage. [50] Formation of MDA is assessed by quantification of thiobarbituric acid reactive species (TBARS). The protein free plasma obtained after centrifugation was treated with thiobarbituric acid (0.33%) in boiling water bath for 30 minutes to form an 1:2 adduct which was measured at 535 nm with spectrophotometer (Schimadzu UV-160A, Cat No. 204-04550-01, Made in Japan). MDA standards were prepared by using 1, 1, 3, 3-tetraethoxy propane. The concentration of MDA equivalents in nmol/ml in samples were interpolated from the standard curve.

G. Estimation of Serum Nitric Oxide (NO)

Nitric Oxide (NO) is an unstable compound, it is rapidly converted to nitrates and nitrites in the body, hence their concentration is parallel to NO levels. Total production of NO was determined by assaying for nitrate and nitrite. Nitrate and nitrite concentrations were then estimated by the Griess method [52]. In this method nitrate is first reduced to nitrite which is treated with sulfanilamide and N1-napthyl-ethylene diamine. The colored compound is formed after which its characteristic absorption spectrum was determined. The optical densities of the samples were then obtained on a spectrophotometer at 540 nm (Schimadzu UV-160A, Cat No. 204-04550-01, Made in Japan).

H. Melatonin Assay-Radio Immuno Assay (RIA)

Plasma melatonin was quantitatively measured by Direct Radioimmuno Assay (RIA) [53]. The amount of 125I-labelled antigen bound to the antibody is inversely proportional to the analyte concentration of the sample. Standard melatonin (1000 pg/l) - LDN GmbH & Co. KG, Nordhorn, Germany was used. When the system is in equilibrium, the antibody bound radioactivity is precipitated with a second antibody in the presence of polyethylene glycol. The precipitate is counted in a gamma counter. Quantification of unknown samples is achieved by comparing their activity with a reference curve prepared with known calibrators.

I. Gene expression by real-time PCR (RT-PCR)

Protocol of Schmittgen & Livak [54] was followed for RT-PCR analysis. According to this protocol, the RNA was first isolated from the sample. Then the RNA was exposed with DNAse I, later cDNA was synthesized. In the next step RT-PCR was performed, where triplicates PCRs per gene, per cDNA sample were made. To the triplicates, master mix containing SYBER green reagent was added as per the protocol. PCR was performed using real time instrument. 40 cycles of 15s at 95℃ was followed for SYBER green detection. Further PCR efficiency of gene of interest and control genes was determined.

J. Chromosomal Aberrations-Micronuclei Test (MNT)

Micronuclei (MN) were observed in cytokinesis blocked cells using cytochalasin B (Cyt-B) following the method suggested by Fenech and Morley [55]. About 0.2 ml of PHA was added to 5 ml of RPMI 1640 medium using 1 ml syringe. 15 drops of blood was added to each vial. Samples were initiated in duplicates. The culture vials were incubated for 72 hrs at 37℃ and shaken for proper mixing. Cyt-B (6 µg/ml) was added at 44th hour after initiation of culture and incubated further for another 28 hrs at 37℃ and then cultures were harvested. The cultures were centrifuged at 1000 rpm for 5-10 min. The supernatant was discarded and 5 ml of pre warmed hypotonic solution (0.56%) was added to the pellet drop by drop slowly on by vortexing and incubated for 10 min for 37℃. Then the vials were centrifuged for a minute and supernatant was discarded. Cells were fixed in 5 ml of fixative (3:1 methanol: acetic acid) followed by two more changes of fixative. The slides were prepared in triplicate by gently dropping the cell suspension onto the pre-cleaned slides and flame dried. Slides were stained with 2% Giemsa for 10 min, rinsed in distilled water and air dried. To determine the MN yield, nearly 1000 binucleated (BN) cells were scored for each experimental condition under high magnifications of 400x and finally 1000x from 2 coded slides/culture. Identification of MN was according to the criteria summarized by Countryman and Heddle [56]. Routinely on average ~2000 BN cells were scored for the presence of MN in each subject and mean values of the results were calculated.

K. Statistical Analysis

Coding and de coding of the slides was done during the statistical analysis. For statistical evaluation, observations of each parameter were pooled and mean $\pm$ SD was calculated. Student's t-test paired and unpaired comparisons were applied to determine the significance of various biochemical changes between samples and controls.

III. RESULTS

The Table 1 shows the result of various parameters in the night shift samples and control groups. The results demonstrated comet tail length as an index of DNA damage (Fig. 1). The mean tail length (8.9$\pm$0.744) was observed in ELF – EMFs and LAN exposed samples. The mean estradiol levels (pg/ml) showed significant increase in ELF–EMFs and LAN exposed samples when compared with controls.

The mean MDA concentration (nmoles/ml) of ELF–EMFs and LAN exposed samples also showed significant increase in MDA levels when compared to controls. The levels of serum Nitric Oxide (NO) determined as nitrites or nitrates showed an increased level of serum NO in the exposed groups. The result also outlines the melatonin levels, which differ significantly between exposed and controls. The mean RT-PCR value (6.99$\pm$6.56) was observed. An increase in the percentage of micronuclei of exposed compared to controls was also found.

Table 1: DNA Damage, MDA, NO, Estradiol, Melatonin levels and RT-PCR in the exposed compared with control group.

DNA Damage Length (arbitrary units)			
Subjects	N	Mean ± SD	P-value
EMF-Exposed	400	8.76 ± 1.31	<0.0001
Control	200	4.30 ± 1.13	
MDA Levels (nmoles/ml)			
Subjects	N	Mean ± SD	P-value
EMF-Exposed	400	2.56 ± 0.82	<0.0001
Control	200	1. 87 ± 1.47	
NO Levels (µmoles/ml)			
Subjects	N	Mean ± SD	P-value
EMF-Exposed	400	2.58 ± 0.09	0.776
Control	200	1.98 ± 0.07	
Estradiol levels (pg/ml)			
Subjects	N	Mean ± SD	P-value
EMF-Exposed	400	284.4 ± 89.08	<0.0001
Control	200	72.10 ± 34.08	
Melatonin levels (pg/ml)			
Subjects	N	Mean ± SD	P-value
EMF-Exposed	50	35.12 ± 2.72	<0.0001
Control	50	96.00 ± 146.59	
RT-PCR (pg/ml)			
Subjects	N	Mean ± SD	P-value
EMF-Exposed	10	7.06±6.67	0.042
Control	10	1.13±1.25	
MNT			
Subjects	N	Mean ± SD	P-value
EMF-Exposed	400	1.18±0.4	0.237
Control	200	1.13±0.34	

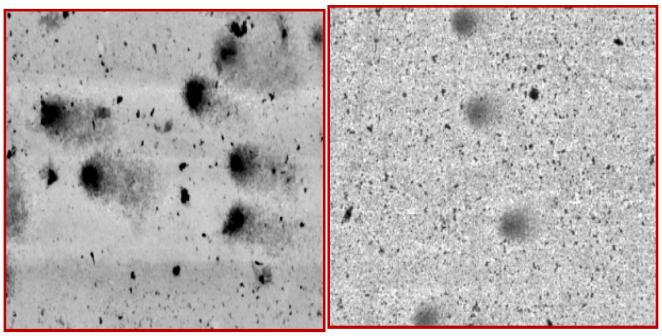

Fig. 1. SCGE- Comet Tails.

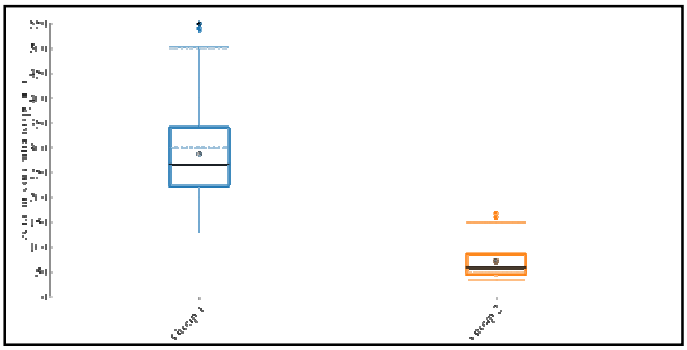

Fig. 2. Box-plot showing Estradiol (pg/ml) concentration. (Group 1- samples- Night shift working women; Group 2- Control).

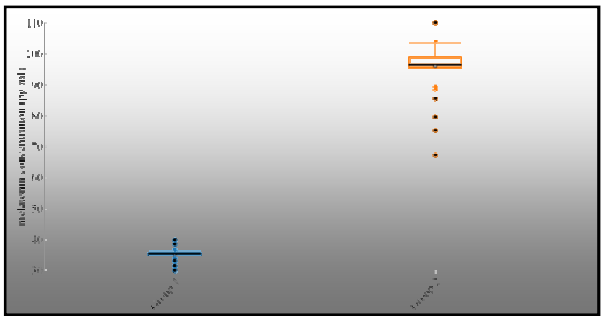

Fig. 3. Box plot - melatonin concentration (pg/ml). (Group 1- samples- Night shift working women; Group 2-Control).

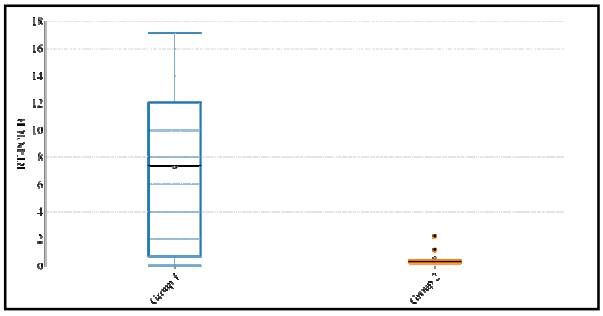

Fig. 4. Box plot-RT-PCR (Group 1: Nightshift working women; Group 2: Controls).

IV. DISCUSSION

The research into the potential health effects of exposure to EMFs has been underway for several decades. A number of independent scientific committees and working groups have reviewed the literature and concluded that exposures to EMFs, may impose adverse health effects in humans. Nonetheless, considering the scientific ‘‘weight of evidence,’’ mostly from human epidemiological data, the International Agency for Research on Cancer (IARC) concluded that EMF exposures act as ‘‘possible human carcinogen’’ in the IARC category of Class 2B [7]. Despite the epidemiologic association of magnetic fields with health hazards, a cause-and-effect relationship cannot be inferred.

The study has shown the possible bioeffects of ELF-EMFs on exposed subjects occupationally exposed to light at night.

A. ELF-EMFs effect on Biomarkers of Genetic Damage

An important basis for assessing a potential cancer risk due to ELF-EMF exposure is knowledge of biological effects on human cells at the DNA level. The findings from alkaline comet assay carried out in peripheral blood lymphocytes showed induction of DNA damage in occupationally exposed subjects, when compared against controls.

B. ELF-EMFs Effect on Oxidant-Antioxidant Balance

The MDA levels in samples of ELF-EMFs occupationally exposed subjects suggest a statistical significance in LPO activity. Owing to the great number of biological actions of free radicals, the LPO activity could have an important implication in the comprehension of the biological stress response to ELF-EMFs. Oxidative DNA damage and LPO activity in exposure to ELF-EMFs was reported in certain *in vivo* experimentations and animal models. A time-dependent significant higher TBARS levels ($p<0.001$) and induced DNA damage ($p<0.05$) in the exposure group (50Hz, 0.97 mT) was observed [57]. It is also observed that nitric oxide synthase is stimulated by the EMF exposure [58, 59]. This compound can be involved in the stimulation of lipid peroxidation indirectly. This hypothesis could also explain the increased TBARS production in our exposed group as well.

Similarly, the influence of ELF-EMFs on oxidative-antioxidative balance in cells was demonstrated [60]. Since free radicals are able to damage many biomolecules, including DNA, enzymes, lipids and proteins, we can hypothesize that modulation in antioxidative enzyme activities observed in our study are related to overproduction of oxidants NO and MDA under ELF-EMFs exposure. However, the precise mechanisms involved in the effect on oxidative stress are not clear yet.

C. ELF-EMFs Effect on Hormones and Stress parameters

Very few studies were carried out on estradiol and melatonin hormone in occupationally ELF-EMFs exposed subjects. The effect of ELF-EMFs occupational exposure on production of gonadal and pineal gland hormone, melatonin was evaluated in our study. The mean estradiol and melatonin concentration of the exposed subjects was significantly different from those of healthy individuals (Table 1). The exposed night shift workers have relatively higher levels of estradiol and suppressed melatonin levels against the control group (Fig. 2, 3). The Box Plot of RT-PCR predicts the higher gene expression in samples (Fig. 4).

IV. CONCLUSION

The possibility of breast cancer in the women night shift workers is so far supported due to the increase in estradiol levels (E2) and decrease in melatonin levels. The genotoxic activities of the ELF-EMFs and LAN cause the DNA damage which may lead to increase in the micronuclei conditions in the night shift working women. Along with them the MDA and NO levels in serum were also increased significantly. This probably raises the risk of oxidative stress and breast cancer possibilities. The gene expression in real-time PCR supports the estradiol level rise in the women who are exposed to EMFs mostly during the night times. Therefore, it is concluded that estradiol level rise in blood may lead to the possibility of breast cancer.

Melatonin levels in the exposed subjects were observed to be low when compared to the control population. So the decrease in the melatonin levels in the night shift workers indirectly indicates the effect of ELF-EMFs and LAN in the night shift women.

So far in the study, we were able to evaluate that EMFs cause decrease in melatonin and increase in estradiol level in the night shift working women. This inturn may lead to the increase in other parameters like DNA damage and oxidative stress, predicting the possibility of breast cancer. Previous studies in this field were not

sufficient and hence the association between night shift works was found in few studies only [24, 26, 27, 28].

V. FUTURE SCOPE

Though our results show a significant effect associated with longer duration of intense night shifts to ELF-EMFs, pooled collaborative meta-analysis studies from individuals, is required to further clarify the association of ELF-EMFs with women in night shift work and risk of breast cancer.

ACKNOWLEDGEMENTS

The authors thank University Grants Commission (UGC) for funding this Major Research project. The team of authors expresses their profuse thanks to the management of Bharatiya Vidya Bhavans New Science College for their support and cooperation.

Conflict of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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How to cite this article: Varak, K., Surender, V., Ahuja, Y. and Tiwari, R. (2019). Occupational Exposure to Extremely Low Frequency-Electromagnetic Fields (ELF-EMFS) and Light at Night (LAN) and Risk of Breast Cancer in Human Subjects. *International Journal on Emerging Technologies*, **10**(3): 337–344.